

UCSF

UC San Francisco Electronic Theses and Dissertations

Title

Developing protein-protein interaction inhibitors of the herpesvirus protease family

Permalink

<https://escholarship.org/uc/item/564079wq>

Author

Gable, Jonathan Edward

Publication Date

2015

Peer reviewed|Thesis/dissertation

Developing protein-protein interaction inhibitors of the herpesvirus
protease family

by

Jonathan Edward Gable

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biophysics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Copyright 2015
by
Jonathan E. Gable

Acknowledgements

UCSF was truly an amazing place to be, to learn, and to do science. Aspects of UCSF's location, architecture, and design contributed to this, but the people are truly to thank—administrators, staff, students, and faculty alike. Here I want to thank and acknowledge those who made my achievements and my current scientific state of mind possible.

I want to start by thanking Charles Craik, my advisor on this journey. Charly gave me the freedom to explore science and to expand my knowledge and skills; his enthusiasm never wavering through the inevitable failures, his simultaneous appreciation and desire to push one step further never dulled by successes. Broadly, but especially as a graduate student, this freedom is the greatest gift a student can receive and the greatest gift an educator can give. He let my interests drive my learning and my science while providing the resources to endeavor in nearly any biophysical, biochemical, or biological line of inquiry I could imagine. I never would have thought I would have the opportunity to try so many techniques: HTS, virtual screening, ITC, crystallography, enzyme kinetics, protein purification, peptide synthesis, SPR, Octet, thermophoresis, mammalian cell culture, NMR and the list goes on (even those seemingly unrelated online courses, which turned out to come in handy after all). As a scientist, as an avid learner, as a student what could be better? There may never be another time in my life that combines so magnificently the freedom to explore and the resources for that exploration.

Importantly I was at once free and not without guidance. Charly never let the big picture escape and since I can easily become mired in details and nuances (something which I would like to think improved during my graduate career) his perspective was extremely valuable. He, and indeed much of the training at UCSF, ingrained in me the importance of narrative in scientific discovery. He gave me exposure to grant writing, a skill set that will no doubt have great utility

throughout my scientific career. Charly was generous in sharing his network and his years of experience, both in and out of academia. Never once did I feel as though Charly lost sight of the fact that I was a student first and foremost. For all of these things and more, I thank him.

My experience working on the project described in this text would not have been the same, and indeed the project would not exist in its current state, were it not for Tina, Greg, Aida, and Tim. Tina and Greg mentored me during my rotation. Despite my struggles during this, my first rotation at UCSF, Tina was always helpful and encouraging. She helped me during the rotation and just as important expressed her support to Charly for my joining the lab. Having an advocate that early on at UCSF was truly an honor. Greg has continued to be a critical part of the project and my education throughout my time in the Craik lab. He has taught me a tremendous amount about NMR and we have had countless interesting discussions about the details of protease biophysics. On top of all that, as my neighbor, he kindly shared (allowed me to steal?) literally liters of his buffer stocks (which, in my defense, I tried to replenish when they ran low). I thank him for teaching me so much and letting me be a “klepto” when it came to 1 M potassium phosphate (among other solutions). Aida, through tremendous trials and tribulations, solved the first KSHV Pr-DD2 crystal structure that was the basis for much of the work described in this dissertation. Tim joined the lab and the project part way through my time in the Craik lab, but in doing so had a profound effect on the project, my experience, and my knowledge. From day one it was apparent that Tim was driven and interested in all aspects of what the project was, and what it could become. I learned a great deal about small molecules and drug discovery from him with more chemical detail than I had ever previously considered. In addition to this, he was a fantastic colleague to have around for discussion. Many a vibrant and

unabashedly honest scientific conversation took place, all of which I thoroughly enjoyed and learned from.

In addition to those working on the herpesvirus project, all of the Craik lab members over the years have been amazing. The lab dynamics, although evolving over time, were always a true joy. Star and Melody joined the lab at the same time as me and although the three of us were all relatively reserved in lab I felt as if there was a tacit support throughout the arch of graduate school. Everyone was consistently willing to help and discuss. Anthony has an encyclopedic knowledge of proteases and is always engaged with creative ideas to share. Michael, Cheryl, Greg, and JungMin were fantastic neighbors, happy to indulge in fun and informative scientific musings (with a dash of sarcasm now and again, just for good measure). Cathal and Star taught me everything I know about cell culture and western blots, which is no small feat given that I knew nothing about either when I arrived at UCSF. Truly every person in the Craik lab over the past ~5 ½ years has contributed to my education and my experience. I cannot thank them enough for making my time in the Craik lab as amazing as it was. It would not have been the same without them.

The faculty at UCSF also dramatically enhanced my experience and learning. First were my thesis committee members, Jim Wells and Joe DeRisi. Jim wells shared some of his amazing breadth of experience, both in and out of academia, as well as his expertise in proteases. Joe is one of the most creative scientists I have ever met and an amazing educator; whether through watching his TED Talk, taking and helping teach bootcamp, or TAing for his Illustrator course I learned a great deal about how to make science exciting and understandable. Joe's expertise in virology was also a tremendous help. My rotation mentors impacted the arch of my graduate experience. Andrej Sali gave me my first experience doing computational biology, something

that I dabbled in throughout my time in the Craik lab and will now focus on extensively as I start my work at Schrödinger. David Agard is a truly rigorous and learned scientist. His wealth of knowledge, rigorous critical thinking, and desire to fully understand whatever he studies seems unparalleled. It was a pleasure to work with and be taught by him both in his lab and during the Marco course. Yifan Cheng enabled me for the first time ever to truly see the molecule I was working with, the 20S proteasome. There is something remarkable and memorable about that. It has been a pleasure to watch his many successes. What I learned from Yifan and David about EM has allowed me to better understand and better contribute to many group meetings in the Craik lab as we collaborated with him in the use of antibodies to stabilize and visualize challenging protein targets.

One of the great things about UCSF is that you do not need a formal association with faculty in order to interact with them. This is the unusually fantastic collaborative environment UCSF fosters. Brian Shoichet and Henry Lin collaborated with me to perform virtual screening. When I narrowed down that I wanted to pursue computational chemistry/biology after UCSF Brian was always willing to chat, discussions that were extremely useful for me. Matt Jacobson is a fantastic scientist and educator. As the head of the biophysics program he always worked on behalf of the students. He was engaged during my presentations at retreats always asking deeply insightful questions I had not previously considered. He was also a great help as I pursued computational positions for my post-doctoral work. He was always honest and clear, something I greatly appreciated. I mentioned before how meaningful it is to have an advocate early in one's experience at a new institution. Bob Stroud fulfilled this role. Through our interactions in macro, his minicourse, and later as I TAed for the macro course, Bob made clear his support for my

progression as a student and scientist, ultimately becoming a reference for me as I applied to post-doctoral work.

Collaborations abound at and outside of UCSF, from advise on a challenging kinetics analysis to screening hundreds of thousands of compounds. Michelle Arkin and Chris Wilson enabled my first foray into high-throughput screening. Kip Guy furthered that experience, allowing me to visit Saint Jude Children's Research Hospital and helping me learn about screening, chemistry, and how one actually bridges the massive divide between a screening hit and a drug. Adam Renslo and Priya Jaishankar always had insightful ideas when it came to chemistry and helped enable my first publication at UCSF. Chris Farady, as a former Craik lab member now at Novartis, not only facilitated fragment-based screening efforts between Novartis and us he also always made time to meet when he was in town or have a phone call from Basel whether to discuss data or discuss my interest in transitioning into industry after graduate school. Chris Waddling taught me much of what I know when it comes to the practicalities of getting from a tube of protein to a protein modeled into an electron density, spending hours helping in the crystallography lab and even helping me collect data in Berkeley including data that was critical to my first publication. Mark Kelley was always willing to lend a helping hand or suggest a paper to read when it came to NMR. Geeta Narlikar and Sue Miller helped me figure out the complexities of both equilibrium and kinetics in our unique system, including thinking about Zhang Poorman analysis. John Gross was a fantastic neighbor and resource for better understanding NMR and thinking about the challenges of monitoring protein-protein interaction. June Lee was an important mentor as the Director of CTSI. She not only helped keep clinical and business considerations in mind while considering how to progress our project, she also gave me the opportunity to intern at CTSI where I was able to think critically about not just my own

project, but a host of clinical translational work at UCSF. June was also generous when it came to sharing her network with me, something I greatly appreciated both at networking events as well as when I reached out to her about potential digital health speakers for the UCSF business club. Finally the administration, without which iPQB would not run, were absolutely fantastic. Rebecca Brown did everything in her power to make the experience for biophysics students a positive one. Julia Molla and Nicole Flowers were also ready to step in to help at any time, despite not formally being administrators for the biophysics program. In combination, Matt, Rebecca, Julia, and Nicole advocated and fought for us, the students, and in doing so greatly enhanced my experience.

Of course, my scientific journey started long before I arrived at UCSF. Carol Gontang, my high school biology teacher, facilitated my interest in biology and biochemistry and it was under her guidance that I was first struck by the amazing notion that physics can help explain chemistry, and chemistry biology, and biology is every living thing around us. She also facilitated my first real lab experience under the mentorship of Brian Kobilka at Stanford. Brian was kind enough to take me on as a senior in high school. I distinctly recall struggling to do simple serial dilutions, yet he and the postdocs I worked with were patient in helping me learn. With my interest in membrane proteins established I joined Judy Kim's lab at UCSD. Judy taught me physical chemistry and laser spectroscopy, but more importantly she taught me how to be truly rigorous and detail oriented. I still remember the first time I looked at a tryptophan fluorescence spectrum and she was trying to point out the shoulder at 290 nm, which seemed inconsequential and not really there to me at the time. By the end (and sooner) of my time in the Kim lab, the 290 nm shoulder was clear as day and I cannot imagine how I missed such an obvious feature of the spectrum those years ago. My time in the Kim lab and at UCSD gave me a

new level of critical thinking ability, part of the hours-long lab meetings we had discussing intricacies of quantum yield or appropriate approaches to background correction and peak identification, among many other things. It was in the Kim lab that I made my first public entrance into the scientific world by contributing to and ultimately writing my own scientific manuscript. Carol Gontang, Brian Kobilka, and Judy Kim profoundly influenced my scientific trajectory, and I thank them for that.

The students at UCSF are amazing. I cannot name them all but my interactions with students from every year and every program crafted my experience at UCSF and made it all the better. My roommates Aram and Charles were always fun to chat with, whether about science or science fiction, and just great guys to be around. Hanging out with Peter, Erik, Charles, and Aram was always a blast. Many a wonderful whiskey was had. It meant a great deal to have them as friends. My entire class as well as those I interacted with from CCB and Tetrad were fantastic. Of course, one student stands out above all the rest: Carrie Shiau. I am so thankful that we met; it has been such a joy to have you, and indeed your whole family, in my life. Thank you for your support, for traveling this road to a PhD ahead of me and providing me with your guidance, and for being so thoughtful, wonderful, and caring.

I would not be where I am or who I am without my family. My father, also a scientist, instilled in me an interest in the nature of all things scientific and the science of all nature (and of course how the “unnatural” can affect the natural). My stepmother shared with me her humor and creativity, as well as her love of nature. My mother was a constant source of support, care, and meaningful conversation. My stepfather introduced me to programming, something that clearly impacted my entire education, as well as countless obscure but interesting words and factoids. All of my parents embraced me and the path I choose. They showed interest in what I was doing,

however unintelligible, and encouraged me to persist and explore. My brother has been a lifelong friend. My entire family, the Gable, Saeks, Mitchell, and Bell families, all contributed to my experience. Special thanks to my grandparents; you touched my life directly and made my parents who they are and in doing so made me who I am.

Thank you to all of my supporters, mentors, friends, and family.

This work was supported by the National Center for Research Resources and the National Center for Advancing Translational Sciences, National Institutes of Health, through the UCSF Clinical and Translational Science Institute (CTSI) Grant Numbers UL1 TR000004 and UL1 RR024131. This work was also funded in part by grants from the National Institutes of Health (R01-AI090592 and P50-GM08250) and NCI and the Helen Diller Family Comprehensive Cancer Center (P30 CA 82103-13). I was supported by the NIH Structural Biology Training Grant (GM008284) and the NSF Graduate Research fellowship program (1144247). We are thankful to the UC Office of the President, Multicampus Research Programs and Initiatives grant MR-15-328599 and the Program for Breakthrough Biomedical Research, which is partially funded by the Sandler Foundation for their support of the Advanced Light Source and Beamline 8.3.1 which was used to acquire the crystallographic data herein.

Abstract

The herpesviruses are an exceptionally interesting pathogen and the cause of lifelong infection for more than 90% of the human population. They are also a significant cause of morbidity and mortality with limited treatment options; many herpesviruses have no approved treatment and all approved treatments target the same viral enzyme suffering from resistance and toxicity. This text begins with a review of both the biology and the current and future treatment options for the nine human herpesviruses. Particular emphasis is placed on the herpesvirus proteases as potential therapeutic targets, the focus of the remaining 8 chapters. I report high throughput screening (HTS) assays and results for standard HTS, fragment-based screening, and disulfide tethering identifying novel inhibitors of both Kaposi's sarcoma-associated herpesvirus (KSHV) and cytomegalovirus (CMV) proteases. Detailed structural analysis reveals at atomic level the unique mode of binding for a subset of dimer disruptors—protein-protein interaction antagonists that bind a transient cryptic pocket at the dimer interface thereby allosterically inhibiting the active site. Kinetic analysis shows that these analogs are slow time-dependent inhibitors, a fact that could aid in achieving cellular efficacy. Finally I detail our first evidence of a dimer disruptor successfully inhibiting viral reinfection in human cells. As a whole, this text provides a powerful case study of developing a protein-protein interaction antagonist against a highly dynamic essential viral protein and advances progress toward novel methods of herpesvirus inhibition.

Table of contents

	Page
Chapter 1	1
Current and potential treatments for Ubiquitous but Neglected Herpesvirus Infections	
Chapter 2	115
A high-throughput screening pipeline for identification of herpesvirus protease protein-protein interaction inhibitors	
Chapter 3	142
Screening for novel human cytomegalovirus protease inhibitors	
Chapter 4	168
Fragment-based protein-protein interaction antagonists of a dimeric protease	
Chapter 5	194
Activity-based disulfide tethering for the identification of novel human cytomegalovirus protease dimer disruptors	
Chapter 6	220
Broad-spectrum allosteric inhibition of herpesvirus proteases	
Chapter 7	281
Kinetic and crystallographic analysis of DD2 analogs	
Chapter 8	304
Characterization of a fluorescence lifetime substrate for the human herpesvirus proteases and its apparent activation of KSHV protease	
Chapter 9	319
Inhibition of viral replication in mammalian cells by a protein-protein interaction antagonist of KSHV protease	
Concluding remarks	353

List of tables

Table 1-1	114
Herpesvirus genes and their associated inhibitors	
Table 2-1	139
Hits with robust dose response	
Table 2-2	140
Disubstituted pyrazoles inhibition of KSHV, EBV, and CMV proteases	
Table 2-3	141
Disubstituted pyrazole aggregation by dynamic light scattering	
Table 3-1	158
Representative hit compounds	
Table 3-S1	160-7
Reagent information	
Table 4-1	183
Confirmed primary screen hits	
Table 4-2	184
Indole R-group table	
Table 4-3	185
Aminothiazole R-group table	
Table 4-4	186
Trifluorobenzene R-group table	
Table 4-5	187
Sulfur tricycle R-group table	

Table 4-6	188
Oxygen tricycle R-group table	
Table 5-1	214
Enzyme construct activity comparision	
Table 5-2	215
Summary statistics for distributions of inhibition	
Table 5-3	216
Representative hit compounds	
Table 6-1	259
DD2 analogs	
Table 6-S1	262
Ile-to-Val site-directed mutagenesis primers	
Table 6-S2	279
Data collection and refinement statistics for compound 2	
Table 6-S3	280
Data collection and refinement statistics for compound 3	
Table 7-1	298-9
DD2 analogs	
Table 7-S1	302
Optimized 24-well grid screen	
Table 7-S2	303
Conditions for crystal growth	

List of figures

Figure 1-1	92
Human herpesviruses, diseases, and antiherpesvirus treatments organized by subfamilies.	
Figure 1-2	93
“Druggable” viral replication cycle	
Figure 1-3	94
Approved guanosine analog inhibitors of herpesvirus replication.	
Figure 1-4	95
Additional inhibitors of the viral DNA polymerase	
Figure 1-5	96
Bicyclic nucleoside analogs and a lipid ester analog of cidofovir. These inhibitors target viral replication	
Figure 1-6	97
Helicase-primase inhibitors	
Figure 1-7	98
Terminase inhibitors	
Figure 1-8	99
Portal vertex inhibitors	
Figure 1-9	100
Putative HCMV fusion inhibitors	
Figure 1-10	101
Ribonucleotide reductase protein-protein interaction inhibitors and inhibitors of unknown mechanism	

Figure 1-11	102
Roscovitine, a cyclin dependent kinase (CDK) inhibitor, and HIV and HCV protease inhibitors	
Figure 1-12	103
Dimeric herpesvirus protease and its shallow substrate binding pocket.	
Figure 1-13	104
Overlay of known herpesvirus protease structures	
Figure 1-14	106
M- and R-site alignments for herpesvirus protease substrate specificity	
Figure 1-15	107
General protease inhibitors and an HCMV pentafluoroethyl ketone inhibitor	
Figure 1-16	108
β - and <i>trans</i> -lactam herpesvirus protease inhibitors	
Figure 1-17	109
Azetidine, benzoxazinone, thieno[2,3-d]oxazinone, spirocyclopropyl oxazolones, and benzylidene N-sulphonyloxyimidazolone mechanism-based herpesvirus protease inhibitors	
Figure 1-18	110
Order-to-disorder transition of conserved oxyanion hole arginines upon dimer disruption.	
Figure 1-19	111
Dimer disruptor 2 (DD2), an allosteric herpesvirus protease inhibitor	
Figure 1-20	113
Proposed mechanism of dimer disruption and allosteric inhibition of herpesvirus proteases	
Figure 2-1	132
Z' and Z factor values across all 323 plates	

Figure 2-2	133
Scatter plot of percent inhibition.	
Figure 2-3	134
Scatter plot of autofluorescence versus percent inhibition	
Figure 2-4	135
Nutlin-like Murcko fragments	
Figure 2-5	136
Disubstituted pyrazole analogs.	
Figure 2-6	137
Disubstituted pyrazole IC ₅₀ curves	
Figure 2-7	138
NMR determination of dimer disruption and binding site mapping	
Figure 3-1	154
HTS validation and quality control	
Figure 3-2	155
Screening ROC analysis	
Figure 3-3	156
Example time-dependent dose response	
Figure 3-4	157
Zhang-Poorman analysis	
Figure 3-S1	159
Lineweaver Burk analysis of known protease inhibitor 1	

Figure 4-1	189
¹³ C NMR mapping of fragment binding and determination of mechanism of action	
Figure 4-2	190
¹⁵ N NMR mapping of fragment binding	
Figure 4-S1	191-3
¹³ C NMR mapping of all fragments with selective Ile labeling fragment binding	
Figure 5-1	206
CMV protease cysteines in crystal structure and model	
Figure 5-2	207
Potential cysteine mutations	
Figure 5-3	208
Final CMV protease construct	
Figure 5-4	209
Example BME50 curves	
Figure 5-5	210
Primary screen scatter plot	
Figure 5-6	211
Distribution of inhibition at varying BME concentrations	
Figure 5-7	212
Example MS data confirming covalent binding	
Figure 5-8	213
Comparison of tethering and KSHV Pr FBS hits	

Figure 5-S1	217
Correlation between mean percent inhibition and BME50	
Figure 5-S2	218
Histograms of percent inhibition	
Figure 5-S3	219
Normal quantile-quantile plots for percent inhibition	
Figure 6-1	250
IC50 values across multiple HHV proteases.	
Figure 6-2	251
Zhang–Poorman analysis of DD2 and compound 2.	
Figure 6-3	252
HHV domain diagram and ¹³ C– ¹ H HSQC spectrum of KSHV protease with selectively labeled Met197	
Figure 6-4	253
¹³ C– ¹ H HSQC Ile spectra in the presence of compound 2	
Figure 6-5	254
¹³ C– ¹ H HSQC Ile spectra in the presence of compound 3	
Figure 6-6	255
Asymmetric unit with the inhibitor bound	
Figure 6-7	256
Compound 2 and 3 binding pockets	
Figure 6-8	257
Compound binding mode with and without crystal packing	

Figure 6-9	258
Model of the conserved mechanism of inhibition	
Figure 6-S1	270
Dynamic light scattering of non-ionic compounds	
Figure 6-S2	271
Combined chemical shift perturbations for ^{13}C - ^1H HSQC Ile spectra	
Figure 6-S3	272
^{15}N -HSQC spectra for truncated KSHV protease with inhibitors	
Figure 6-S4	273
HSV-2 Pr D213 ^{13}C - ^1H HSQC isoleucine assignments	
Figure 6-S5	274
CMV Pr D221 ^{13}C - ^1H HSQC isoleucine assignments	
Figure 6-S6	275
Overlay of monomer A and monomer B	
Figure 6-S7	276
Symmetry-mate bridging molecule perturbs inhibitor binding to monomer A	
Figure 6-S8	277
Overlay of PLOP-minimized model and crystal structure	
Figure 6-S9	278
DD2 inhibition of KSHV Pr R82Q	
Figure 7-1	292
Time dependent inhibition with DD2-177	

Figure 7-2	293
Evidence of an enzyme isomerization model	
Figure 7-3	294
Overlay of DD2 analog crystal structure interfacial pockets	
Figure 7-4	295
DD2 analog crystal structure variable region analysis	
Figure 7-5	296
DD2-29 monomer B crystal structure	
Figure 7-6	297
DD2-29 monomer A crystal structure	
Figure 7-S1	300
Summary of crystallography trials	
Figure 7-S2	301
Comparison between enzyme isomerization and irreversible inhibition model fits	
Figure 8-1	311
KSHV protease substrates	
Figure 8-2	312
Hits from fluorescence lifetime pilot screen	
Figure 8-3	313
FRET vs ACC substrate IC50 curves	
Figure 8-4	314
HPLC traces of unlabeled substrate turnover and inhibition	

Figure 8-5	315
Overlay of WT KSHV protease and KSHV S114A protease in the presence of FLT substrate crystal structures	
Figure 8-S1	316
LC-MS confirmation of FLT substrate cleavage	
Figure 8-S2	317
MALDI-TOF MS confirmation of unlabeled substrate cleavage	
Figure 8-S3	318
Unlabeled substrates and their cleavage products	
Figure 9-1	336
Reinfection assay overview	
Figure 9-2	337
Example flow cytometry curves	
Figure 9-3	338
Reinfection bar plot	
Figure 9-4	339
DD2-29 reinfection dose response	
Figure 9-5	340
DD2 and DD2-29	
Figure 9-6	341
Reinfection with and without serum during treatment	
Figure 9-7	342
Theoretical western blot outcomes	

Figure 9-8	343
Anti-protease western blot optimization	
Figure 9-9	344
Anti-AP western time course	
Figure 9-10	345
Anti-protease western time course	
Figure 9-11	346
Anti-AP western varying treatment and induction strategy	
Figure 9-S1	347
Summary of MTS assay data	
Figure 9-S2	348
Summary of Sytox red data	
Figure 9-S3	349
Summary of reinfection data	
Figure 9-S4	350
HEK293 dox-inducible GFP flow data	
Figure 9-S5	351
HEK293 dox-inducible GFP flow data continued	
Figure 9-S6	352
MTS assay cell number optimization	

CHAPTER 1: Current and potential treatments for Ubiquitous but Neglected Herpesvirus Infections

Jonathan E. Gable, Timothy M. Acker, Charles S. Craik

This chapter contains previously published material from:

Gable, J. E., Acker, T. M., & Craik, C. S. (2014). Current and Potential Treatments for Ubiquitous but Neglected Herpesvirus Infections. *Chemical reviews*, 114(22), 11382-11412.

Copyright 2014 American Chemical Society.

1. Introduction

2. Herpesvirus biology

2.1 Viral classification

2.2 Subfamilies

2.3 Cell tropism

2.4 Viral replication cycle

3. Unmet clinical need – KSHV- and HCMV-associated disease

3.1 Kaposi's sarcoma associated herpesvirus (KSHV)/HHV 8

3.2 HHV-8 associated disease

3.2.1 Kaposi's sarcoma

3.2.2 B cell malignancies – Primary effusion lymphoma and multicentric

Castleman's disease

3.2.3 Current treatments

3.3 Cytomegalovirus disease

3.3.1 Congenital setting

3.3.2 Organ-transplant setting

3.3.3 HIV-AIDS setting

4. Non-protease drug targets

4.1 Current treatments and their molecular mechanisms of action

4.2 Improving existing treatments

4.2.1 Nucleoside analogs

4.2.2 Nucleoside phosphonate

4.3 Non-nucleoside DNA replication inhibitors – targeting helicase-primase

4.4 Targeting encapsidation and packaging

4.4.1 Terminase inhibitors

4.4.2 Portal vertex inhibitors

4.5 Other viral targets and inhibitors with unknown mechanism of action

4.5.1 Targeting viral entry

4.5.2 Targeting viral kinases

4.5.3 Targeting the ribonucleoside reductase

4.5.4 Inhibitors with unknown mechanism of action

4.5.5 Vaccine development

4.5.6 Targeting latency and immune evasion

4.6 Host targets for herpesvirus inhibition

5. Success of other protease-targeting drugs

5.1 HIV Pr drugs

5.2 HCV Pr drugs

6. HHV protease

6.1 Biological role in viral replication

6.2 HHV protease structure

6.3 Substrate binding and specificity

6.4 Active-site inhibitors of HHV proteases

6.4.1 Generic serine protease inhibitors

6.4.2 Activated carbonyl warheads

6.4.3 Beta lactam inhibitors

6.4.4 Trans-lactams

6.4.5 Azetidine inhibitors

6.4.6 Benzoxazinone and thienof[2,3-d]oxazinone mechanism-based inhibitors

6.4.7 spirocyclopropyl oxazolones and the benzylidine N-sulphonyloxyimidazolones mechanism-based inhibitors

6.5 Thiol-reactive inhibitors

6.6 HHV dimerization and activation

6.7 Dimer disruption as an alternative to active-site inhibitors

7. Conclusion

Introduction:

More than 90% of the world's population is infected with a herpesvirus.⁽¹⁾ Despite this staggering fact, only a handful of approved drugs exist for the general treatment of herpesvirus infections. To date, all of these drugs inhibit the same enzyme, the viral DNA polymerase. Nine human herpesviruses have been identified, and each has been associated with disease. In immune-competent individuals, herpesvirus infections are the causes of unpleasant but typically non-life-threatening diseases such as oral and genital herpes, chickenpox and shingles, skin rash in infants (roseola infantum), and infectious mononucleosis (also known simply as mono). In individuals with immature or compromised immune systems, herpesvirus infection can be devastating. Developmental disabilities, loss of sight and hearing, cancer, life-threatening pneumonia, encephalitis (inflammation of the brain), and death comprise only a partial list of the cost herpesviruses have on well being in this subset of the population.

The tremendous complexity of herpesvirus biology brings with it many potential avenues for therapeutic interventions that remain in their infancy. However, the past two decades have seen progress toward novel treatments for herpesviruses; this is the subject of the current review. Previous reviews of the subject are either more than 10 years old or cover a subsection of the field. Herein we provide a comprehensive review of herpesvirus drug discovery with an emphasis on the most recent advances in the field and their progression from early discovery to clinical development. The focus is on small-molecule inhibitor development so we do not cover biologics and vaccine development in as much detail. There is little work on antiherpes biologics outside the context of vaccine development, which is reviewed elsewhere.⁽²⁾ We will, however, discuss some exciting biologics targeting viral polypeptides that appear to drive oncogenesis, though they are not required for the viral replication cycle. The necessary herpesvirus biology is

introduced, and a more detailed review of that biology/virology can be found elsewhere.⁽³⁾ By highlighting the exciting recent work in herpesvirus drug development, and the historical studies that enabled it, we hope to spur interest in the many potential therapeutic targets for this ubiquitous but neglected virus family.

2. Herpesvirus biology:

2.1 Viral Classification

All herpesviruses are large enveloped double-stranded DNA viruses. The viral genome is composed of a linear chain of 125–290 kbp and contains ~70–200 protein coding genes, depending on the specific virus. Herpesvirus virions (the infectious particles) have three major components: the nucleocapsid, the tegument, and the envelope. Herpesviruses have an icosahedral nucleocapsid ($T = 16$) composed of 162 capsomeres (150 hexons and 12 pentons) where the viral genome resides. A matrix of viral proteins called the tegument exists between the lipid bilayer envelope and the nucleocapsid. The envelope contains glycoproteins critical to cell attachment and entry. Virions are approximately 200 nm in diameter.⁽⁴⁾

2.2 Subfamilies

The taxonomic family *Herpesviridae* consists of herpesviruses that infect mammals, birds, and reptiles. This family does not include herpesviruses infecting fish and frogs (*Alloherpesviridae*) or bivalves (*Malacoherpesviridae*). Human herpesviruses are further broken down into three subfamilies, the α -, β -, and γ -herpesviruses (Figure 1-1). Differences in biology and genetics give rise to these classifications. To date nine human herpesviruses have been identified: herpes simplex virus 1 and 2 (HSV1 and 2, α), Varicella zoster virus (VZV, α), human cytomegalovirus (HCMV, β), human herpesvirus 6a, 6b, and 7 (HHV6a, 6b, 7, β),

Epstein–Barr virus (EBV, γ) and Kaposi’s sarcoma-associated herpesvirus (KSHV AKA human herpesvirus 8 or HHV8, γ). All of these viruses establish life-long latent infections with potential for periodic lytic reactivation—and all can cause disease (Figures 1-[1](#) and 1-[2](#)).

2.3 Cell Tropism

The α -human herpesviruses (HSV1, HSV2, and VZV; Figure 1-[1](#)) establish latent infection in cells of the peripheral nervous system. HSV1, the primary cause of oral herpes, resides primarily in the trigeminal ganglia while HSV2, the primary cause of genital herpes, tends to reside in sacral ganglia. VZV, the etiologic agent of chickenpox and shingles, latently infects both the trigeminal ganglia and the dorsal basal ganglia. Primary infection with HSV1 and HSV2 occurs in mucoepithelial cells where lytic replication also takes place. Unlike the herpes simplex viruses, VZV requires transport from mucoepithelial cells in the upper respiratory tract where infection is established to skin cells where disease most often manifests. Infection of T-cells is thought to enable this transport.

The β -herpesviruses (HCMV, HHV7, and HHV6; Figure 1-[1](#)) have a complex cell tropism infecting an array of immune cells as well as endothelial cells and fibroblasts. Polymorphonuclear leukocytes (PMNLs), peripheral blood mononucleated cells (PBMCs) including macrophages and monocytes, endothelial cells, and fibroblasts can all support both lytic and latent infection *in vivo*. Hematopoietic stem cells and CD14+ monocytes are thought to be the main source of reactivation.[\(5\)](#) The trafficking of human β -herpesviruses from among different cell types is an active area of research, particularly for human cytomegalovirus.[\(6\)](#) The γ -herpesviruses infect epithelial, endothelial, and B cells. For EBV both lytic and latent infection can take place in B cells. EBV specifically infects B cells through interaction between EBV envelope glycoproteins and complement receptor 2 (CD21) on host cells. The exact site of

primary infection and the mechanism by which EBV is transferred from B cells to epithelial cells is still an area of active research. Primary EBV infection may be mediated by epithelial cells; however, some have postulated tonsillar B cells are the site of primary infection as B cells are much more efficiently infected than are epithelial cells.[\(7\)](#) It is also possible that both are implicated in primary infection. Numerous methods have been proposed for cell-to-cell transfer of EBV, and this process is thought to be critical for infection of epithelial cells.[\(7\)](#) Nasopharyngeal carcinoma and oral hairy leukoplakia both provide direct evidence for the importance of EBV infection in epithelial cells. KSHV establishes and maintains latent infection in CD19+ B cells. Multicentric Castleman's disease and primary effusion lymphoma are examples where disease manifests in B cells. However Kaposi's sarcoma arises from endothelial infection, again highlighting the importance of the complex cell tropism of γ -herpesviruses. These KSHV-associated diseases are further discussed in section [3.2](#).

2.4Viral Replication Cycle

The viral replication cycle is depicted in Figure 1-[2](#). During entry, the nucleocapsid is released from the envelope upon fusion with the host cell membrane and is internalized. Dynein/dynactin motor protein complexes then transport the nucleocapsid to the nucleus along tubulin microtubules. Capsids bind nuclear pores, and their DNA genome is released into the nucleus (Figure 1-[2](#)).

In productive infection, the viral genome must be replicated. A viral replisome composed of a DNA polymerase, the helicase-primase complex, and an ssDNA-binding protein perform this task (Figure 1-[2](#), replication). Cellular RNA Polymerase II generates viral transcripts. A viral transactivator (i.e., HSV1 VP16), introduced into the cell as part of the tegument, kick-starts a cascade of viral gene expression controlled by the intermediate-early promoters. A variety of

viral proteins, such as endoribonucleases, rapidly affect host mRNA stability, resulting in decreased expression of host proteins and a competitive advantage for the translation of viral transcripts.[\(8\)](#) The major capsid protein (MCP), assembly protein (AP), and maturational protease (Pr) are expressed in the cytoplasm and translocate to the nucleus where they assemble to form the immature capsid. Processing by the viral protease allows for maturation of the capsid, formation of the portal vertex, and ultimately encapsidation of the viral genome by the terminase complex (Figure 1-[2](#), capsid maturation).[\(9\)](#) Once the mature nucleocapsid has formed, it must associate with tegument proteins, become enveloped, and egress from the cell (Figure [2](#), egress). Acquisition of the tegument was recently reviewed.[\(10\)](#) The viral capsid is too large to be transported through the nuclear pore. Instead, herpesviruses remodel host membranes, a process mediated in part by viral kinases (i.e., HCMV pUL97, Figure 1-[2](#)). Primary envelopment occurs with transport into the inner nuclear membrane, de-envelopment with transport out of the perinuclear space and into the cytoplasm, and finally secondary envelopment moving from the cytoplasm to the extracellular space (Figure 1-[2](#), egress). This process requires a range of viral proteins and was recently reviewed.[\(11\)](#) The role of host and viral proteins in HCMV maturation, and its comparison to that of other herpesviruses, was also recently reviewed.[\(12\)](#)

During latency, maintenance of the viral episome varies between herpesviruses, in large part based on whether the cells they latently infect replicate. The α -herpesviruses infect cells of the peripheral nervous system that do not normally replicate, so no DNA replication or segregation is required. β - and γ -Herpesviruses establish latency in replicative cells and thus require mechanisms to ensure the viral genome is properly replicated and segregated into daughter cells. Proteins such as latency-associated nuclear antigen (LANA) in KSHV have

evolved to do this, for instance, by tethering the viral episome to host chromosomes during segregation and enabling replication of the viral episome by host replisome machinery.⁽¹³⁾ EBV protein Epstein-Barr virus nuclear antigen 1 (EBNA-1) plays a similar role.⁽¹⁴⁾ The β -herpesviruses (Figure 1-1), such as HCMV, have no known analog of LANA/EBNA-1. The mechanism by which the β -herpesviruses maintain life-long latent infection is still relatively poorly understood and an area of active research. Recently Mücke et al. determined that HCMV major immediate early 1 protein (ME1P) binds host nucleosomes in a way analogous to that of LANA and postulated ME1P may play the role of LANA/EBNA-1 for HCMV.⁽¹⁵⁾

3. Unmet clinical need

Although all of the human herpesviruses cause disease and would benefit from novel treatments, KSHV and HCMV cause especially devastating disease in immunocompromised individuals. These patients may be living in developing nations with limited resources, thus diminishing the incentive for drug discovery targeting the diseases that affect them. In other cases, such as congenital cytomegalovirus, regulatory concerns and the difficulty associated with clinical trial recruitment can disincentivize research in the field. For these reasons, and given the theme of this journal issue, we focus our description of disease on those associated with KSHV and HCMV infection. We also highlight drug discovery efforts in this area, many of which are directly applied or highly relevant to the other seven human herpesviruses.

3.1 Kaposi's Sarcoma-Associated Herpesvirus (KSHV)/HHV8

KSHV was first discovered in 1994 during the height of the AIDS pandemic in the United States. On the basis of epidemiological data the scientific and medical communities suspected Kaposi's sarcoma (KS) had an infectious etiology. At that time, human

cytomegalovirus (HCMV), human herpesvirus 6 (HHV6), hepatitis B virus, HIV, and *Mycoplasma penetrans* were all considered possible causes; however, etiologic association between KS and these infections was not established.[\(16\)](#) Researchers at Columbia University, New York, used representational difference analysis to identify novel sequences found in KS lesions but not in normal-tissue samples from the same patient.[\(16h\)](#) Two novel sequences were identified and characterized. The first had a 51% sequence identity to herpesvirus saimiri capsid protein, a γ -herpesvirus that infects New World Monkeys and causes lymphoma. This sequence also had moderate sequence identity (39%) to Epstein–Barr Virus (EBV) capsid protein, a known human herpesvirus-associated with lymphoproliferative disorders.[\(17\)](#) The second sequence was homologous to protein in the tegument between the nucleocapsid and the virus envelope of both herpesvirus saimiri and EBV. Later, the full genome of KSHV was sequenced.[\(18\)](#)

The presence of KSHV DNA in KS lesions described in 1994 was not, however, sufficient to provide a causal link, and this point remained contentious as of 1995.[\(19\)](#) Ganem and colleagues provided critical evidence in support of the etiologic connection between KSHV and KS. They first showed that most KS cells exhibited latent infection. This, in combination with establishing a cell line for the study of KSHV, enabled the development of a serologic test against the latency-associated nuclear antigen (LANA), mirroring an assay previously developed for the EBV homologue EBNA-1.[\(20\)](#) Use of this assay determined whether the presence of KSHV mirrored KS risk in different populations. For example, KS risk was known to be higher for HIV-positive homosexual men than HIV-positive patients who contracted HIV through exposure to blood products. Indeed, this study established that KSHV infection tracks with KS risk and suggested that KSHV can be sexually transmitted and does not always accompany HIV infection (i.e., HIV-positive patients who contracted infection from blood products had a rate of KSHV

infection similar to the HIV-negative population).[\(20a\)](#) By 1997, a strong case for a causal link between KSHV and KS had been made. KSHV DNA was present in KS lesions, KSHV infection preceded development of KS, KSHV infection tracked with risk of developing KS, and KSHV was shown to infect the tissue implicated in disease not only for KS but also for multicentric Castleman's disease and primary effusion lymphoma.[\(21\)](#)

KSHV, like all other herpesviruses, is a large enveloped double-stranded DNA virus. It has a ~165 kb genome and encodes 86 proteins. The lytic stage of KSHV has a complex gene expression pattern and includes formerly unappreciated small ORFs of unknown function.[\(22\)](#) KSHV establishes latency in B cells but can infect a variety of endothelial, epithelial, and hematopoietic cells. The viral life cycle for KSHV mirrors that of other herpesviruses. Integrins and heparan sulfate have been implicated in KSHV cell entry via interaction with KSHV glycoproteins. Since integrins and heparan sulfate are present on most cell types, it was not immediately apparent why and how KSHV selectively infects endothelial cells and B cells. Recently, it was shown that the ephrin receptor tyrosine kinase A2 is critical for viral cell entry and fusion with endothelial cells, however not for cell attachment.[\(23\)](#)

After fusion and release of the nucleocapsid into the cytoplasm, the viral genome is transported to the nucleus (Figure 1-[2](#), entry). Once in the nucleus, viral DNA is transcribed. KSHV has distinct genetic programs corresponding to latency and the lytic cycle. Within the lytic cycle KSHV—like all other herpesviruses—has immediate-early, early, and late genes.[\(22\)](#) Latently expressed genes are involved in immune evasion and maintenance of the latent infection. Lytic genes are involved in large part with generation of new capsids and the productive spread of those capsids to neighboring cells. Proteins involved in capsid assembly are expressed in the cytoplasm, but viral capsid assembly occurs in the nucleus of the cell. In a process mediated by

the assembly protein-protease fusion (AP/Pr, KSHV ORF17), a procapsid forms which then angularizes with the removal of the assembly protein by cleavage from the major capsid protein (Figure 1-2, capsid maturation). Liberation of the assembly protein allows for conformational changes and packaging of viral DNA into the nucleocapsid. The nucleocapsid then exits from the nucleus, joins tegument proteins, acquires glycoproteins and enveloping lipids, and ruptures from the cell (Figure 1-2, egress).

3.2HHV8-Associated Disease

3.2.1Kaposi's Sarcoma (KS)

Four primary types of KS are observed: AIDS-KS, classic KS, iatrogenic KS, and endemic KS. Endemic KS exists primarily in Central and Eastern Africa and was observed prior to the HIV pandemic. This form of KS often affects children with disseminated lymphadenopathy. Classic KS typically affects elderly men of Mediterranean or Ashkenazi Jewish origin. Iatrogenic KS occurs in patients that are immunosuppressed for medical reasons, such as organ transplant. AIDS-KS is by far the most prevalent form of KS, though its frequency in western nations has diminished drastically with the availability of highly active antiretroviral therapy (HAART). AIDS-KS remains an enormous health burden in much of Sub-Saharan Africa where effective HIV/AIDS treatments are not available and HIV/AIDS incidence is high.

3.2.2B Cell Malignancies: Primary Effusion Lymphoma and Multicentric Castleman's Disease

Both primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) arise from KSHV-infection of B cells. Their diagnosis, clinical manifestations, and viral etiology have been recently reviewed.[\(24\)](#) Most instances of PEL occur in HIV/AIDS patients. These cases make up 4% of all HIV-related non-Hodgkin's lymphomas. Interestingly, PEL cancer cells

have a B-cell genotype but do not express B-cell antigens. This affects PEL treatment strategies (see section [3.2.3](#) below). Extensive mutations are found in immunoglobulin genes of PEL B-cells. KSHV is found in all PEL samples, but many HIV-positive KSHV-positive patients never develop PEL. This has led to the conclusion that KSHV is necessary but not sufficient for PEL. The high frequency of coinfection with EBV in PEL cancer cells has led some to suggest EBV may be a cofactor that acts in concert with KSHV to give rise to this lymphoma. This is highlighted by an abundance of EBV latency protein EBNA1 and EBV microRNA expression, both thought to play a role in tumorigenesis. [\(25\)](#)

KSHV is not necessary for MCD; however, KSHV infection is found in roughly half of the cases in immunocompetent patients and nearly all cases in the immunocompromised population. There are two types of MCD, hyaline vascular and plasma cell types. The plasma cell type is more common, especially in the disseminated multicentric Castleman's disease (as opposed to unicentric CD). [\(24d\)](#) A hallmark of MCD is cytokine dysregulation, especially interleukin 6 (IL-6). This is due both to upregulation of human IL-6 as well as expression of viral IL-6, an IL-6 mimic encoded by the KSHV genome. [\(24b\)](#) Recent success in treating MCD with anti-IL-6 or anti-IL-6 receptor antibodies strongly suggests a role for IL-6 in disease. [\(26\)](#) A host of viral proteins that suppress apoptosis are also implicated in tumorigenesis. Interestingly, many of these are lytic genes suggesting that treatment with antiherpesvirus drugs that target replicating (lytic) virus could be efficacious. [\(24a\)](#)

3.2.3 Current Treatments

Current treatments for KS depend on epidemiological classification of the disease. AIDS-KS treatment focuses primarily on anti-HIV/AIDS therapeutics—though even with HAART only ~50% of patients experience complete remission. For this reason, chemotherapies and radiation

are often also used to treat KS. Liposomal anthracyclines such as Doxil and DaunoXome are the most commonly used chemotherapy for KS. The anthracyclines function through DNA intercalation. Paclitaxel (Taxol), gemcitabine (Gemzar), and vinorelbine (Navelbine) can also be used. Paclitaxel and vinorelbine are mitotic inhibitors, while gemcitabine is a fluorinated nucleoside analog. Due to the side effects of these more traditional chemotherapies an interest in immunotherapies for KS treatment has arisen. For some time interferon α was used; however, this too suffers from severe side effects. Clinical trials are ongoing for drugs such as bevacizumab, interleukin-12, lenalidomide, pomalidomide, bortezomib, and sorafenib though these are not yet approved for treatment. Work on HIV vaccines and decreasing cost of antiretrovirals may still be the best hope for much of the population suffering from AIDS-KS. KSHV *in vitro* susceptibility to antiherpesvirus treatments (Figure 1-3) has been extensively tested with the general conclusion that ganciclovir (**1**) was the most potent guanosine nucleoside analog drug (discussed in section 4.1).⁽²⁷⁾ Only recently, it was shown in a randomized double-blind clinical trial that valganciclovir (**2**), the prodrug of ganciclovir (**1**), reduces viral load *in vivo*.⁽²⁸⁾ This is consistent with previous reports that note a reduction in KS frequency in HIV/AIDS patients being treated with ganciclovir (**1**) for HCMV infection, though no measurements of KSHV were made.⁽²⁹⁾ Antiherpesvirus treatments have also shown utility in the iatrogenic/organ transplant setting.⁽³⁰⁾ In combination, these studies suggest antiherpesvirus treatments such as ganciclovir (**1**) and valganciclovir (**2**) aid in the treatment of KS but are not efficacious enough to be used as monotherapies.

In an HIV/AIDS setting, HAART is recommended for treatment of KS, PEL, and MCD. PEL and MCD are both lymphoproliferative disorders; however, their treatment varies due to expression of B-cell antigens in MCD and the lack thereof in PEL. For MCD (but not PEL),

rituximab, a chimeric monoclonal antibody that targets CD20 on B cells, is often used in treatment.[\(31\)](#) Valganciclovir **(2)** as a component of combination therapy has also shown some promise in clinical trials for KSHV-positive MCD treatment, consistent with a strong correlation between viral replication and disease progression and severity.[\(32\)](#) Due to involvement of IL-6 and viral IL-6 (a KSHV-encoded IL-6 mimic), anti-IL-6 and anti-IL 6 receptor antibodies are also being investigated as possible treatments.[\(26\)](#) This year an anti-IL-6 antibody, siltuximab, was approved for the treatment of HIV-negative KSHV-negative MCD. Unfortunately, siltuximab does not bind tightly to viral IL-6, and so approval did not include KSHV-positive MCD that makes up about half of cases in the immunocompetent population and nearly all cases in immunocompromised individuals.[\(24a, 31\)](#)

PEL is not treated with rituximab because PEL tumors do not express most B-cell antigens, including CD20. Instead, first-line PEL treatment consists of standard chemotherapy combination treatments. Autologous stem cell transplant is an additional option. Some temporary remission has been seen with administration of antiherpesvirus agents directly into the pleural cavity. However, this approach is thought to be hampered by the fact that only a small population of infected cells in PEL are undergoing lytic replication and are thus sensitive to current antiherpesvirus agents.[\(33\)](#)

3.3Cytomegalovirus Disease

HCMV infects a large segment of the population with overall age-adjusted seroprevalence in the United States estimated at 50.4%.[\(34\)](#) In countries such as Brazil, Chile, South Africa, Turkey, and India, seroprevalence is estimated to exceed 90%.[\(35\)](#) Importantly, seroprevalence increases substantially with age. During their reproductive years many women and their partners are seronegative. This leaves many pregnant women at risk of primary HCMV

infection, a high risk factor for congenital HCMV. This is especially the case in countries with relatively low overall seroprevalence, such as the United States, where the percent of seropositive women can nearly double between women ages 12–19 and women ages 30–39.⁽³⁴⁾ Transmission of infectious HCMV is through body fluids into which the virus is shed such as saliva, tears, breast milk, urine, genital secretions, semen, and blood. In the case of congenital HCMV, transfer can be intrauterine. HCMV infection is largely asymptomatic for individuals with a healthy immune system; however, in neonates, organ transplant patients, and individuals with HIV/AIDS this infection has devastating consequences as discussed below.

3.3.1 Congenital Setting

The United States Centers for Disease Control and Prevention (CDC) estimates that 8000 children born in the United States each year suffer from permanent health problems such as hearing and/or vision loss, mental disability, seizures, or in rare cases death due to congenital HCMV infection.⁽³⁶⁾ This outpaces the incidence of Down syndrome (4000/yr), fetal alcohol syndrome (5000/yr), or spina bifida (3500/yr) while receiving considerably less attention and awareness from the public. In a recent review, Manicklal et al. describe how scientists, doctors, and the public alike neglect congenital HCMV infection not just in the United States, but also globally.⁽³⁷⁾ The biology that gives rise to HCMV neuropathies and their resultant developmental disabilities remains elusive and is reviewed elsewhere.⁽³⁸⁾ Mounting evidence from clinical trials suggests treating neonates confirmed to have HCMV disease with antivirals such as ganciclovir (1) and valganciclovir (2) (discussed in section 4.1) limits disease manifestation, though it cannot reverse damage caused before birth or prior to treatment.⁽³⁹⁾ Pregnant mothers cannot be treated with ganciclovir (1) due to documented mutagenic character of this drug in animal studies, though valaciclovir (3) may provide some

benefit to these patients and their children.[\(40\)](#) These limited treatment options have no approved guidelines in the United States. Given the potential for severe toxicity in neonates and pregnant mothers, parents are left with the challenge of weighing the risks of potential clinical manifestation of HCMV in their children and the potential harm of current treatments.

3.3.2 Organ Transplant Setting

HCMV infection causes significant morbidity and mortality in organ transplant patients, operating primarily through symptomatic viremia (CMV syndrome), invasion and damage of specific tissues, HCMV pneumonia, as well as an increased chance of graft rejection. Improved diagnostics, prophylaxis, and treatment of HCMV have significantly reduced mortality for organ transplant patients infected with HCMV. As recently as the 1980s and early 1990s, mortality due to HCMV pneumonia in bone marrow recipients was 70–95%.[\(41\)](#) Today the incidence of HCMV pneumonia has been much reduced, and mortality in that patient population is 15–50%, still extremely significant but much improved.[\(42\)](#) Overall mortality stands at 1–2%.[\(43\)](#) The guanosine nucleotide analog ganciclovir **(1)** and its prodrug valganciclovir **(2)** have been critical to this achievement and can be part of the standard of care for transplant patients. Strategies and guidelines both in the United States and internationally for managing HCMV infection in the solid organ transplantation setting were recently reviewed.[\(44\)](#) Despite the relative success of these drugs, toxicity and emerging resistance are substantial limitations of the current treatment paradigms.[\(45\)](#)

3.3.3 HIV/AIDS Setting

End-organ disease in the gastrointestinal track and eye are common manifestations of HCMV infection in HIV/AIDS patients and uncommon in other settings. It is unclear what gives rise to gastrointestinal symptoms in HIV/AIDS patients and not in organ transplant or congenital

settings. Current studies cannot rule out contributions from pathogens other than HCMV. HCMV retinitis however is a well-known symptom of both congenital HCMV infection and infection in HIV/AIDS patients, though not in organ transplant recipients. In severe cases of HCMV retinitis, loss of vision can occur. Prior to the development of highly active antiretroviral therapy (HAART), both systemic and topical antivirals were used to treat HCMV infection in HIV/AIDS patients. One particularly interesting treatment, fomivirsen, was the first antisense drug to be approved by the FDA in August 1998. This antisense oligonucleotide prevents HCMV replication by binding mRNA encoding the major immediate-early transcription factor, a critical regulator of the viral lifecycle.⁽⁴⁶⁾ Unfortunately this treatment is limited to HCMV retinitis due to poor pharmacokinetics that require intraocular injection as the route of administration.

3.4 Assays for Drug Discovery

Three broad types of assays are available for herpesvirus drug discovery: *in vitro* assays with recombinant protein, cell culture viral assays, and animal models of viral infection and disease. The first type is highly varied, reflecting the multitude of targets available in herpesvirus drug discovery. They are discussed throughout and include measurements of kinase activity by Western blot, protease activity through fluorogenic substrates, and fluorescence polarization to monitor protein–protein interaction, to name a few.

Yield reduction assays (YRA), plaque reduction assays (PRA), and cytopathic effect assays (CPE) are common cell culture viral assays.⁽⁴⁷⁾ CPE can be measured by visual microscopic inspection or by any of a number of dyes: Crystal Violet dye stains the remaining attached cells in a plate, neutral red dye stains the lysosomes of live cells, and MTT/XTT/MTS dyes measure mitochondrial activity. If a test compound prevents viral infection or the lytic cycle, then fewer cells will die. CPE assays use a higher multiplicity of infection than PRA

assays. Plaque reduction assays also measure cell death. Inoculums contain fewer virions such that infection of a monolayer of cells gives rise to individual plaques that can be enumerated. Each plaque is indicative of a viral infection. The plaque forms as neighboring cells are infected and die.[\(48\)](#) The yield reduction assay relies on extent of reinfection as a measure of virion yield. Serial dilution of spent media containing virions is used to reinfect fresh monolayers of cells, and the extent of reinfection correlates with how many infectious virions were produced in the presence of potential inhibitor. Extent of reinfection can be measured in numerous ways including number of plaques, presence of a virally encoded fluorophore such as GFP, or cell death.[\(47a\)](#) Alternatively, an YRA can be performed via ELISA to directly quantify the amount of virus in the media when appropriate antibodies are available.

Animal models for herpesviruses rely on the conservation of this family of viruses throughout evolutionary history.[\(49\)](#) For example in CMV, murine CMV (MCMV), rat CMV (RCMV), guinea pig CMV (gpCMV), and rhesus CMV (RhCMV) are critical models in drug and vaccine development. As in most fields, different animal models afford different benefits. Mice and rat models of CMV have a long history with well-understood immunology and great availability of reagents.[\(49\)](#) However, MCMV and RCMV cannot cross the placental barrier and thus are not useful as models of congenital CMV. In contrast, the ability of gpCMV to infect the fetus provides a congenital model, although fewer reagents are available and gestation periods are considerably longer.[\(49\)](#) Animal models for HCMV are reviewed in detail elsewhere.[\(49, 50\)](#) Throughout this review we reference studies employing many of these assays in the process of herpesvirus drug discovery.

4 Nonprotease Drug Targets

4.1 Current Treatments and Their Molecular Mechanisms of Action

All currently approved treatments in the United States target the viral DNA polymerase (with the exception of the antisense oligonucleotide fomivirsen which is limited to intraocular injections for treatment of HCMV retinitis in HIV/AIDS patients). Ganciclovir (1), valganciclovir (2), valaciclovir (3), acyclovir (4), penciclovir (5), and famciclovir (6) are all guanosine analogs (Figure 1-3). For the prodrug forms, valaciclovir (3), valganciclovir (2), and famciclovir (6), a valyl ester or an ester acetate group is cleaved to release the parent compound. A viral kinase phosphorylates the drug to a monophosphate form. Host kinases then convert the monophosphate to the active triphosphate. The identity of the viral kinase responsible for that initial phosphorylation event varies between herpesviruses and can be a source of resistance mutations.⁽⁵¹⁾ The active triphosphate preferentially inhibits the viral DNA polymerase and incorporates into the viral DNA, preventing viral DNA replication. Acyclovir (4) and ganciclovir (1) (as well as their respective prodrugs) have different affinities for herpesvirus kinases. For instance, ganciclovir (1) is a good substrate for HCMV UL97, but acyclovir (4), while still phosphorylated by UL97, is a worse substrate. Acyclovir (4) is a better substrate for the herpes simplex virus thymidine kinases than it is for HCMV UL97.^(51a-51c, 52) It is this variability that makes ganciclovir (1) a more selective treatment for HCMV, while acyclovir is commonly used to treat the α -herpesviruses. Penciclovir (5) is a topical agent for the treatment of oral herpes caused by HSV-1. Famciclovir (6), a prodrug of penciclovir (5) with improved oral availability, is used primarily to treat shingles (VZV, herpes zoster) and to a lesser extent recurrent HSV-1 and HSV-2 infections.

Brivudine (7), foscarnet (8), and cidofovir (9) are also approved herpesvirus treatments targeting the viral DNA polymerase; however, they are not guanosine analogs (Figure 1-4). Brivudine [(*E*)-5-(2-bromovinyl)-2'-deoxyuridine, 7] is a thymidine analog used primarily for

the treatment of shingles (VZV, herpes zoster). It functions similarly to acyclovir and is active as a triphosphate, monophosphorylated by the VZV thymidine kinase and subsequently acted on by cellular kinases. Brivudine (7) is approved in some European and Central American countries.(53) Cidofovir (9) is a cytidine analog. In contrast to the guanosine analogs, it does not require the viral kinase for phosphorylation to the active form. Host kinases phosphorylate cidofovir (9) to the active diphosphate form where it binds to and competitively inhibits the viral DNA polymerase, and like the guanosine analogs, it is also incorporated into the viral DNA, although two consecutive cidofovir incorporations are required to fully inhibit HCMV DNA elongation.(54) These two actions prevent viral DNA replication.(55) Foscarnet (8) is phosphonoformic acid, a phosphonic acid derivative. It acts at the pyrophosphate binding site of the viral DNA polymerase, preventing chain elongation. It too does not require phosphorylation by a viral kinase.(56) Both cidofovir (9) and foscarnet (8) are second-line treatments for resistant herpesvirus infection. Because they rely solely on host kinases for conversion to the active form, they can readily be used to treat patients with viral infections where resistance mutations have arisen in a viral kinase, blocking guanosine analog treatments. Unfortunately, foscarnet (8) and cidofovir (9) also both require intravenous administration and exhibit severe dose-limiting toxicities. Both can cause severe nephrotoxicity, and cidofovir (9) can also cause myelosuppression.(55, 56)

4.2Improving Existing Treatments

Viral DNA polymerase inhibitors are enormously successful drugs. While there is a need for drugs with novel mechanisms of action, the track record of success, well-understood ADMET characteristics, and clear opportunity for improvement have driven continued development of molecules based on these existing approved treatments.

4.2.1 Nucleoside Analogs

Efforts to improve the original nucleoside analog, acyclovir, gave rise to compounds such as valaciclovir (**3**), ganciclovir (**1**), and valganciclovir (**2**) that are now standard of care treatments for herpesviral infection. Much of this history has been previously reviewed in the literature.⁽⁵⁷⁾ In the current review, we focus on guanosine nucleoside analogs that have recently entered clinical trials for the first time. Cyclopropavir (**10**) and valomaciclovir (**11**) are both novel guanosine nucleoside analogs (Figure 1-4).⁽⁵⁸⁾ Cyclopropavir (**10**) is a dihydroxymethyl methylenecyclopropane nucleoside analog that binds tightly to and is phosphorylated by the HCMV protein kinase UL97. The monophosphate form is then converted to the active triphosphate by cellular guanosine monophosphate kinase. The triphosphate form inhibits the viral DNA polymerase and prevents viral replication. As with other guanosine analogs used to treat HCMV, resistance mutations for cyclopropavir (**10**) map to UL97 kinase. Cyclopropavir's potent binding to UL97 makes it not only an inhibitor of viral DNA synthesis, but also a competitive inhibitor of UL97. Cyclopropavir (**10**) is active against HCMV in cell culture and murine CMV in mice. A phase 1 study to determine safety and pharmacokinetics of cyclopropavir (**10**) in healthy volunteers was completed in August 2013. Preclinical development on this chemical scaffold is ongoing. Monohydroxymethyl (rather than dihydroxymethyl) methylenecyclopropane nucleoside analogs with a broader antiherpetic activity and improved resistance profiles were recently reported and appear to function similarly through a UL97-mediated process.⁽⁵⁹⁾

Valomaciclovir (**11**), unlike cyclopropavir, is active against the α -herpesviruses (HSV-1, HSV-2, and VZV) and EBV, but not the β -herpesviruses or KSHV. Valomaciclovir (**11**) is a prodrug form of ([*(R)*-9[4-hydroxy-2-(hydroxymethyl)butyl]guanine] (H2G), an antiherpesviral

compound identified in the early 1990s. H2G, like valomaciclovir (**11**), is most potent against the α -herpesviruses and EBV, although some weak inhibition of HHV-6 and KSHV was observed.⁽⁶⁰⁾ H2G had poor bioavailability in animal models, and thus, analogs with short-chain alkyl esters, valine and divaline esters, mono- or difatty acid esters, and diesters with an amino acid and a long-chain fatty acid were generated to improve aqueous solubility and cell permeability. Ultimately the stearyl/valyl diester found in valomaciclovir (**11**) was found to be optimal.^(60c, 61) Valomaciclovir phase 2 clinical trials for the treatment of shingles (herpes zoster, VZV) and infectious mononucleosis (EBV) were completed in 2009 and 2010, respectively. In August 2012 the results of a phase 2b randomized, double-blind, active-controlled trial versus valaciclovir (**3**) for the treatment of shingles were reported.⁽⁶²⁾ Noninferiority was achieved with the two highest doses of once-daily valomaciclovir (**11**) when compared to the approved active competitor, 3-times-daily valaciclovir (**3**). Small sample sizes (given the large coefficient of variation in some measures) and a lack of placebo control were noted as concerns for the study and reason to pursue additional trials with this compound. No study results for the infectious mononucleosis trial have yet been published, and the sponsor, Epiphany Biosciences, had not initiated additional trials in the United States or European Union at the time this review was written.⁽⁶³⁾

Bicyclic nucleoside analogs (BCNAs, Figure 1-5) have recently been developed with high specificity for VZV, selectively targeting the VZV thymidine kinase over even closely related HSV-1 and HSV-2 thymidine kinases. BCNAs are not broken down by catabolic enzymes, a problem that was observed with VZV inhibitor brivudine [**7**, (*E*)-5-(2-bromovinyl)-2'-deoxyuridine, BVDU]. That BCNAs are not broken down by catabolic enzymes was a welcome result, providing additional stability and avoiding significant increases in fluorouracil

observed with BVDU (**7**) due to inhibition of dihydropyrimidine dehydrogenase by the free base of BVDU (**7**), (*E*)-5-(2-bromovinyl)uracil. Unexpectedly, the triphosphate forms of BCNAs are not detected in cells, and thus, it is unclear whether BCNAs act via direct inhibition of DNA polymerase. A valyl-ester prodrug, FV-100 (**12**), was developed from the highly potent BCNA cf-1743 (3-(2-deoxy- β -D-ribofuranosyl)-6-(p-pentylphenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one) (**13**). A phase 2 active comparator clinical trial against valaciclovir (**3**) in patients with shingles (VZV, herpes zoster) was completed in 2010. While FV-100 (**12**) appeared to show some improvement over valaciclovir (**3**) in reduction of pain related to shingles, this result was not statistically significant and did not meet the primary end point. After changing hands a number of times, FV-100 (**12**) is now being pursued by ContraVir. Careful clinical trial design and outcome measurements could enable FV-100 (**12**) to progress into the clinic. It will be exciting to see if these relative newcomers in the long history of antiherpesvirus nucleoside analogs make it to the clinic.

FV-100 (**12**), valaciclovir (**3**), and valganciclovir (**2**) are all peptidyl prodrugs of their parent compounds (cf-1743 (**13**), acyclovir (**4**), and ganciclovir (**1**), respectively). Taking this a step further, Velázquez and colleagues have developed “double-prodrugs” of cf-1743 (**13**) and acyclovir (**4**). In these prodrugs two enzymatic events must take place, cleavage of a peptide by proteases and cleavage of a peptidyl-ester by any of a number of hydrolases.⁽⁶⁴⁾ Previously this group established that peptides cleaved preferentially by dipeptidyl peptidase could serve as the “first” prodrug component. A variety of peptidyl esters, including the valyl-ester used in valaciclovir, served as the “second” prodrug component. These compounds exhibited better solubility, serum stability, cell permeability, and oral bioavailability in mice. The novel prodrugs

were converted to their active compounds and had substantial antiviral activity in cell culture.[\(64, 65\)](#)

4.2.2 Nucleoside Phosphonates

CMX001 (Brincidofovir) (**14**), a lipid ester analog of cidofovir (**9**), is an additional example of leveraging a tried-and-true mechanism of action and a previously approved drug to produce a new chemical entity with improved pharmacological qualities and efficacy. Cidofovir (**9**) is a nucleoside phosphonate that targets viral DNA replication both by inhibition of the viral DNA polymerase as well as incorporation into viral DNA, preventing replication.[\(66\)](#) Lipid ester analogs of cidofovir not only improve bioavailability, but also improve antiviral activity by 3–4 orders of magnitude against HSV-1 and 2, VZV, HCMV, murine CMV, HHV-6, EBV, and KSHV.[\(67\)](#) Hexadecyloxypropyl cidofovir (CMX001) (**14**) also showed activity against HCMV strains resistant to standard of care, including unmodified cidofovir (**9**). Importantly, the incorporation of lipid esters enabled oral bioavailability for a drug that previously could only be administered intravenously. On top of that, it had significantly reduced accumulation in the kidney, reducing the likelihood of the dose limiting nephrotoxicity characteristic of cidofovir (**9**).[\(68\)](#) Excitingly, CMX001 (**14**) has progressed through phase 1 and 2 trials and is now being assessed in phase 3 trials for the treatment of adenovirus and HCMV infection ([ClinicalTrials.gov](https://clinicaltrials.gov) identifiers NCT02087306 and NCT01769170).

4.3 Non-Nucleoside DNA Replication Inhibitors: Targeting Helicase-Primase

The helicase-primase complex performs three critical functions for herpesviruses during viral DNA replication; it (1) unwinds the viral DNA, (2) forms the replication fork, and (3) primes the leading and lagging strands (Figure 1-[2](#), replication). This complex has been best studied in HSV-1 where it was first identified and determined to consist of gene products UL5,

UL8, and UL52 (Table 1).⁽⁶⁹⁾ Biochemical studies revealed that UL5 has DNA helicase activity, UL52 has RNA polymerase (primase) activity, and UL8 has ATPase activity that is stimulated by ssDNA.⁽⁷⁰⁾ The first example of a HSV helicase inhibitor, a 2-aminothiazole, was published in 1998.⁽⁷¹⁾ Kleymann et al. from Bayer AG and Crute et al. from Boehringer Ingelheim Pharmaceuticals, Inc., independently published two structurally similar thiazole-based inhibitors of the helicase-primase complex in 2002 (Figure 1-6).⁽⁷²⁾ The optimal Bayer compound *N*-[5-(aminosulfonyl)-4-methyl-1,3-thiazol-2-yl]-*N*-methyl-2-[4-(2-pyridinyl)phenyl]acetamide, BAY-57-1293 (also known as AIC316 or Pritelivir, **15**), exhibited ED₅₀ values of 0.5 mg/kg in a mouse lethal challenge model for both HSV-1 and HSV-2. Generation of escape mutants and biochemical analysis with recombinant helicase-primase suggest BAY 57-1293 (**15**) simultaneously binds UL5 and UL52 and stabilizes the helicase-primase complex at the replication fork, stalling DNA replication.^(72b) The Boehringer Ingelheim compound 1-benzyl-1-cyclohexanecarbonyl-3-[4-(2-methyl-1,3-thiazol-5-yl)phenyl]urea, BILS 179 BS (**16**), was also shown to be a potent inhibitor of HSV, acts on the helicase-primase complex, and was effective in animal models.^(72a) Replacing the methyl group in **16** with an amino group brought the potency in cell culture from an IC₅₀ of about 30 nM to 6 nM.⁽⁷³⁾ Studies on the potential for escape mutants for helicase-primase inhibitors were recently summarized and are ongoing.⁽⁷⁴⁾ In terms of clinical development, BAY 57-1293 (**15**) has shown the most promise and is now being developed by AiCuris as AIC316 (**15**) or Pritelivir. In January of 2014 results from a double-blind randomized, placebo controlled, phase 2 study in otherwise healthy individuals with HSV-2 positive genital herpes showed significant reduction in viral shedding and hinted at reduced occurrence of genital lesion outbreaks. In May of 2013 the Food and Drug Administration placed a hold on further clinical development of AIC316 due to dermal and

hematologic toxicities in monkeys that could not be readily explained. These monkeys received roughly 15–250 times the highest dose administered in the phase 2 trial (and 70–900 times more than the most effective dose determined by this trial, which was not the highest). No such toxicities were observed in the phase 2 trial; however, research into the reasons for these toxicities in monkeys is reportedly underway.⁽⁷⁵⁾

4.4 Targeting Encapsidation and Packaging

4.4.1 Terminase Inhibitors

Targeting the terminase complex has great potential for the inhibition of HCMV replication. The terminase, composed of UL89 and UL56 (Table 1) in HCMV, is functionally conserved across herpesviruses.⁽⁷⁶⁾ It cuts the replicating viral genome into genome-length segments and packages them into the capsid. Human cells do not share any analogous complex, nor do they process their DNA in this way. This makes the terminase an exciting target in terms of selectively inhibiting viral replication, while limiting host off-target effects.

Inhibitors of the terminase (Figure 1-7) began to surface in 1995 with the identification of 2,5,6-trichloro- β -D-ribofuranosyl benzimidazole (TCRB, 17), initially intended as an anticancer agent, and its 2-bromo analog 2-bromo-5,6-dichloro-1-(β -D-ribofuranosyl)benzimidazole (BDCRB 18). As nucleoside analogs, these inhibitors were expected to undergo phosphorylation and inhibit viral DNA synthesis. Surprisingly, neither of these expectations held true. TCRB (17) and BDCRB (18) act without chemical modification, and resistance mutations map to UL56 and UL89 (Table 1) of the HCMV viral terminase.⁽⁷⁷⁾ TCRB (17) and BDCRB (18) are potent inhibitors of the β -herpesvirus HCMV, but not of α -herpesviruses (HSV-1, 2, and VZV), β -herpesvirus HHV-6, or the γ -herpesviruses (EBV and KSHV/HHV8).⁽⁷⁸⁾ At a concentration of 125 μ M, BDCRB (18) inhibits the nuclease activity of UL89, though only slightly. This modest

effect observed with recombinant UL89 is insufficient to explain BDCRB (**18**) activity in cell culture.⁽⁷⁹⁾ This may reflect proposed binding of both UL89 and UL56 (Table 1) by this class of molecules.⁽⁸⁰⁾ The development and improvement of these benzimidazole terminase inhibitors and how they ironically gave rise to an inhibitor of kinase UL97, maribavir (**19**), were recently reviewed by Biron et al.⁽⁸¹⁾ Further discussion of maribavir (**19**) can be found below (section 4.5.2).

In 2001 Bayer reported a significantly different chemical scaffold linked to terminase inhibition. The sulfonamide 3-hydroxy-2,2-dimethyl-*N*-[4([5-(dimethylamino)-1-naphthyl]sulfonyl]amino)-phenyl]propanamide, BAY 38-4766 (**20**), inhibits HCMV, and like the benzimidazoles TCRB (**17**) and BDCRB (**18**), resistance mutations map to UL56 and UL89.⁽⁸⁰⁾ BAY 38-4766 (**20**) is well-tolerated and effective in murine and guinea pig CMV infection models, including the prevention of guinea pig CMV in immunocompromised animals. It is also effective against some monkey CMV strains, though to a lesser extent than the rodent models.⁽⁸²⁾ BAY 38-4766 (**20**) was taken into phase 1 trials and showed safety in healthy volunteers at doses up to 2 g. However, there is no evidence that BAY 38-4766 (**20**) progressed further in clinical development.⁽⁴⁵⁾

The benzimidazole terminase inhibitors suffer from metabolic instability; the glycosidic bond is cleaved *in vivo*.⁽⁸³⁾ Substitution of the furan sugar found in BDCRB for β -D-ribose gives GW275175X (**21**, 2-bromo-5,6-dichloro-1- β -D-ribopyranosyl-1*H*-benzimidazole) and eliminates the major metabolic liability of BDCRB and TCRB. Developed and sponsored by GlaxoSmithKline, this compound advanced through a phase 1 trial but was dropped to pursue maribavir (**19**).^(45, 84)

The most advanced terminase inhibitor to date is AIC246 (**22**) (Letermovir), a 3,4-dihydro-quinazoline-4-yl-acetic acid derivative that was developed by AiCuris as a potent inhibitor of HCMV through extensive hit-to-lead optimization.⁽⁸⁵⁾ In cell culture across three different HCMV strains, AIC246 (**22**) shows low (4–5 nM) EC₅₀ as measured by cytopathic effect reduction and GFP reduction in fibroblasts. Ganciclovir (**1**), the standard of care for HCMV, is more than 400-fold less potent in these same assays. AIC246 (**22**) is effective against HCMV strains resistant to ganciclovir. This is consistent with the fact that AIC246 (**22**) activity is independent of the viral kinases and the viral DNA polymerase, the two primary sources of resistance mutations for nucleoside analog inhibitors. AIC246 (**22**) also shows potent antiviral activity in a mouse xenograft model of HCMV. In this assay AIC246 (**22**) is about 5-fold more potent than valganciclovir (**2**), an orally bioavailable form of ganciclovir (**1**). Murine CMV and guinea pig CMV models could not be used since AIC246 (**22**) is inactive against these viruses in cell culture.^(85c) AIC246 (**22**) is thought to act on UL56 of the HCMV terminase. This is based on generation and genotyping of mutant viruses that escape AIC246 (**22**). These data leave open the possibility that AIC246 (**22**) binds UL89 and mutations in UL56 (Table 1) perturb this binding indirectly, though the simplest explanation is direct interaction with UL56. Interestingly, AIC246-resistant viruses were not resistant to putative sulfonamide and benzimidazole terminase inhibitors, suggesting distinct structural determinants of binding.^(85a, 85b) In December 2011 AIC246 (**22**) completed a phase 2 study, and in April 2012 AiCuris announced that it had passed all primary efficacy end points. The drug has received Orphan Drug status in the European Union and Fast Track Designation in the United States. Late in 2012 AiCuris announced they have signed on to an exclusive worldwide licensing agreement with Merck for AiCuris' portfolio of anti-HCMV leads, including AIC246 (**22**).

4.4.2 Portal Vertex Inhibitors

Researchers at Wyeth (now part of Pfizer) identified and developed thiourea derivatives that inhibit correct formation of the portal vertex (Figure 1-8). One series of compounds was relatively selective for HSV-1 while exhibiting some inhibition of HSV-2 and HCMV. (86) Another related series selectively targeted VZV. (87) The initial thiourea compound identified, *N*-(3-chloro-4-(3-(5-chloro-2,4-dimethoxyphenyl)thioureido)phenyl)formamide (**23**), inhibits HSV-1 virus production with an IC_{50} of 7.9 μ M for the Patton strain, and 24.5 μ M in the E377 strain. Substitution of the formamide for the 2-fluoro-phenyl ring resulted in WAY-150138 (**24**) and improved potency ~20-fold for both viral strains. Mechanism of action was determined by interrogating five critical aspects of inhibitor effect: time-dependence (relative to time of infection), viral DNA replication, viral DNA cleavage, capsid morphology, and resistance mutations. WAY-150138 (**24**) did not prevent viral DNA replication since it inhibits viral production after DNA replication is expected to be complete, suggesting it acts later in the viral replication cycle. Southern blot analysis revealed viral DNA cleavage was not taking place in the presence of inhibitor. Consistent with those data, no C-capsids were observed in the presence of inhibitor. Finally three single point mutations that conferred resistance were identified, all mapped to the portal protein (HSV-1 UL6). (86b) A follow-up study further investigated the mechanism of action of WAY-150138 (**24**). It ruled out selective inhibition of UL6 (Table 1) protein synthesis, degradation of UL6 after translation, and extraction of UL6 from already formed B-capsids. Western blots performed on capsids from compound-treated cells revealed depletion of UL6 and UL15. The authors propose that WAY-150138 (**24**) prevents incorporation of UL6 into capsids. Two key arguments are made in favor of this over the alternative conclusion

of interaction with UL15. First, resistance mutations mapped only to UL6 and not UL15 (Table 1). Second, it has been shown that UL15 associates with capsids only in the presence of UL6. Thus, if only UL15 incorporation was prevented, one would expect to still observe the presence of UL6. In summary, these thiourea derivatives appear to prevent incorporation of the portal vertex (UL6) into capsids, thus preventing DNA encapsidation.^(86a) Closely related compounds (**25**, **26**, and **27**) are reported to function in a similar way against VZV, though mechanism of action was not as thoroughly pursued, so additional studies are warranted for further development of this series.⁽⁸⁷⁾ On the basis of reports on related thiourea inhibitors of HCMV (section 4.5.1) these compounds were likely dropped due to a lack of stability from hydrolysis of the thiourea as well as poor bioavailability.⁽⁸⁸⁾

4.5 Other Viral Targets and Inhibitors with Unknown Mechanism of Action

Inhibitors of entry and attachment, of viral kinases, and vaccine development are additional potential approaches to herpesvirus treatment. Progress has been made in each case and is discussed below.

4.5.1 Targeting Viral Entry

Structural and biochemical studies of entry and attachment have paved the way for inhibitor development, though much remains to be done in this field, and no currently approved drugs act via this mechanism. The majority of molecules that inhibit entry or attachment are peptides or large charged molecules such as heparin. While such compounds benefit from having extracellular targets such as herpesvirus glycoproteins, they suffer from poor oral bioavailability and metabolic stability. Arguably the most advanced-stage example of a compound thought to function by blocking viral entry is SP-303 (also known as Virend), a proanthocyanidin oligomer tested in phase 2 clinical trials as a topical treatment for anal and genital herpes. This compound

showed some promise; however, there is no evidence that it was further pursued for FDA approval. Connolly et al. further review advances in our structural and functional understanding of herpesvirus entry and the potential for targeting the process therapeutically.⁽⁸⁹⁾

Another interesting example of entry inhibitors is a thiourea derivative that is reported to block HCMV glycoprotein B-mediated fusion with host cells (Figure 1-9). This small molecule came out of SAR studies from the hit compounds against HSV-1 that gave rise to the portal vertex inhibitors described in section 4.4.2. Substitutions at the acyl group in **28** demonstrate how SAR for HSV-1 and HCMV rapidly diverged. Introduction of a phenyl group improved potency against both viruses by 10-fold; however, a 2-furoyl (**29**), and other heteroaromatic groups, increased potency against HCMV but lost activity against HSV-1. The 2-furoyl compound (**29**) was selective and potent with an IC_{50} against HCMV of 0.2 $\mu\text{g/mL}$ and an IC_{50} against HSV-1 of $>10 \mu\text{g/mL}$. This became the starting point for additional SAR around ring 1. In a series of alkyl and electron withdrawing groups that had improved potency, introduction of trifluoromethyl and chloro groups meta and para, respectively, to the thiourea nitrogen was optimal (**30**, IC_{50} 0.03 $\mu\text{g/mL}$). No substitutions on ring 2 were tolerated, though changes in selectivity were observed. For instance, the 3,6-dimethoxy analog (**31**) had modest activity against VZV and no activity against HCMV. Introduction of a thiazole (**32**) in place of the furoyl resulted in further improvement in potency, with an IC_{50} of 0.008 $\mu\text{g/mL}$. Unfortunately, the thiourea group in this series was readily hydrolyzed under acidic conditions at elevated temperatures.⁽⁹⁰⁾

A follow-up study sought to overcome this stability problem. The authors presumed that thiourea hydrolysis took place via protonation of the thiourea nitrogen bound directly to ring 1. They further hypothesized that the electronegative character of the ring 1 substituents (i.e.,

trifluoromethyl) made for a better leaving group and that insertion of a linker that is not electron withdrawing between the substituted phenyl and the thiourea would improve stability. A simple methylene spacer was sufficient to improve stability. Nearly all of compound **33** remained intact after 7 days at 37 °C, while 66% of compound **32** decomposed under those same conditions. Likewise, compound **33** had improved stability under both acidic conditions (0.1 or 1 N HCl) and basic conditions (0.1 N NaOH) at 37 °C. Fortuitously, this spacer also improved potency 10- to 20-fold across all seven reported linkers.[\(88\)](#)

Somewhat surprisingly, mechanism of action studies were carried out with the less stable thiourea series represented by **32**. As described by Jones et al., there is significant evidence that this series inhibits glycoprotein-B mediated fusion with the host cell membrane.[\(91\)](#) Resistance mutations were generated with compound **32** that could be confirmed via marker transfer experiments to map to the viral glycoprotein B (UL55, Table [1](#)). As the authors acknowledge, however, they did not look for mutations in other glycoproteins involved in fusion and thus cannot rule out additional targets beyond glycoprotein B. To further strengthen their case that these compounds specifically block fusion, they assessed the effect of time-of-addition of inhibitor, initial events in the replication cycle such as detegumentation and cytoplasm-to-nucleus transfer, interaction of inhibitor with host cell membrane proteins, and direct measurement of fusion in lipid mixing assays. Inhibitor reduced viral yield when administered at the time of infection; however, no effect was observed if treatment occurred just 2 h post infection. This strongly supports activity very early in the replication cycle. Detegumentation and cytoplasm-to-nucleus transport, which normally occur rapidly after fusion, did not occur in the presence of compound as measured by the lack of abundant tegument protein pp65 in the nucleus. Pretreatment of host cells with inhibitor prior to infection had no effect, suggesting no

highly stable interaction with the host is made, though weaker interactions are still quite possible. Finally, the use of virions labeled with fluorescent lipids enabled direct measurement of fusion inhibition. In the presence of compound, in contrast to vehicle alone, fluorescent lipids were retained on the virion and not transferred to the host cell.[\(91\)](#) In summary, there is strong evidence that these inhibitors prevent HCMV fusion and interact with viral envelope glycoprotein B; however, additional targets for this compound series cannot be ruled out. Many other targets in viral entry exist, and Connolly et al. further review advances in our structural and functional understanding of herpesvirus entry and the potential for targeting the process therapeutically.[\(89\)](#)

For KSHV specifically, the ephrin receptor tyrosine kinase A2 (EphA2) recently emerged as a potential therapeutic target. KSHV requires binding to EphA2 for entry into endothelial cells.[\(23\)](#) This could be relevant to the treatment of Kaposi's sarcoma, though likely not B cell malignancies PEL and MCD (section [3.2.2](#)). Both EphA2 binding alone and downstream signaling by EphA2 contribute to KSHV cell entry. The former greatly enhances entry, while the latter appears necessary in at least a subset of endothelial cells.[\(23a\)](#) This makes EphA2 an attractive target both for kinase inhibitors targeting the intracellular tyrosine kinase domain and for small molecules, peptides, or antibodies that directly block KSHV glycoprotein–EphA2 interaction.[\(23\)](#) Other proteins involved in EphA2 signaling may also prove to be novel therapeutic targets.[\(92\)](#) The discovery that KSHV requires binding to EphA2 for entry in some cell types and recent discoveries about herpesvirus entry were recently described.[\(23, 89, 92\)](#) Targets for the viral entry pathway of HSV-1 and HSV-2 are discussed elsewhere.[\(93\)](#)

4.5.2 Targeting Viral Kinases

Maribavir (**19**) [2-isopropylamino-5,6-dichloro-1(β -D-ribofuranosyl)benzimidazole] was identified during the development of benzimidazole inhibitors of the herpesvirus terminase (section [4.4.1](#)). Maribavir (**19**) inhibits viral kinases, not the terminase that its chemical predecessors targeted. The full history of maribarvir's discovery and development has been extensively reviewed elsewhere.[\(81, 94\)](#) In the current review, we focus on some highlights of early development, maribavir's (**19**) unique mechanism of action, specificity among the herpesviruses, and recent progress in its clinical development.

As with many drug development campaigns, resistance mutations were generated to guide identification of the drug target. Biron et al. identified mutations mapping to UL97 HCMV protein kinase (Table [1](#)) that conferred resistance to maribavir (**19**). Inhibition of recombinant UL97 (Table [1](#)) confirmed that it is maribavir's target. Maribavir (**19**) inhibits wild type UL97 (Table [1](#)) potently, with an IC_{50} of 3 nM in a histone phosphorylation assay. Somewhat alarmingly, the recombinant UL97 (Table [1](#)) with the resistance mutation identified in the study, Leu397Arg, resulted in a 20 000-fold decrease in potency (IC_{50} of 60 μ M).[\(95\)](#) Maribavir (**19**) is reported to competitively inhibit ATP binding to recombinant UL97 (Table [1](#)) with a nanomolar K_i (though the Lineweaver–Burk presented shows an inversion of the typical trend between 15 and 20 nM maribavir and thus warrants careful replication).[\(96\)](#) UL97 (Table [1](#)) is also the kinase responsible for phosphorylating many nucleoside analog drugs, such as ganciclovir (**1**). Ganciclovir-resistant HCMV mutants are susceptible to maribavir (**19**). This supports structural models based on resistance mutations that suggest maribavir (**19**) interacts directly with the ATP-binding pocket. Likewise, maribavir-resistant mutants are susceptible to ganciclovir (**1**).[\(94, 97\)](#) Interestingly, however, cyclopropavir (**9**) is reported to compete with maribavir (**19**) for binding to UL97 (Table [1](#)), conflicting with the notion that maribavir does not

interfere with guanosine nucleotide analogs binding as substrates. Gentry et al. note this unexpected result and hypothesize it could be indicative of conformational differences between ganciclovir (**1**) and cyclopropavir (**9**) binding, owing to the methylenecyclopropane of cyclopropavir.[\(98\)](#) Indeed, cyclopropavir (**9**) was recently used to generate HCMV strains resistant to ganciclovir (**1**) and maribavir (**19**), in addition to cyclopropavir (**9**).[\(99\)](#) These data hint at sites of potential overlap on UL97 between all three drugs or conformational changes to UL97 (Table [1](#)) that preclude their binding.

Later studies found mutations conferring resistance to maribavir (**19**) were more commonly found in the viral gene pUL27, not the kinase, though resistance was mild compared to UL97 mutations. Study of the biological function of UL27 and its relation to UL97 and maribavir (**19**) resistance is in its infancy. Reitsma et al. provide the most compelling evidence of the relationship between UL97 and UL27 to date.[\(100\)](#) UL27 was shown to enhance proteasomal degradation of the acetyltransferase Tip60. Of note, the HIV virulence factor Tat also targets Tip60 for degradation, and Tat expression in cells infected with UL27-driven maribavir-resistant HCMV restores susceptibility to maribavir. Degradation of Tip60 results in increased levels of cyclin-dependent kinase (CDK) inhibitor p21^{waf/CIP1}, leading to a halt of cell cycle progression. Normally, protein kinase UL97 functions to drive cells into the S phase so that viral DNA replication can take place.[\(100\)](#) When maribavir (**19**) inhibits UL97 (Table [1](#)), progression to the S phase is hindered leading to reduced viral replication (though this is only one of numerous effects of UL97 inhibition, Figure 1-[2](#), viral kinases). If UL27 no longer drives the degradation of Tip60, then p21 expression is blocked and activity of CDKs allows progression to the S phase, overcoming that aspect of UL97 (Table [1](#)) inhibition.[\(100\)](#) These findings are in agreement with previous work that showed differences in maribavir (**19**) susceptibility based on cell state and

cellular kinase modulation.[\(101\)](#) This intriguing and complex mechanism of drug action and resistance still leaves numerous unanswered questions that will no doubt be the subject of future work.[\(102\)](#)

Maribavir **(19)** inhibits EBV as well as HCMV; however, the target for EBV is less clear. Multiple reports indicate that Maribavir **(19)** inhibits EBV protein kinase (also known as BGLF4 or EBV-PK, Table [1](#)) in cell culture; however, it showed no inhibition of recombinant EBV-PK *in vitro*.[\(103\)](#) It is possible that the known EBV-PK substrates that are no longer phosphorylated in cells in the presence of maribavir can be acted on by other kinases, which are the true target of maribavir. This may also be an example of indirect (e.g., through blocking a cofactor) or substrate-dependent inhibition of EBV-PK. It is clear, however, that maribavir **(19)** prevents phosphorylation of the EBV DNA polymerase processivity factor (EA-D), a substrate for EBV-PK, and alters transcription of key viral genes leading to a reduction in viral fitness.[\(103, 104\)](#)

Maribavir **(19)** exhibits acceptable ADMET characteristics that enabled progression into clinical trials. Phase 1 and phase 2 clinical trials were successful and established maribavir as having fewer deleterious side effects than current HCMV treatment options. Phase 3 clinical trials for prophylaxis in HCMV solid organ transplant and bone marrow transplant settings were established. Unfortunately, neither of these trials was successful in meeting their primary or secondary outcomes. No data were published for these trials, so it is unclear if study design, lack of full biological understanding of mechanism of action, or something inherent to the use of maribavir **(19)** in the selected patient populations is the most likely cause for these clinical failures.[\(94\)](#)

4.5.3 Targeting the Viral Ribonucleotide Reductase

Human herpesviruses encode a viral ribonucleotide reductase for the conversion of adequate amounts of deoxyribonucleoside diphosphates from the corresponding ribonucleoside diphosphates during active viral DNA synthesis.[\(105\)](#) The importance of the viral reductase in producing infectious particles has been the subject of conflicting studies. The reductase is composed of two subunits, R1 and R2, and their association is required for catalytic activity.[\(106\)](#) Temperature sensitive mutants with a lesion in the R1 subunit of the virally encoded reductase established 100-fold decrease in viral production in a tissue culture setting.[\(106\)](#) However, there were several reports that showed drastic differences in the importance of the viral reductase, and these differences were species specific in animal models.[\(107\)](#) Nevertheless, the viral reductase was seen as a tractable therapeutic target, and it was pursued for many years. Unfortunately, those efforts did not culminate in the approval of novel therapeutics and have waned in recent years. Below, we discuss examples of inhibiting the protein–protein interaction between the R1 and R2 subunits as this was the first example of an effective protein–protein interaction antagonist *in vivo*.

Two studies published simultaneously first described the inhibition of the viral reductase protein–protein interaction (PPI) with slightly different synthetic peptides incorporating similar sequences from the C-terminus of subunit 2.[\(108\)](#) Follow-up studies by researchers at Boehringer Ingelheim developed peptidomimetic compounds, such as BILD 1263 (**34**, Figure 1-[10](#)), that improved potency over shorter synthetic peptides by over 200 000-fold and that displayed *in vivo* efficacy in a mouse ocular model of infection: the first such example for a PPI antagonist.[\(109\)](#) The evolution of viral resistance to related compounds and identification of the sites on the R1 subunit of the HSV-1 reductase sufficient for resistance (A1091S and P1090L) were also described.[\(109b\)](#)

The SAR of the compounds was also reported and described in detail.^(109c) The authors of the study noted an important observation regarding IC₅₀ values and cellular efficacy, namely that the radioligand binding assay used to establish IC₅₀ was only accurate to 1 nM and that the compounds showing cellular efficacy all displayed IC₅₀s lower than the sensitivity of their assay, making meaningful SAR interpretation difficult within the most potent series.^(109c) Beginning at the N-terminal (R₁) position of compounds related to **35**, it was found that increasing lipophilicity improved potency and that the stereochemistry around methyl-substituted cyclohexyl moieties was important (**36**). Ultimately, a (dimethylcyclohexyl) amino group improved potency over 7-fold (2 nM). This improved compound was used to probe the C-terminal (R₂, **35**) position and was shown to improve *in vivo* potency in an HSV-1 induced keratitis mouse model. Compounds containing the hydroxymethyl moiety at R₂ such as is found in BILD 1263 (**34**) were significantly less potent in the radioligand binding assay, but, for seemingly obvious permeability reasons, were more potent in the cell culture assays. The authors also describe a series of substitutions related to neopentylamine and discovered that the addition of a stereochemically defined methyl- or ethyl-substituted neopentylamine was optimal for activity in the cellular-based assay. Next, the authors explored the N-substituted pyrrolidine of BILD 1263 (**34**) and the amide linkage in the N-terminal direction of this side chain (**X** in **35**). The amide was replaced with a methylene linkage in order to lower desolvation, and the potency in the cellular assays was also found to improve by replacing the pyrrolidine with a *tert*-butyl group (i.e., **36**). These studies led to the discovery of BILD 1351 (**36**), which is significantly smaller than the previously described BILD 1263 (**34**) and equipotent in the cellular-based assays. BILD 1351 (**36**) and another congener more similar to BILD 1263 (**34**) both showed

improved *in vivo* efficacy, but unfortunately, no public record of these compounds advancing further toward clinical trials has been published.(109c)

4.5.4 Inhibitors with Unknown Mechanism of Action

A number of recent publications and patents report herpesvirus inhibitors with as of yet unknown mechanisms of action. To provide a comprehensive review and encourage further investigation of these compounds, they are discussed in brief below.

Patents disclosed by Wunberg et al. describe a series of dihydroquinazolines that exhibit anti-HCMV activity in cell culture.(110) The most potent inhibitors, similar to compound **37**, had EC₅₀ values of 0.01 μ M with a 50% cytotoxic concentration (CC₅₀) of 15 μ M, 1500 times that of the EC₅₀ value. No statement regarding the mechanism of action for these compounds is provided in the patent, though detailed SAR is described. The approved cancer treatment and kinase inhibitor gefitinib shares the quinazoline scaffold and was previously reported to have anti-HCMV activity in cell culture, though no activity in an animal model.(111) The quinazolines described by Wunberg et al. may also be acting on host or viral kinases, though testing of this hypothesis has not been reported in the literature.

Another example of antiviral compounds with unknown mechanism of action comes from a recent publication by De Castro et al. describing a series of 4-benzyloxy- γ -sultone (**38**) derivatives.(112) These compounds inhibit VZV and HCMV in cell culture with EC₅₀ values for the most potent compounds of approximately 10 μ M. They are inactive against HSV-1 and HSV-2 as well as a number of other viruses. Activity against HHV6 and 7, EBV, and KSHV was not assayed. Extensive SAR revealed that the 4 and 5 positions of the γ -sultone are critical to antiviral activity. Specifically, a benzyloxyl group at the 4 position, as shown, and one benzyl group at the 5 position were required for both HCMV and VZV inhibition. The authors do not

explore the stereochemical preferences for the antiviral activity. These compounds were equally active against HCMV strains resistant to ganciclovir, foscarnet, cidofovir, and acyclovir as well as mutant strains lacking HCMV protein kinase UL97 and VZV thymidine kinase (VZV-TK).(112) On the basis of these data, it is likely this series of inhibitors does not act through traditional DNA polymerase inhibition or inhibition of UL97 or VZV-TK. As we hope this review has made clear, this leaves many possible mechanisms by which inhibition could be taking place and additional research is warranted.

Yamanouchi Pharmaceutical Co., Ltd. (now a part of Astellas Pharma Inc.), disclosed tetrahydro-2*H*-thiopyran-4-carboxamide analogs (**39**) with potent antiviral activity against the α -herpesviruses (HSV1 and 2, and VZV). Their most potent inhibitor (**39**) has an EC₅₀ of 0.033 μ M in a VZV plaque reduction assay and 0.006 μ M in an HSV-1 cytopathic effect inhibition assay. These compounds also show activity in an HSV-1 mouse model with 70–100% inhibition of lesions with a 10 mg/kg twice-daily dosing regimen over 5 days.(113) A more recent patent application from the same group also claims a reduction in pain caused by herpesvirus lesions.(114) To our knowledge, no information on the mechanism of action has been published. Our final example of a herpesvirus inhibitor with an unknown mechanism is also an example of repurposing. Leflunomide (**40**), an immunosuppressive and inhibitor of mitochondrial dihydroorotate dehydrogenase in the pyrimidine synthesis pathway, is approved for the treatment of arthritis but was found to have antiviral activity against resistant strains of HCMV and HSV-1. Its use in the treatment of HCMV in the organ transplant setting was recently reviewed, including *in vitro* studies on its potential mechanisms of action.(115) In a study by Waldman et al., electron microscopy revealed treatment with leflunomide in HCMV-infected cells results in formation of a mature nucleocapsid, but the tegument and envelope are not acquired.(116) The

same was found to be true in HSV-1-infected cells treated with leflunomide.(117) Studies of the active metabolite of leflunomide (A771726, 41) and an analog thereof (FK778, 42), both of which also inhibit HCMV replication, propose a different mechanism of action. These molecules were found to interfere with HCMV signaling events and thus prevent some aspects of viral DNA replication and protein production, as well as apoptosis of HCMV-infected cells.(118) Differences in viral strain and cell type have been proposed as a potential explanation for some of the discrepancies between the studies.(118a) It is possible that treatments that so drastically affect cell metabolism could impact viral replication in multiple ways. While it is clear both biochemically and clinically that leflunomide can be useful in preventing HCMV replication, many questions about its mechanism of action remain unanswered.

4.5.5 Vaccine Development

Tremendous effort continues toward the goal of developing herpesvirus vaccines. To date, only the development of VZV vaccines (chickenpox and shingles) has been successful. Vaccine development has focused primarily on HSV-1 and 2, HCMV, and EBV. Both modified viruses as well as specific antigens are being used in this work.(2a) Development of HSV vaccines continues to be hampered by a poor understanding of why previous attempts at vaccination have failed, and, equally, if not more importantly, why some seropositive individuals are largely or completely asymptomatic while others have frequent clinical episodes. Across all herpesviruses it appears that while natural immunity is protective against reinfection and in some cases clinical manifestation of disease, it often does not prevent reactivation and shedding of the virus and thus transmission. In fact, concerns have been raised that vaccination could reduce symptoms but not shedding, leading to increased transmission. Past and current development and research to overcome these challenges have been reviewed elsewhere.(2a-2c, 119)

4.5.6 Targeting Latency and Immune Evasion

All current herpesvirus treatments target only replicating virus, while an ideal treatment also would target latency. To accomplish this one would need to block the action of those proteins responsible for maintaining latency. These can be broken into two categories: direct maintenance of latency (e.g., enabling segregation of DNA into daughter cells) and immune evasion. Both present interesting and challenging drug discovery opportunities. The macromolecules required for maintenance of latency vary substantially among the herpesviruses as one might expect given the diverse cell tropisms. Direct maintenance has been best studied in EBV and KSHV, where one can readily establish latent infection in cell culture. In recent years many papers have been published on herpesvirus latency, laying the groundwork for future therapeutic development. From a small-molecule discovery standpoint, the herpesvirus-encoded GPCRs offer exciting potential targets given the vast knowledge for targeting GPCRs in other indications.⁽¹²⁰⁾ Directly targeting mediators of latency such as LANA could prove more challenging from a druggability standpoint due to the protein–protein and protein–DNA interactions involved; however, promising research in this area is ongoing.⁽¹²¹⁾ Finally, as we learn more about differences between uninfected and latently infected cells we are able to take advantage not only of viral proteins expressed during latency, but also differences in host protein expression that enable specific targeting of latently infected cells.⁽¹²²⁾ It will be exciting to watch as these strategies—alone or in combination—are brought to bear on the challenging goal of targeting herpesvirus latency.

4.6 Host Targets for Herpesvirus Inhibition

As with other viruses, such as HIV, some attempts to target host proteins for the inhibition of herpesviruses have emerged. In theory this approach could substantially reduce, if not eliminate,

development of resistance to treatment. However, targeting host proteins raises serious concerns about toxicities, both on- and off-target. The three major host targets being currently explored are the protein kinases cyclin-dependent kinase (CDK) and mammalian target of rapamycin (mTOR), and cyclooxygenase 2 (COX2). Many other kinases are up-regulated during HCMV infection and are potential therapeutic targets, some with known small-molecule inhibitors.⁽¹²³⁾ Another recent potential target is the ephrin receptor tyrosine kinase A2 (EphA2). KSHV cell entry requires binding to EphA2, and its down-regulation or addition of soluble ephrin ligand prevented viral entry.^(23, 92) This leaves open the possibility of targeting EphA2 both at the extracellular receptor as well as the intracellular kinase domain. The use of CDK inhibitors to prevent viral replication in cell culture provided the first example of a host-targeted herpesvirus inhibitor, showing efficacy against at least HSV 1 and 2, VZV, and HCMV.⁽¹²⁴⁾ The exact mode of inhibition appears to differ between herpesviruses, stalling their replication at different stages of the viral lifecycle.^(124a, 125) Broadly, CDK inhibitors such as roscovitine (**43**, Figure 1-11) appear to inhibit viruses that require a nuclear phase of replication.

After anecdotal evidence emerged that organ transplant patients receiving immunosuppressive agents targeting mTOR had fewer and less severe complications from HCMV, more extensive molecular and clinical studies were established to interrogate this observation. The underlying mechanism of action is complex and likely varies between herpesviruses and disease manifestations. Mechanistic studies using rapamycin recently revealed that the mTOR pathway is critical for modulation of HCMV late genes in infected macrophages.⁽¹²⁶⁾ Everolimus, an approved immunosuppressant and rapamycin analog, is now in clinical trials for treatment of HCMV disease in renal transplant patients ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00828503) identifier NCT00828503). Other

approved mTOR inhibitors are in clinical trials for EBV- and KSHV-caused cancers, where molecular mechanism of action is likely much more complex ([ClinicalTrials.gov](https://clinicaltrials.gov) identifiers NCT00918333 and NCT02110069). Li et al. recently reviewed the use of kinase inhibitors for the treatment of herpesvirus-associated disease, including but not limited to CDK, mTOR, and EphA2.(127) Brennan et al. summarize our understanding of the effect of immunosuppressive drugs on virus pathobiology, including the effect of mTOR inhibitors on herpesviruses.(128) COX2 is one of the many genes that are upregulated during HCMV infection. This upregulation of COX2 directly results in a >50-fold increase in prostaglandin E₂. Treatment with noncytotoxic COX2 inhibitors decreases the accumulation of prostaglandin E₂ and reduces the production of HCMV virions from infected fibroblasts by more than 100-fold. Treatment with both a COX2 inhibitor and exogenous prostaglandin E₂ rescues viral production, highlighting an important role for prostaglandin E₂ in HCMV replication. More recently, Schröer et al. demonstrated that COX2 inhibition by approved anti-inflammatory drugs tolfenamic acid and indomethacin diminished direct cell-to-cell spread of HCMV in fibroblast cell culture and that this effect was reversed with the addition of prostaglandin E₂. These studies highlight a potential host target in the control of herpesvirus infection and build on a long history of research showing prostaglandins play a critical role in herpesvirus biology.(129)

5. Successes in targeting viral proteases

To our knowledge, industry largely (if not entirely) halted efforts to develop HHV protease inhibitors in the late 1990s and early 2000s. Since then, myriad antiprotease therapies have entered the clinic, particularly HCV protease inhibitors and a variety of improved HIV protease inhibitors. Many lessons can be learned from these discovery efforts and applied to HHV

protease inhibitor development. In combination with increasing structural, biochemical, and biological understanding of the HHV proteases, the success of HIV and HCV protease inhibitor development revives a long-standing interest in targeting the highly functionally conserved HHV protease family.

5.1 HIV Protease Drugs

The development of HIV protease inhibitors is one of the most successful examples of structure-based drug design to date and is expertly reviewed in the literature.[\(130\)](#) In 1981 reports first surfaced of what we now know as symptoms of HIV/AIDS, namely the severe opportunistic infections Kaposi's sarcoma and pneumocystis pneumonia.[\(131\)](#) Two years later HIV was isolated and deemed the likely cause of AIDS, and by 1985 full genome sequences for the virus had been published.[\(132\)](#) Amazingly, just 10 years after the HIV genome was sequenced, the US FDA approved the first HIV protease inhibitor, saquinavir (**44**).[\(133\)](#) Part of this rapid success came from synthetic compounds developed and lessons learned during failed attempts to develop inhibitors of renin, an aspartyl protease involved in control of blood pressure.[\(134\)](#)

There are 10 HIV protease inhibitors currently marketed in the United States, and at least one new therapeutic, a deuterated form of the peptidomimetic inhibitor atazanavir (**45**), is currently in clinical development. All but one of these are peptidomimetic competitive active-site inhibitors with a hydroxyethylene core that acts as a transition state mimetic, displacing a critical water molecule from the active site and preventing proteolysis.[\(135\)](#) Tipranavir (**46**) is the exception, a nonpeptidomimetic inhibitor that uses a dihydropyrone ring in place of the hydroxyethylene core.[\(136\)](#) As described below, a similar focus on peptidomimetic inhibitors has dominated drug discovery efforts for herpesvirus protease inhibitors.

In retrospect, HIV protease is an excellent drug target. However, HIV protease inhibitors faced skepticism in early development, in part due to inherent challenges in targeting proteases and in part due to the success of reverse transcriptase inhibitors that entered the market prior to saquinavir's (44) approval. As resistance mutations developed against the reverse transcriptase inhibitors, another line of attack against these highly mutagenic viruses was necessary; protease inhibitors continue to play that central role in combination treatments for HIV/AIDS. The herpesvirus market mirrors, to some extent, the early HIV market where nucleoside analogs dominate and pharmaceutical companies, after significant effort, have largely abandoned the viral protease as a drug target. Ultimately, the success of HIV protease inhibitors, and later HCV protease inhibitors, continues to drive interest in and provide critical lessons for the development of small molecules targeting the highly conserved HHV proteases.

5.2HCV Protease Drugs

In the late 1990s Vertex pharmaceuticals, the developer of HIV protease inhibitor amprenavir (Agenerase, 47), initiated preclinical efforts to develop a clinical hepatitis C virus protease inhibitor. The combined efforts of academia and industry were able to overcome numerous substantial challenges: the target was not yet validated *in vivo*, the viral lifecycle was poorly understood, no robust preclinical models in cell culture or animals were available, and no structure of the viral protease had been solved. In a remarkable story that is expertly reviewed in the literature, scientists were able to achieve approval of telaprevir (48), the first HCV protease inhibitor, just 22 years after the virus was discovered in 1989.(137) Since then many other companies have clinical trials for or have approved HCV protease inhibitors.(138) Parallels can be drawn between the herpesvirus proteases and HCV protease. When first screened *in vitro*, no known serine protease inhibitors were effective against HCV protease. This was attributed to the

active site being flat and solvent exposed, lacking a well-defined pocket into which an inhibitor could bind.[\(137d, 139\)](#) In addition, it was shown that the protease was dynamic—binding to different inhibitors yielding different conformations of the enzyme. The same has been said of the human herpesvirus proteases, as described below (section [6](#)). Initial inhibitor development against HCV protease focused on decamer peptidomimetics derived from the natural substrate. To simultaneously obtain low molecular weight and “drug-like” properties while maintaining potency, the peptidomimetics were truncated to 5-mers spanning the P4–P1’ sites and a reversible covalent tetra-aldehyde moiety was introduced.[\(137f, 140\)](#) Use of an α -ketoamide warhead was determined to be optimal, and eventually exploration of that scaffold arrived at Telaprevir (**48**). Detailed discussion of medicinal chemistry for HCV protease inhibitors can be found elsewhere.[\(137f, 140, 141\)](#) A similar approach to HCMV protease inhibitors was taken, starting with peptidomimetics and utilizing covalent inhibitors to overcome lack of potency. An additional parallel can be drawn in the recent reports of allosteric regulation of HCV protease, specifically compounds that trap a closed and autoinhibited conformation of the helicase-protease fusion NS3-4A.[\(142\)](#) A similar allosteric inhibition of herpesviruses proteases involving the trapping of an inactive monomeric conformation of the enzyme is reviewed in detail below (sections [6.2–6.3](#) and [6.5–6.6](#)).

6. Human herpesvirus protease

6.1 Biological Role in Viral Replication

Herpesvirus proteases have long been known to be essential for viral replication.[\(143\)](#) The protease (also known as assemblin and Pr) and the assembly protein are expressed from two overlapping open reading frames (i.e., KSHV ORF17 and ORF17.5, Table [1](#)). The assembly protein alone (i.e., KSHV ORF17.5, Table [1](#)) is expressed approximately 10-fold more than the

protease–assembly protein fusion. The viral protease is expressed in the cytoplasm of virally infected cells as a monomeric fusion to the assembly protein (AP). The Pr-AP fusion binds the major capsid protein (MCP) and is translocated to the nucleus where the procapsid forms. Concentration-dependent dimerization in the nucleus allosterically activates protease, leading to protease-mediated cleavage of the release site (R-site) that frees the protease from AP. AP is proteolyzed from the MCP by hydrolysis at the maturation site (M-site). Cleavage at the M-site releases the internal assembly protein from the outer protein layer, enabling a conformational change in the outer shell as well as the portal vertex. These conformational changes allow packaging of viral DNA, and the mature capsid is formed (Figure 1-2, capsid maturation).[\(9a, 144\)](#) In typical infections three forms of the viral capsid are produced. A-capsids are empty, lacking both the assembly protein and viral DNA. B-capsids contain the inner lining of assembly protein ORF17.5 but no viral DNA. C-capsids have expelled the scaffolding protein and contain viral DNA. Only C-capsids progress directly to mature virions. This is true across multiple herpesviruses.[\(145\)](#) When the viral protease is not present to perform its function, only B-capsids are formed and DNA packaging fails.[\(143b, 146\)](#) The lack of A-capsid formation suggests A-capsids may be the result of premature release/cleavage of the assembly protein from B-capsids. This suggests that activators of the protease could block viral maturation, though further research is needed to confirm this hypothesis. Lacking genetic material and being morphologically malformed, these B-capsids are incapable of going on to become infectious virions. The results of herpesvirus protease knockout, as well as knockdown of the protease in murine cytomegalovirus that results in reduced viral load, provide genetic validation of HHV proteases as potential therapeutic targets.[\(147\)](#)

6.2HHV Protease Structure

Many years of work have contributed to our current understanding of the HHV proteases. Cytomegalovirus protease was the first herpesvirus protease for which a crystal structure was determined, with four groups independently publishing HCMV protease structures in 1996.[\(148\)](#) These structures revealed a novel serine protease fold and a Ser-His-His catalytic triad providing a structural framework for a unique enzyme with low proteolytic activity due in part to the noncanonical catalytic triad (Scheme [1](#), Figure 1-[12](#)).[\(149\)](#) Consistent with biochemical studies suggesting numerous herpesvirus proteases form dimers, the HCMV protease crystal structure revealed a dimeric enzyme where each monomer contains an independent active site (Figure 1-[12](#)). The monomeric unit consists of a core β -barrel of mostly antiparallel β -sheets partially surrounded by seven α -helices. Subsequently, structures of VZV, HSV1 and 2, KSHV, and EBV proteases were solved.[\(150\)](#) All six known HHV protease structures share the same fold, dimeric state, and Ser-His-His catalytic triad (Figure 1-[13](#)). These are the only proteins for which structures are known that have this fold, comprising their own superfamily in the SCOP database (MEROPS Clan SH, Family S21). Despite their structural and functional homology, the HHV proteases vary widely in sequence identity from 29% to 97% identical. Despite this unique fold, the Ser-His-His catalytic triad adopts a conformation very similar to that of the chymotrypsin-fold serine protease catalytic triad (MEROPS Clan PA, Family S1). The substrate binding pocket is shallow, dynamic, and solvent-exposed, as was discovered with HCV protease (Figure 1-[12](#)). The core β -barrel is conserved while helices are more divergent, and loops even more so. The structural configuration of the protease suggests autoproteolytic processing to liberate protease from assembly protein happens in trans, not in cis. Namely, the distance between the active site and the release site as well as structural constraints required for enzyme activity likely preclude intramolecular proteolysis.

6.3 Substrate Binding and Specificity

All herpesvirus proteases share a P1–P1' specificity for Ala-Ser. Alignment of all M and R sites across the human herpesviruses reveals a profile sequence showing the strict requirement for alanine in the P1 pocket and serine in the P1' pocket. There is also a strong preference for tyrosine in the P4 pocket (Figure 1-14). While much of the specificity between these enzymes is shared, there are distinct differences that produce functional biochemical consequences. For example, HCMV protease is able to cleave its own endogenous substrate, HCMV assembly protein, as well as HSV2 assembly protein. Conversely, HSV2 protease is unable to efficiently cleave HCMV assembly protein. KSHV protease can cleave itself at the dimer interface leading to loss of activity resulting in additional regulation of proteolysis. The herpesviruses are recalcitrant to most standard serine protease inhibitors as described in section 6.4.1 and as was observed for HCV protease. This is likely due to their poor catalytic activity.(149) Antichaotropic agents such as high concentrations of sulfate or citrate, glycerol, and others can improve herpesvirus protease activity 10–100-fold.(151) At their most active, these highly selective processing enzymes are significantly less active than many other proteases with catalytic efficiencies 2–3 orders of magnitude less than typical digestive enzymes depending on substrates and conditions.

HSV2 protease was the first protease to be cocrystallized with an active-site inhibitor, diisopropyl phosphate (DIP, 49). This allowed for direct identification of the oxyanion hole, involving two arginines and two leucines that are absolutely conserved across all known human herpesvirus proteases. Surface loops that are the most varied in sequence and structure are proposed to play a role in substrate recognition as is true for chymotrypsin-fold

enzymes.[\(150a\)](#) Loops and helices, which are markedly less structurally conserved than the core β -barrel, contribute to defining the extended binding pocket.

For KSHV and HCMV proteases, substrate binding has been shown to occur through an induced-fit model.[\(152\)](#) Two distinct but related features have been reported: the effect of substrate binding on the active site and the effect of substrate binding on dimerization.

Comparison of crystallographic structures between HCMV protease in its apoenzyme state and protease bound to a transition state-analog inhibitor reveals rearrangements of the conserved oxyanion hole arginine residues upon substrate binding. This result is supported by changes in tryptophan fluorescence of reporter Trp42, which upon substrate binding is packed against part of loop 9 that contributes a conserved arginine to stabilize the oxyanion hole (Figure 1-[18](#)). In KSHV protease, crystallographic studies reveal a large pocket in the S4 position to accommodate a P4 tyrosine when present; however, this pocket is not apparent from the apoenzyme state.[\(152d\)](#) In addition, substrate binding promotes dimerization. The literature to date does not make it clear whether substrate binding to a binding-competent monomer promotes a conformation that more readily dimerizes or whether substrate only binds the dimer but in doing so stabilizes the interface. In either case, the observation that addition of increasing concentration of substrate or peptidyl-inhibitor results in an increasing dimer population holds. Addition of a phosphonate inhibitor to KSHV protease results in a “super dimer” that no longer undergoes measurable monomer–dimer equilibrium. While this does not address whether substrate can bind to monomer and promote dimerization, it does confirm that substrate binding stabilizes the dimeric state of the enzyme.[\(152d\)](#)

6.4 Active-Site Inhibitors of HHV Proteases

6.4.1 Generic Serine Protease Inhibitors

HCMV protease has been the most actively pursued target of the HHV proteases both in academia and industry. As was the case for HCV protease, HCMV protease (and indeed all HHV proteases) is poorly inhibited by traditional serine protease inhibitors. A typical mechanism-based serine protease inhibitor, 3,4-dichloroisocoumarin (**50**), required high concentrations (0.1–1 mM) to achieve inhibition and was ultimately found to react with thiol groups of HCMV's five cysteine residues, not the active-site serine.⁽¹⁵³⁾ Widely used chloromethyl-ketones such as the chymotrypsin inhibitor tosylamido-2-phenylethyl-chloromethyl ketone (TPCK **51**) achieves only 33% inhibition of HCMV protease at 250 μ M. Tosyl-L-lysine chloromethyl ketone (TLCK **52**), phenylmethylsulfonyl fluoride (PMSF **53**), and benzamidine (**54**) show no inhibition of HCMV protease at 250 μ M. Diisopropylfluorophosphate (DFP **55**) inhibits both HCMV and HSV-1, albeit weakly, providing some of the early evidence that HHV proteases are serine proteases.⁽¹⁵⁴⁾

Macromolecular protease inhibitors such as lima bean and soybean trypsin inhibitors, ovomucoid, Bowman–Birk inhibitor, and bovine pancreatic trypsin inhibitor likewise do not inhibit HCMV protease as expected due to the unique structural fold of the enzyme. Peptide aldehydes chymostatin, elastatinal, leupeptin, and antipain also fail to inhibit HCMV protease presumably due to the weak nucleophilicity of the active-site serine as a result of the noncanonical catalytic triad.⁽¹⁵³⁾ HCMV protease—HHV proteases in general—are clearly nontrivial protease targets.

6.4.2 Activated Carbonyl Warheads

Similar to the early development of HCV protease inhibitors, initial attempts to achieve specific inhibitors of HCMV protease relied on peptides and peptidomimetics, taking advantage of the substantial work describing substrate specificity for this enzyme. An 11-mer peptide spanning P6

to P5' (GVVVNA/SCRLA) of the native M-site was both a substrate and competitive inhibitor with K_i of 0.225 mM and K_M of 0.515 mM. A similar peptide where the P1' serine is replaced by alanine (GVVNA/ACRLA) showed substantial improvement in both K_i (72 μ M) and K_M (0.112 mM).⁽¹⁵⁵⁾ Use of a reduced peptide bond at the cleavage site did relatively little to improve the native M-site peptide as an inhibitor.⁽¹⁵⁶⁾ The limited success of competitive noncovalent peptidic inhibitors led to the pursuit of mechanism-based inhibitors of HCMV protease. These utilized covalent warheads, such as trifluoromethyl ketone (**56**). In 1997 a group from Boehringer Ingelheim utilized a trifluoromethyl ketone warhead linked to M-site-derived peptides to interrogate the influence of peptide length and side-chain on inhibition.⁽¹⁵⁷⁾ They found that a tetramer was the minimal peptide that would achieve low micromolar IC_{50} values. Lengthening the P1 side chain by a single carbon from methyl to ethyl was tolerated and somewhat improved inhibition; however adding two carbons to yield a propyl were not tolerated and decreased activity. Extensive SAR around the P2 side chain suggested that the S2 site was permissive while the S3 pocket preferred a bulkier *tert*-butyl glycine (Tbg, also known as *tert*-leucine) over the native valine residue. With the unnatural amino acid Tbg in place, the P4 capping group was explored and tolerant of a variety of substitutions. Several warheads were tolerated in place of the trifluoro methyl ketone. Interestingly, α -ketobenzoxazoles were not specific to HCMV protease, while the other warheads maintained selectivity against a panel of serine proteases. The pentafluoroethyl ketone inhibitor was the most potent with an IC_{50} of 110 nM (**57**). Unfortunately, none of these compounds were efficacious in cell culture. The authors suspect this is due to cell permeability and/or metabolic stability.⁽¹⁵⁷⁾

6.4.3 β -Lactam Inhibitors

In light of still limited success with activated carbonyls, an alternative strategy was pursued. As Waxman and Darke describe and as was noted by the group out of Boehringer Ingelheim that published extensively on protease active-site inhibitors, monocyclic β -lactam inhibitors of human leukocyte elastase were co-opted as a starting point for HCMV protease inhibitor development.^(153, 158) Human leukocyte elastase, like the HHV proteases, has a substrate preference dominated by small alkyl side chains.⁽¹⁵⁹⁾ Specifically, researchers at Merck had shown that N-carboxylamino derived β -lactams (**58**) inhibit leukocyte elastase.⁽¹⁶⁰⁾ Déziel and Malenfant reported peptidic substrate-based β -lactams built both from their previous work on substrate-based inhibitors described above and on the β -lactam scaffold.^(157, 158) These inhibitors incorporated the optimized peptidyl portion from the previous work onto the C4 position of the β -lactam through either an ether or thioether bond and probed substitutions at the β -lactam nitrogen leading to a 70 nM inhibitor (**59**). Replacing the peptidyl portion of the compounds with a variety of substituents failed to improve potency and/or selectivity across other proteases such as leukocyte elastase. However, some less potent analogs showed limited activity in cell culture (EC_{50} of 60 μ M in a plaque reduction assay).^(158a) Further elaboration of this scaffold by the same group provides interesting SAR but ultimately failed to increase potency in enzyme assays or cell culture.^(158b) Bonneau et al. elegantly show that these β -lactams are indeed acting through an acyl enzyme intermediate in the case of HCMV protease.⁽¹⁶¹⁾ Using kinetic analyses with a fluorogenic β -lactam they were able to measure the rate of acylation and deacylation, and relate that to IC_{50} . These studies highlight the importance of whether the C4 position is occupied by a leaving group as well as the overall electrophilicity of the carbonyl carbon as it is influenced by the substitutions on the β -lactam ring.⁽¹⁶¹⁾

6.4.4 *trans*-Lactam Inhibitors

Derived from euphane triterpene natural product inhibitors of serine proteases, the 5,5-translactam scaffold similar to **60** has been optimized to achieve potent inhibition of HCMV protease. The work of Borthwick et al. skillfully explored and optimized this novel class of inhibitors and is expertly reviewed elsewhere.[\(162\)](#) In the present review we address the key findings of these studies and relevant details of the approach that aided in the inhibitor development against this challenging target.

A rational design was used beginning from the highly conserved cleavage sequence for HCMV protease, Val-Xxx-Ala/Ser where the slash denotes the site of cleavage. A methyl group was introduced α to the lactam carbonyl in order to mimic the alanine methyl that would hypothetically occupy the S1 pocket. Given the apparent importance of this alanine, Borthwick et al. maintained this substituent and synthesized stereospecific analogs to determine which stereochemistry would best position the methyl group in the S1 pocket, ultimately favoring the *cis*-substitution as depicted in **60**.[\(163\)](#) The subsequent SAR focused first on the absolute stereochemistry for the active diastereomer of the translactam, which is also as depicted in compound **60**. Further functionalization of the lactam nitrogen from which the S1' site is accessed showed a preference for trigonal substituents (not tetrahedral), with potency tracking with electron withdrawing ability, but also showing some preferences for the sterics of the various substituents that were analyzed.[\(164\)](#) In the same work, the authors utilize array chemistry to probe tolerable substitutions at the pyrrole nitrogen of the *trans*-lactam system and identify the monocyclic pyrrole derived from *S*-proline as an ideal substituent (**61**). With absolute stereochemistry assigned and evaluated, the authors modified the R₁ and R₂ positions of compound **61** and discovered the dansyl-cyclopropyl substituents as depicted in compound **62** to be optimal. The authors report a K_i of 20 nM and an IC₅₀ of 340 nM for **62** and provide a

thorough mechanistic study as well as docking results.⁽¹⁶⁴⁾ Substantial effort was then dedicated to establishing the fine balance of potency, pharmacokinetic properties, and selectivity necessary to advance a small molecule as a drug candidate.⁽¹⁶⁵⁾ The authors moved to a 4-isopropylphenylurea in place of the dansyl group and replaced the cyclopropyl with a benzothiazole derivative to obtain **63**, which showed a favorable PK and activity profile including a half-life greater than 24 h, 2 orders of magnitude selectivity for HCMV protease over acetylcholine esterase, elastase, and thrombin, and an excellent IC₅₀.⁽¹⁶⁶⁾ Importantly, this compound had high nanomolar to low micromolar antiviral activity in cell culture against lab strain AD169, equivalent to ganciclovir (**1**), and low cytotoxicity, creating an excellent therapeutic index. Finally, an unsubstituted benzothiazole derivative showed acceptable bioavailability in dogs and good brain and ocular penetration in guinea pigs.^(165b) This exciting work is a quintessential example of hit-to-lead drug discovery utilizing a combination of rational SAR, combinatorial chemistry, and structure-based drug design to address not only *in vitro* potency, but also pharmacological considerations critical to drug discovery. Unfortunately, to our knowledge, there is no literature describing the progression of the *trans*-lactams into animal disease models or clinical trials.

6.4.5 Azetidine Inhibitors

Several papers have aimed to develop efficacious noncovalent active-site inhibitors against HCMV protease that move away from substrate-based β -lactam compounds.⁽¹⁶⁷⁾ The first examples of the switch from covalent inhibitor to noncovalent inhibitor against HCMV protease were published in 2004. The authors first hypothesize that derivatives at the C4 position with carbonyl functionality (**64**) will introduce an interaction with Arg165 and Arg166, which function to stabilize the oxyanion hole.^(158b, 167a) Interestingly, the absolute stereochemistry of

the covalent inhibitors had little effect on potency in either the AD-169 or Davies strain. The first compounds showed a narrow effective concentration range, however, and all displayed significant cellular toxicity. Removing the electrophile from the β -lactam led to the discovery of azetidine **65**, which is hypothesized to be the first noncovalent competitive inhibitor of the HCMV protease.[\(167a\)](#) The compound displays an excellent cellular EC₅₀, comparable to ganciclovir (**1**), but cellular toxicity remains an issue. These compounds were not assayed against isolated protease, so it is difficult to assess any relationship between biochemical and cellular potencies.

Subsequent efforts to more fully understand the structure–activity relationships describe modifications to the lactam nitrogen substituents as well as the C4 carbonyl substitutions. The authors explore conformational rigidity and stereochemical consequences of lactam and azetidine compounds and extend the cellular assays to include VZV.[\(167b, 167c\)](#) However, the SAR is complicated. The benzyl carbamate **65** remains the preferential substituent over urea derivatives, and the simpler *tert*-butyl esters are preferred over amino acid coupled esters and amides at the C4 carbonyl position.[\(167b, 167c\)](#) Overall, the balance between potency and toxicity remains an issue for these compounds and further work on the mechanism of action is warranted.

6.4.6 Benzoxazinone and Thieno[2,3-*d*]oxazinone Mechanism-Based Inhibitors

Scientists at SmithKline Beecham (now GlaxoSmithKline) and Searle (now a part of Pfizer) took an alternative approach focused on benzoxazinones typified by **66** and related compounds which are mechanism-based serine protease inhibitors, particularly of human leukocyte elastase.[\(168\)](#) In 1996 Jarvest et al. from SmithKline Beecham developed benzoxazinones that inhibit HSV-1 protease. The authors began their optimization efforts by synthesizing derivatives that contain the alanine from the HSV-1 VNA/S cleavage recognition sequence.[\(168a\)](#) The

initial efforts verified that inhibition takes place through an acyl-enzyme intermediate, stereochemistry around the amino acid moieties is relevant, specific SAR exists at various positions of the benzoxazinone, and the aqueous half-life could be optimized. The optimized compound from the initial work contained an isopropyl amine in the 2- position of the benzoxazinone and a Cbz-Ala-NH in the 7- position (**67**). This inhibitor had an IC_{50} of 5 μ M on HSV-1 protease and an aqueous half-life of 171 h.[\(168a\)](#) Similar inhibitors designed from the known elastase inhibitors were concurrently developed against HCMV protease.[\(168b\)](#) The most potent benzoxazinone inhibitor (**68**) of HCMV protease had an IC_{50} of 0.22 μ M; however, it was not selective (e.g., IC_{50} against chymotrypsin of 0.065 μ M). Selective inhibitors were about 10-fold less potent.[\(168b\)](#)

Optimization of related thienoxazinone compounds typified by **69** as a follow-up study by the scientists at SmithKline Beecham yielded nanomolar inhibitors of HSV-1, HSV-2, and HCMV proteases.[\(168c\)](#) SAR in the series varied between the proteases, and selectivity remained challenging. A follow-up report described reoriented thienophenes as in **70**. The simplest compound in the study, an unsubstituted cinnamoyl group at the R position of **70** inhibits HSV-2, VZV, and HCMV proteases with 300, 38, and 500 nM IC_{50} values, respectively. The authors reported reasonable cytotoxicity data but do not report selectivity over other relevant proteases. A second lead that used a 2-bromo-5-methyl thiophene in place of the benzyl group of the initial cinnamoyl hit inhibits HSV-2, VZV, and HCMV proteases with 270, 8, and 210 nM IC_{50} s, respectively.[\(168d\)](#) Both lead compounds showed no inhibition of elastase and trypsin up to 100 μ M and inhibited HSV-2 protease in cells with $\sim$ 8 μ M potency—though the assay was not described and the citation which had been submitted for publication was never published, leaving the assay design and interpretation unclear.[\(168c, 168d\)](#) It was later reported by these same

authors that additional unpublished data revealed that these inhibitors did not show antiviral activity in a yield reduction assay.⁽¹⁶⁹⁾ Without publication of the cell culture assays and data it is difficult to make sense of these ostensibly conflicting results (though the nanomolar IC₅₀s in enzymatic assays remain interesting).

6.4.7 Spirocyclopropyl Oxazolones and the Benzyldiene *N*-Sulphonyloxyimidazolones

Mechanism-Based Inhibitors

The scientists from SmithKline Beecham also identified two new classes of serine protease inhibitors, the spirocyclopropyl oxazolones (**71**) and *N*-substituted benzyldiene imidazolones (**72**).⁽¹⁷⁰⁾ The SAR shows excellent selectivity over the canonical mammalian serine proteases elastase, trypsin, and chymotrypsin for the oxazolones. Several of the oxazolone analogs with an exocyclic double bond or acyclic aliphatic chain replacing the cyclopropyl moiety highlight the importance of the cyclopropyl group which is hypothesized to properly orient the optimal phenyl substituent (i.e., R₃ = Ph, **71**). The most potent spirocyclopropyl oxazolone had IC₅₀ values of 500 nM and 200 nM against HSV-2 and HCMV proteases, respectively, while showing modest inhibition at elastase, trypsin, and chymotrypsin at 100 μM. Interestingly, the reported imidazolones were generally less potent, showing only minimal activity of HSV-2 protease. These compounds were initially hypothesized to inhibit the enzymes through the previously described nucleophilic attack/elimination/Löschen rearrangement reported by Groutas et al. for the structurally similar succinamide inhibitors of elastase.⁽¹⁷¹⁾ However, mechanistic studies of the imidazolones did not observe the expected acyclic product, but rather isolated an imidazole leading to the proposed mechanism in Scheme 2.⁽¹⁷⁰⁾ A sulfonate of the imidazolone with an ethenylbenzene in place of the phenylcyclopropyl moiety was also active against HCMV protease with an IC₅₀ of 0.4 μM, while showing little inhibition of HSV-2 protease.⁽¹⁷⁰⁾

6.5 Human Herpesvirus Protease Dimerization and Activation

Dimerization is an essential step in the activation of all human herpesvirus proteases.[\(150c, 172\)](#) The dimer interface is composed largely of helix 5–helix 5 intermonomeric interactions, with additional binding surface contributed by helix 1 of each monomer. On average these bury 2000 Å² surface area. Unlike HIV protease, each monomer contains its own Ser-His-His catalytic triad, and these active sites are functional and independent of one another. Titration of inactive catalytic serine-to-alanine mutants into wild type monomeric enzyme drives dimerization and activates the wild type monomer of the heterodimeric species.[\(173\)](#) Two key studies provide a mechanism by which dimerization is allosterically linked to activation at the catalytic site and support the notion that this mechanism is conserved across HHV proteases. Batra et al. demonstrate by mutagenesis and structure determination that mutation of the dimer interface of HCMV protease can result in a dimerization-competent protein that is nonetheless inactive due to a rearrangement of the conserved residues that help form the oxyanion hole. A single point mutation, S225Y, results in a dimer where conserved residues Arg165 and Arg166 are disordered. Arg165 is part of the oxyanion hole, and Arg166 contributes to its stabilization. This loss of the oxyanion hole explains the roughly 1700-fold reduction in k_{cat} for this mutant. Interestingly, the catalytic triad (which resides on the core β -barrel of these proteases) is unperturbed in the S225Y variant. In support of this model, the K_{M} of substrate binding and the disassociation constant (K_{d}) of dimerization are unchanged as well. As a first hint of allosteric networks throughout these proteases, the binding of an activated peptidomimetic inhibitor caused a structural change in the variant protease that reverted it to a wild type conformation.[\(151b\)](#) The structure of the monomer for these variants, however, remained elusive. Pray et al. interrogated conformational changes between monomer and dimer using circular dichroism and tryptophan

fluorescence. These data confirmed an increase in helical content upon dimerization leading to the hypothesis that dimerization orders the two c-terminal helices and that this results in activation.[\(174\)](#) Indeed, subsequent studies validated that hypothesis. ^{13}C HSQC NMR chemical shift index measurements and NMR hydrogen/deuterium exchange experiments show the two C-terminal helices of KSHV protease are ordered in the dimer and largely disordered in the monomer. Furthermore, an engineered disulfide bond between the C-terminal helix and oxyanion loop acts as a redox switch that can activate the enzyme. Even the obligate monomeric enzyme, KSHV protease M197D, can be activated in this way showing that the monomer is catalytically active when in the appropriate conformation.[\(175\)](#)

How then is dimerization regulated? The most obvious answer is through changes in concentration of the enzyme. This is consistent with the current model of HHV protease activation, wherein it is concentrated into the nucleus and ultimately the immature capsid as a fusion to the capsid assembly protein. As was suggested by the work of Batra et al., communication between the substrate binding site and the dimer interface also regulates dimerization and thus activity. This was illustrated by the use of an optimized hexapeptide diphenylphosphonate inhibitor of KSHV protease. In an initially puzzling result observed by Marnett et al., addition of this covalent inhibitor up to roughly 0.4 mol equiv relative to protease concentration actually increased enzyme activity, followed by a decrease at higher inhibitor concentrations. An NMR assay using a selectively labeled methionine at the dimer interface revealed that inhibitor addition promoted dimerization. Size exclusion chromatography confirmed this, and circular dichroism indicated an increase in helical content upon dimerization. Thus, in the absence of inhibitor the majority of enzyme is monomeric and inactive. As inhibitor is increased up to roughly 0.5 mol equiv a population of stabilized dimers in which only a single

monomer is inhibited emerges, leaving the other monomer in a highly active conformation and able to process substrate. Further increase in the inhibitor concentration leads to an increasing population of enzyme for which both monomers are inhibited and overall enzyme activity is reduced. This study solidified a model in which enzymatic activity is linked to quaternary structure and an allosteric link exists between the dimer interface and the substrate binding site. Interestingly, these data also suggest that substoichiometric active-site inhibition of HHV proteases may lead to overall activation, a potential contributor to the challenges realized by those developing active-site inhibitors of these unusual enzymes.⁽¹⁷⁶⁾ The cocrystal structure of KSHV protease and the hexapeptide phosphonate inhibitor supports these findings.^(152d) The crystallization of an HHV protease monomer for the first time further supported this model of a significant structural change between monomer and dimer and loss of the oxyanion hole as a mechanism by which dimerization regulates activity (Figure 1-20). Truncation of the two C-terminal helices from KSHV protease established an obligate monomeric HHV protease. In the presence of a small-molecule protein–protein interaction inhibitor, discussed in section 6.6, Lee et al. crystallized this monomeric KSHV protease. Consistent with the findings from HCMV protease, monomeric KSHV protease had a disordered oxyanion hole with no structural perturbation of the catalytic triad. In the case of KSHV protease, however, the substrate binding pocket was also occluded by a loop (residues 17–21, Figure 1-18).⁽¹⁷⁷⁾

6.6 Dimer Disruption as an Alternative to Active-Site Inhibitors

Although a great deal of work has been put toward developing active-site inhibitors of the HHV proteases, compounds targeting the protease have yet to advance to the clinic. An alternative approach that could potentially overcome limitations inherent to targeting the shallow and dynamic active site is identifying small molecules that prevent dimerization, and thus inhibit one

or more of the HHV proteases. Proof of concept for this approach was first achieved using a peptide instead of small molecules. Interfacial helix 5 of KSHV protease was grafted onto the avian pancreatic polypeptide (APP). The avian pancreatic polypeptide scaffold provided stability for the interfacial helix, whereas a peptide composed of the helix 5 residues alone is unstructured and shows no inhibition within the solubility limit of the peptide. This grafted helix 5 APP inhibited KSHV protease with a 300 μM IC_{50} . Size exclusion chromatography was used to confirm that addition of the grafted helix 5 peptide results in loss of the dimer species, and a 7-amino-4-carbamoylmethyl coumarin (ACC) fluorogenic substrate was used to monitor inhibition of protease activity.[\(173b\)](#) While providing proof of concept, this peptide inhibitor lacked potency and would not be suitable for showing antiviral efficacy in cell culture due to its likely being unable to cross the cell membrane. To pursue a small-molecule inhibitor of KSHV protease dimerization a 183-compound helical mimetic library was screened using an optimized fluorogenic ACC substrate. From an initial hit, a series of 4-benzoylaminobenzoic acid compounds were synthesized, and a 3 μM inhibitor named dimer disruptor 2 (DD2, **73**) was identified.[\(178\)](#) Again, size exclusion chromatography was used to show a loss of the dimeric state of KSHV protease with increasing concentrations of inhibitor. Two-dimensional protein NMR was also used to confirm dimer disruption and map binding of the compound. Met197, a residue in helix 5, was selectively ^{13}C labeled, and a ^{13}C -HSQC was used to probe dimerization state. The monomer–dimer equilibrium showed slow exchange kinetics on the NMR time scale revealing distinct monomer and dimer peaks that could be used to monitor the monomer and dimer populations in solution. Addition of DD2 resulted in a complete loss of the dimer peak and a chemical shift perturbation of the monomer peak. Uniform ^{15}N labeling and ^{15}N HSQC showed significant peak broadening for Trp109 suggesting DD2 binds at or near Trp109, an aromatic

hotspot at the dimer interface.⁽¹⁷⁸⁾ Truncation of helix 6 and all but a single turn of helix 5 enabled crystallization of the monomer bound to DD2. This crystal structure confirmed that the small molecule bound at the dimer interface in a transient hydrophobic pocket formed by rotation of the tryptophan indole side chain causing the structural changes diagramed in Figure 1-20.⁽¹⁷⁷⁾ While this approach validated the dimer interface as a druggable site on KSHV protease, solubility and permeability of DD2 appear to have prevented its use in cell culture. Therefore, KSHV protease, as of yet, has not been validated pharmacologically though it is presumed that inhibition of the enzyme would prevent viral replication as has been shown for other HHV proteases.⁽¹⁷⁸⁾ While this lack of cell culture activity is disappointing, disrupting dimerization to allosterically inactivate the herpesviruses still is a promising approach, as is the DD2 scaffold. Recently, a combination of kinetics, NMR, and crystallography revealed that DD2 and two carboxylate bioisostere analogs inhibit not only KSHV protease, but also HCMV, EBV, and to a lesser extent HSV-2 proteases.⁽¹⁷⁹⁾ These data suggest that elaboration of the DD2 scaffold could give rise to potent cell-permeable allosteric inhibitors of HHV proteases. Such a compound would be one of few protein–protein interaction inhibitors in an infectious disease setting and an exciting advance in allosteric modulation of traditionally challenging targets.

7. Conclusion

Enormous progress has been made in the field of herpesvirus drug discovery, though approved treatments are still limited to a single viral target. Herpesviruses such as Kaposi's sarcoma-associated herpesvirus and Epstein–Barr Virus have no approved antiviral treatments—despite causing severe disease. Cytomegalovirus infection has no approved treatment in the congenital setting, and treatments for the organ transplant and HIV/AIDS settings suffer from resistance and dose-limiting toxicities.

Encouragingly, many scientists in both academia and industry are stepping up to the challenge. Small molecules against multiple herpesviruses with targets throughout the viral replication cycle are being developed. Many of these have activity in cell culture; some are making it to clinical trials. These inhibitors span the gamut in both target and mechanism: competitive kinase inhibitors, prodrug DNA polymerase inhibitors, allosteric protease inhibitors, and host–protein inhibitors, to name only a few. The field is clearly on the move. As we increase our understanding of herpesvirus biology and improve our ability to target “undruggable” proteins, even the lauded goal of targeting latency may become a reality.

The complexity of herpesviruses presents both challenges and opportunities for drug discovery. These viruses encode more than 100 proteins, many with unclear biology. Small open reading frames and noncoding RNA are abundant and varied, with little known about their function.[\(22, 180\)](#) These challenges also represent opportunities. Indeed, every one of these macromolecules provides a chance to both better understand and prevent human disease through chemical intervention.

Acknowledgement

We gratefully acknowledge UCSF Clinical and Translational Science Institute (CTSI) Grants UL1 TR000004 and UL1 RR024131 and NIH RO1 GM104659 for financial support. J.E.G. was supported by the NIH Structural Biology Training Grant GM008284 and NSF Graduate Research fellowship 1144247. T.M.A. was supported by NIH NIGMS NRSA fellowship F32 GM111012.

1. Wald, A.; Corey, L. Persistence in the population: Epidemiology, transmission. In Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis; Arvin, A., Campadelli-Fiume, G., Mocarski, E., , Eds.; Cambridge University Press, Cambridge, U.K., 2007.

2. (a) Chentoufi, A. A.; Kritzer, E.; Yu, D. M.; Nesburn, A. B.; Benmohamed, L. Clin. Dev. Immunol. 2012, 2012, 187585, (b) Coleman, J. L.; Shukla, D. Hum. Vaccines Immunother. 2013, 9, 729, (c) Fu, T. M.; An, Z.; Wang, D. Vaccine 2014, 32, 2525(d) Boppana, S. B.; Britt, W. J. Recent approaches and strategies in the generation of antihuman cytomegalovirus vaccines. In Human Cytomegaloviruses Methods and Protocols; Yurochko, A. D.; Miller, W. E., Eds.; Humana Press: New York, 2014.
3. Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis; Arvin, A.; Campadelli-Fiume, G.; Mocarski, E., Jr.; Moore, P. S.; Roizman, B.; Whitley, R.; Yamanishi, K., Eds.; Cambridge University Press: Cambridge, U.K., 2007.
4. (a) Davison, A. J. Overview of classification. In Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis, 2011/02/25 ed.; Arvin, A.; Campadelli-Fiume, G.; Mocarski, E., Jr.; Moore, P. S.; Roizman, B.; Whitley, R.; Yamanishi, K., Eds.; Cambridge University Press: Cambridge, U.K., 2007.(b) Davison, A. J.; Eberle, R.; Ehlers, B.; Hayward, G. S.; McGeoch, D. J.; Minson, A. C.; Pellett, P. E.; Roizman, B.; Studdert, M. J.; Thiry, E. Arch. Virol. 2009, 154, 171(c) McGeoch, D. J.; Dolan, A.; Ralph, A. C. J. Virol. 2000, 74, 10401
5. (a) Goodrum, F.; Reeves, M.; Sinclair, J.; High, K.; Shenk, T. Blood 2007, 110, 937(b) Hargett, D.; Shenk, T. E. Proc. Natl. Acad. Sci. U.S.A. 2010, 107, 20039
6. Sattentau, Q. Nat. Rev. Microbiol. 2008, 6, 815
7. (a) Bayliss, G. J.; Wolf, H. Nature 1980, 287, 164(b) Bayliss, G. J.; Wolf, H. Proc. Natl. Acad. Sci. U.S.A. 1981, 78, 7162(c) Chang, Y.; Tung, C. H.; Huang, Y. T.; Lu, J.; Chen, J. Y.; Tsai, C. H. J. Virol. 1999, 73, 8857, (d) Imai, S.; Nishikawa, J.; Takada, K. J. Virol. 1998, 72, 4371, (e) Speck, P.; Longnecker, R. J. Natl. Cancer Inst. 2000, 92, 1849(f) Shannon-Lowe, C. D.;

Neuhierl, B.; Baldwin, G.; Rickinson, A. B.; Delecluse, H. J. *Proc. Natl. Acad. Sci. U.S.A.* 2006, 103, 7065(g) Shannon-Lowe, C.; Rowe, M. *PLoS Pathog.* 2011, 7, e1001338

8. (a) Abernathy, E.; Clyde, K.; Yeasmin, R.; Krug, L. T.; Burlingame, A.; Coscoy, L.; Glaunsinger, B. *PLoS Pathog.* 2014, 10, e1003882(b) Read, G. S. *Wiley Interdiscip. Rev. RNA* 2013, 4, 693(c) Shu, M.; Taddeo, B.; Zhang, W.; Roizman, B. *Proc. Natl. Acad. Sci. U.S.A.* 2013, 110, E1669

9. (a) Singer, G. P.; Newcomb, W. W.; Thomsen, D. R.; Homa, F. L.; Brown, J. C. *J. Virol.* 2005, 79, 132(b) Welch, A. R.; Woods, A. S.; McNally, L. M.; Cotter, R. J.; Gibson, W. *Proc. Natl. Acad. Sci. U.S.A.* 1991, 88, 10792

10. Henaff, D.; Remillard-Labrosse, G.; Loret, S.; Lippe, R. *J. Virol.* 2013, 87, 4895

11. (a) Johnson, D. C.; Baines, J. D. *Nat. Rev. Microbiol.* 2011, 9, 382(b) Mettenleiter, T. C.; Muller, F.; Granzow, H.; Klupp, B. G. *Cell Microbiol.* 2013, 15, 170

12. Tandon, R.; Mocarski, E. S. *Trends Microbiol.* 2012, 20, 392

13. Mercier, A.; Arias, C.; Madrid, A. S.; Holdorf, M. M.; Ganem, D. *J. Virol.* 2014, 88, 6762

14. Hodin, T. L.; Najrana, T.; Yates, J. L. *J. Virol.* 2013, 87, 13020

15. Mucke, K.; Paulus, C.; Bernhardt, K.; Gerrer, K.; Schon, K.; Fink, A.; Sauer, E. M.; Asbach-Nitzsche, A.; Harwardt, T.; Kieninger, B.; Kremer, W.; Kalbitzer, H. R.; Nevels, M. *J. Virol.* 2014, 88, 1228

16. (a) Drew, W. L.; Conant, M. A.; Miner, R. C.; Huang, E. S.; Ziegler, J. L.; Groundwater, J. R.; Gullett, J. H.; Volberding, P.; Abrams, D. I.; Mintz, L. *Lancet* 1982, 2, 125(b) Siddiqui, A. *Proc. Natl. Acad. Sci. U.S.A.* 1983, 80, 4861(c) Bovenzi, P.; Mirandola, P.; Secchiero, P.; Strumia, R.; Cassai, E.; Di Luca, D. *Lancet* 1993, 341, 1288(d) Vogel, J.; Hinrichs, S. H.; Reynolds, R. K.; Luciw, P. A.; Jay, G. *Nature* 1988, 335, 606(e) Wang, R. Y.; Shih, J. W.;

Weiss, S. H.; Grandinetti, T.; Pierce, P. F.; Lange, M.; Alter, H. J.; Wear, D. J.; Davies, C. L.; Mayur, R. K.; Lo, S. Clin. Infect. Dis. 1993, 17, 724(f) Van den Berg, F.; Schipper, M.; Jiwa, M.; Rook, R.; Van de Rijke, F.; Tigges, B. J. Clin. Pathol. 1989, 42, 128(g) Johnston, G. S.; Jockusch, J.; McMurtry, L. C.; Shandera, W. X. Cancer Detect. Prev. 1990, 14, 337, (h) Chang, Y.; Cesarman, E.; Pessin, M. S.; Lee, F.; Culpepper, J.; Knowles, D. M.; Moore, P. S. Science 1994, 266, 1865

17. Hanto, D. W.; Frizzera, G.; Purtilo, D. T.; Sakamoto, K.; Sullivan, J. L.; Saemundsen, A. K.; Klein, G.; Simmons, R. L.; Najarian, J. S. Cancer Res. 1981, 41, 4253,

18. Russo, J. J.; Bohenzky, R. A.; Chien, M. C.; Chen, J.; Yan, M.; Maddalena, D.; Parry, J. P.; Peruzzi, D.; Edelman, I. S.; Chang, Y.; Moore, P. S. Proc. Natl. Acad. Sci. U.S.A. 1996, 93, 14862

19. Cohen, J. Science 1995, 269, 745

20. (a) Kedes, D. H.; Operskalski, E.; Busch, M.; Kohn, R.; Flood, J.; Ganem, D. Nat. Med. 1996, 2, 918(b) Zhong, W.; Wang, H.; Herndier, B.; Ganem, D. Proc. Natl. Acad. Sci. U.S.A. 1996, 93, 6641(c) Renne, R.; Zhong, W.; Herndier, B.; McGrath, M.; Abbey, N.; Kedes, D.; Ganem, D. Nat. Med. 1996, 2, 342

21. Ganem, D. Cell 1997, 91, 157

22. Arias, C.; Weisburd, B.; Stern-Ginossar, N.; Mercier, A.; Madrid, A. S.; Bellare, P.; Holdorf, M.; Weissman, J. S.; Ganem, D. PLoS Pathog. 2014, 10, e1003847

23. (a) Chakraborty, S.; Veettil, M. V.; Bottero, V.; Chandran, B. Proc. Natl. Acad. Sci. U.S.A. 2012, 109, E1163(b) Hahn, A. S.; Kaufmann, J. K.; Wies, E.; Naschberger, E.; Panteleev-Ivlev, J.; Schmidt, K.; Holzer, A.; Schmidt, M.; Chen, J.; Konig, S.; Ensser, A.; Myoung, J.; Brockmeyer, N. H.; Sturzl, M.; Fleckenstein, B.; Neipel, F. Nat. Med. 2012, 18, 961

24. (a) Cesarman, E. *Annu. Rev. Pathol.: Mech. Dis.* 2014, 9, 349(b) El-Osta, H. E.; Kurzrock, R. *Oncologist* 2011, 16, 497(c) Muzes, G.; Sipos, F.; Csomor, J.; Sreter, L. *Pathol. Oncol. Res.* 2013, 19, 345(d) Waterston, A.; Bower, M. *Acta Oncol.* 2004, 43, 698
25. (a) Horenstein, M. G.; Nador, R. G.; Chadburn, A.; Hyjek, E. M.; Inghirami, G.; Knowles, D. M.; Cesarman, E. *Blood* 1997, 90, 1186, (b) Szekely, L.; Chen, F.; Teramoto, N.; Ehlin-Henriksson, B.; Pokrovskaja, K.; Szeles, A.; Manneborg-Sandlund, A.; Lowbeer, M.; Lennette, E. T.; Klein, G. J. *Gen. Virol.* 1998, 79 (Pt 6) 1445, (c) Xia, T.; O'Hara, A.; Araujo, I.; Barreto, J.; Carvalho, E.; Sapucaia, J. B.; Ramos, J. C.; Luz, E.; Pedroso, C.; Manrique, M.; Toomey, N. L.; Brites, C.; Dittmer, D. P.; Harrington, W. J., Jr. *Cancer Res.* 2008, 68, 1436
26. (a) Muzes, G.; Sipos, F.; Csomor, J.; Sreter, L. *APMIS* 2013, 121, 668(b) Nishimoto, N. *Clin. Rev. Allergy Immunol.* 2005, 28, 221(c) Nishimoto, N.; Sasai, M.; Shima, Y.; Nakagawa, M.; Matsumoto, T.; Shirai, T.; Kishimoto, T.; Yoshizaki, K. *Blood* 2000, 95, 56,
27. (a) Friedrichs, C.; Neyts, J.; Gaspar, G.; De Clercq, E.; Wutzler, P. *Antiviral Res.* 2004, 62, 121(b) Glesby, M. J.; Hoover, D. R.; Weng, S. G.; Graham, N. M. H.; Phair, J. P.; Detels, R.; Ho, M. T.; Saah, A. J. *J. Infect. Dis.* 1996, 173, 1477(c) Kedes, D. H.; Ganem, D. J. *Clin. Invest.* 1997, 99, 2082(d) Medveczky, M. M.; Horvath, E.; Lund, T.; Medveczky, P. G. *AIDS* 1997, 11, 1327
28. Casper, C.; Krantz, E. M.; Corey, L.; Kuntz, S. R.; Wang, J.; Selke, S.; Hamilton, S.; Huang, M. L.; Wald, A. J. *J. Infect. Dis.* 2008, 198, 23
29. (a) Martin, D. F.; Kuppermann, B. D.; Wolitz, R. A.; Palestine, A. G.; Li, H.; Robinson, C. A. N. *Engl. J. Med.* 1999, 340, 1063(b) Mocroft, A.; Youle, M.; Gazzard, B.; Morcinek, J.; Halai, R.; Phillips, A. N. *AIDS* 1996, 10, 1101
30. Schwartz, R. A. *J. Surg. Oncol.* 2004, 87, 146

31. Dalla Pria, A.; Nelson, M.; Bower, M. *Expert Opin. Orphan Drugs* 2013, 1, 189,
32. Uldrick, T. S.; Polizzotto, M. N.; Aleman, K.; O'Mahony, D.; Wyvill, K. M.; Wang, V.; Marshall, V.; Pittaluga, S.; Steinberg, S. M.; Tosato, G.; Whitby, D.; Little, R. F.; Yarchoan, R. *Blood* 2011, 117, 6977
33. (a) Chen, Y. B.; Rahemtullah, A.; Hochberg, E. *Oncologist* 2007, 12, 569(b) Halfdanarson, T. R.; Markovic, S. N.; Kalokhe, U.; Luppi, M. *Ann. Oncol.* 2006, 17, 1849(c) Luppi, M.; Trovato, R.; Barozzi, P.; Vallisa, D.; Rossi, G.; Re, A.; Ravazzini, L.; Potenza, L.; Riva, G.; Morselli, M.; Longo, G.; Cavanna, L.; Roncaglia, R.; Torelli, G. *Leukemia* 2005, 19, 473
34. Bate, S. L.; Dollard, S. C.; Cannon, M. J. *Clin. Infect. Dis.* 2010, 50, 1439
35. Cannon, M. J.; Schmid, D. S.; Hyde, T. B. *Rev. Med. Virol.* 2010, 20, 202
36. Cannon, M. J.; Davis, K. F. *BMC Public Health* 2005, 5, 70
37. Manicklal, S.; Emery, V. C.; Lazzarotto, T.; Boppana, S. B.; Gupta, R. K. *Clin. Microbiol. Rev.* 2013, 26, 86
38. Cheeran, M. C. J.; Lokensgard, J. R.; Schleiss, M. R. *Clin. Microbiol. Rev.* 2009, 22, 99
39. Swanson, E. C.; Schleiss, M. R. *Pediatr. Clin. North Am.* 2013, 60, 335
40. Jacquemard, F.; Yamamoto, M.; Costa, J. M.; Romand, S.; Jaqz-Aigrain, E.; Dejean, A.; Daffos, F.; Ville, Y. *BJOG* 2007, 114, 1113
41. (a) Meyers, J. D.; Flournoy, N.; Thomas, E. D. *Rev. Infect. Dis.* 1982, 4, 1119(b) Meyers, J. D. *Am. J. Med.* 1986, 81, 27(c) Meyers, J. D.; Flournoy, N.; Thomas, E. D. *J. Infect. Dis.* 1986, 153, 478(d) Schmidt, G. M.; Horak, D. A.; Niland, J. C.; Duncan, S. R.; Forman, S. J.; Zaia, J. A. *N. Engl. J. Med.* 1991, 324, 1005
42. Machado, C. M.; Dulley, F. L.; Boas, L. S.; Castelli, J. B.; Macedo, M. C.; Silva, R. L.; Pallota, R.; Saboya, R. S.; Pannuti, C. S. *Bone Marrow Transplant.* 2000, 26, 413

43. Ruell, J.; Barnes, C.; Mutton, K.; Foulkes, B.; Chang, J.; Cavet, J.; Guiver, M.; Menasce, L.; Dougal, M.; Chopra, R. *Bone Marrow Transplant.* 2007, 40, 55
44. (a) Kotton, C. N. *Nat. Rev. Nephrol.* 2010, 6, 711, (b) Kotton, C. N.; Kumar, D.; Caliendo, A. M.; Asberg, A.; Chou, S. W.; Snyderman, D. R.; Allen, U.; Humar, A.; Consensus, T. S. I. C. *Transplantation* 2010, 89, 779(c) Kotton, C. N.; Kumar, D.; Caliendo, A. M.; Asberg, A.; Chou, S. W.; Danziger-Isakov, L.; Humar, A. *Transplantation* 2013, 96, 333(d) Le Page, A. K.; Jager, M. M.; Kotton, C. N.; Simoons-Smit, A.; Rawlinson, W. D. *Transplantation* 2013, 95, 1455
45. Biron, K. K. *Antiviral Res.* 2006, 71, 154
46. Perry, C. M.; Balfour, J. A. *Drugs* 1999, 57, 375
47. (a) Prichard, M. N.; Turk, S. R.; Coleman, L. A.; Engelhardt, S. L.; Shipman, C., Jr.; Drach, J. C. *J. Virol. Methods* 1990, 28, 101(b) Safrin, S.; Phan, L.; Elbeik, T. *Clin. Diagn. Virol.* 1995, 4, 81(c) Baldanti, F.; Biron, K. K.; Gerna, G. *J. Infect. Dis.* 1998, 177, 823(d) Cotarelo, M.; Catalan, P.; Sanchez-Carrillo, C.; Menasalvas, A.; Cercenado, E.; Tenorio, A.; Bouza, E. J. *Antimicrob. Chemother.* 1999, 44, 705
48. (a) Dulbecco, R.; Vogt, M.; Strickland, A. G. *Virology* 1956, 2, 162(b) Doceul, V.; Hollinshead, M.; van der Linden, L.; Smith, G. L. *Science* 2010, 327, 873
49. Dogra, P.; Sparer, T. E. What we have learned from animal models of HCMV. In *Human Cytomegaloviruses Methods and Protocols*; Yurochko, A. D.; Miller, W. E., Eds.; Humana Press: New York, 2014.
50. Cekinovic, D.; Lisnic, V. J.; Jonjic, S., Rodent models of congenital cytomegalovirus infection. In *Human Cytomegaloviruses Methods and Protocols*; Yurochko, A. D.; Miller, W. E., Eds.; Humana Press: New York, 2014.

51. (a) Smee, D. F.; Boehme, R.; Chernow, M.; Binko, B. P.; Matthews, T. R. *Biochem. Pharmacol.* 1985, 34, 1049(b) Littler, E.; Stuart, A. D.; Chee, M. S. *Nature* 1992, 358, 160(c) Sullivan, V.; Talarico, C. L.; Stanat, S. C.; Davis, M.; Coen, D. M.; Biron, K. K. *Nature* 1992, 359, 85(d) Meng, Q.; Hagemeyer, S. R.; Fingerroth, J. D.; Gershburg, E.; Pagano, J. S.; Kenney, S. C. *J. Virol.* 2010, 84, 4534
52. Talarico, C. L.; Burnette, T. C.; Miller, W. H.; Smith, S. L.; Davis, M. G.; Stanat, S. C.; Ng, T. I.; He, Z.; Coen, D. M.; Roizman, B.; Biron, K. K. *Antimicrob. Agents Chemother.* 1999, 43, 1941,
53. Keam, S. J.; Chapman, T. M.; Figgitt, D. P. *Drugs* 2004, 64, 2091
54. (a) Xiong, X.; Smith, J. L.; Chen, M. S. *Antimicrob. Agents Chemother.* 1997, 41, 594, (b) De Clercq, E. *Clin. Microbiol. Rev.* 2003, 16, 569
55. Lea, A. P.; Bryson, H. M. *Drugs* 1996, 52, 225
56. Chrisp, P.; Clissold, S. P. *Drugs* 1991, 41, 104
57. De Clercq, E.; Field, H. J. *Br. J. Pharmacol.* 2006, 147, 1
58. Price, N. B.; Prichard, M. N. *Curr. Opin. Virol.* 2011, 1, 548,
59. (a) Prichard, M. N.; Williams, J. D.; Komazin-Meredith, G.; Khan, A. R.; Price, N. B.; Jefferson, G. M.; Harden, E. A.; Hartline, C. B.; Peet, N. P.; Bowlin, T. L. *Antimicrob. Agents Chemother.* 2013, 57, 3518(b) Komazin-Meredith, G.; Chou, S.; Prichard, M. N.; Hartline, C. B.; Cardinale, S. C.; Comeau, K.; Williams, J. D.; Khan, A. R.; Peet, N. P.; Bowlin, T. L. *Antimicrob. Agents Chemother.* 2014, 58, 274
60. (a) Neyts, J.; Andrei, G.; De Clercq, E. *Antimicrob. Agents Chemother.* 1998, 42, 3285, (b) Neyts, J.; De Clercq, E. *Antimicrob. Agents Chemother.* 1997, 41, 2754, (c) Soike, K. F.; Bohm, R.; Huang, J. L.; Oberg, B. *Antimicrob. Agents Chemother.* 1993, 37, 1370(d) Akesson-

Johansson, A.; Harmenberg, J.; Wahren, B.; Linde, A. *Antimicrob. Agents Chemother.* 1990, 34, 2417(e) Abele, G.; Eriksson, B.; Harmenberg, J.; Wahren, B. *Antimicrob. Agents Chemother.* 1988, 32, 1137(f) Abele, G.; Karlstrom, A.; Harmenberg, J.; Shigeta, S.; Larsson, A.; Lindborg, B.; Wahren, B. *Antimicrob. Agents Chemother.* 1987, 31, 76

61. (a) Lake-Bakaar, D. M.; Lindborg, B.; Datema, R. *Antimicrob. Agents Chemother.* 1989, 33, 110(b) Lowe, D. M.; Alderton, W. K.; Ellis, M. R.; Parmar, V.; Miller, W. H.; Roberts, G. B.; Fyfe, J. A.; Gaillard, R.; Ertl, P.; Snowden, W. *Antimicrob. Agents Chemother.* 1995, 39, 1802(c) Engelhardt, P.; Hogberg, M.; Johansson, N.; Lindborg, B.; Linden, K.; Sahlberg, C.; Stahle, L.; Zhou, X.-X. *Antiviral Res.* 1998, 37, 76

62. Tying, S. K.; Plunkett, S.; Scribner, A. R.; Broker, R. E.; Herrod, J. N.; Handke, L. T.; Wise, J. M.; Martin, P. A. *J. Med. Virol.* 2012, 84, 1224

63. (a) U.S. National Institutes of Health. *ClinicalTrials.gov*. www.clinicaltrials.gov, 2014.(b) European Medicines Agency. *EU Clinical Trials Register*. www.clinicaltrialsregister.eu, 2014.

64. Diez-Torrubia, A.; Cabrera, S.; De Meester, I.; Camarasa, M. J.; Balzarini, J.; Velazquez, S. *ChemMedChem* 2012, 7, 1612

65. Diez-Torrubia, A.; Cabrera, S.; de Castro, S.; Garcia-Aparicio, C.; Mulder, G.; De Meester, I.; Camarasa, M. J.; Balzarini, J.; Velazquez, S. *Eur. J. Med. Chem.* 2013, 70, 456

66. Safrin, S.; Cherrington, J.; Jaffe, H. S. *Rev. Med. Virol.* 1997, 7, 145

67. (a) Beadle, J. R.; Hartline, C.; Aldern, K. A.; Rodriguez, N.; Harden, E.; Kern, E. R.; Hostetler, K. Y. *Antimicrob. Agents Chemother.* 2002, 46, 2381(b) Williams-Aziz, S. L.; Hartline, C. B.; Harden, E. A.; Daily, S. L.; Prichard, M. N.; Kushner, N. L.; Beadle, J. R.; Wan, W. B.; Hostetler, K. Y.; Kern, E. R. *Antimicrob. Agents Chemother.* 2005, 49, 3724

68. Kern, E. R.; Collins, D. J.; Wan, W. B.; Beadle, J. R.; Hostetler, K. Y.; Quenelle, D. C. *Antimicrob. Agents Chemother.* 2004, 48, 3516
69. (a) Crute, J. J.; Mocarski, E. S.; Lehman, I. R. *Nucleic Acids Res.* 1988, 16, 6585 (b) Crute, J. J.; Tsurumi, T.; Zhu, L. A.; Weller, S. K.; Olivo, P. D.; Challberg, M. D.; Mocarski, E. S.; Lehman, I. R. *Proc. Natl. Acad. Sci. U.S.A.* 1989, 86, 2186
70. (a) Crute, J. J.; Lehman, I. R. *J. Biol. Chem.* 1991, 266, 4484, (b) Zhu, L. A.; Weller, S. K. *J. Virol.* 1992, 66, 458,
71. Spector, F. C.; Liang, L.; Giordano, H.; Sivaraja, M.; Peterson, M. G. *J. Virol.* 1998, 72, 6979,
72. (a) Crute, J. J.; Grygon, C. A.; Hargrave, K. D.; Simoneau, B.; Faucher, A. M.; Bolger, G.; Kibler, P.; Liuzzi, M.; Cordingley, M. G. *Nat. Med.* 2002, 8, 386 (b) Kleymann, G.; Fischer, R.; Betz, U. A.; Hendrix, M.; Bender, W.; Schneider, U.; Handke, G.; Eckenberg, P.; Hewlett, G.; Pevzner, V.; Baumeister, J.; Weber, O.; Henninger, K.; Keldenich, J.; Jensen, A.; Kolb, J.; Bach, U.; Popp, A.; Maben, J.; Frappa, I.; Haebich, D.; Lockhoff, O.; Rubsamen-Waigmann, H. *Nat. Med.* 2002, 8, 392
73. Liuzzi, M.; Kibler, P.; Bousquet, C.; Harji, F.; Bolger, G.; Garneau, M.; Lapeyre, N.; McCollum, R. S.; Faucher, A. M.; Simoneau, B.; Cordingley, M. G. *Antiviral Res.* 2004, 64, 161
74. (a) Biswas, S.; Field, H. J. *Antiviral Chem. Chemother.* 2008, 19, 1, (b) Biswas, S.; Kleymann, G.; Swift, M.; Tiley, L. S.; Lyall, J.; Aguirre-Hernandez, J.; Field, H. J. *J. Antimicrob. Chemother.* 2008, 61, 1044 (c) Biswas, S.; Tiley, L. S.; Zimmermann, H.; Birkmann, A.; Field, H. J. *Antiviral Res.* 2008, 80, 81

75. Wald, A.; Corey, L.; Timmler, B.; Magaret, A.; Warren, T.; Tyring, S.; Johnston, C.; Kriesel, J.; Fife, K.; Galitz, L.; Stoelben, S.; Huang, M. L.; Selke, S.; Stobernack, H. P.; Ruebsamen-Schaeff, H.; Birkmann, A. N. *Engl. J. Med.* 2014, 370, 201
76. (a) Przech, A. J.; Yu, D.; Weller, S. K. *J. Virol.* 2003, 77, 9613 (b) Mocarski, E., Jr. Comparative analysis of herpesvirus-common proteins. In *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis*; Arvin, A.; Campadelli-Fiume, G.; Mocarski, Jr, E.; Moore, P. S.; Roizman, B.; Whitley, R.; Yamanishi, K., Eds.; Cambridge University Press: Cambridge, U.K., 2007.
77. (a) Townsend, L. B.; Devivar, R. V.; Turk, S. R.; Nassiri, M. R.; Drach, J. C. *J. Med. Chem.* 1995, 38, 4098 (b) Krosky, P. M.; Underwood, M. R.; Turk, S. R.; Feng, K. W.; Jain, R. K.; Ptak, R. G.; Westerman, A. C.; Biron, K. K.; Townsend, L. B.; Drach, J. C. *J. Virol.* 1998, 72, 4721, (c) Townsend, L. B.; Revankar, G. R. *Chem. Rev.* 1970, 70, 389 (d) Underwood, M. R.; Harvey, R. J.; Stanat, S. C.; Hemphill, M. L.; Miller, T.; Drach, J. C.; Townsend, L. B.; Biron, K. K. *J. Virol.* 1998, 72, 717,
78. Williams, S. L.; Hartline, C. B.; Kushner, N. L.; Harden, E. A.; Bidanset, D. J.; Drach, J. C.; Townsend, L. B.; Underwood, M. R.; Biron, K. K.; Kern, E. R. *Antimicrob. Agents Chemother.* 2003, 47, 2186
79. Scheffczik, H.; Savva, C. G.; Holzenburg, A.; Kolesnikova, L.; Bogner, E. *Nucleic Acids Res.* 2002, 30, 1695
80. Buerger, I.; Reefschlaeger, J.; Bender, W.; Eckenberg, P.; Popp, A.; Weber, O.; Graeper, S.; Klenk, H. D.; Ruebsamen-Waigmann, H.; Hallenberger, S. *J. Virol.* 2001, 75, 9077
81. Biron, K. K.; Gudmundsson, K. S.; Drach, J. C., Benzimidazole ribonucleosides: Novel drug candidates for the prevention and treatment of cytomegalovirus diseases. In *Antiviral Drugs:*

From Basic Discovery Through Clinical Trials; Kazmierski, W. M., Ed.; Wiley: New York, 2011.

82. (a) Reefschlaeger, J.; Bender, W.; Hallenberger, S.; Weber, O.; Eckenberg, P.; Goldmann, S.; Haerter, M.; Buerger, I.; Trappe, J.; Herrington, J. A.; Haebich, D.; Ruebsamen-Waigmann, H. J. *Antimicrob. Chemother.* 2001, 48, 757(b) Schleiss, M. R.; Bernstein, D. I.; McVoy, M. A.; Stroup, G.; Bravo, F.; Creasy, B.; McGregor, A.; Henninger, K.; Hallenberger, S. *Antiviral Res.* 2005, 65, 35

83. Townsend, L. B.; Gudmundsson, K. S.; Daluge, S. M.; Chen, J. J.; Zhu, Z.; Koszalka, G. W.; Boyd, L.; Chamberlain, S. D.; Freeman, G. A.; Biron, K. K.; Drach, J. C. *Nucleosides Nucleotides* 1999, 18, 509

84. Underwood, M. R.; Ferris, R. G.; Selleseth, D. W.; Davis, M. G.; Drach, J. C.; Townsend, L. B.; Biron, K. K.; Boyd, F. L. *Antimicrob. Agents Chemother.* 2004, 48, 1647

85. (a) Goldner, T.; Hempel, C.; Ruebsamen-Schaeff, H.; Zimmermann, H.; Lischka, P. *Antimicrob. Agents Chemother.* 2014, 58, 610(b) Goldner, T.; Hewlett, G.; Ettischer, N.; Ruebsamen-Schaeff, H.; Zimmermann, H.; Lischka, P. *J. Virol.* 2011, 85, 10884(c) Lischka, P.; Hewlett, G.; Wunberg, T.; Baumeister, J.; Paulsen, D.; Goldner, T.; Ruebsamen-Schaeff, H.; Zimmermann, H. *Antimicrob. Agents Chemother.* 2010, 54, 1290

86. (a) Newcomb, W. W.; Brown, J. C. *J. Virol.* 2002, 76, 10084(b) van Zeijl, M.; Fairhurst, J.; Jones, T. R.; Vernon, S. K.; Morin, J.; LaRocque, J.; Feld, B.; O'Hara, B.; Bloom, J. D.; Johann, S. V. *J. Virol.* 2000, 74, 9054

87. Visalli, R. J.; Fairhurst, J.; Srinivas, S.; Hu, W.; Feld, B.; DiGrandi, M.; Curran, K.; Ross, A.; Bloom, J. D.; van Zeijl, M.; Jones, T. R.; O'Connell, J.; Cohen, J. I. *J. Virol.* 2003, 77, 2349

88. Bloom, J. D.; Dushin, R. G.; Curran, K. J.; Donahue, F.; Norton, E. B.; Terefenko, E.; Jones, T. R.; Ross, A. A.; Feld, B.; Lang, S. A.; DiGrandi, M. J. *Bioorg. Med. Chem. Lett.* 2004, 14, 3401
89. Connolly, S. A.; Jackson, J. O.; Jardetzky, T. S.; Longnecker, R. *Nat. Rev. Microbiol.* 2011, 9, 369
90. Bloom, J. D.; DiGrandi, M. J.; Dushin, R. G.; Curran, K. J.; Ross, A. A.; Norton, E. B.; Terefenko, E.; Jones, T. R.; Feld, B.; Lang, S. A. *Bioorg. Med. Chem. Lett.* 2003, 13, 2929
91. Jones, T. R.; Lee, S. W.; Johann, S. V.; Razinkov, V.; Visalli, R. J.; Feld, B.; Bloom, J. D.; O'Connell, J. J. *Virol.* 2004, 78, 1289
92. Bandyopadhyay, C.; Valiya-Veetil, M.; Dutta, D.; Chakraborty, S.; Chandran, B. *PLoS Pathog.* 2014, 10, e1003941
93. (a) Gianni, T.; Salvioli, S.; Chesnokova, L. S.; Hutt-Fletcher, L. M.; Campadelli-Fiume, G. *PLoS Pathog.* 2013, 9, e1003806 (b) Stampfer, S. D.; Heldwein, E. E. *Curr. Opin. Virol.* 2013, 3, 13, (c) Zheng, K.; Xiang, Y.; Wang, X.; Wang, Q.; Zhong, M.; Wang, S.; Fan, J.; Kitazato, K.; Wang, Y. *mBio* 2014, 5, e00958,
94. Biron, K. K., Maribavir: A novel benzimidazole ribonucleoside for the prevention and treatment of cytomegalovirus diseases. In *Antiviral Drug Strategies*; De Clercq, E., Ed.; Wiley-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2011.
95. Biron, K. K.; Harvey, R. J.; Chamberlain, S. C.; Good, S. S.; Smith, A. A., 3rd; Davis, M. G.; Talarico, C. L.; Miller, W. H.; Ferris, R.; Dornsife, R. E.; Stanat, S. C.; Drach, J. C.; Townsend, L. B.; Koszalka, G. W. *Antimicrob. Agents Chemother.* 2002, 46, 2365
96. Shannon-Lowe, C. D.; Emery, V. C. *Herpesviridae* 2010, 1, 4,
97. Chou, S.; Marousek, G. I. J. *Virol.* 2008, 82, 246

98. Gentry, B. G.; Kamil, J. P.; Coen, D. M.; Zemlicka, J.; Drach, J. C. *Antimicrob. Agents Chemother.* 2010, 54, 3093
99. Chou, S.; Komazin-Meredith, G.; Williams, J. D.; Bowlin, T. L. *Antimicrob. Agents Chemother.* 2014, 58, 1809
100. Reitsma, J. M.; Savaryn, J. P.; Faust, K.; Sato, H.; Halligan, B. D.; Terhune, S. S. *Cell Host Microbe.* 2011, 9, 103
101. Chou, S.; Van Wechel, L. C.; Marousek, G. I. *Antimicrob. Agents Chemother.* 2006, 50, 2557
102. Kamil, J. P.; Coen, D. M. *Cell Host Microbe* 2011, 9, 85
103. Whitehurst, C. B.; Sanders, M. K.; Law, M.; Wang, F. Z.; Xiong, J.; Dittmer, D. P.; Pagano, J. S. *J. Virol.* 2013, 87, 5311
104. Gershburg, E.; Pagano, J. S. *J. Virol.* 2002, 76, 998
105. Conner, J.; Cross, A.; Murray, J.; Marsden, H. J. *Gen. Virol.* 1994, 75 (Pt 12) 3327
106. Preston, V. G.; Palfreyman, J. W.; Dutia, B. M. *J. Gen. Virol.* 1984, 65 (Pt 9) 1457
107. (a) Idowu, A. D.; Fraser-Smith, E. B.; Poffenberger, K. L.; Herman, R. C. *Antiviral Res.* 1992, 17, 145(b) Turk, S. R.; Kik, N. A.; Birch, G. M.; Chiego, D. J., Jr.; Shipman, C., Jr. *Virology* 1989, 173, 733(c) Cameron, J. M.; McDougall, I.; Marsden, H. S.; Preston, V. G.; Ryan, D. M.; Subak-Sharpe, J. H. *J. Gen. Virol.* 1988, 69 (Pt 10) 2607
108. (a) Cohen, E. A.; Gaudreau, P.; Brazeau, P.; Langelier, Y. *Nature* 1986, 321, 441(b) Dutia, B. M.; Frame, M. C.; Subak-Sharpe, J. H.; Clark, W. N.; Marsden, H. S. *Nature* 1986, 321, 439
109. (a) Liuzzi, M.; Deziel, R.; Moss, N.; Beaulieu, P.; Bonneau, A. M.; Bousquet, C.; Chafouleas, J. G.; Garneau, M.; Jaramillo, J.; Krogsrud, R. L.; Lagace, L.; McCollum, R. S.; Nawoot, S.; Guindon, Y. *Nature* 1994, 372, 695(b) Bonneau, A. M.; Kibler, P.; White, P.;

- Bousquet, C.; Dansereau, N.; Cordingley, M. G. J. *Virol.* 1996, 70, 787, (c) Moss, N.; Beaulieu, P.; Duceppe, J. S.; Ferland, J. M.; Garneau, M.; Gauthier, J.; Ghiron, E.; Goulet, S.; Guse, I.; Jaramillo, J.; Llinas-Brunet, M.; Malenfant, E.; Plante, R.; Poirier, M.; Soucy, F.; Wernic, D.; Yoakim, C.; Deziel, R. *J. Med. Chem.* 1996, 39, 4173
110. (a) Wunberg, T.; Baumeister, J.; Jeske, M.; Nikolic, S.; Submeier, F.; Zimmermann, H.; Grosser, R.; Henninger, K.; Hewlett, G.; Keldenich, J. Substituted Quinazolines as Antiviral Agents, Especially against Cytomegaloviruses. U.S. Patent Appl., U.S. Patent US20060235032 A1. 2006.(b) Wunberg, T.; Baumeister, J.; Betz, U.; Jeske, M.; Kleymann, G.; Lampe, T.; Nikolic, S.; Reefschrager, J.; Schohe-Loop, R.; Submeier, F.; Zimmermann, H.; Grosser, R.; Henninger, K.; Hewlett, G.; Keldenich, J.; Kramer, T.; Nell, P.; Lin, T. 2-(3-phenyl-2-piperazinyl-3,4-dihydroquinazolin-4-yl)acetic acids as antiviral agents, especially against cytomegaloviruses. U.S. Patent 7960387. 2011.
111. Schleiss, M.; Eickhoff, J.; Auerochs, S.; Leis, M.; Abele, S.; Rechter, S.; Choi, Y.; Anderson, J.; Scott, G.; Rawlinson, W.; Michel, D.; Ensminger, S.; Klebl, B.; Stamminger, T.; Marschall, M. *Antiviral Res.* 2008, 79, 49
112. De Castro, S.; Garcia-Aparicio, C.; Andrei, G.; Snoeck, R.; Balzarini, J.; Camarasa, M. J.; Velazquez, S. *J. Med. Chem.* 2009, 52, 1582
113. Kontani, T.; Miyata, J.; Hamaguchi, W.; Kawano, T.; Kamikawa, A.; Suzuki, H.; Sudo, K. Tetrahydro-2H-thiopyran-4-carboxamide Derivative. U.S. Patent 6903125, 2005.
114. Suzuki, H.; Chono, K.; Noto, T.; Katsumata, K.; Nakamura, Y. Agent for Preventing or Treating Zoster-Associated Pain. U.S. Patent Appl. 20110201659, 2011.
115. Chacko, B.; John, G. T. *Transplant Infect. Dis.* 2012, 14, 111

116. Waldman, W. J.; Knight, D. A.; Lurain, N. S.; Miller, D. M.; Sedmak, D. D.; Williams, J. W.; Chong, A. S. *Transplantation* 1999, 68, 814
117. Knight, D. A.; Hejmanowski, A. Q.; Dierksheide, J. E.; Williams, J. W.; Chong, A. S.; Waldman, W. J. *Transplantation* 2001, 71, 170
118. (a) Evers, D. L.; Wang, X.; Huong, S. M.; Andreoni, K. A.; Huang, E. S. *Antiviral Res.* 2005, 65, 1(b) Qi, R.; Hua-Song, Z.; Xiao-Feng, Z. *Eur. J. Med. Res.* 2013, 18, 3
119. (a) Stanberry, L. R.; Cunningham, A. L.; Mindel, A.; Scott, L. L.; Spruance, S. L.; Aoki, F. Y.; Lacey, C. J. *Clin. Infect. Dis.* 2000, 30, 549(b) Dasgupta, G.; Chentoufi, A. A.; Nesburn, A. B.; Wechsler, S. L.; BenMohamed, L. *Expert Rev. Vaccines* 2009, 8, 1023(c) Dasgupta, G.; Nesburn, A. B.; Wechsler, S. L.; BenMohamed, L. *Future Microbiol.* 2010, 5, 1(d) Schiller, J. T.; Lowy, D. R. *Annu. Rev. Microbiol.* 2010, 64, 23(e) Smith, C.; Khanna, R. *Hum. Vaccines* 2010, 6, 1062(f) Wang, D.; Fu, T. M. *Curr. Opin. Virol.* 2014, 6C, 13
120. Vischer, H. F.; Siderius, M.; Leurs, R.; Smit, M. J. *Nat. Rev. Drug Discovery* 2014, 13, 123
121. (a) Corte-Real, S.; Collins, C.; Aires da Silva, F.; Simas, J. P.; Barbas, C. F., 3rd; Chang, Y.; Moore, P.; Goncalves, J. *Blood* 2005, 106, 3797(b) Beauchemin, C.; Moerke, N. J.; Faloon, P.; Kaye, K. M. *J. Biomol Screening* 2014, 19, 947
122. (a) Weekes, M. P.; Tan, S. Y.; Poole, E.; Talbot, S.; Antrobus, R.; Smith, D. L.; Montag, C.; Gygi, S. P.; Sinclair, J. H.; Lehner, P. J. *Science* 2013, 340, 199(b) Wills, M. R.; Poole, E.; Lau, B.; Krishna, B.; Sinclair, J. H. *Cell Mol. Immunol* 2014,
123. Schubart, D.; Habenberger, P.; Stein-Gerlach, M.; Bevec, D. *Cellular Kinases Involved in Cytomegalovirus Infection and Their Inhibition*. U.S. Patent 6849409, 2005.
124. (a) Bresnahan, W. A.; Boldogh, I.; Chi, P.; Thompson, E. A.; Albrecht, T. *Virology* 1997, 231, 239(b) Schang, L. M.; Bantly, A.; Knockaert, M.; Shaheen, F.; Meijer, L.; Malim, M. H.;

- Gray, N. S.; Schaffer, P. A. J. *Viol.* 2002, 76, 7874(c) Coen, D. M.; Schaffer, P. A. *Nat. Rev. Drug Discovery* 2003, 2, 278
125. Schang, L. M.; Phillips, J.; Schaffer, P. A. J. *Viol.* 1998, 72, 5626,
126. Poglitsch, M.; Weichhart, T.; Hecking, M.; Werzowa, J.; Katholnig, K.; Antlanger, M.; Krmpotic, A.; Jonjic, S.; Horl, W. H.; Zlabinger, G. J.; Puchhammer, E.; Saemann, M. D. *Am. J. Transplant.* 2012, 12, 1458
127. Li, R.; Hayward, S. D. *Trends Microbiol.* 2013, 21, 286
128. Brennan, D. C.; Aguado, J. M.; Potena, L.; Jardine, A. G.; Legendre, C.; Saemann, M. D.; Mueller, N. J.; Merville, P.; Emery, V.; Nashan, B. *Rev. Med. Virol.* 2013, 23, 97
129. (a) Blyth, W. A.; Hill, T. J.; Field, H. J.; Harbour, D. A. J. *Gen. Virol.* 1976, 33, 547(b) Hill, T. J.; Blyth, W. A. *Lancet* 1976, 1, 397(c) Harbour, D. A.; Blyth, W. A.; Hill, T. J. *Gen. Virol.* 1978, 41, 87(d) Giron, D. J. *Proc. Soc. Exp. Biol. Med.* 1982, 170, 25(e) Gebhardt, B. M.; Varnell, E. D.; Kaufman, H. E. J. *Ocul. Pharmacol. Ther.* 2005, 21, 114
130. Wlodawer, A.; Vondrasek, J. *Annu. Rev. Biophys. Biomol. Struct.* 1998, 27, 249
131. Friedman-Kien, A.; Laubenstein, L.; Marmor, M.; Hymes, K.; Green, J.; Ragaz, A.; Gottlieb, J.; Muggia, F.; Demopoulos, R.; Weintraub, M. *MMWR. Morb. Mortal. Wkly. Rep.* 1981, 30, 305
132. (a) Ratner, L.; Haseltine, W.; Patarca, R.; Livak, K. J.; Starcich, B.; Josephs, S. F.; Doran, E. R.; Rafalski, J. A.; Whitehorn, E. A.; Baumeister, K.; Ivanoff, L.; Petteway, S. R. J.; Pearson, M. L.; Lautenberger, J. A.; Papas, T. S.; Ghayeb, J.; Chang, N. T.; Gallo, R. C.; Wong-Staal, F. *Nature* 1985, 313, 277(b) Wain-Hobson, S.; Sonigo, P.; Danos, O.; Cole, S.; Alizon, M. *Cell* 1985, 40, 9
133. Flexner, C. *Nat. Rev. Drug Discovery* 2007, 6, 959

134. Jensen, C.; Herold, P.; Brunner, H. R. *Nat. Rev. Drug Discovery* 2008, 7, 399
135. De Lucca, G. V.; Jadhav, P. K.; Waltermire, R. E.; Aungst, B. J.; Erickson-Viitanen, S.; Lam, P. Y. *Pharm. Biotechnol.* 1998, 11, 257
136. Wensing, A. M.; van Maarseveen, N. M.; Nijhuis, M. *Antiviral Res.* 2010, 85, 59
137. (a) Chen, K. X.; Njoroge, F. G. *Curr. Opin. Invest. Drugs* 2009, 10, 821, (b) Reiser, M.; Timm, J. *Expert Rev. Anti-Infect. Ther.* 2009, 7, 537(c) Chary, A.; Holodniy, M. *Rev. Recent Clin. Trials* 2010, 5, 158(d) Kwong, A. D.; Kauffman, R. S.; Hurter, P.; Mueller, P. *Nat. Biotechnol.* 2011, 29, 993(e) Manns, M. P.; von Hahn, T. *Nat. Rev. Drug Discovery* 2013, 12, 595(f) Perni, R. B.; Pitlik, J.; Britt, S. D.; Court, J. J.; Courtney, L. F.; Deininger, D. D.; Farmer, L. J.; Gates, C. A.; Harbeson, S. L.; Levin, R. B.; Lin, C.; Lin, K.; Moon, Y. C.; Luong, Y. P.; O'Malley, E. T.; Rao, B. G.; Thomson, J. A.; Tung, R. D.; Van Drie, J. H.; Wei, Y. *Bioorg. Med. Chem. Lett.* 2004, 14, 1441
138. (a) Carrion, A. F.; Gutierrez, J.; Martin, P. *Expert Opin. Pharmacother.* 2014, 15, 711(b) De Nicola, S.; Aghemo, A. *Liver Int.* 2014, 34, e168(c) Scola, P. M.; Sun, L. Q.; Wang, A. X.; Chen, J.; Sin, N.; Venables, B. L.; Sit, S. Y.; Chen, Y.; Cocuzza, A.; Bilder, D. M.; D'Andrea, S. V.; Zheng, B.; Hewawasam, P.; Tu, Y.; Friborg, J.; Falk, P.; Hernandez, D.; Levine, S.; Chen, C.; Yu, F.; Sheaffer, A. K.; Zhai, G.; Barry, D.; Knipe, J. O.; Han, Y. H.; Schartman, R.; Donoso, M.; Mosure, K.; Sinz, M. W.; Zvyaga, T.; Good, A. C.; Rajamani, R.; Kish, K.; Tredup, J.; Klei, H. E.; Gao, Q.; Mueller, L.; Colonno, R. J.; Grasela, D. M.; Adams, S. P.; Loy, J.; Levesque, P. C.; Sun, H.; Shi, H.; Sun, L.; Warner, W.; Li, D.; Zhu, J.; Meanwell, N. A.; McPhee, F. J. *Med. Chem.* 2014, 57, 1730(d) Scola, P. M.; Wang, A. X.; Good, A. C.; Sun, L. Q.; Combrink, K. D.; Campbell, J. A.; Chen, J.; Tu, Y.; Sin, N.; Venables, B. L.; Sit, S. Y.; Chen, Y.; Cocuzza, A.; Bilder, D. M.; D'Andrea, S.; Zheng, B.; Hewawasam, P.; Ding, M.;

Thuring, J.; Li, J.; Hernandez, D.; Yu, F.; Falk, P.; Zhai, G.; Sheaffer, A. K.; Chen, C.; Lee, M. S.; Barry, D.; Knipe, J. O.; Li, W.; Han, Y. H.; Jenkins, S.; Gesenberg, C.; Gao, Q.; Sinz, M. W.; Santone, K. S.; Zvyaga, T.; Rajamani, R.; Klei, H. E.; Colonna, R. J.; Grasela, D. M.; Hughes, E.; Chien, C.; Adams, S.; Levesque, P. C.; Li, D.; Zhu, J.; Meanwell, N. A.; McPhee, F. J. *Med. Chem.* 2014, *57*, 1708

139. Kim, J. L.; Morgenstern, K. A.; Lin, C.; Fox, T.; Dwyer, M. D.; Landro, J. A.; Chambers, S. P.; Markland, W.; Lepre, C. A.; O'Malley, E. T.; Harbeson, S. L.; Rice, C. M.; Murcko, M. A.; Caron, P. R.; Thomson, J. A. *Cell* 1996, *87*, 343

140. Perni, R. B.; Britt, S. D.; Court, J. C.; Courtney, L. F.; Deininger, D. D.; Farmer, L. J.; Gates, C. A.; Harbeson, S. L.; Kim, J. L.; Landro, J. A.; Levin, R. B.; Luong, Y. P.; O'Malley, E. T.; Pitlik, J.; Rao, B. G.; Schairer, W. C.; Thomson, J. A.; Tung, R. D.; Van Drie, J. H.; Wei, Y. *Bioorg. Med. Chem. Lett.* 2003, *13*, 4059

141. (a) Landro, J. A.; Raybuck, S. A.; Luong, Y. P.; O'Malley, E. T.; Harbeson, S. L.; Morgenstern, K. A.; Rao, G.; Livingston, D. J. *Biochemistry* 1997, *36*, 9340(b) Llinas-Brunet, M.; Bailey, M.; Fazal, G.; Goulet, S.; Halmos, T.; Laplante, S.; Maurice, R.; Poirier, M.; Poupart, M. A.; Thibeault, D.; Wernic, D.; Lamarre, D. *Bioorg. Med. Chem. Lett.* 1998, *8*, 1713(c) Steinkuhler, C.; Biasiol, G.; Brunetti, M.; Urbani, A.; Koch, U.; Cortese, R.; Pessi, A.; De Francesco, R. *Biochemistry* 1998, *37*, 8899(d) Perni, R. B.; Kwong, A. D. *Prog. Med. Chem.* 2002, *39*, 215(e) Perni, R. B.; Farmer, L. J.; Cottrell, K. M.; Court, J. J.; Courtney, L. F.; Deininger, D. D.; Gates, C. A.; Harbeson, S. L.; Kim, J. L.; Lin, C.; Lin, K.; Luong, Y. P.; Maxwell, J. P.; Murcko, M. A.; Pitlik, J.; Rao, B. G.; Schairer, W. C.; Tung, R. D.; Van Drie, J. H.; Wilson, K.; Thomson, J. A. *Bioorg. Med. Chem. Lett.* 2004, *14*, 1939(f) Yip, Y.; Victor, F.; Lamar, J.; Johnson, R.; Wang, Q. M.; Barket, D.; Glass, J.; Jin, L.; Liu, L.; Venable, D.;

Wakulchik, M.; Xie, C.; Heinz, B.; Villarreal, E.; Colacino, J.; Yumibe, N.; Tebbe, M.; Munroe, J.; Chen, S. H. *Bioorg. Med. Chem. Lett.* 2004, 14, 251(g) Lin, C.; Kwong, A. D.; Perni, R. B. *Infect. Disord.: Drug Targets* 2006, 6, 3(h) Perni, R. B.; Chandorkar, G.; Cottrell, K. M.; Gates, C. A.; Lin, C.; Lin, K.; Luong, Y. P.; Maxwell, J. P.; Murcko, M. A.; Pitlik, J.; Rao, G.; Schairer, W. C.; Van Drie, J.; Wei, Y. *Bioorg. Med. Chem. Lett.* 2007, 17, 3406

142. Saalau-Bethell, S. M.; Woodhead, A. J.; Chessari, G.; Carr, M. G.; Coyle, J.; Graham, B.; Hiscock, S. D.; Murray, C. W.; Pathuri, P.; Rich, S. J.; Richardson, C. J.; Williams, P. A.; Jhoti, H. *Nat. Chem. Biol.* 2012, 8, 920

143. (a) Preston, V. G.; Coates, J. A.; Rixon, F. J. J. *Virol.* 1983, 45, 1056, (b) Gao, M.; Matusick-Kumar, L.; Hurlburt, W.; DiTusa, S. F.; Newcomb, W. W.; Brown, J. C.; McCann, P. J., 3rd; Deckman, I.; Colonno, R. J. J. *Virol.* 1994, 68, 3702, (c) Matusick-Kumar, L.; McCann, P. J., III; Robertson, B. J.; Newcomb, W. W.; Brown, J. C.; Gao, M. J. *Virol.* 1995, 69, 7113,

144. (a) Liu, F. Y.; Roizman, B. J. *Virol.* 1991, 65, 5149, (b) Martinez, R.; Sarisky, R. T.; Weber, P. C.; Weller, S. K. J. *Virol.* 1996, 70, 2075, (c) Newcomb, W. W.; Thomsen, D. R.; Homa, F. L.; Brown, J. C. J. *Virol.* 2003, 77, 9862(d) Yang, K.; Wills, E. G.; Baines, J. D. *Virology* 2012, 429, 63

145. (a) Gibson, W.; Roizman, B. J. *Virol.* 1972, 10, 1044, (b) Nealon, K.; Newcomb, W. W.; Pray, T. R.; Craik, C. S.; Brown, J. C.; Kedes, D. H. J. *Virol.* 2001, 75, 2866

146. (a) McNab, A. R.; Desai, P.; Person, S.; Roof, L. L.; Thomsen, D. R.; Newcomb, W. W.; Brown, J. C.; Homa, F. L. J. *Virol.* 1998, 72, 1060, (b) Newcomb, W. W.; Trus, B. L.; Cheng, N.; Steven, A. C.; Sheaffer, A. K.; Tenney, D. J.; Weller, S. K.; Brown, J. C. J. *Virol.* 2000, 74, 1663

147. Jiang, X.; Gong, H.; Chen, Y. C.; Vu, G. P.; Trang, P.; Zhang, C. Y.; Lu, S.; Liu, F. *Proc. Natl. Acad. Sci. U.S.A.* 2012, 109, 13070
148. (a) Chen, P.; Tsuge, H.; Almassy, R. J.; Gribskov, C. L.; Katoh, S.; Vanderpool, D. L.; Margosiak, S. A.; Pinko, C.; Matthews, D. A.; Kan, C. C. *Cell* 1996, 86, 835(b) Qiu, X.; Culp, J. S.; DiLella, A. G.; Hellmig, B.; Hoog, S. S.; Janson, C. A.; Smith, W. W.; Abdel-Meguid, S. S. *Nature* 1996, 383, 275(c) Shieh, H. S.; Kurumbail, R. G.; Stevens, A. M.; Stegeman, R. A.; Sturman, E. J.; Pak, J. Y.; Wittwer, A. J.; Palmier, M. O.; Wiegand, R. C.; Holwerda, B. C.; Stallings, W. C. *Nature* 1996, 383, 279(d) Tong, L.; Qian, C.; Massariol, M. J.; Bonneau, P. R.; Cordingley, M. G.; Lagace, L. *Nature* 1996, 383, 272
149. Khayat, R.; Batra, R.; Massariol, M. J.; Lagace, L.; Tong, L. *Biochemistry* 2001, 40, 6344
150. (a) Hoog, S. S.; Smith, W. W.; Qiu, X.; Janson, C. A.; Hellmig, B.; McQueney, M. S.; O'Donnell, K.; O'Shannessy, D.; DiLella, A. G.; Debouck, C.; Abdel-Meguid, S. S. *Biochemistry* 1997, 36, 14023(b) Qiu, X.; Janson, C. A.; Culp, J. S.; Richardson, S. B.; Debouck, C.; Smith, W. W.; Abdel-Meguid, S. S. *Proc. Natl. Acad. Sci. U.S.A.* 1997, 94, 2874(c) Reiling, K. K.; Pray, T. R.; Craik, C. S.; Stroud, R. M. *Biochemistry* 2000, 39, 12796(d) Buisson, M.; Hernandez, J. F.; Lascoux, D.; Schoehn, G.; Forest, E.; Arlaud, G.; Seigneurin, J. M.; Ruigrok, R. W.; Burmeister, W. P. J. *Mol. Biol.* 2002, 324, 89
151. (a) Hall, D. L.; Darke, P. L. J. *Biol. Chem.* 1995, 270, 22697(b) Batra, R.; Khayat, R.; Tong, L. *Nat. Struct. Biol.* 2001, 8, 810
152. (a) Batra, R.; Khayat, R.; Tong, L. *Protein Pept. Lett.* 2001, 8, 333, (b) Tong, L.; Qian, C.; Massariol, M. J.; Deziel, R.; Yoakim, C.; Lagace, L. *Nat. Struct. Biol.* 1998, 5, 819(c) LaPlante, S. R.; Bonneau, P. R.; Aubry, N.; Cameron, D. R.; Deziel, R.; Grand-Maitre, E.; Plouffe, C.; Tong, L.; Kawai, S. H. J. *Am. Chem. Soc.* 1999, 121, 2974[ACS Full Text ACS Full Text], (d)

Lazic, A.; Goetz, D. H.; Nomura, A. M.; Marnett, A. B.; Craik, C. S. J. *Mol. Biol.* 2007, 373, 913

153. Waxman, L.; Darke, P. L. *Antiviral Chem. Chemother.* 2000, 11, 1,

154. (a) DiIanni, C. L.; Mapelli, C.; Drier, D. A.; Tsao, J.; Natarajan, S.; Riexinger, D.; Festin, S. M.; Bolgar, M.; Yamanaka, G.; Weinheimer, S. P.; Meyers, C. A.; Colonno, R. J.; Cordingley, M. G. J. *Biol. Chem.* 1993, 268, 25449, (b) Stevens, J. T.; Mapelli, C.; Tsao, J.; Hail, M.; O'Boyle, D., 2nd; Weinheimer, S. P.; DiIanni, C. L. *Eur. J. Biochem.* 1994, 226, 361(c)

Holwerda, B. C. *Antiviral Res.* 1997, 35, 1

155. LaFemina, R. L.; Bakshi, K.; Long, W. J.; Pramanik, B.; Veloski, C. A.; Wolanski, B. S.; Marcy, A. I.; Hazuda, D. J. J. *Virol.* 1996, 70, 4819,

156. Holskin, B. P.; Bukhtiyarova, M.; Dunn, B. M.; Baur, P.; de Chastonay, J.; Pennington, M. W. *Anal. Biochem.* 1995, 227, 148

157. Ogilvie, W.; Bailey, M.; Poupart, M. A.; Abraham, A.; Bhavsar, A.; Bonneau, P.; Bordeleau, J.; Bousquet, Y.; Chabot, C.; Duceppe, J. S.; Fazal, G.; Goulet, S.; Grand-Maitre, C.; Guse, I.; Halmos, T.; Lavallee, P.; Leach, M.; Malenfant, E.; O'Meara, J.; Plante, R.; Plouffe, C.; Poirier, M.; Soucy, F.; Yoakim, C.; Deziel, R. J. *Med. Chem.* 1997, 40, 4113

158. (a) Deziel, R.; Malenfant, E. *Bioorg. Med. Chem. Lett.* 1998, 8, 1437(b) Yoakim, C.; Ogilvie, W. W.; Cameron, D. R.; Chabot, C.; Guse, I.; Hache, B.; Naud, J.; O'Meara, J. A.; Plante, R.; Deziel, R. J. *Med. Chem.* 1998, 41, 2882

159. Harper, J. W.; Cook, R. R.; Roberts, C. J.; McLaughlin, B. J.; Powers, J. C. *Biochemistry* 1984, 23, 2995

160. Finke, P. E.; Shah, S. K.; Fletcher, D. S.; Ashe, B. M.; Brause, K. A.; Chandler, G. O.; Dellea, P. S.; Hand, K. M.; Maycock, A. L.; Osinga, D. G.; Underwood, D. J.; Weston, H.; Davies, P.; Doherty, J. B. J. *Med. Chem.* 1995, 38, 2449
161. Bonneau, P. R.; Hasani, F.; Plouffe, C.; Malenfant, E.; LaPlante, S. R.; Guse, I.; Ogilvie, W. W.; Plante, R.; Davidson, W. C.; Hopkins, J. L.; Morelock, M. M.; Cordingley, M. G.; Deziel, R. *J. Am. Chem. Soc.* 1999, 121, 2965[ACS Full Text ACS Full Text],
162. Borthwick, A. D. *Med. Res. Rev.* 2005, 25, 427
163. Borthwick, A. D.; Angier, S. J.; Crame, A. J.; Exall, A. M.; Haley, T. M.; Hart, G. J.; Mason, A. M.; Pennell, A. M. K.; Weingarten, G. G. *J. Med. Chem.* 2000, 43, 4452
164. Borthwick, A. D.; Crame, A. J.; Ertl, P. F.; Exall, A. M.; Haley, T. M.; Hart, G. J.; Mason, A. M.; Pennell, A. M. K.; Singh, O. M. P.; Weingarten, G. G.; Woolven, J. M. J. *Med. Chem.* 2002, 45, 1
165. (a) Borthwick, A. D.; Crame, A. J.; Ertl, P. F.; Exall, A. M.; Haley, T. M.; Hart, G. J.; Mason, A. M.; Pennell, A. M.; Singh, O. M.; Weingarten, G. G.; Woolven, J. M. J. *Med. Chem.* 2002, 45, 1(b) Borthwick, A. D.; Davies, D. E.; Ertl, P. F.; Exall, A. M.; Haley, T. M.; Hart, G. J.; Jackson, D. L.; Parry, N. R.; Patikis, A.; Trivedi, N.; Weingarten, G. G.; Woolven, J. M. J. *Med. Chem.* 2003, 46, 4428
166. Borthwick, A. D.; Exall, A. M.; Haley, T. M.; Jackson, D. L.; Mason, A. M.; Weingarten, G. G. *Bioorg. Med. Chem. Lett.* 2002, 12, 1719
167. (a) Gerona-Navarro, G.; Perez de Vega, M. J.; Garcia-Lopez, M. T.; Andrei, G.; Snoeck, R.; Balzarini, J.; De Clercq, E.; Gonzalez-Muniz, R. *Bioorg. Med. Chem. Lett.* 2004, 14, 2253(b) Gerona-Navarro, G.; Perez de Vega, M. J.; Garcia-Lopez, M. T.; Andrei, G.; Snoeck, R.; De Clercq, E.; Balzarini, J.; Gonzalez-Muniz, R. *J. Med. Chem.* 2005, 48, 2612(c) Perez-Faginas, P.;

Aranda, M. T.; Garcia-Lopez, M. T.; Snoeck, R.; Andrei, G.; Balzarini, J.; Gonzalez-Muniz, R. Bioorg. Med. Chem. 2011, 19, 1155

168. (a) Jarvest, R. L.; Parratt, M. J.; Debouck, C. M.; Gorniak, J. G.; Jennings, L. J.; Serafinowska, H. T.; Strickler, J. E. Bioorg. Med. Chem. Lett. 1996, 6, 2463, (b) Abood, N. A.; Schretzman, L. A.; Flynn, D. L.; Houseman, K. A.; Wittwer, A. J.; Dilworth, V. M.; Hippenmeyer, P. J.; Holwerda, B. C. Bioorg. Med. Chem. Lett. 1997, 7, 2105, (c) Jarvest, R. L.; Connor, S. C.; Gorniak, J. G.; Jennings, L. J.; Serafinowska, H. T.; West, A. Bioorg. Med. Chem. Lett. 1997, 7, 1733, (d) Jarvest, R. L.; Pinto, I. L.; Ashman, S. M.; Dabrowski, C. E.; Fernandez, A. V.; Jennings, L. J.; Lavery, P.; Tew, D. G. Bioorg. Med. Chem. Lett. 1999, 9, 443(e) Pinto, I. L.; Jarvest, R. L.; Clarke, B.; Dabrowski, C. E.; Fenwick, A.; Gorczyca, M. M.; Jennings, L. J.; Lavery, P.; Sternberg, E. J.; Tew, D. G.; West, A. Bioorg. Med. Chem. Lett. 1999, 9, 449

169. Jarvest, R. L.; Dabrowski, C. E. Herpes virus and cytomegalovirus proteinase. In Proteinase and Peptidase Inhibition: Recent Potential Targets for Drug Development; Smith, H. J.; Simons, C., Eds.; Taylor & Francis: London, 2003.

170. Pinto, I. L.; West, A.; Debouck, C. M.; DiLella, A. G.; Gorniak, J. G.; O'Donnell, K. C.; O'Shannessy, D. J.; Patel, A.; Jarvest, R. L. Bioorg. Med. Chem. Lett. 1996, 6, 2467,

171. Groutas, W. C.; Brubaker, M. J.; Stanga, M. A.; Castrisos, J. C.; Crowley, J. P.; Schatz, E. J. J. Med. Chem. 1989, 32, 1607

172. (a) Cole, J. L. Biochemistry 1996, 35, 15601(b) Darke, P. L.; Cole, J. L.; Waxman, L.; Hall, D. L.; Sardana, M. K.; Kuo, L. C. J. Biol. Chem. 1996, 271, 7445(c) Margosiak, S. A.; Vanderpool, D. L.; Sisson, W.; Pinko, C.; Kan, C. C. Biochemistry 1996, 35, 5300(d) Schmidt, U.; Darke, P. L. J. Biol. Chem. 1997, 272, 7732

173. (a) Khayat, R.; Batra, R.; Bebernitz, G. A.; Olson, M. W.; Tong, L. *Biochemistry* 2004, 43, 316(b) Shimba, N.; Nomura, A. M.; Marnett, A. B.; Craik, C. S. *J. Virol.* 2004, 78, 6657
174. Pray, T. R.; Reiling, K. K.; Demirjian, B. G.; Craik, C. S. *Biochemistry* 2002, 41, 1474
175. Nomura, A. M.; Marnett, A. B.; Shimba, N.; Dotsch, V.; Craik, C. S. *Nat. Struct. Mol. Biol.* 2005, 12, 1019
176. Marnett, A. B.; Nomura, A. M.; Shimba, N.; Ortiz de Montellano, P. R.; Craik, C. S. *Proc. Natl. Acad. Sci. U.S.A.* 2004, 101, 6870
177. Lee, G. M.; Shahian, T.; Baharuddin, A.; Gable, J. E.; Craik, C. S. *J. Mol. Biol.* 2011, 411, 999
178. Shahian, T.; Lee, G. M.; Lazic, A.; Arnold, L. A.; Velusamy, P.; Roels, C. M.; Guy, R. K.; Craik, C. S. *Nat. Chem. Biol.* 2009, 5, 640
179. Gable, J. E.; Lee, G. M.; Jaishankar, P.; Hearn, B. R.; Waddling, C. A.; Renslo, A. R.; Craik, C. S. *Biochemistry* 2014, 53, 4648
180. Stern-Ginossar, N.; Weisburd, B.; Michalski, A.; Le, V. T.; Hein, M. Y.; Huang, S. X.; Ma, M.; Shen, B.; Qian, S. B.; Hengel, H.; Mann, M.; Ingolia, N. T.; Weissman, J. S. *Science* 2012, 338, 1088

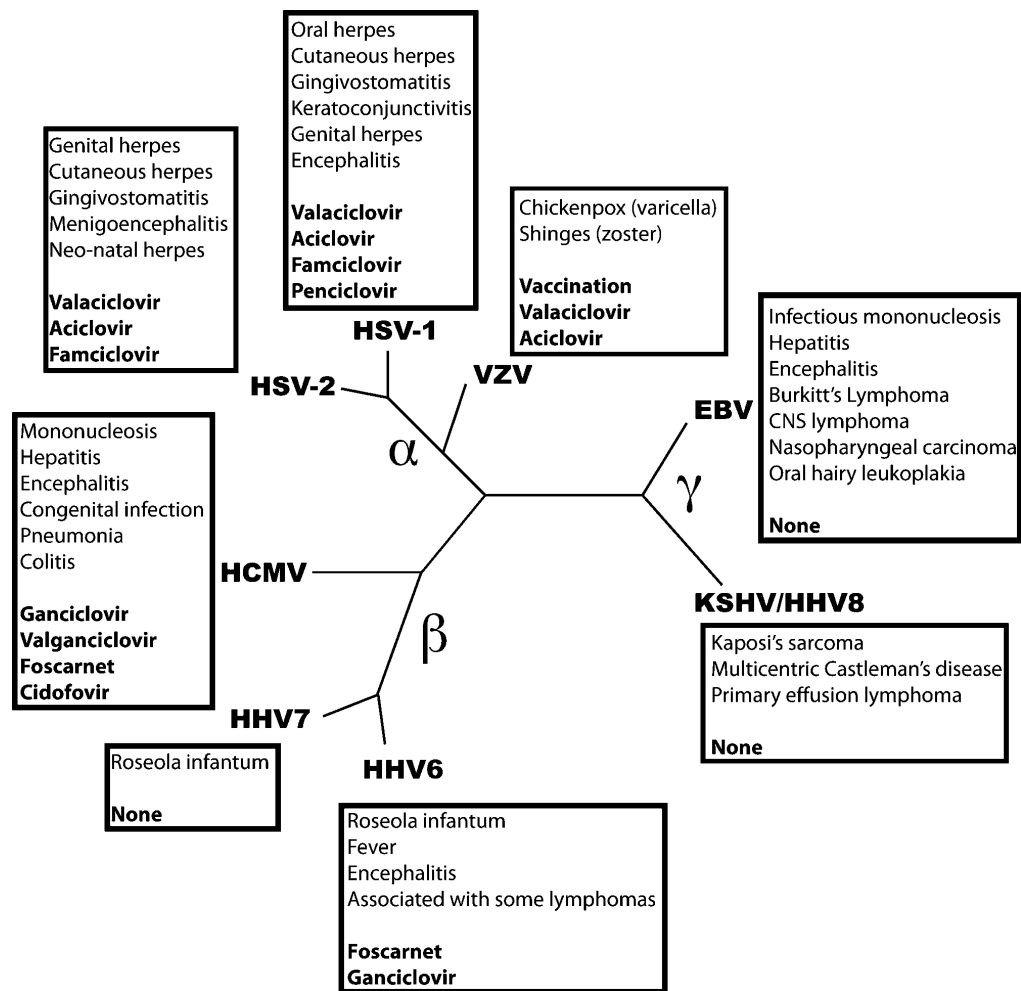


Figure 1-1. Human herpesviruses, diseases, and antiherpesvirus treatments organized by subfamilies.

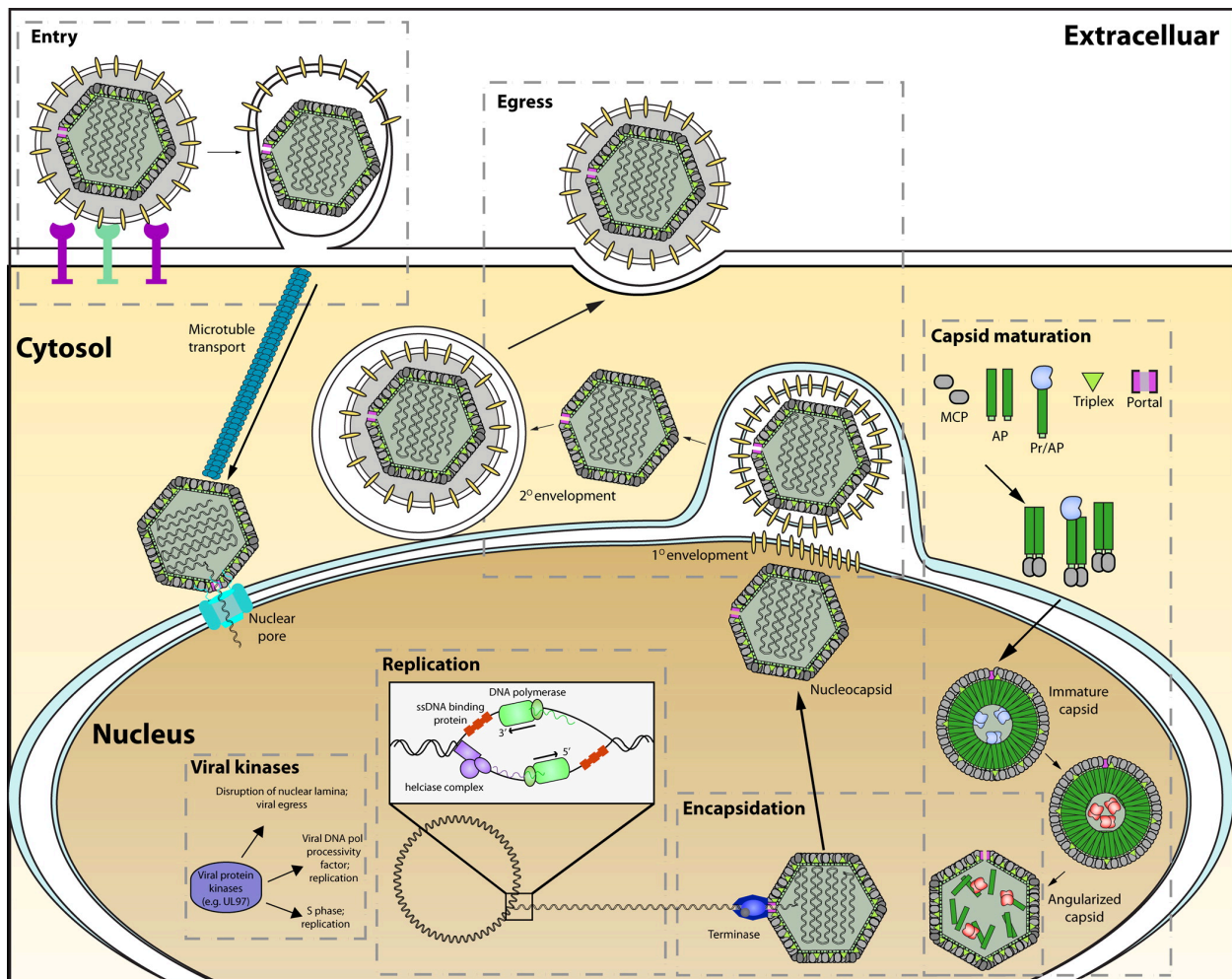


Figure 1-2. “Druggable” viral replication cycle. The processes of entry, viral DNA replication, encapsidation, capsid maturation, egress, and the role of viral kinases are depicted diagrammatically. Each process offers opportunities for inhibition of the viral replication cycle.

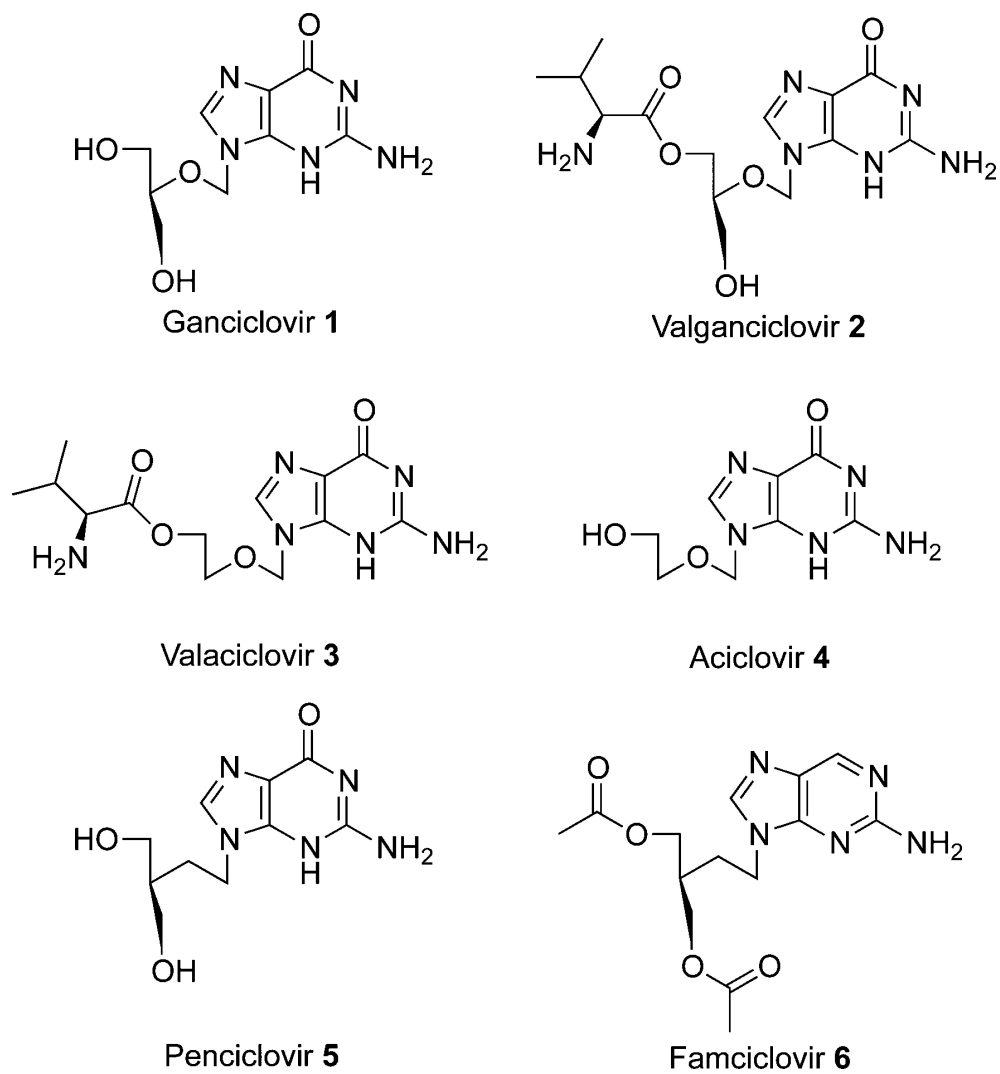


Figure 1-3. Approved guanosine analog inhibitors of herpesvirus replication (Figure 1-2, replication).

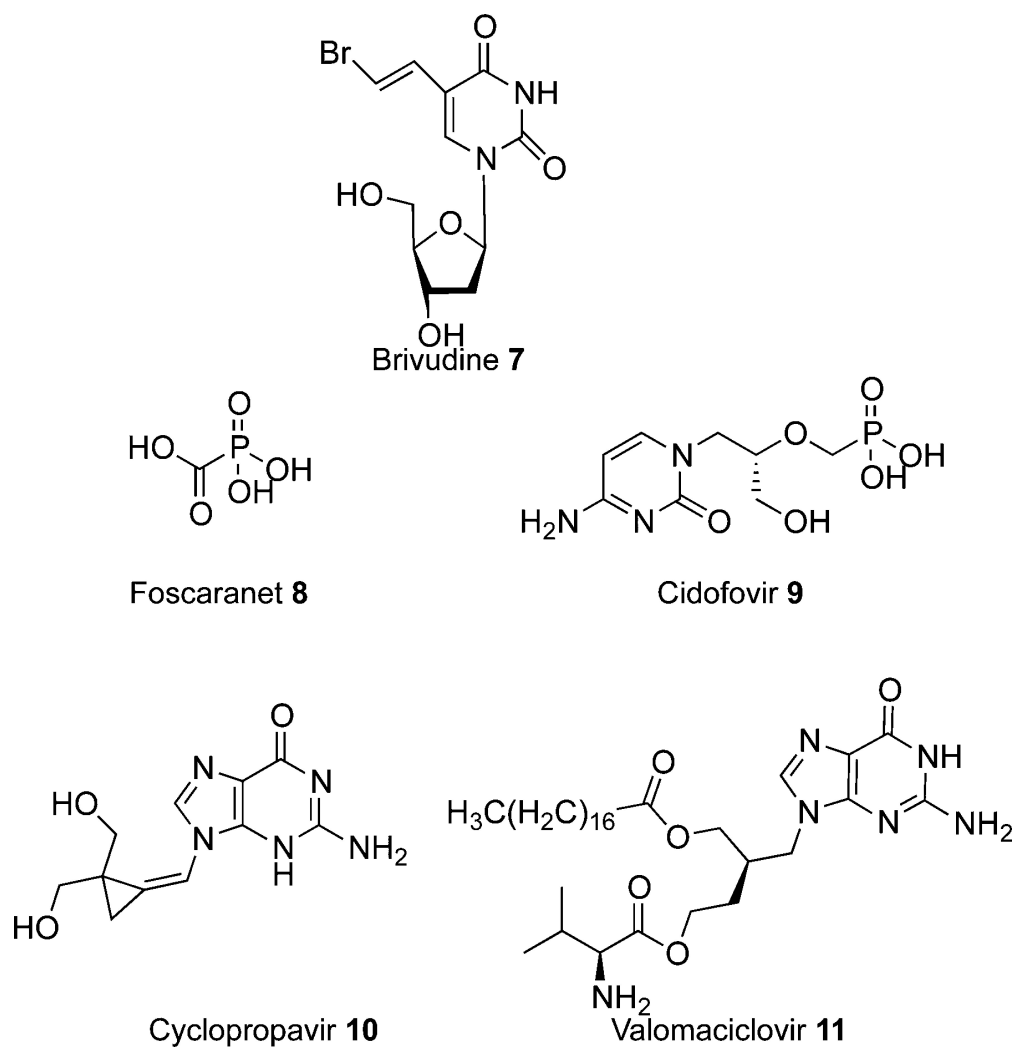


Figure 1-4. Additional inhibitors of the viral DNA polymerase (Figure 1-2, replication).

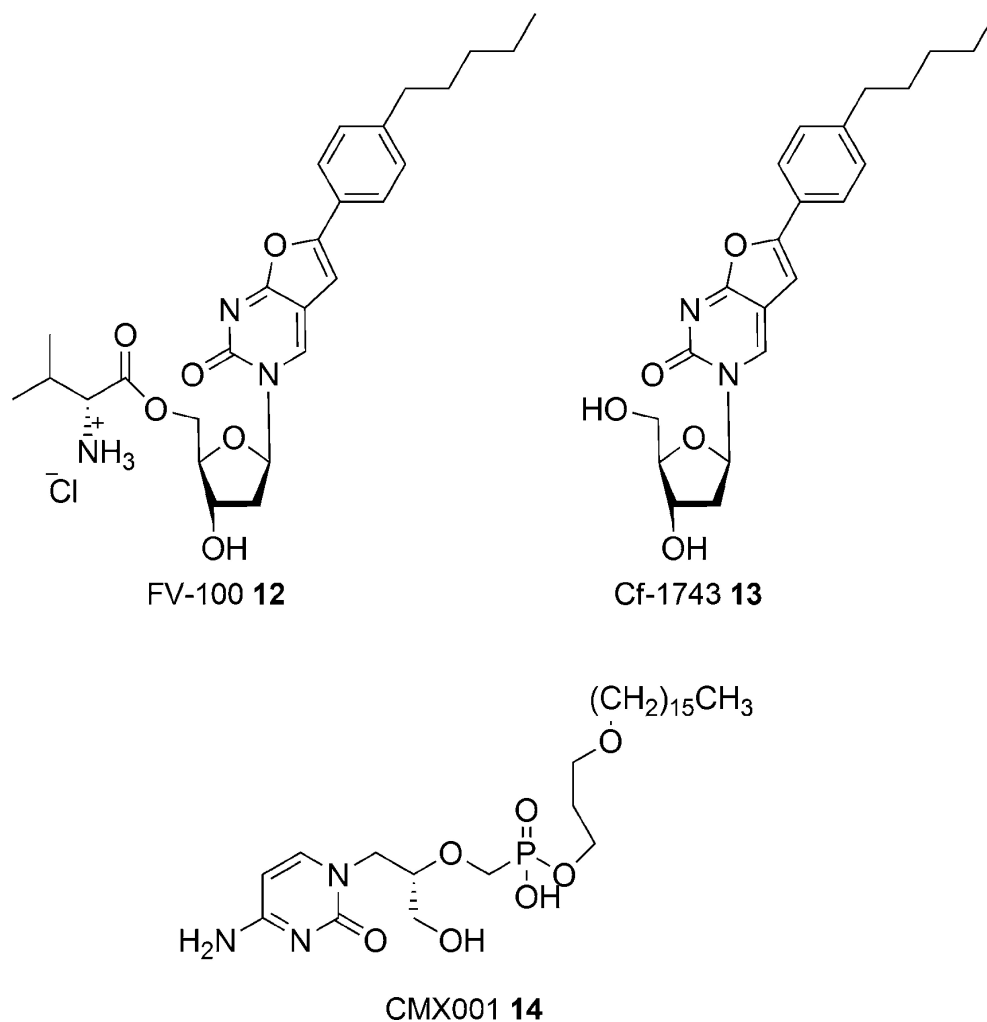


Figure 1-5. Bicyclic nucleoside analogs and a lipid ester analog of cidofovir. These inhibitors target viral replication (Figure 1-2, replication).

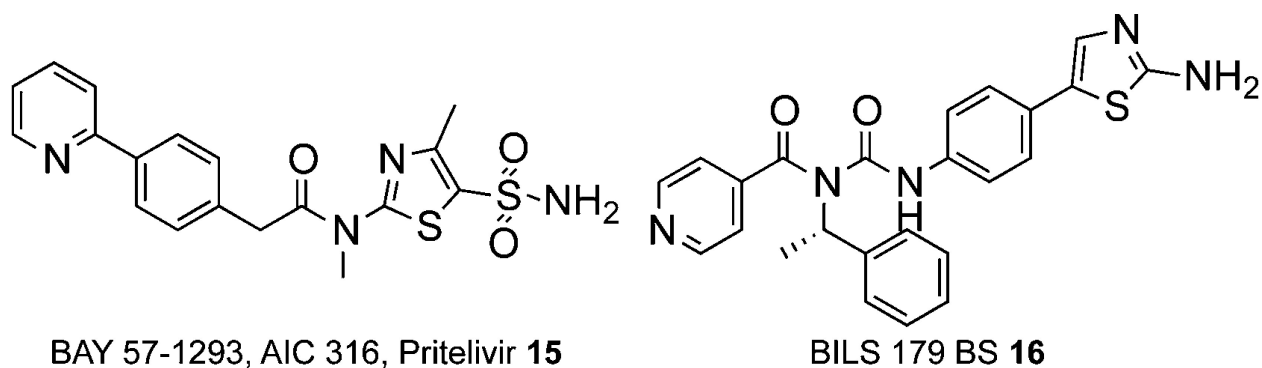
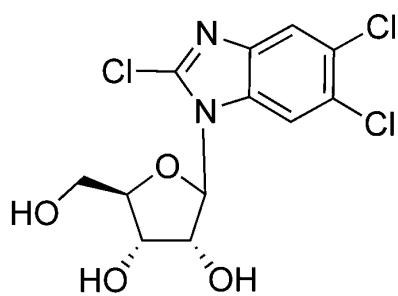
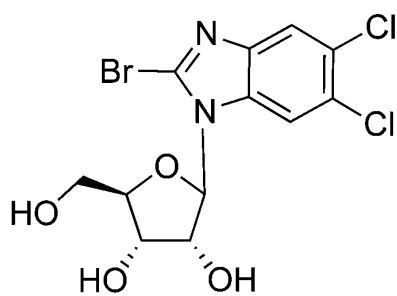


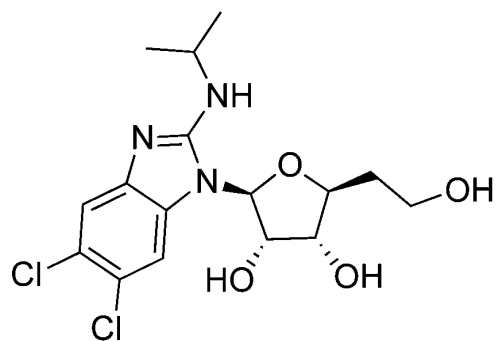
Figure 1-6. Helicase-primase inhibitors (Figure 1-2, replication).



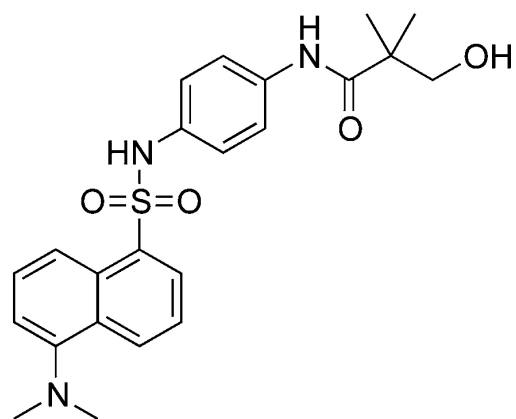
TCRB 17



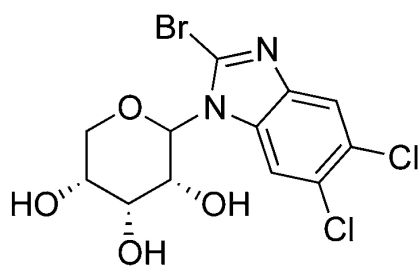
BDCRB 18



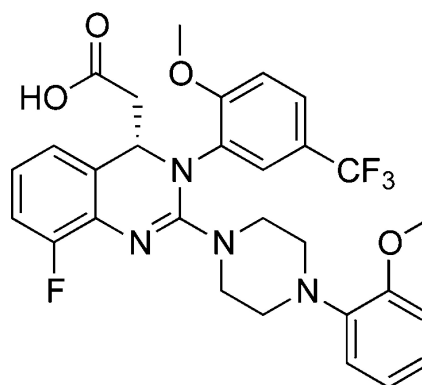
Maribavir 19



BAY 38-4766 20

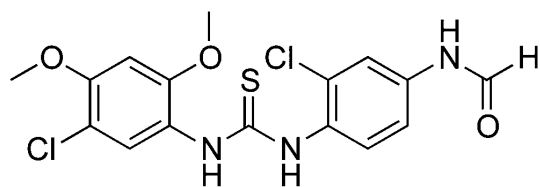


GW275175X 21

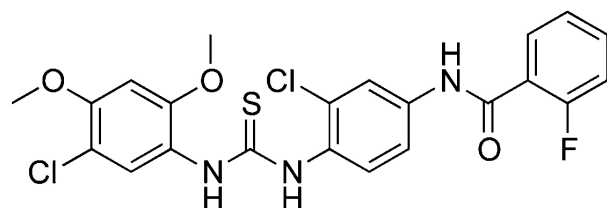


AIC246, Letemovir 22

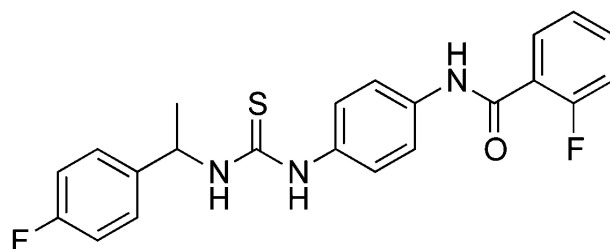
Figure 1-7. Terminase inhibitors (Figure 1-2, encapsidation).



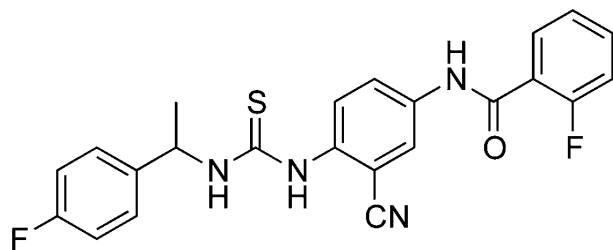
Thiourea **23**



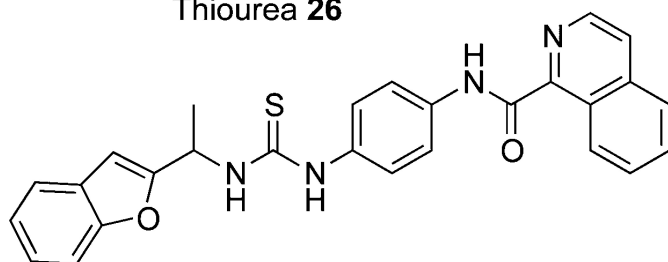
WAY-150138 **24**



Thiourea **25**

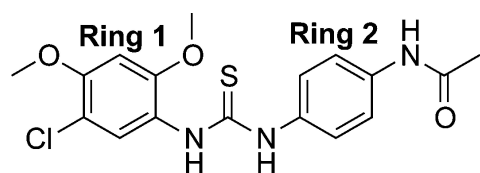


Thiourea **26**

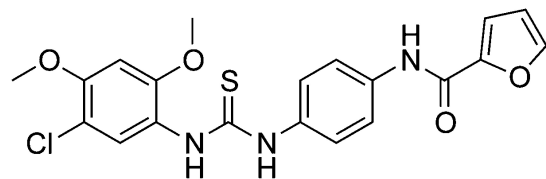


Thiourea **27**

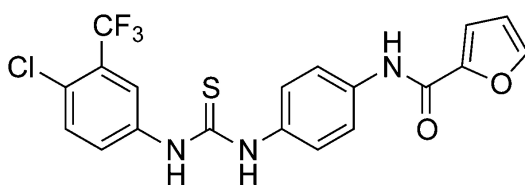
Figure 1-8. Portal vertex inhibitors (Figure 1-2, encapsidation).



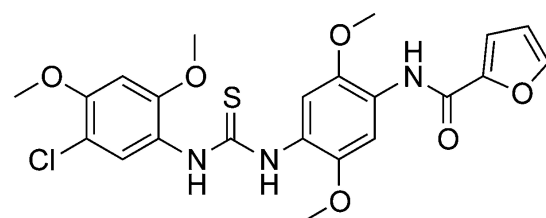
Thiourea 28



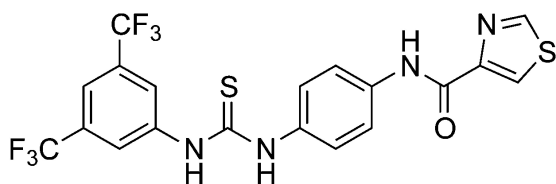
Thiourea 29



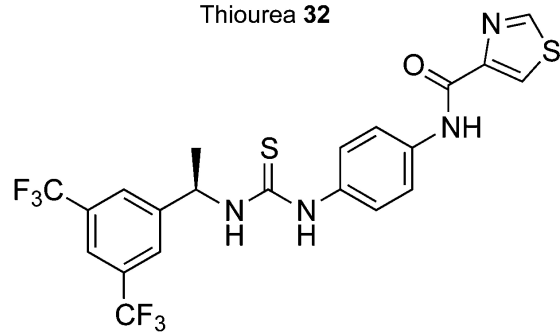
Thiourea 30



Thiourea 31



Thiourea 32



Thiourea 33

Figure 1-9. Putative HCMV fusion inhibitors (Figure 1-2, entry).

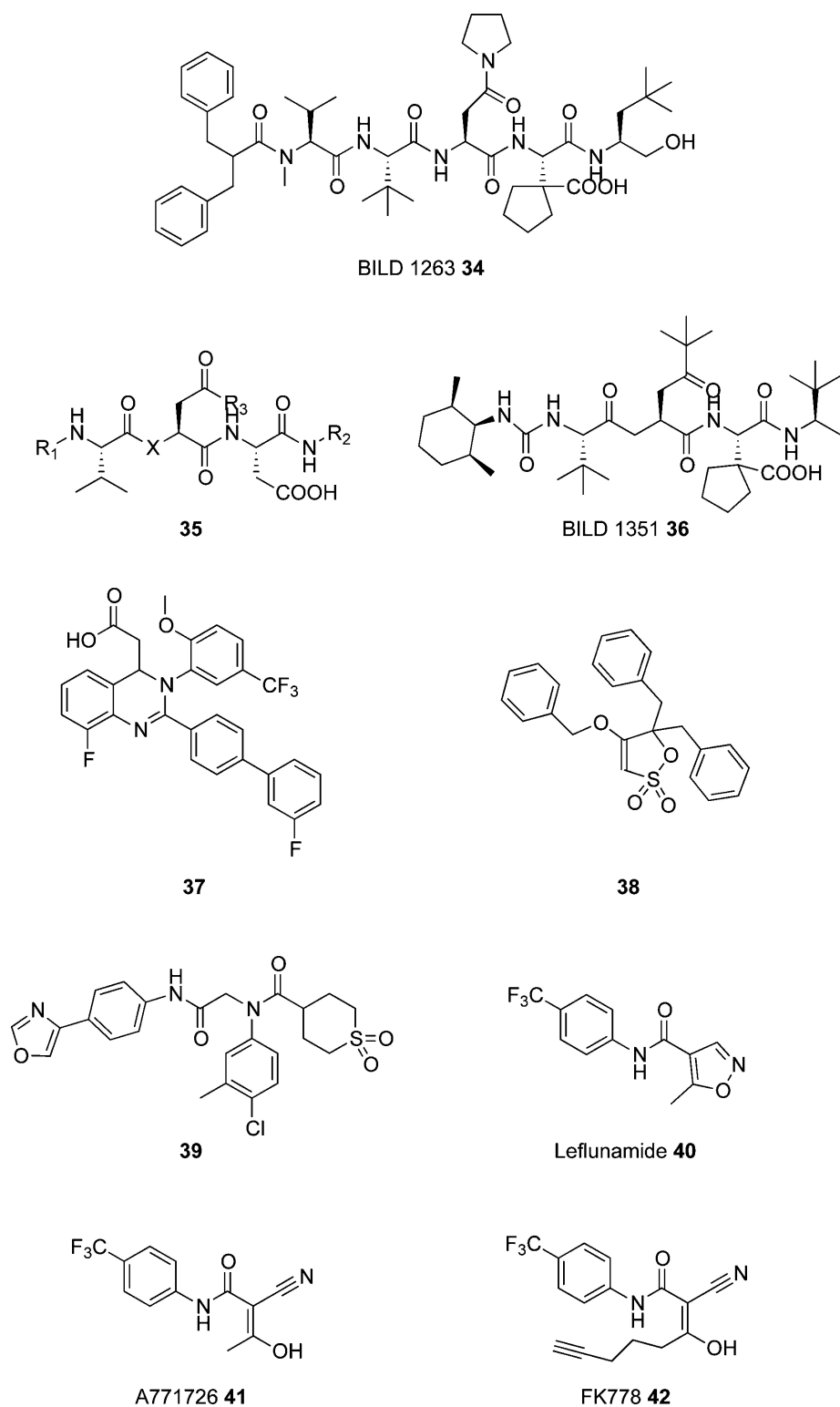
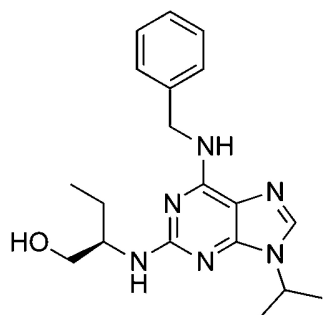
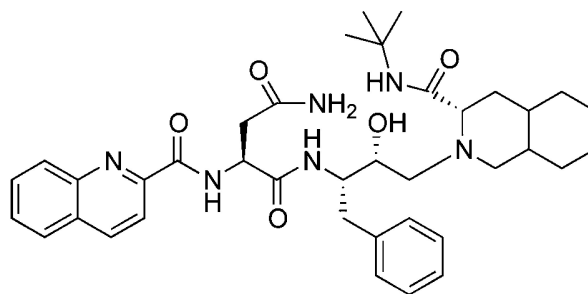


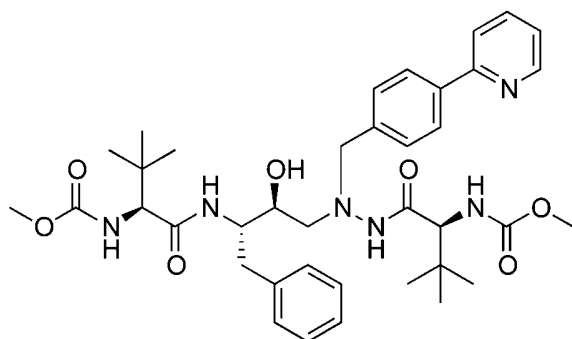
Figure 1-10. Ribonucleotide reductase protein-protein interaction inhibitors and inhibitors of unknown mechanism.



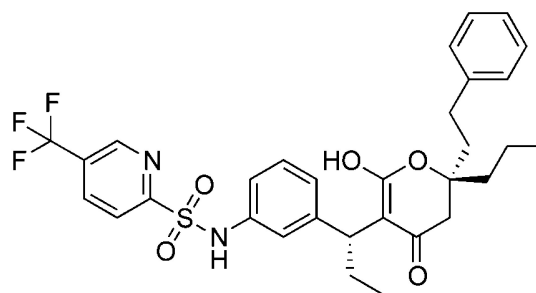
Roscovitine **43**



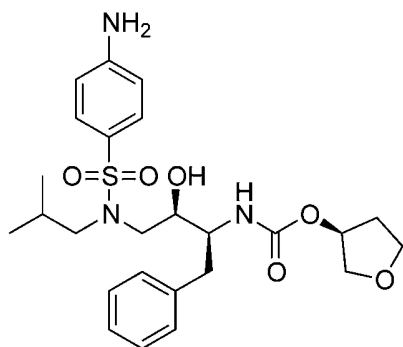
Saquinavir **44**



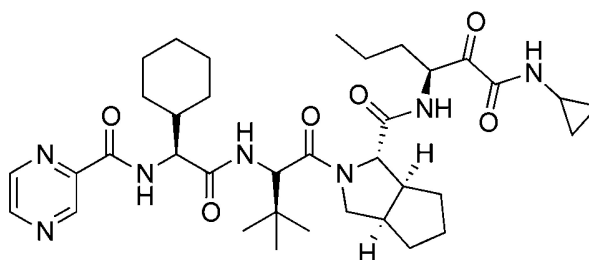
Atazanavir **45**



Tipranavir **46**



Amprenavir; Agenerase **47**



Telaprevir **48**

Figure 1-11. Roscovitine, a cyclin dependent kinase (CDK) inhibitor, and HIV and HCV protease inhibitors.

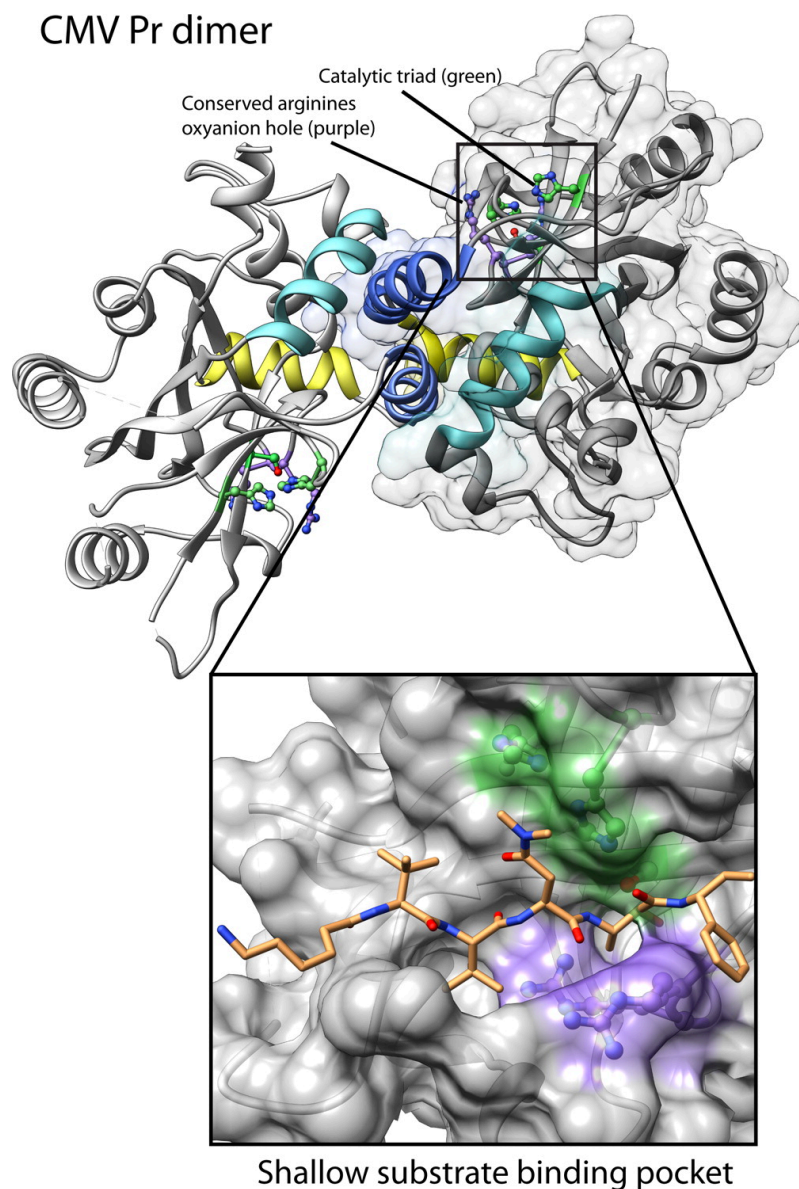


Figure 1-12. Dimeric herpesvirus protease and its shallow binding pocket. HCMV protease (PDB: 1NJU) is depicted. Helices 2 (cyan), 5 (blue), and 6 (yellow) are critical to dimerization and allosteric activation of the enzyme. The shallow binding pocket (inset) is shown with a peptidomimetic inhibitor bound. The catalytic triad (Ser-His-His) is shown in green and the conserved arginines of the oxyanion whole in purple.

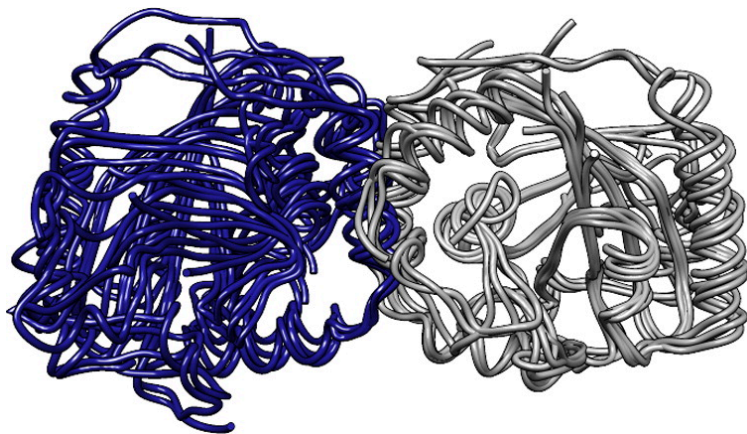
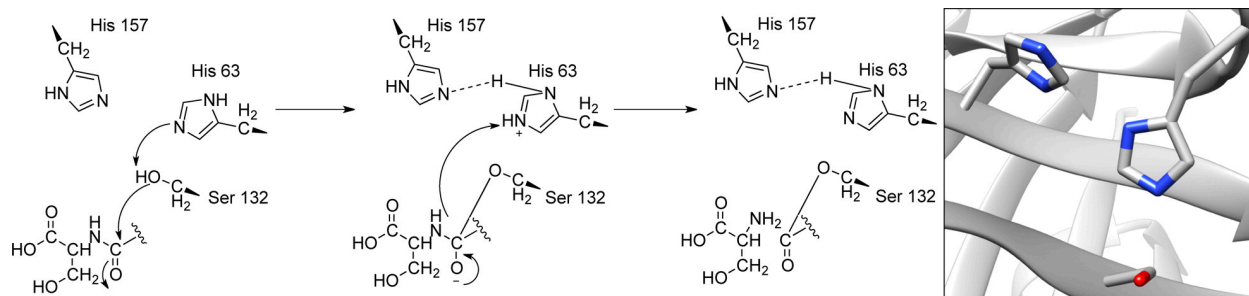


Figure 1-13. Overlay of known herpesvirus protease structures (HCMV, HSV2, EBV, VZV, and KSHV proteases).



Scheme 1-1. Herpesvirus protease catalytic mechanism and Ser-His-His Catalytic triad.

M- and R-site alignment logo

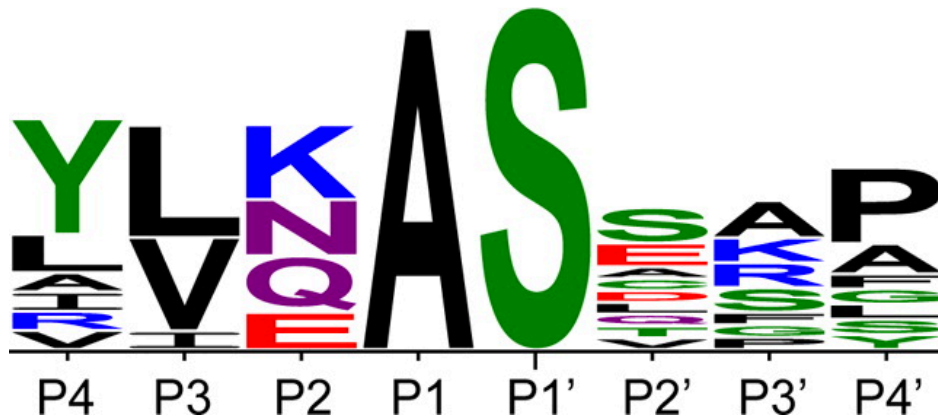
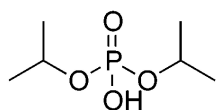
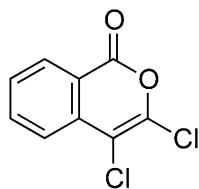


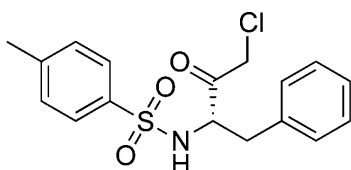
Figure 1-14. M- and R-site alignments for herpesvirus protease substrate specificity. WebLogo 3.3 was used to generate a specificity profile based on the M- and R-cleavage sites of KSHV, EBV, HHV-6, HCMV, HSV-1, and VZV assembly protein–protease fusion sequences.



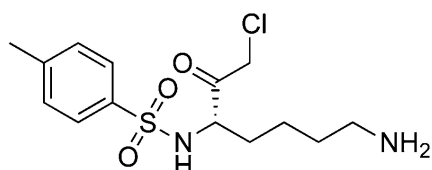
Diisopropyl Phosphate **49**



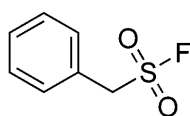
50



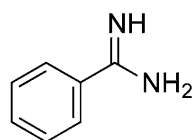
TPCK **51**



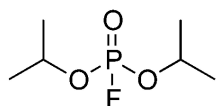
TLCK **52**



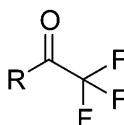
PMSF **53**



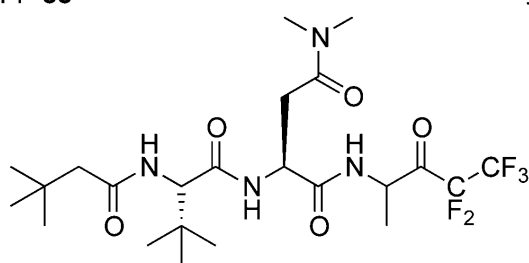
Benzamidine **54**



DFP **55**

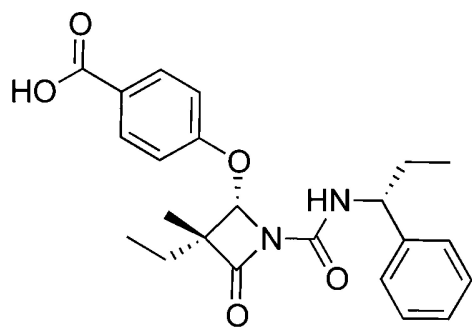


Trifluoromethyl ketone **56**

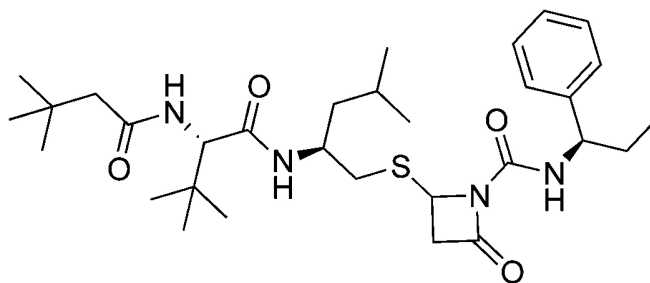


57

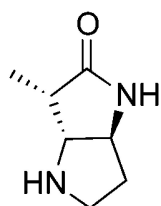
Figure 1-15. General protease inhibitors and an HCMV pentafluoroethyl ketone inhibitor (Figure 1-2, capsid maturation).



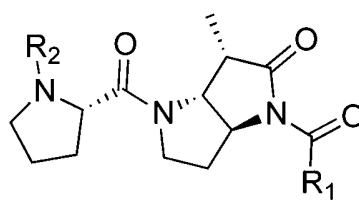
58



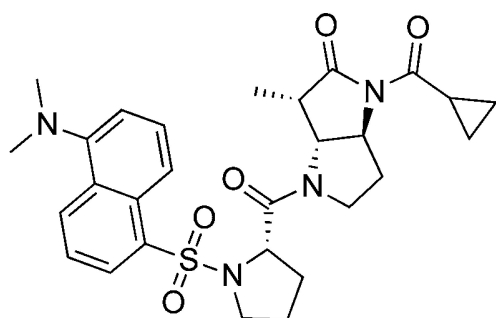
59



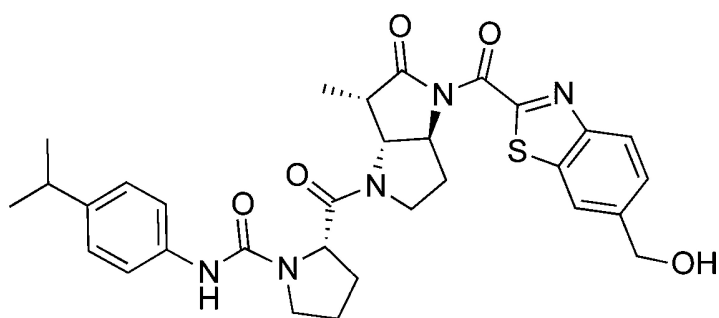
60



61



62



63

Figure 1-16. β - and *trans*-lactam herpesvirus protease inhibitors (Figure 1-2, capsid maturation).

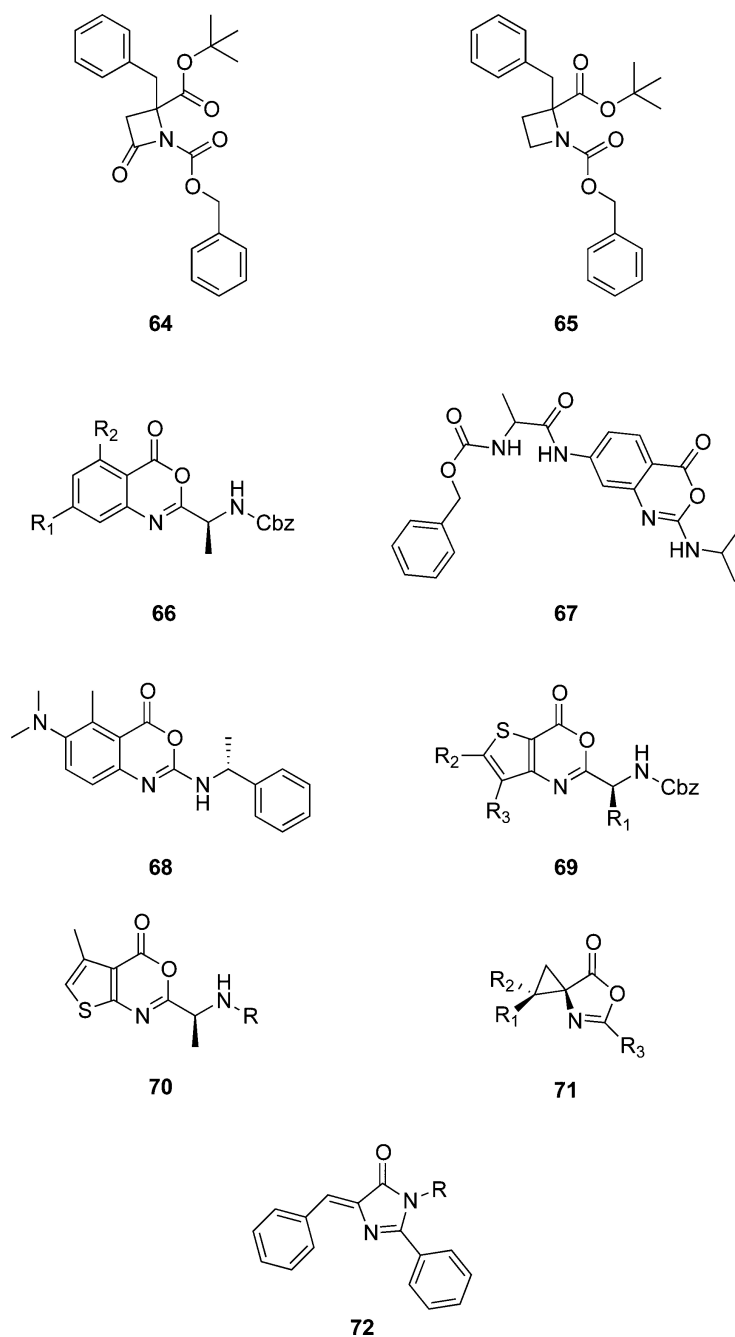


Figure 1-17. Azetidine, benzoxazinone, thieno[2,3-d]oxazinone, spirocyclopropyl oxazolones, and benzylidene N-sulphonyloxyimidazolone mechanism-based herpesvirus protease inhibitors (Figure 1-2, capsid maturation).

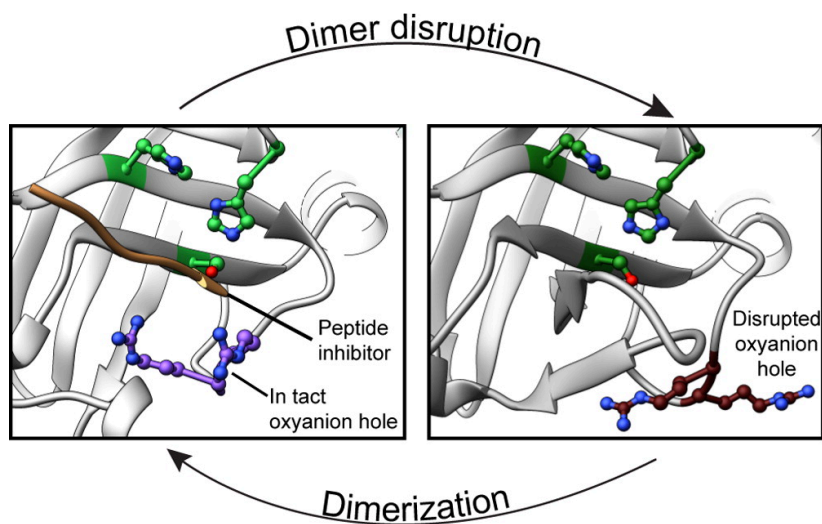


Figure 1-18. Order-to-disorder transition of conserved oxyanion hole arginines upon dimer disruption.

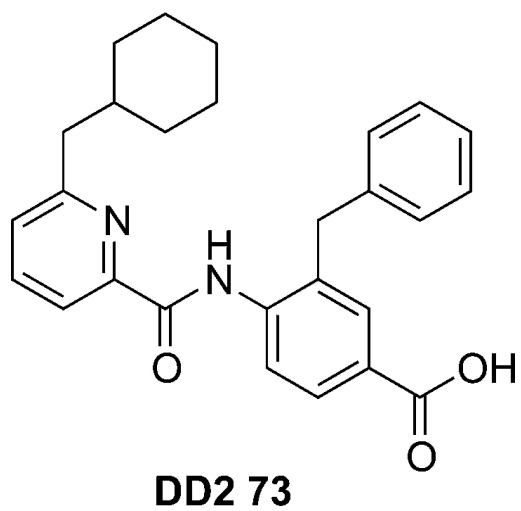
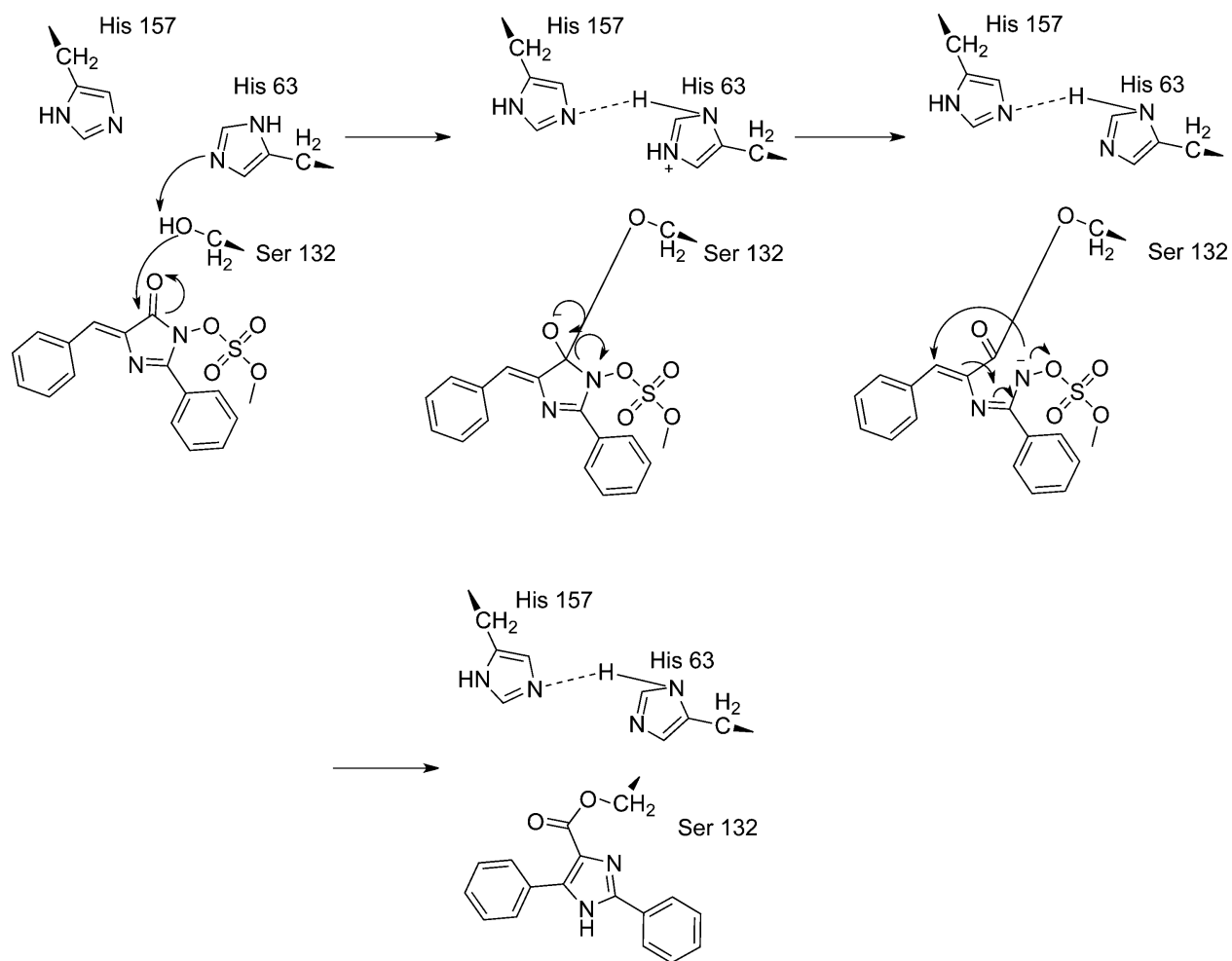


Figure 1-19. Dimer disruptor 2 (DD2), an allosteric herpesvirus protease inhibitor (Figure 1-2, capsid maturation).



Scheme 1-2. Potential mechanism of covalent protease inhibition by imidazolones.

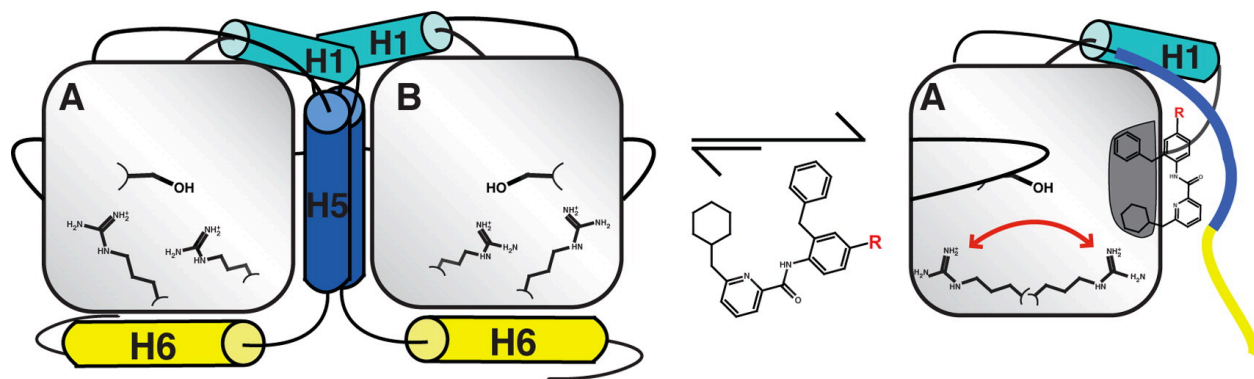


Figure 1-20. Proposed mechanism of dimer disruption and allosteric inhibition of herpesvirus proteases. Inhibitor binding in the core of the protein precludes folding of the C-terminal helices, preventing both dimerization and proper formation of the oxyanion holes formed by two conserved arginines. The catalytic serine is unperturbed, but the substrate binding site may be occluded. Reprinted from ref 176. Copyright 2014 American Chemical Society.

Table 1. Herpesvirus Genes and Their Associated Inhibitors^a

common name	HSV ORF	VZV ORF	HCMV ORF	HHV6/7 ORF	EBV ORF	KSHV ORF	inhibitor/ compd no.	function
DNA polymerase	UL30	28	UL54	38	BALF5	9	nucleoside analogs and related (comps 1–14)	viral DNA replication
processivity factor	UL54	16	UL44	27	BMRF1	59		viral DNA replication
helicase-primease: helicase	UL5	55	UL105	77	BBLF2	40	pritelivir (15), BILS 179 BS (16)	helicase activity, viral DNA replication
helicase-primease: ATPase	UL8	52	UL102	74	BBLF3	41		ATPase activity, viral DNA replication
helicase-primease: RNA polymerase (primase)	UL52	6	UL70	43	BSLF1	56		primase activity, viral DNA replication
ssDNA binding protein	UL29	29	UL57	U41	BALF2	6		viral DNA replication
ribonucleotide reductase large subunit	UL39	19	UL45	U28	BORF2	61	BILD 1263 and BILD 1351 (34, 36)	metabolism
ribonucleotide reductase small subunit	UL40	18	N/A	N/A	BarF1	60		metabolism
Major capsid protein	UL19	40	UL86	U57	BcLF1	25		capsid structure
capsid triplex protein 1	UL38	20	UL46	U29	BORF1	62		capsid structure
capsid triplex protein 2	UL18	41	UL85	U56	BdLF1	26		capsid structure
assembly protein (AP)	UL26.5	33.5	UL80.5			17.5		capsid assembly
maturational protease (PR)	UL26	33	UL80	U53	BVRF2	17	see Figures 15–17, 19	capsid assembly and DNA encapsidation
portal vertex	UL6	54	UL104	U76	BBRF1	43	WAY-150138 and related (23–27)	capsid structure and DNA encapsidation
terminase ATPase subunit 1	UL15	42, 45	UL89	U66	BGRF1 BDRF1	29	TCRB (17), BDCRB (18), BAY 38–4766 (20)	viral DNA replication and encapsidation, putative role in DNA translocation
terminase ATPase subunit 2	UL28	30	UL56	U40	BALF3	7	TCRB (17), BDCRB (18), BAY 38–4766 (20), GW275175X (21), AIC246 (22)	viral DNA replication and encapsidation, DNA binding and cleavage
terminase binding protein	UL33	25	UL51	UL35	BFRF1A	67		viral DNA replication and encapsidation
glycoprotein B	UL27	31	UL55	U39	BALF4	8	comps 28–33	fusion and entry, heparin-binding
CDK-like protein kinases	N/A	N/A	UL97	U69	BGLF4	36	cyclopropavir (10), maribivir (19)	multifunctional, prodrug activation
thymidine kinases	UL23	36	N/A	N/A	BXLF1	21		metabolism, prodrug activation

^aAdapted with permission from ref 76b. Copyright 2007 Cambridge University Press.

CHAPTER 2: A high-throughput screening pipeline for identification of herpesvirus protease protein-protein interaction inhibitors

Jonathan E. Gable, Chris Wilson, Michelle Arkin, Charles S. Craik

ABSTRACT

Nine human herpesviruses (HHVs) are the etiological agents for a variety of severe medical conditions with inadequate treatment options. All HHVs rely on a genetically validated conserved dimeric viral protease for replication. To date, there are no therapeutics targeting this protease nor compounds suitable for use in cell-culture as chemical tools. We have developed a high-throughput screening assay for Kaposi's sarcoma-associated herpesvirus protease utilizing an optimized fluorescent substrate as a readout of enzymatic activity. Inclusion of substrate in 100-fold molar excess of the enzyme and use of enzyme concentrations near the K_d for dimerization is intended to bias the screen toward the identification of dimer disruptors, for which we have identified a specific pocket. The screen achieved Z- and Z'-factor values consistently >0.5 in 384-well format. A total of 122K compounds and 3K fragments were screened against KSHV protease and numerous low-micromolar inhibitors were identified. This assay can be used for further screening of KSHV protease, but importantly can also be adapted to any of the other eight HHV proteases.

Introduction

Herpesviruses make up one of the most prevalent viral families including nine human types that cause a variety of severe illnesses.¹ All current treatments for herpesviral infection target a single process, viral DNA replication, and suffer from poor efficacy due to viral resistance mutations, require frequent intravenous injection, and/or cause severe side effects such as myelosuppression.^{2, 3} A dimeric viral protease is conserved across all HHVs and is critical for viral maturation, making it a potential therapeutic target.⁴⁻⁷ Efforts by pharmaceutical companies to target the active-site of the essential serine protease have yet to yield clinical leads.⁸⁻¹² Given the evidence supporting an allosteric link between protease dimerization and activation, we have focused our efforts on identifying molecules that target the dimer interface.^{3, 13-23} Furthermore, the dimerization affinity is weak with a reported K_d of 1.7 μ M for KSHV Pr.²² We have developed a high-throughput screen well suited for, but not limited to, the identification of these small-molecule protein-protein interaction inhibitors. The substrate used is an optimized hexapeptide attached to 7-amino-4-carbamoylmethyl coumarin (ACC), where cleavage at the scissile bond releases the ACC group resulting in increased fluorescence.¹⁴ We successfully used this assay in 96-well plate format to identify the small molecule inhibitor DD2 (**Fig. 3**), which binds a novel allosteric pocket at the dimer interface of KSHV Pr and traps it in an inactive monomeric state.^{14, 16, 22} The assay was then adapted to a 384-well format with the intent of finding new inhibitors, especially those directed at the DD2 pocket. The assay described here can readily be adapted to at least two other HHV proteases, cytomegalovirus (CMV) protease and Epstein Barr virus (EBV) protease. Medium-throughput NMR assays that allow for determination of dimer disruption are also described.

Materials and methods

Materials

Wild-type (WT) KSHV protease was expressed and purified as previously described.²² After purification protease was flash-frozen at a concentration $> 40 \mu\text{M}$ ($> 1 \text{ mg/mL}$) in assay buffer (described below) and stored at -80°C . The KSHV protease construct used contains the S204G mutation that prevents autolysis while maintaining full catalytic activity. The 7-amino-4-carbamoyl-methylcoumarin (ACC) fluorescent substrate (Ac-Pro-Val-Tyr-tBug-Gln-Ala-ACC) was purchased crude from Anaspec and purification was performed as previously described using standard high-performance liquid chromatography (HPLC).²⁴ Substrate was stored at a concentration of 10 mM in 100% DMSO at -20°C . Known inhibitor dimer disruptor 2 (DD2) was synthesized as previously described.²² Initial optimization was carried out in black round-bottom 96-well plates. Fluorescence high-throughput screening (HTS) assays were carried out in black 384-well microplates. Both plate formats were purchased from Corning Life Sciences (Acton, MA). During initial assay optimization fluorescence measurements monitoring activity were taken on the Spectra MAX Gemini EM Fluorescence Microplate Reader from Molecular Devices (Sunnyvale, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Assay conditions

All optimized fluorescence assays were conducted in a buffer with the following composition: 25 mM potassium phosphate pH 8, 150 mM KCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, and a final DMSO concentration of 1-2% (v/v) after substrate and inhibitor addition. Prior to the addition of substrate or compound, concentrated stock protease was diluted into assay buffer to a final concentration of $1.2 \mu\text{M}$. After the addition of 50 nL or 1 μL DMSO stock compound for 384-well and 96-well plates, respectively, plates were incubated at 30°C for

30 minutes. The reaction was started with the addition of substrate and measurements were made at 30 °C.

Optimization for HTS

Optimization for automated HTS was carried out on a screening system integrated by High Resolution Engineering (Woburn, MA). A Staubli T60 robot arm was used to transfer plates from instrument to instrument. Assay buffer containing protease was dispensed in bulk using Matrix Wellmates (Matrix Technologies, Hudson, NH). An on-line dispenser performed substrate addition. Compound transfers were performed using a 384-well pin tool with 10 nL hydrophobic surface-coated pins (V&P Scientific, San Diego, CA). These pins transferred on average 25 nL of compound stock in DMSO with coefficients of variation of less than 10%. Fluorescence measurements were made using an EnVision Multilabel Plate Reader (PerkinElmer) with an excitation wavelength of 380 nm and a static and polarized 480 nm filter.

Automation

High-throughput screening was performed at the UCSF Small Molecule Discovery Center (SMDC). A WellMate bulk dispenser (Thermo Scientific) was used to dispense 20 µL of protease assay master-mix (columns 2-22 and 24), and master-mix containing positive control inhibitor DD2 (30 µM, columns 1 and 23), to barcoded 384 well black fluorescence plates (Corning #3820).

High-throughput kinetic assays were performed using an automated screening platform available at SMDC. Fifty nanoliters of compounds (10 or 50 mM in 100% DMSO) were added by 384 slotted pin-tool (VP Scientific) to pre-dispensed assay plates, using a Biomek FXP liquid handler (Beckman Coulter). Seven assay plates were prepared sequentially in this manner, and equilibrated at 30 °C in a temperature and humidity controlled plate incubator. Protease reaction

was initiated in the first assay plate after 30 minutes, through addition of 5 μ l labeled peptide substrate (P6) from an EL406 bulk dispenser (BioTek Instruments). Final reaction conditions were: 25 μ l per well, 20 or 100 μ M test compound, 0.2% (v/v) DMSO.

Sample fluorescence was measured immediately following, and then repeatedly in sequence for every plate, with an Envision 2104 multi-mode reader (Perkin Elmer; monochromator Ex = 380 nm/ Em = 435 nm, 30°C chamber temperature). The average cycle time (kinetic data interval) of an assay plate was 20 minutes.

Data upload, processing and presentation through a web-based interface (QB3 HiTS) was performed through Pipeline Pilot (Accelrys Biovia). Raw fluorescence signal, plate barcode ID and embedded measurement time-stamp were extracted, and reaction rate (fluorescence change per minute) derived from a linear fit. Per-plate Z' assay performance statistics were calculated from positive (cols 1 & 23) and negative (cols 2 & 24) control wells. Test compound reaction rates were normalized into percent inhibition between positive (100% inhibition) and negative (0% inhibition) control limits. Initial hit selection used a lower inhibition limit defined by "Mean test compound inhibition + (3SD of Mean)": small molecules with percent inhibition greater than Mean+3SD were prioritized for cherry picking and further study in dose response.

Compound library screened

A pilot screen was performed with the first seven plates of the ChemBridge Premium set. In total a diverse library was screened consisting of 23,762 compounds from the ChemBridge library, 50,000 compounds from the ChemDiv library, 30,106 compounds from the SPECS library, and 30,000 compounds from the ChemBridge Premium library. Fragments screened were the Maybridge RO3 library (995 fragments), the Life Chemicals library (1465 fragments), and the Asinex natural-product like molecules (259 fragments).

Triage

A 3σ cutoff was used to define hit compounds. These were then analyzed for autofluorescence artifacts by plotting the “time 0” measurement versus percent inhibition. Compounds whose inhibition could be explained by autofluorescence were discarded (Figure 2-3). Compounds with known fluorescence quenching moieties such as nitrophenyl were discarded.²⁵ Automated dose response was performed on the remaining 352 compounds. Each of seven plates contained a different concentration of all 352 compounds. The conditions and screening protocol were the same as the primary screen, again collecting 5 time points after substrate addition for each plate from which a slope is computed. IC_{50} values were determined from fitting percent inhibition vs compound concentration to the Hill equation. For compounds likely to undergo aggregation (high cLogP, high cLogSW) dynamic light scattering was performed on a DynaPro MS/X equipped with a 55 mW laser at 826.6 nm. The integration time was 100 s (10x10 sec) and laser power was 100% unless reduction in laser power was necessary to avoid saturating the detector. The detector angle was 90 degrees. Measurements were performed at 25 degrees Celsius.

Results

Optimization for high-throughput screening

In order to screen large small-molecule libraries our existing 96-well fluorogenic activity assay was adapted to a 384-well format. The reaction volume was reduced from 100 μ l to 25 μ l. The volumes of the protease, control compounds, and substrate were 19 μ l, 1 μ l, and 5 μ l, respectively. Optimal protease and substrate concentrations were determined to be 1.2 and 100 μ M, respectively. These concentrations resulted in >60 minutes of robust linear signal. At concentrations of up to 5% DMSO no significant change in enzymatic activity was observed

(data not shown). High and Low signal control assays were run in parallel on a 384-well plate, with at least one entire column allocated to each. Initial optimization achieved a Z' of >0.6 with a 60 minute reaction, immediate quenching with 25 μ L of 100% DMSO, and centrifugation prior to acquiring an endpoint measurement. As a prerequisite to HTS in a 384-well plate format, signal drift and plate uniformity were assessed across the entire plate.

Out of concern regarding potential spectrophotometric interference from compounds given the low excitation wavelength for coumarin (Ex: 380 nm, Em:460 nm) we pursued a screening approach in which multiple time points were taken rather than a single endpoint assay. Using automation we were able to design a protocol wherein an initial measurement was taken immediately after substrate addition after which four additional measurements were made roughly every 15 minutes (timestamps allowed for accurate velocity determination). This scheme resulted in a velocity for each well in the assay. Use of these velocities rather than endpoint achieved $Z' >0.7$ (Figure 2-1) and provided a means by which artifacts could be rapidly identified and removed.

Primary screen data analysis

Hits from the primary screen of 122,000 compounds and 3,000 fragments were defined as those that at 20 μ M compound or 100 μ M fragment exhibited greater than 18% inhibition ($>3\sigma$ more inhibition than DMSO alone, Figure 2-2). This resulted in 1013 hits (0.81% hit rate).

For the 1013 hits identified a plot of autofluorescence (fluorescence immediately after substrate addition) vs. percent inhibition yielded a striking correlation. Many compounds appeared to be hits due largely if not solely to their fluorescence properties (Figure 2-3). Compounds of interest are those that have very little autofluorescence and/or fall above the prediction bands. The extent of inhibition observed for these compounds is unlikely to be

explained by their background fluorescence alone. Of the 1013 hits 473 of them were removed due their background fluorescence. This approach may have led to some false negatives. Some compounds also exhibited fluorescence quenching. Compounds containing a nitrophenyl group are known quenchers of coumarin and were observed to quench our substrate in this assay. The 352 compounds that remained after filtering for autofluorescence and fluorescence quenching progressed to an automated confirmation screen consisting of IC₅₀ determination. Ten compounds and fragments showed a clear dose response (Table 2-1).

Trisubstituted pyrazoles

Use of Murcko fragment analysis revealed that many of the trisubstituted pyrazoles in the library were also hits, even after extensive filtering (Figure 2-4).^{26, 27} Of the 60 trisubstituted pyrazoles screened 38 were hits (63% hit rate as compared to an overall 0.8% hit rate). This was an intriguing result due to their similarity to the nutlin scaffold, a known scaffold for protein-protein interaction inhibition of p53-mdm2, which is also dominated by a helical interaction much like that of the KSHV protease dimer interface.²⁸ However, given the possible conformers of these molecules and the geometry of the DD2 pocket we hypothesized that a disubstituted pyrazole would be sufficient for inhibition. Removing one of the phenyl groups would improve solubility and, if potency was maintained, also ligand efficiency.

A selection of commercially available disubstituted pyrazoles were purchased and tested for protease inhibition. Of the nine initially purchased, only disubstituted pyrazole **2** showed a dose response with an IC₅₀ of about 600 μ M (Figures 2-5 and 2-6a). Based on this, additional disubstituted pyrazoles were purchased resembling disubstituted pyrazole **2** (Figure 2-5, bottom). Of these, disubstituted pyrazoles **11** and **13** exhibited a dose response against KSHV protease

(Figure 2-6). These compounds also inhibited Epstein Barr Virus and CMV proteases (Figure 2-6).

NMR was used to determine whether the disubstituted pyrazoles disrupt dimerization and if so, where binding occurs. $^{13}\text{C}/^1\text{H}$ HSQC of selectively labeled ^{13}C methionine wildtype KSHV protease showed dimer disruption in the presence of disubstituted pyrazoles **2**, **11**, and **13**. In the presence of these compounds the Met197 dimer peak disappears (broadens below the detection limit) and the Met197 monomer peak shifts as shown for representative **2** in Figure 2-7a. $^{13}\text{C}/^1\text{H}$ HSQC of truncated obligate monomeric KSHV Δ 196 selectively labeled with ^{13}C isoleucine shows chemical shift perturbations of Ile105 and Ile44 but not the remaining isoleucines. This indicates direct binding to the previously identified DD2 binding pocket. Using $^{15}\text{N}/^1\text{H}$ HSQC of uniformly labeled KSHV Δ 196 binding we mapped binding to the DD2 pocket, consistent with the ^{13}C Ile results (Figure 2-7c,d).

Given the high concentrations of compound used in these experiments we were concerned about the potential for aggregation, despite the specific perturbations observed by NMR.²⁹ Indeed, mild but detectable uniform peak broadening was apparent in the ^{13}C Ile experiments consistent with some degree of aggregation or loss of protein (Figure 2-7b). Dynamic light scattering experiments revealed that all three disubstituted pyrazoles exhibited some aggregation at or above 100 μM , with **2** exhibiting the least aggregation (Table 2-3). Disubstituted pyrazoles **4** and **8** exhibited aggregate formation similar to that of **2** but did not inhibit, suggesting the inhibition with **2** may be specific. Disubstituted pyrazole **2** inhibited trypsin at greater than 200 μM , supporting some degree of non-specific inhibition. The addition of 0.1% CHAPS restored trypsin activity at concentrations of up to 600 μM **2**. In the presence of 0.1% CHAPS colloid size increased consistent with detergent coating the colloidal aggregate

thus preventing adherence of protein to the colloid. Disubstituted pyrazoles **2**, **11**, and **13** exhibited less inhibition of KSHV protease in the presence of 0.1% CHAPS, but still inhibited to a significant degree (data not shown, see JEG_GM120529). The addition of 0.1% Prionex carrier protein that was present in the primary screen also reduced but did not eliminate inhibition by these compounds.

Discussion

We have established a robust high-throughput screening pipeline including validation of protein-protein interaction inhibition by NMR for the identification of KSHV protease inhibitors. The screen of 125K molecules identified multiple low-micromolar hits. SAR by catalogue based on fragments identified dimer disruptors that bind to the previously identified interfacial pocket of KSHV protease.

While the disubstituted pyrazoles identified through SAR by catalogue provide a potential starting point for the development of dimer disruptors we were surprised by the lack of protein-protein interaction inhibitors identified in our screen. Part of this is likely due to bias inherent in the screening deck used, and indeed in most screening decks, where “typical” drug targets such as kinases drive the selection of compounds for inclusion in these commercially available libraries, rather than a focus on molecules likely to bind to and disrupt protein-protein interfaces.³⁰ High concentration of substrate was used in this screen (10X K_M) in order to out-compete potential active site inhibitors, thereby biasing the screen toward protein-protein interaction antagonists. However, in retrospect, this high concentration of substrate likely promoted dimerization making it difficult to identify any inhibitors and leaving primarily reactive compounds as hits. Compound **1** contains a thiazolidinone with a double-bond in the 5-position comprising a Michael acceptor. Similar compounds are reported inhibitors of cruzipain,

a protease, as well as aldehyde dehydrogenase 1A1 and inositol monophosphatase 1 (ChEMBL 1599746). Compound **2** has a very electron deficient aldehyde carbon that could undergo nucleophilic attack. The acid anhydride in **3** is highly reactive. Compound **4** has no known targets reported in ChEMBL and while the aldehyde is again of some concern, this molecule seems relatively benign but is also a weak inhibitor with an IC₅₀ of 100 μ M. The remaining hits, **5-10**, would require additional follow-up to ensure their mechanism of inhibition is not artifactual, especially given their high IC₅₀ values.

The disubstituted pyrazoles highlight the complexity of protein-protein interaction antagonist discovery and high throughput screening in general. These compounds exhibit both non-specific and specific inhibition of KSHV protease. NMR spectroscopy clearly identified specific binding to the interfacial DD2 pocket while simultaneously suggesting some degree of aggregation, observed as uniform broadening in the HSQC. Dynamic light scattering confirmed aggregation/colloid formation and tests against trypsin showed non-specific inhibition. The inclusion of detergent restored but not all proteolytic activity for KSHV protease, consistent with a combination of specific and non-specific inhibition.

The screen presented here can be readily modified for use with at least CMV and EBV proteases. Reduction in the concentration of substrate and inclusion of CHAPS as well as Prionex could help identify specific dimer disruptors. This report represents both a case study in the challenging task of screening for protein-protein interaction antagonists and a highly robust pipeline for the identification of a novel class of HHV protease inhibitors.

References

- [1] Wald, A., and Corey, L. (2007) Persistence in the population: epidemiology, transmission, In *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (Arvin, A., Campadelli-Fiume, G., Mocarski, E., and al., e., Eds.), Cambridge University Press, Cambridge, Cambridge.
- [2] Coen, D. M., and Schaffer, P. A. (2003) Antiherpesvirus drugs: a promising spectrum of new drugs and drug targets, *Nat. Rev. Drug Discov.* 2, 278-288.
- [3] Gable, J. E., Acker, T. M., and Craik, C. S. (2014) Current and Potential Treatments for Ubiquitous but Neglected Herpesvirus Infections, *Chem. Rev.*
- [4] Tong, L. (2002) Viral proteases, *Chem. Rev.* 102, 4609-4626.
- [5] Gao, M., Matusick-Kumar, L., Hurlburt, W., DiTusa, S. F., Newcomb, W. W., Brown, J. C., McCann, P. J., 3rd, Deckman, I., and Colonno, R. J. (1994) The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth, *J. Virol.* 68, 3702-3712.
- [6] Matusick-Kumar, L., McCann, P. J., 3rd, Robertson, B. J., Newcomb, W. W., Brown, J. C., and Gao, M. (1995) Release of the catalytic domain N(o) from the herpes simplex virus type 1 protease is required for viral growth, *J. Virol.* 69, 7113-7121.
- [7] Preston, V. G., Coates, J. A., and Rixon, F. J. (1983) Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA, *J. Virol.* 45, 1056-1064.
- [8] Borthwick, A. D., Crame, A. J., Ertl, P. F., Exall, A. M., Haley, T. M., Hart, G. J., Mason, A. M., Pennell, A. M. K., Singh, O. M. P., Weingarten, G. G., and Woolven, J. M. (2002) Design and synthesis of pyrrolidine-5,5-trans-lactams (5-oxohexahydropyrrolo[3,2-

- b]pyrroles) as novel mechanism-based inhibitors of human cytomegalovirus protease. 2. Potency and chirality, *Journal of Medicinal Chemistry* 45, 1-18.
- [9] Borthwick, A. D., Davies, D. E., Ertl, P. F., Exall, A. M., Haley, T. M., Hart, G. J., Jackson, D. L., Parry, N. R., Patikis, A., Trivedi, N., Weingarten, G. G., and Woolven, J. M. (2003) Design and synthesis of pyrrolidine-5,5'-trans-lactams (5-oxo-hexahydropyrrolo[3,2-b]pyrroles) as novel mechanism-based inhibitors of human cytomegalovirus protease. 4. Antiviral activity and plasma stability, *Journal of Medicinal Chemistry* 46, 4428-4449.
- [10] Borthwick, A. D., Exall, A. M., Haley, T. M., Jackson, D. L., Mason, A. M., and Weingarten, G. G. (2002) Pyrrolidine-5,5-trans-lactams as novel mechanism-based inhibitors of human cytomegalovirus protease. Part 3: potency and plasma stability, *Bioorg. Med. Chem. Lett.* 12, 1719-1722.
- [11] Ogilvie, W., Bailey, M., Poupart, M. A., Abraham, A., Bhavsar, A., Bonneau, P., Bordeleau, J., Bousquet, Y., Chabot, C., Duceppe, J. S., Fazal, G., Goulet, S., Grand-Maitre, C., Guse, I., Halmos, T., Lavallee, P., Leach, M., Malenfant, E., O'Meara, J., Plante, R., Plouffe, C., Poirier, M., Soucy, F., Yoakim, C., and Deziel, R. (1997) Peptidomimetic inhibitors of the human cytomegalovirus protease, *J. Med. Chem.* 40, 4113-4135.
- [12] Waxman, L., and Darke, P. L. (2000) The herpesvirus proteases as targets for antiviral chemotherapy, *Antivir. Chem. Chemother.* 11, 1-22.
- [13] Batra, R., Khayat, R., and Tong, L. (2001) Molecular mechanism for dimerization to regulate the catalytic activity of human cytomegalovirus protease, *Nat. Struct. Biol.* 8, 810-817.

- [14] Gable, J. E., Lee, G. M., Jaishankar, P., Hearn, B. R., Waddling, C. A., Renslo, A. R., and Craik, C. S. (2014) Broad-spectrum allosteric inhibition of herpesvirus proteases, *Biochemistry* 53, 4648-4660.
- [15] Lazic, A., Goetz, D. H., Nomura, A. M., Marnett, A. B., and Craik, C. S. (2007) Substrate modulation of enzyme activity in the herpesvirus protease family, *J. Mol. Biol.* 373, 913-923.
- [16] Lee, G. M., Shahian, T., Baharuddin, A., Gable, J. E., and Craik, C. S. (2011) Enzyme inhibition by allosteric capture of an inactive conformation, *J. Mol. Biol.* 411, 999-1016.
- [17] Marnett, A. B., Nomura, A. M., Shimba, N., Ortiz de Montellano, P. R., and Craik, C. S. (2004) Communication between the active sites and dimer interface of a herpesvirus protease revealed by a transition-state inhibitor, *Proc. Natl. Acad. Sci. U.S.A.* 101, 6870-6875.
- [18] Nealon, K., Newcomb, W. W., Pray, T. R., Craik, C. S., Brown, J. C., and Kedes, D. H. (2001) Lytic replication of Kaposi's sarcoma-associated herpesvirus results in the formation of multiple capsid species: isolation and molecular characterization of A, B, and C capsids from a gammaherpesvirus, *J. Virol.* 75, 2866-2878.
- [19] Nomura, A. M., Marnett, A. B., Shimba, N., Dotsch, V., and Craik, C. S. (2005) Induced structure of a helical switch as a mechanism to regulate enzymatic activity, *Nat. Struct. Mol. Biol.* 12, 1019-1020.
- [20] Pray, T. R., Reiling, K. K., Demirjian, B. G., and Craik, C. S. (2002) Conformational change coupling the dimerization and activation of KSHV protease, *Biochemistry* 41, 1474-1482.

- [21] Reiling, K. K., Pray, T. R., Craik, C. S., and Stroud, R. M. (2000) Functional consequences of the Kaposi's sarcoma-associated herpesvirus protease structure: regulation of activity and dimerization by conserved structural elements, *Biochemistry* 39, 12796-12803.
- [22] Shahian, T., Lee, G. M., Lazic, A., Arnold, L. A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a viral enzyme by a small-molecule dimer disruptor, *Nat. Chem. Biol.* 5, 640-646.
- [23] Shimba, N., Nomura, A. M., Marnett, A. B., and Craik, C. S. (2004) Herpesvirus protease inhibition by dimer disruption, *J. Virol.* 78, 6657-6665.
- [24] Harris, J. L., Backes, B. J., Leonetti, F., Mahrus, S., Ellman, J. A., and Craik, C. S. (2000) Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries, *Proc. Natl. Acad. Sci. U.S.A.* 97, 7754-7759.
- [25] Paliwal, S., Wales, M., Good, T., Grimsley, J., Wild, J., and Simonian, A. (2007) Fluorescence-based sensing of p-nitrophenol and p-nitrophenyl substituent organophosphates, *Analytica chimica acta* 596, 9-15.
- [26] Bemis, G. W., and Murcko, M. A. (1996) The properties of known drugs. 1. Molecular frameworks, *J. Med. Chem.* 39, 2887-2893.
- [27] Bemis, G. W., and Murcko, M. A. (1999) Properties of known drugs. 2. Side chains, *J. Med. Chem.* 42, 5095-5099.
- [28] Vassilev, L. T., Vu, B. T., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N., and Liu, E. A. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2, *Science* 303, 844-848.

- [29] McGovern, S. L., Caselli, E., Grigorieff, N., and Shoichet, B. K. (2002) A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening, *J. Med. Chem.* *45*, 1712-1722.
- [30] Hert, J., Irwin, J. J., Laggner, C., Keiser, M. J., and Shoichet, B. K. (2009) Quantifying biogenic bias in screening libraries, *Nat. Chem. Biol.* *5*, 479-483.

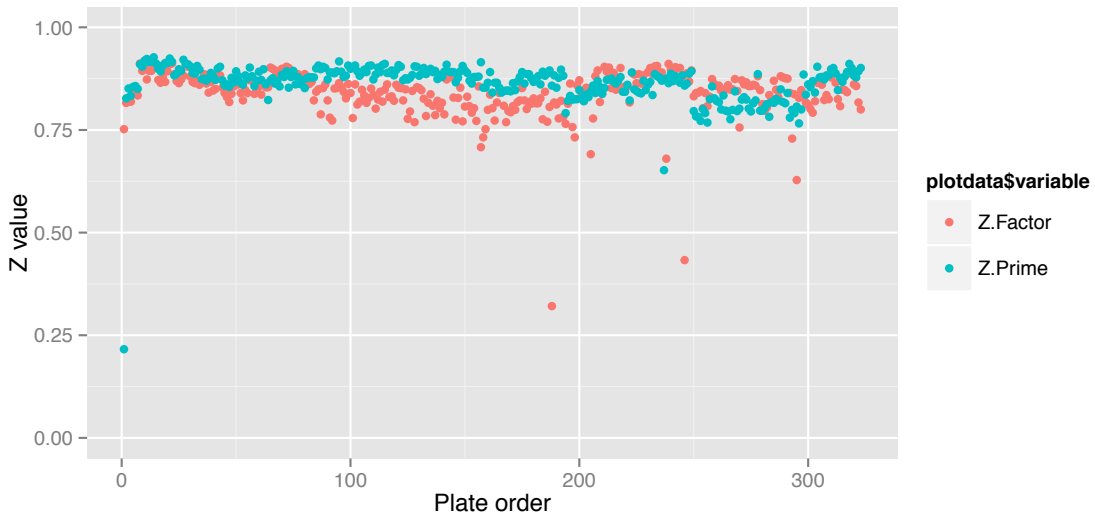


Figure 2-1. Z' and Z factor values across all 323 plates. Only two of the 323 plates had Z factor values less than 0.5. The mean Z prime and Z factor values across all plates were 0.86 and 0.84, respectively.

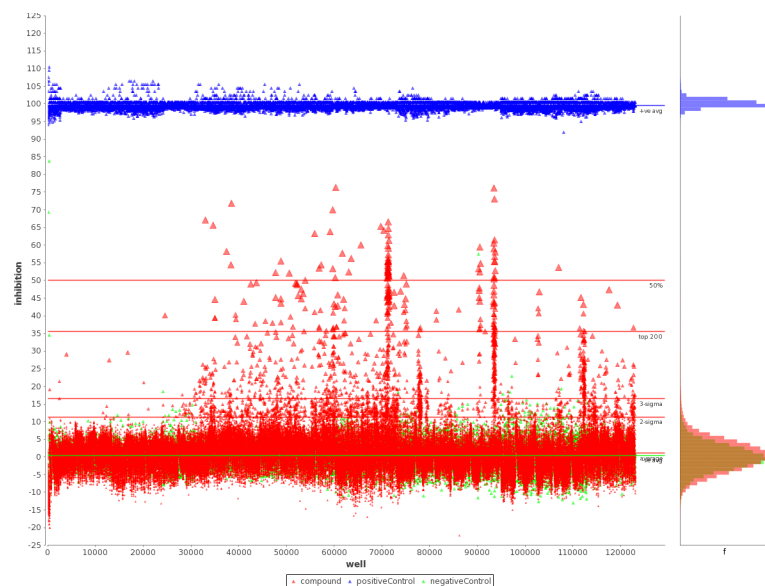


Figure 2-2. Scatter plot of percent inhibition. Negative controls, positive controls, and screening compounds are in green, blue, and red respectively. Red lines indicate 2-sigma, 3-sigma, top 200, and 50% inhibition (bottom to top). A green line indicates the average percent inhibition for all screening compounds.

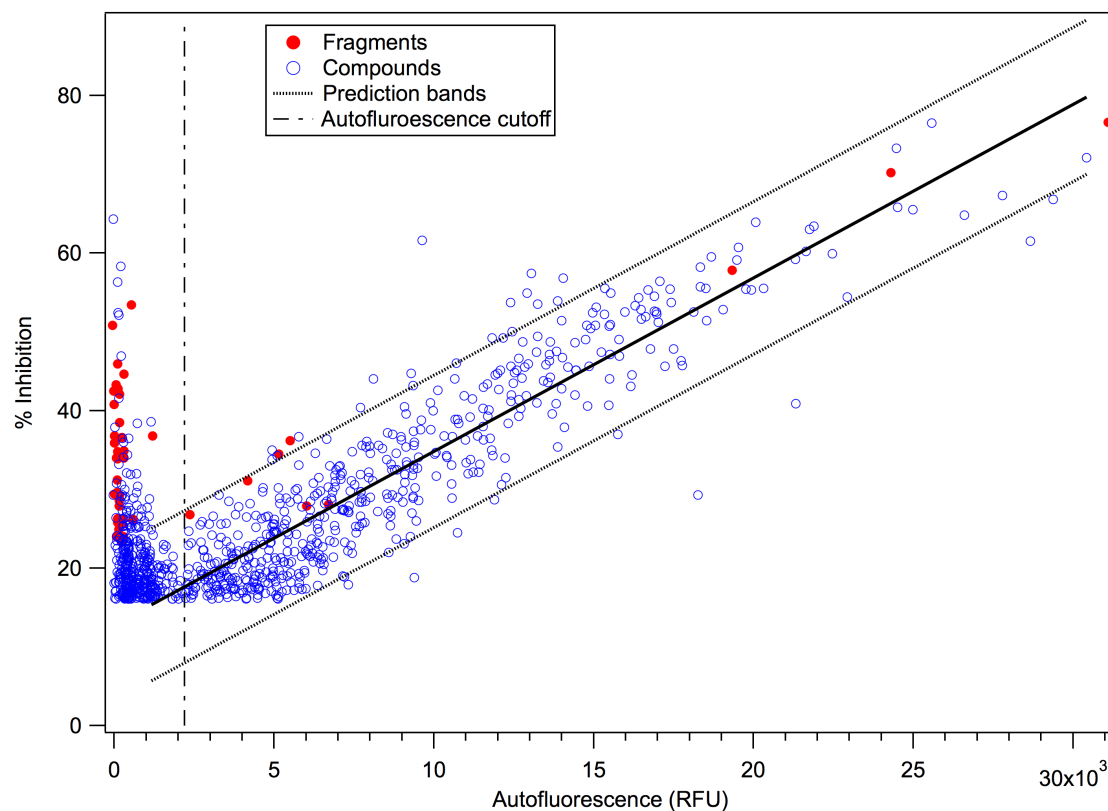
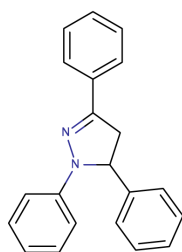
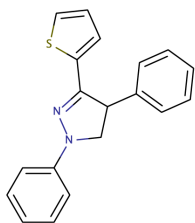


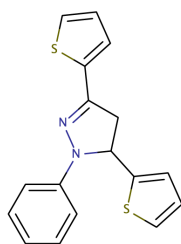
Figure 2-3. Scatter plot of autofluorescence versus percent inhibition. Fluorescence immediately after substrate addition is plotted for the 1013 hit compounds and fragments. The strong correlation between autofluorescence and percent inhibition strongly suggests spectrophotometric artifacts giving rise to apparent inhibition.



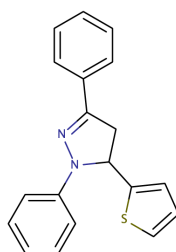
19/35 hit



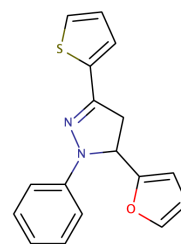
10/12 hit



3/3 hit



3/7 hit



3/3 hit

Figure 2-4. Nutlin-like Murcko fragments.

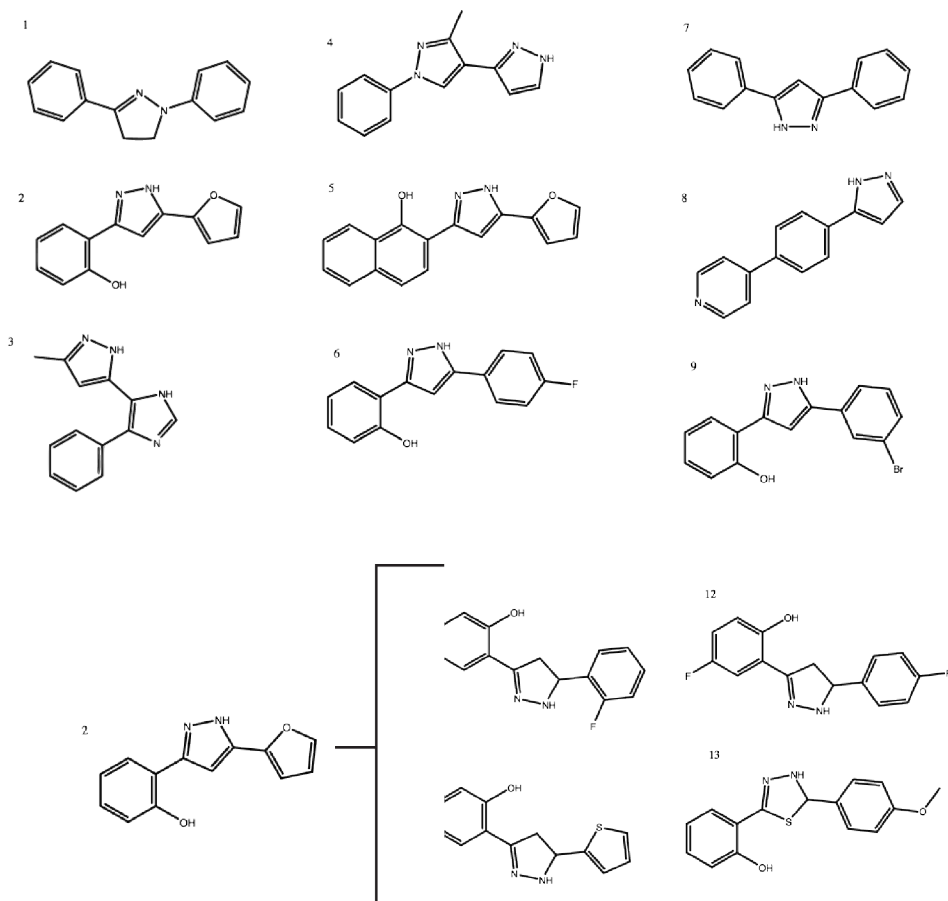


Figure 2-5. Disubstituted pyrazole analogs. Of the 9 analogs initially purchased only **disub 2** showed an appreciable dose response. Four additional analogs were purchased based on this observation (bottom).

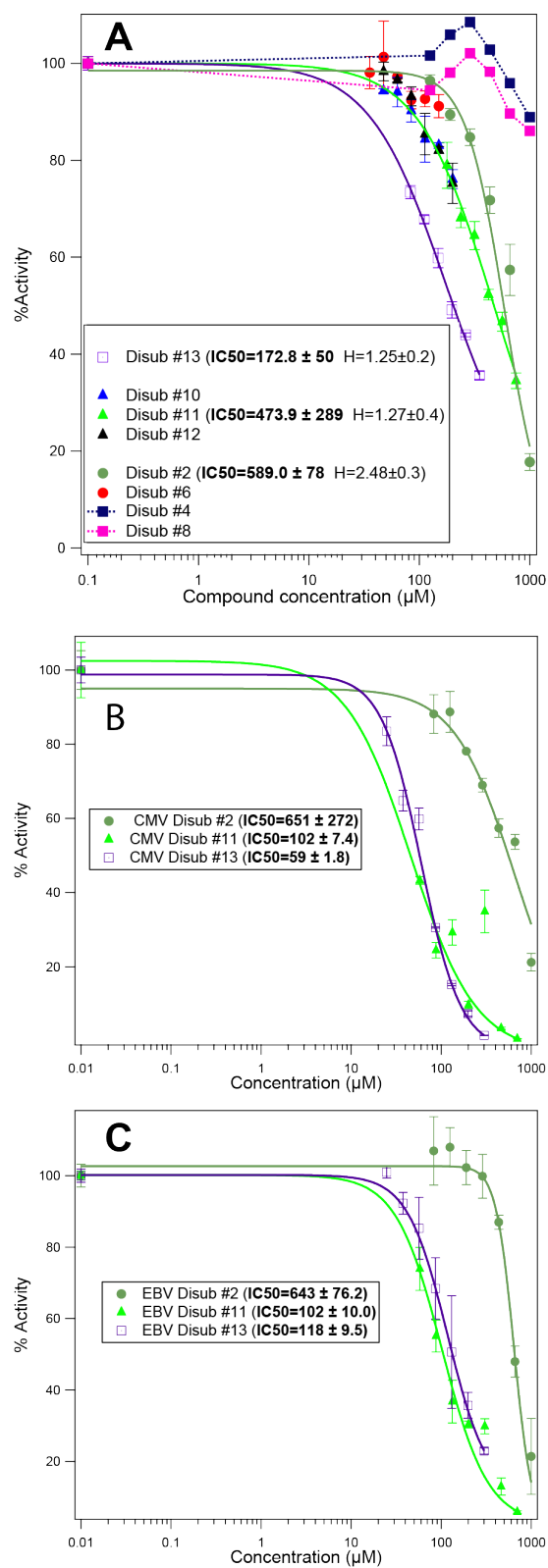


Figure 2-6. Disubstituted pyrazole IC_{50} curves. Disubstituted pyrazoles **2**, **11**, and **13** were tested against KSHV, CMV, and EBV proteases.

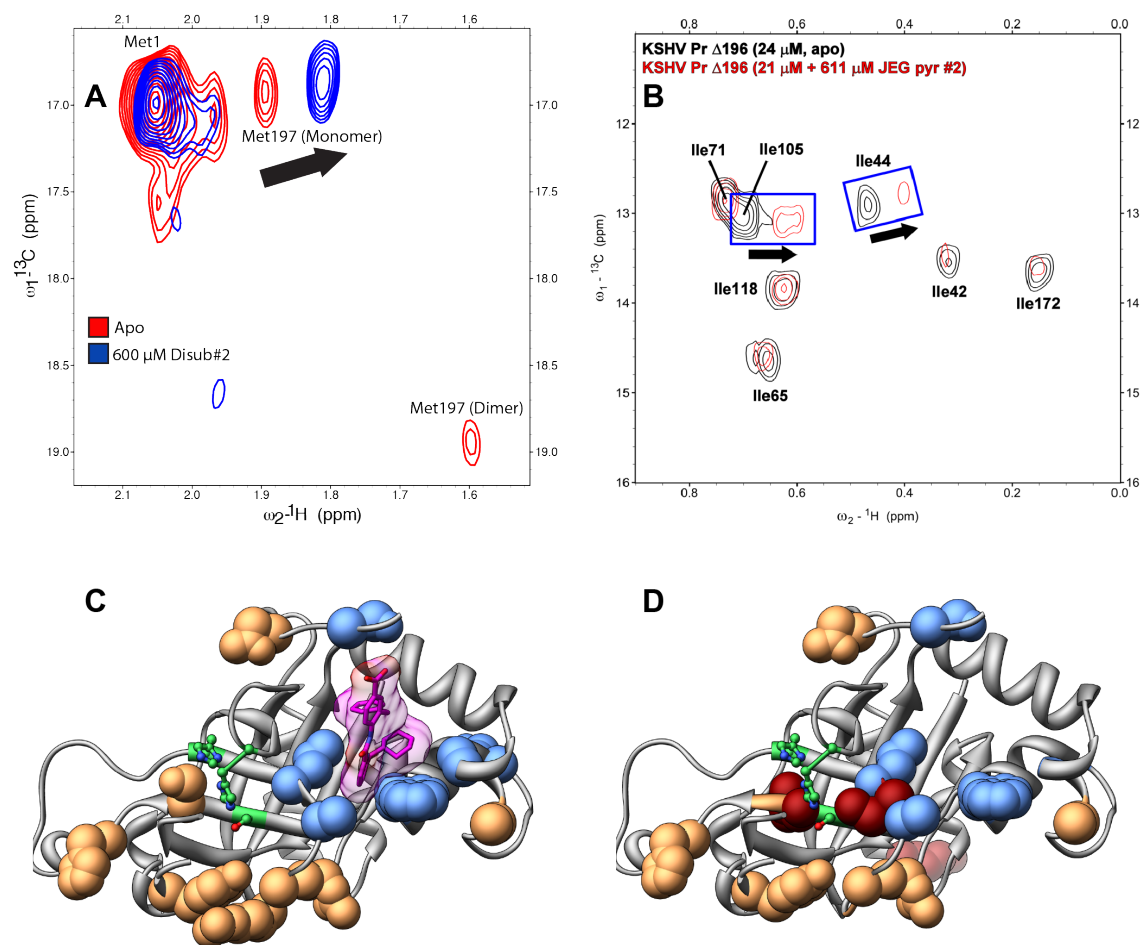


Figure 2-7. NMR determination of dimer disruption and binding site mapping. (A) Selective ^{13}C -Met labeling allows for determination of dimer-monomer equilibrium. In the presence of **disub 2** the dimer peak completely disappears, indicating dimer disruption. (B) ^{13}C -Ile labeling enables mapping of binding to the dimer interface DD2 pocket. Perturbation of Ile105 and Ile44 but not other Ile residues is consistent with binding at the interfacial pocket. (C) DD2 (magenta) binding at the interfacial pocket with ^{15}N HSQC perturbation mapped as CPK residues. Blue residues are in the pocket while tan residues are likely allosterically affected. (D) **Disub 2** ^{15}N -HSQC mapping to the interfacial pocket. Red CPK residues are those residues perturbed by Disub 2 but not DD2. The active site residues are shown in green ball and stick representation.

Table 2-1: Hits with robust dose response

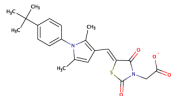
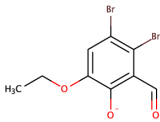
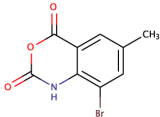
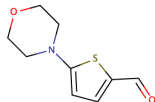
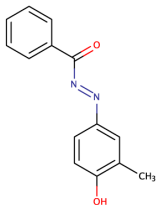
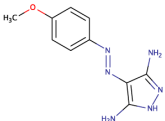
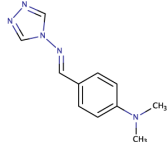
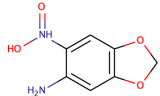
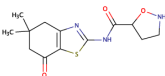
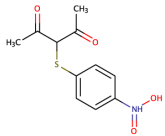
	ID	IC50 (μM)
	1	7
	2	15
	3	90
	4	100
	5	130
	6	140
	7	150
	8	290
	9	300
	10	430

Table 2-2: Disubstituted pyrazoles inhibition of KSHV, EBV, and CMV proteases

	KSHV Pr IC₅₀ (μM)	EBV Pr IC₅₀ (μM)	CMV Pr IC₅₀ (μM)
Disub 2	589 ± 78	643 ± 76	651 ± 272
Disub 11	473 ± 289	102 ± 10	102 ± 7
Disub 13	172 ± 50	118 ± 9	59 ± 2

Table 2-3: Disubstituted pyrazole aggregation by dynamic light scattering

	Radii at 100 μM	Radii at 500 μM	KSHV Pr IC₅₀
Disub 2	0.1 nm and 30 nm	0.1 nm and 30 nm	589 $\pm$ 78
Disub 11	146 nm	Not tested	473 $\pm$ 289
Disub 13	350 nm	Not tested	172 $\pm$ 50
Disub 4	0.1 and 28 nm	0.1 and 25 nm	No inhibition
Disub 8	0.1 and 28 nm	Not tested	No inhibition

CHAPTER 3: Screening for novel human cytomegalovirus protease inhibitors

Jonathan E. Gable, Jaeki Min, Sergio C. Chai, Asli Goktug, Taosheng Chen, R. Kiplin Guy,
Charles S. Craik

Abstract

Human cytomegalovirus is a significant cause of morbidity and mortality, particularly in organ transplant patients and newly born infants. Current FDA-approved treatments for CMV all target the viral polymerase, leading to increasing resistance as well as dose-limiting toxicity. CMV protease has long been considered a potential therapeutic target however to date no high throughput screens have been published for this essential conserved enzyme. Here we report the development and validation of a fluorescence-intensity based assay to identify inhibitors of the CMV protease. We screened roughly 650,000 compounds and identified novel pharmacologically favorable low micromolar inhibitors of CMV protease. Using a kinetic analysis developed by Zhang and Poorman we discovered that our top hit, an LXR agonist, exhibits mixed competitive-dissociative inhibition.

Introduction

Cytomegalovirus (CMV) treatment options are limited. Standard of care options ganciclovir and valganciclovir suffer from dose limiting myelosuppression. In cases where the virus is resistant to these treatments or the patient cannot tolerate them drugs such as cidofovir and foscarnet are used. These drugs require regular intravenous injections and cause severe nephrotoxicity.¹

All FDA approved systemic CMV treatments target a single enzyme, the viral polymerase. No treatments are approved for congenital CMV infection. There is clearly a need for novel therapeutic approaches to CMV treatment.

Human cytomegalovirus protease is a genetically validated target required for viral replication.^{2,3} Despite this, no high throughput screening protocols have been published to enable the scientific community at large to identify novel CMV protease inhibitors. Recent publications reveal a new strategy for CMV protease inhibition—protein-protein interaction antagonism that prevents dimerization and activation of the enzyme.⁴⁻⁶ We hypothesized that large-scale high throughput screening would identify novel protein-protein interaction inhibitors of CMV protease. To test this we developed a fluorescence intensity enzymatic activity-based screen and screened a library of approximately 650,000 compounds against recombinant CMV protease. Here we present the details of the screening protocol and the results of our screen.

Results

Extensive optimization was required to achieve a suitable assay protocol. To reduce fluorescence artifacts observed with coumarin substrates (chapter 2, REF) we used a rhodamine110 substrate, Ac-PVYtBuQA-Rho110-dP.⁷ Substrate concentration optimization, in large part limited by substrate solubility, arrived at a final value of 12.5 μ M. This achieved

greater than 2 hours of linear signal using our optimized conditions. Multiple attempts were made to arrive at a scheme for quenching enzymatic activity prior to endpoint measurements. Addition of DMSO to a final concentration of 50% failed to completely inhibit the enzyme. We had previously shown that Triton-X at concentrations below the critical micellar concentration inhibit enzymatic activity, presumably due to disrupting dimerization. While this appeared to be true Triton-X also caused time-dependent quenching of fluorescence and proved problematic for automation due to bubble formation during dispense steps. Ultimately we decided that given the slow rate of turnover for this enzyme no quenching step was necessary. Related to quenching was selection of a positive control. Initial attempts to use DD2 as a positive control revealed that DMSO stocks at concentrations high enough to enable pin tool addition to a final concentration greater than 80 μ M did not tolerate freeze-thaw cycles, resulting in high variability and poor data quality. PMSF and other standard protease inhibitors are extremely weak inhibitors of CMV protease and also suffer from poor stability.^{8,9} High concentration of ZnCl_2 was selected as our positive control inhibitor for the screen. ZnCl_2 is thought to work by Zn^{2+} coordinating the catalytic histidines of CMV protease.¹⁰ Final assay conditions are reported in Methods and Materials.

The optimized assay conditions gave robust and consistent high, medium, and low signal results across three plates per day over three days (**Figure 3-1A**). A pilot screen of about 10,000 compounds resulted in an excellent Z and Z' factor for all plates (**Figure 3-1B, C**). Expansion of this to an additional approximately 30,000 compounds (about 85 plates) continued to have excellent data quality (**Figure 3-1D,E**) as did all subsequent plates in the approximately 650,000 compounds screened. One hundred and ninety four compounds including 12 compounds with greater than 80% inhibition in the primary screen were cherry picked and automated dose

response was performed to enable receiver operator characteristic (ROC) analysis. Using these data the area under the curve on the ROC plot was 0.942 (a perfect classifier has an area under the curve of 1.0, **Figure 3-2A**). This analysis also allowed us to assess the true positive rate using a variety of percent inhibition cutoffs for defining a hit. A 40% inhibition cutoff was deemed optimal, giving a true positive rate of 89% and a false positive rate of 6% (**Figure 3-2B**). Two example hits from the ROC analysis dose response are shown in **Figure 3-2 C and D**.

As a confirmation screen automated dose response was carried out for the 764 compounds that exhibited greater than 40% inhibition in the primary screen. To better understand mode of inhibition and rule out artifactual inhibition we performed the dose response with five different incubation times prior to addition of substrate: 30 minutes and 1, 2, 3, and 5 hours. **Figure 3-3** shows two example time-dependent dose response experiments. For the first compound the IC_{50} remains roughly constant but the hill slope increases over time. In the second example the hill slope remains roughly constant while the IC_{50} increases slightly.

Representative inhibitors were repurchased and dose response was performed manually, in this case using the rate of kinetic turnover instead of end point measurements to assess IC_{50} (Table 3-1). Compounds **1**, **2**, and **3** are known reversible covalent serine protease inhibitors (e.g CHEMBL396796).¹¹ Repurchased material yielded IC_{50} values comparable to those recorded in automated dose response. Compounds **6** and **7** closely resemble compounds **4** and **5** identified in the screen, yet showed no inhibition in manual dose response. This may be due to the use of TCEP as a reducing agent in the manual IC_{50} determination while DTT was used for automated dose response. Compounds **8**, **9**, and **10** were confirmed to inhibit in manual dose response. These compounds were all colored in solution and thus careful analysis should be performed to address possible deflation of IC_{50} values due to the inner filter effect.¹² All three of these

compounds also contain a quinone functionality that is known to be reactive, particularly with free cysteine thiols, and often non-specifically.¹³ Vitamin B12 (**12**) showed no dose response in manually performed assays. The LXR agonist **11** showed robust and reproducible dose response with no obvious chemical liabilities. No automated dose response was performed for this compound but it was identified as a hit from the primary screen and highlighted here for its favorable pharmacological properties.¹⁴

The mechanism of inhibition for the LXR agonist **11** against CMV protease was not immediately apparent. To interrogate this we first attempted Lineweaver-Burk analysis to distinguish between competitive, non-competitive, and mixed inhibition using **1** as a positive control for competitive inhibition and DD2 as a positive control for mixed inhibition. This analysis failed to give the expected linear plots of inverse velocity vs. inverse substrate concentration (**Figure 3-1S**). This is consistent with previous observations suggesting that the substrate used, P6R, has a K_M far greater than its solubility limit and that the apparent saturation in rate with increasing substrate concentration is in fact due to the limited solubility of the substrate. As an alternative approach we applied the analysis of Zhang and Poorman.¹⁵ This analysis requires that substrate concentration be well below that of K_M and yet above that of dimeric enzyme. Both of these conditions are readily met using the P6R substrate at 12.5 μM . In this analysis a constant intercept with increasing inhibitor concentration indicates competitive inhibition, while an increasing intercept with increasing inhibitor concentration indicates dissociative inhibition (dimer disruption, **Figure 3-4A**). See chapter 6 for additional details on this analysis. As expected, **1** showed competitive inhibition (**Figure 3-4C**). The LXR agonist **11** showed mixed inhibition; at low concentrations of inhibitor inhibition appears competitive while at increased concentrations of inhibitor inhibition becomes dissociative.

Discussion

We identified multiple novel inhibitors of and established a robust 384-well format screen against CMV protease. The LXR agonist **11** (**Table 3-1**) is arguably the most promising hit. This compound appears to function, at least in part, through dissociative inhibition (protein-protein interaction antagonism). Dissociative inhibition is greatly preferred over competitive inhibition in this system as it would allow for binding to and inhibition of the monomeric form of the enzyme and would not need to outcompete the high local concentration of substrate found in the procapsid during viral replication. Compound **11** has tractable chemistry and extensive SAR in the literature that would likely enable a shift away from its LXR agonist functionality while maintaining good cell permeability, bioavailability, and CMV protease inhibition (PubChem CID 16078973).

The exact binding mode of **11** is unclear as are the specifics of its mode of inhibition. Without further structural studies it is difficult to interpret what mechanism of action would result in the mixed competitive and dissociative inhibition observed through Zhang-Poorman analysis (**Figure 3-4**). One could hypothesize that dissociative inhibition at high inhibitor concentration is due to some sort of aggregative process. Dynamic light scattering should be performed to rule this out. NMR HSQC data with this compound have been acquired but require further analysis. These data may clarify mode of binding and thus mechanism of action.

The use of automated time-dependent dose response is rare, but proved highly beneficial. Instances of increasing Hill slope with time rapidly revealed reactive compounds that inhibit non-specifically. Decreasing IC_{50} over time is likely due to compound instability. Indeed, in the case of **1**, **2**, and **3** these compounds are known to hydrolyze in water at pH 8 with half-lives on

the hours scale.¹¹ They may also be hydrolyzed by the protease itself and this could be readily tested using mass spectrometry.

Establishing a robust screening pipeline including rapid kinetic approaches to determining mechanism of action and ruling out promiscuous inhibitors will greatly empower the scientific community to pursue inhibitors of CMV protease. This assay can be readily modified for use with other human herpesvirus proteases as well and the use of a rhodamine substrate offers much-improved false positive rates as compared to the coumarin-based assay described in chapter 2.⁷ Excitingly, we have identified a novel inhibitor of CMV protease with excellent pharmacological properties. We suspect that straightforward SAR studies can both drive potency and limit interaction with LXR in order to generate a small molecule that could for the first time provide pharmacological validation of CMV protease as a therapeutic target.

Materials and Methods

Materials

The rhodamine110 substrate (Ac-Pro-Val-Tyr-tBug-Gln-Ala-Rhod110-dPro) was purchased at >95% purity from Anaspec and prepared as a 10 mM stock in 100% DMSO. Known inhibitor dimer disruptor 2 (DD2) was synthesized as previously described. Initial optimization was carried out in black round-bottom 96-well plates. Fluorescence high-throughput screening (HTS) assays were carried out in black 384-well microplates. Both plate formats were purchased from Corning Life Sciences (Acton, MA). During initial assay optimization fluorescence measurements monitoring activity were taken on the Biotek Synergy H4 multimode plate reader (Ex: 498 nM, Em: 521 nM). Details on chemical vendors are listed in the supplemental material. Protein purification was performed as previously described (see chapter 6).

Assay conditions

All optimized fluorescence assays were conducted in a buffer with the following composition: 25 mM potassium phosphate pH 8, 150 mM KCl, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 0.1% Prionex. Prior to the addition of substrate or compound, concentrated stock protease was diluted into assay buffer to a final concentration of 2.25 μ M. Twenty microliters of dilute protease were added to each well in 384-well plates (Corning 8849BC). Compound was added using the pin tool (50SS) and compound plus enzyme were incubated for 60 minutes at room temperature. A bulk WellMate dispense was used to add 10 μ L 37.5 μ M substrate to each well to start the reaction. Final protein and substrate concentrations were 1.5 μ M and 12.5 μ M, respectively. Additional details on reagent preparation are included in the supplemental methods.

Optimization for HTS

Optimization for automated HTS was carried out on a screening system integrated by High Resolution Engineering (Woburn, MA). A Staubli T60 robot arm was used to transfer plates from instrument to instrument. Assay buffer containing protease was dispensed in bulk using Matrix Wellmates (Matrix Technologies, Hudson, NH). Substrate addition was performed by an on-line dispenser (ask about details). Compound transfers were performed using a 384-well pin tool with 10 nL hydrophobic surface-coated pins (V&P Scientific, San Diego, CA). These pins transferred on average 25 nL of compound stock in DMSO with coefficients of variation of less than 10%. Fluorescence measurements were made using an EnVision Multilabel Plate Reader (PerkinElmer) with an excitation wavelength of 480 nm and emission detection at 521 nm.

Acknowledgements

I would like to acknowledge the UCSF SMDC for their help in partial assay validation at UCSF.

I would also like to thank Kip Guy, Taosheng Chen, and Charles Craik and their funding sources for the opportunity to visit Saint Jude Children's Research hospital during the course of this work.

References

- [1] Torres-Madriz, G., and Boucher, H. W. (2008) Immunocompromised hosts: perspectives in the treatment and prophylaxis of cytomegalovirus disease in solid-organ transplant recipients, *Clin. Infect. Dis.* **47**, 702-711.
- [2] Gao, M., Matusick-Kumar, L., Hurlburt, W., DiTusa, S. F., Newcomb, W. W., Brown, J. C., McCann, P. J., 3rd, Deckman, I., and Colonno, R. J. (1994) The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth, *J. Virol.* **68**, 3702-3712.
- [3] Jiang, X., Gong, H., Chen, Y. C., Vu, G. P., Trang, P., Zhang, C. Y., Lu, S., and Liu, F. (2012) Effective inhibition of cytomegalovirus infection by external guide sequences in mice, *Proc. Natl. Acad. Sci. U.S.A.* **109**, 13070-13075.
- [4] Shahian, T., Lee, G. M., Lazic, A., Arnold, L. A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a viral enzyme by a small-molecule dimer disruptor, *Nat. Chem. Biol.* **5**, 640-646.
- [5] Lee, G. M., Shahian, T., Baharuddin, A., Gable, J. E., and Craik, C. S. (2011) Enzyme inhibition by allosteric capture of an inactive conformation, *J. Mol. Biol.* **411**, 999-1016.
- [6] Gable, J. E., Lee, G. M., Jaishankar, P., Hearn, B. R., Waddling, C. A., Renslo, A. R., and Craik, C. S. (2014) Broad-spectrum allosteric inhibition of herpesvirus proteases, *Biochemistry* **53**, 4648-4660.
- [7] Grant, S. K., Sklar, J. G., and Cummings, R. T. (2002) Development of novel assays for proteolytic enzymes using rhodamine-based fluorogenic substrates, *J Biomol Screen* **7**, 531-540.

- [8] Stevens, J. T., Mapelli, C., Tsao, J., Hail, M., O'Boyle, D., 2nd, Weinheimer, S. P., and Diianni, C. L. (1994) In vitro proteolytic activity and active-site identification of the human cytomegalovirus protease, *Eur. J. Biochem.* 226, 361-367.
- [9] Holwerda, B. C. (1997) Herpesvirus proteases: targets for novel antiviral drugs, *Antiviral Res.* 35, 1-21.
- [10] Polgar, L. (2005) The catalytic triad of serine peptidases, *Cellular and molecular life sciences : CMLS* 62, 2161-2172.
- [11] Schepetkin, I. A., Khlebnikov, A. I., and Quinn, M. T. (2007) N-benzoylpyrazoles are novel small-molecule inhibitors of human neutrophil elastase, *J. Med. Chem.* 50, 4928-4938.
- [12] Liu, Y., Kati, W., Chen, C. M., Tripathi, R., Molla, A., and Kohlbrenner, W. (1999) Use of a fluorescence plate reader for measuring kinetic parameters with inner filter effect correction, *Anal. Biochem.* 267, 331-335.
- [13] Baell, J. B., and Holloway, G. A. (2010) New substructure filters for removal of pan assay interference compounds (PAINS) from screening libraries and for their exclusion in bioassays, *J. Med. Chem.* 53, 2719-2740.
- [14] Collins, J. L., Fivush, A. M., Watson, M. A., Galardi, C. M., Lewis, M. C., Moore, L. B., Parks, D. J., Wilson, J. G., Tippin, T. K., Binz, J. G., Plunket, K. D., Morgan, D. G., Beaudet, E. J., Whitney, K. D., Kliewer, S. A., and Willson, T. M. (2002) Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines, *J. Med. Chem.* 45, 1963-1966.
- [15] Zhang, Z. Y., Poorman, R. A., Maggiora, L. L., Heinrikson, R. L., and Kezdy, F. J. (1991) Dissociative inhibition of dimeric enzymes. Kinetic characterization of the inhibition of HIV-1 protease by its COOH-terminal tetrapeptide, *J. Biol. Chem.* 266, 15591-15594.

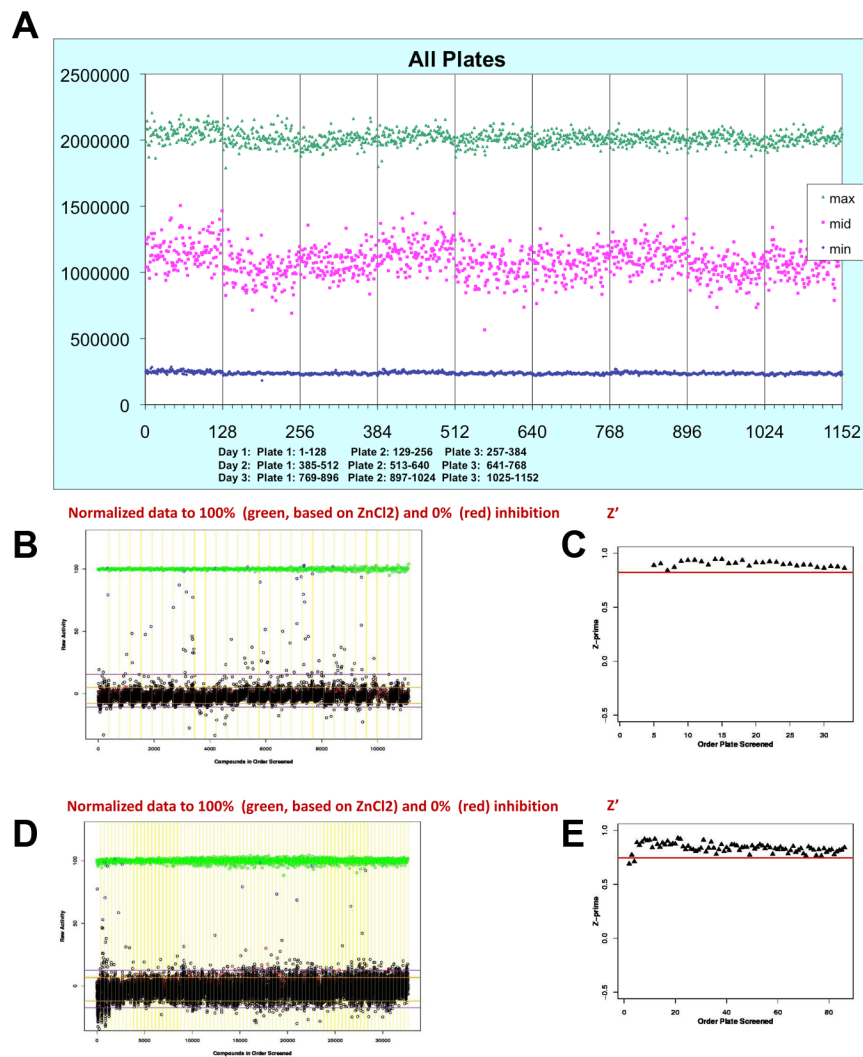


Figure 3-1. HTS validation and quality control. (A) Nine-plate three-day high, medium, and low signal 384-well format assay validation. (B) and (D) show percent activity versus compound number. (C) and (E) show Z' values across a total of approximately 120 plates in total.

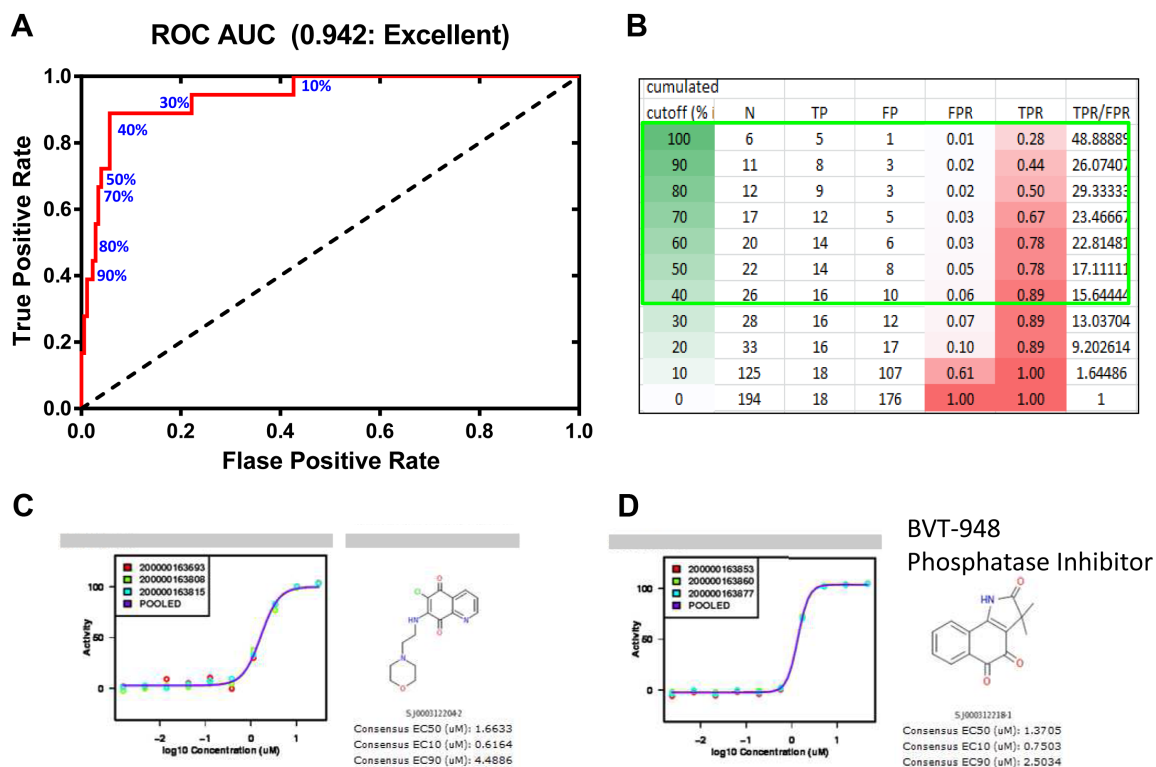


Figure 3-2. Screening ROC analysis. (A) ROC curve plotting false positive rate versus true positive rate. Percent cutoff values are indicated in blue. (B) Table of percent cutoff, total number of compounds (N), true positive count (TP), false positive count (FP), false positive rate (FPR), true positive rate (TPR), and positive likelihood ratio (TPR/FPR). (C) and (D) show example dose response curves used as part of ROC analysis.

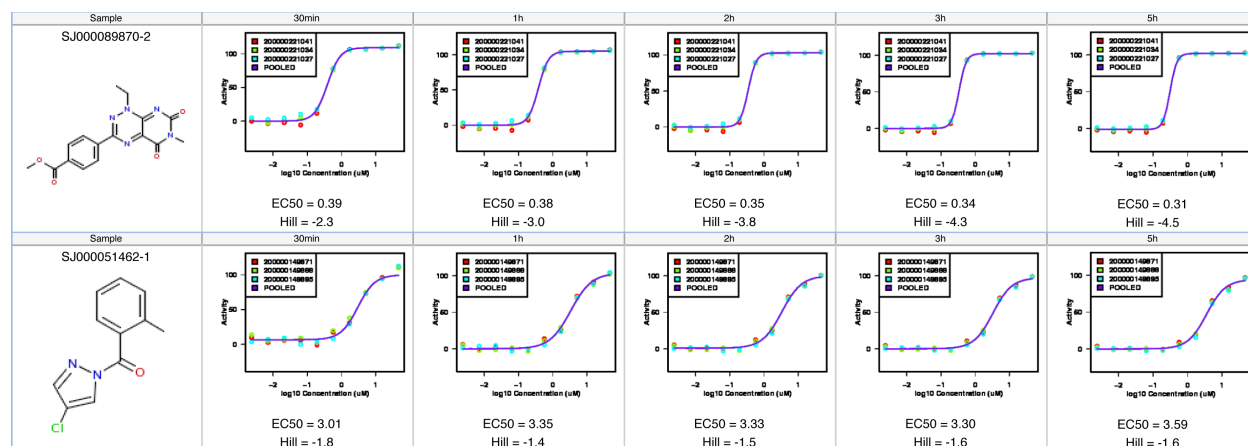


Figure 3-3. Example time-dependent dose response. Dose response curves are shown for 30 min, 1 hr, 2 hr, 3 hr, and 5 hr of compound-enzyme incubation prior to substrate addition. EC₅₀ and Hill slope are reported based on least squares fitting using the Hill equation.

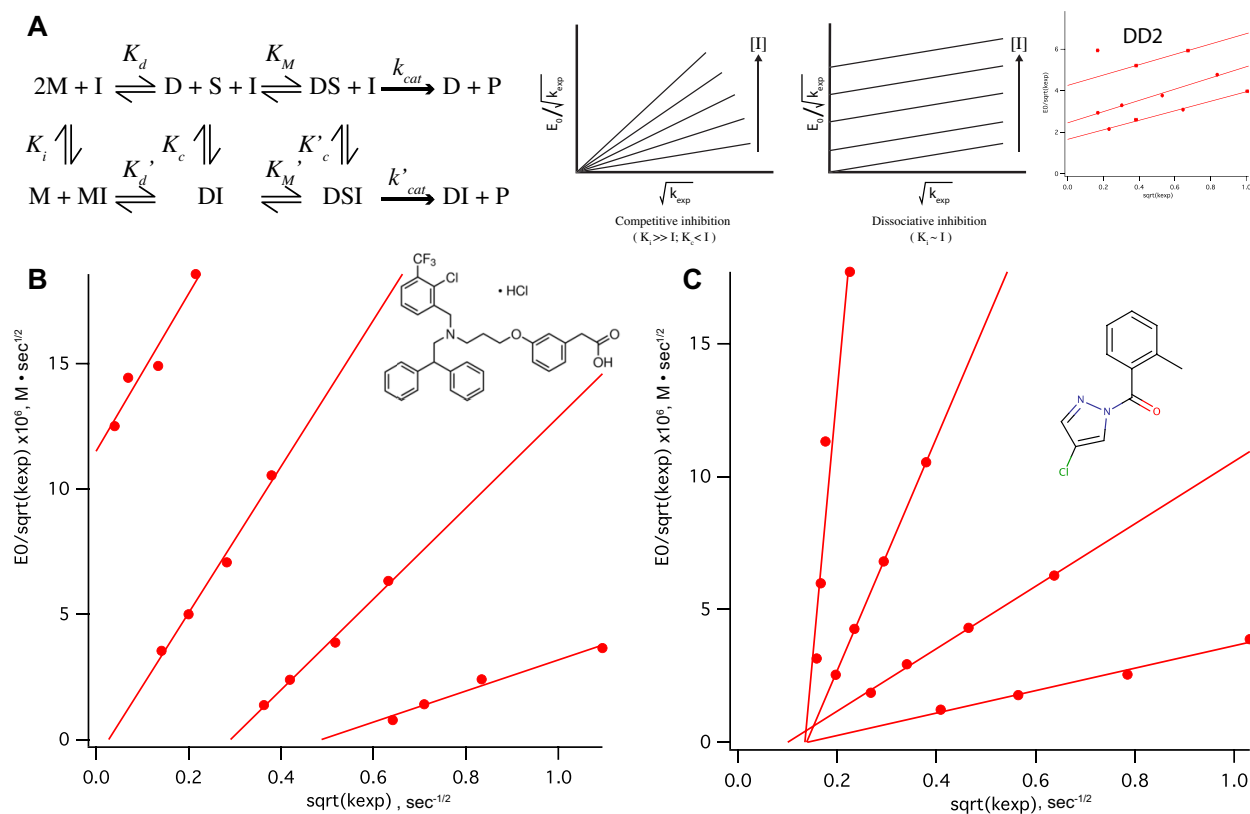
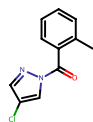
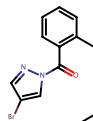
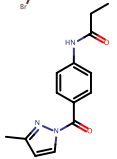
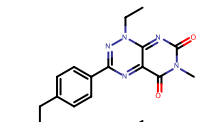
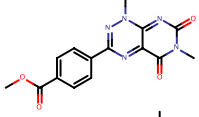
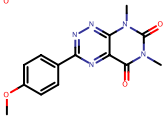
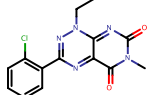
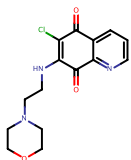
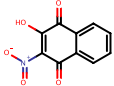
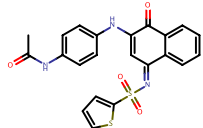
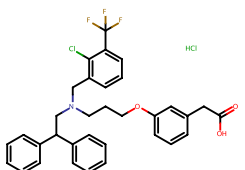


Figure 3-4. Zhang-Poorman analysis. (A) The kinetic scheme from which Zhang-Poorman analysis is derived. The three vertical arms of inhibition from left to right are dissociative, competitive, and uncompetitive. Example plots for competitive and dissociative inhibition are shown in black. Data for DD2, a known dissociative inhibitor, are shown in red. (B) Zhang-Poorman analysis data for the identified LXR agonist and CMV protease inhibitor. (C) Zhang-Poorman analysis for a known covalent-reversible protease inhibitor identified in the screen.

Table 3-1. Representative hit compounds

	ID	IC ₅₀ (auto, 30min)	IC ₅₀ (conf)	Color	Notes
	1	3.01 μ M	5.1 $\pm$ 0.3 μ M	Clear	Known protease inhibitor
	2	3.57 μ M	Not repurchased	N/A	Known protease inhibitor
	3	4.35 μ M	8.9 $\pm$ 0.8 μ M	Clear	Known protease inhibitor
	4	0.70 μ M	Not repurchased	N/A	Redox liabilities, frequent hit
	5	0.39 μ M	Not repurchased	N/A	Redox liabilities, frequent hit
	6	Not screened	No inhibition	Clear	Redox liabilities, frequent hit
	7	Not screened	No inhibition	Clear	Redox liabilities, frequent hit
	8	1.10 μ M	3.7 $\pm$ 0.3 μ M	Red	Need inner filter effect correction Quinone, Michael acceptor Redox sensitive Frequent hit
	9	2.00 μ M	10.8 $\pm$ 1.5 μ M	Orange	Need inner filter effect correction Quinone, Michael acceptor Redox sensitive Frequent hit
	10	1.87 μ M	1.61 $\pm$ 0.1 μ M	Dark red	Need inner filter effect correction Quinone, Michael acceptor Redox sensitive Frequent hit
	11	No auto DR	5.55 $\pm$ 0.3 μ M	Clear	Known LXR agoist
Vitamin B12	12	3.19 μ M	No inhibition	Dark red	Inner filter effect false positive

Supplemental material

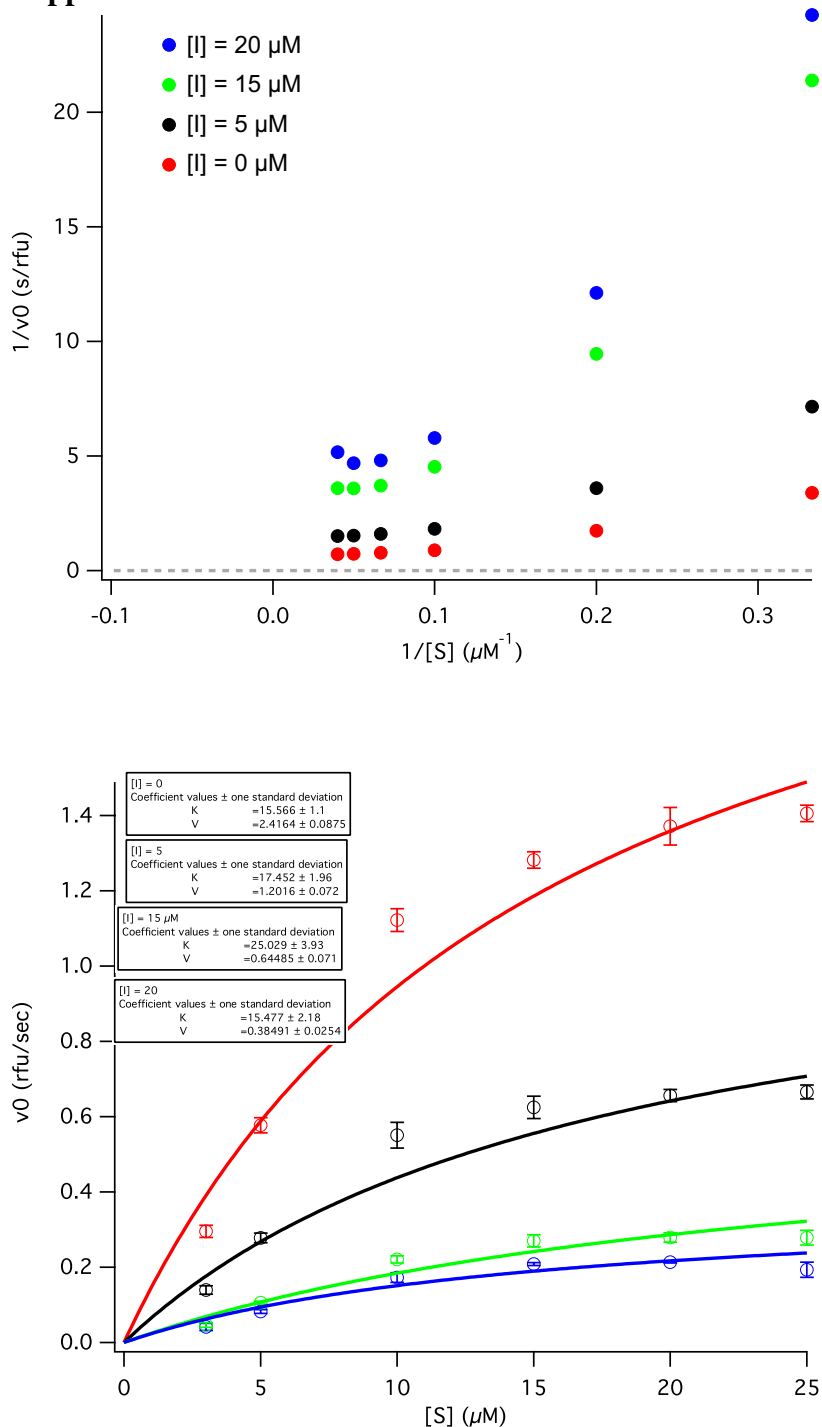


Figure 3-1S. Lineweaver Burk analysis of known protease inhibitor **1**. (Top) The inverse "linear" plot. (Bottom) K_M curves at varying inhibitor concentration. Intended as a positive control, this experiment failed to show clear competitive inhibition likely due to inadequate substrate solubility. The apparent K_M curves are likely due to hitting the solubility limit of the substrate, not actual saturation of substrate binding to the enzyme active sites.

Table 3-S1 Reagent Information**Reagent Details**

Reagent Name	Supplier	Catalog Number	Price Per Unit
Potassium phosphate monobasic	Sigma	P0662	
Potassium phosphate dibasic	Sigma	P3786	
Potassium chloride	Amresco	395	
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate	Sigma	ED4SS	
DL-Dithiothreitol (DTT)	Sigma	D0632	
Prionex	EMD/Calbiochem	529600	
CMV Pr stock	Craik Lab		
P6R substrate (Ac-PVYtLQA(rho110)dP)	Anaspec	Custom synthesis	
MilliQ Water			
Triton X-100	Sigma	X100	

Reagent Sensitivity and Stability

Stock Reagent Sensitivity and Stability

Reagent (Stock)	Sensitivity (light, temperature, etc.)	Storage conditions (temperature, stirring, buffer)	Reagent Lifespan	Other comments
Potassium phosphate monobasic		RT	> 1yr	
Potassium phosphate dibasic		RT	> 1yr	
Potassium chloride		RT	> 1yr	
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate		RT	> 1yr	
DL-Dithiothreitol (DTT)	oxidation	-20 C	> 1 yr	Prepare 1M stock in water
Prionex	temperature	4 C	> 6 months	10% stock solution
CMV Pr stock	temperature, oxidation	-80 C	> 1 yr	351 μ M stock

P6R substrate (Ac-PVYtLQA(rho110)dP)	light	-20 C	> 1yr	Prepare 10 mM stock in fresh DMSO
MilliQ Water				
Triton X-100		RT	> 2 yrs	

7.2.2 Working Reagent Sensitivity and Stability

Reagent (Working)	Sensitivity (light, temperature, etc.)	Storage conditions (temperature, stirring, buffer)	Reagent Lifespan	Other comments
Potassium phosphate monobasic		RT	> 1yr	1.5 mM as component of assay buffer
Potassium phosphate dibasic		RT	> 1yr	23.5 mM as component of assay buffer
Potassium chloride		RT	> 1yr	150 mM as component of assay buffer
Ethylenediaminetetraacetic acid tetrasodium salt dihydrate		RT	> 1yr	0.1 mM as component of assay buffer
DL-Dithiothreitol (DTT)	oxidation	4 C	> 1 yr	5 mM as component of assay buffer

Prionex	temperature	4 C	> 6 months	0.1% as component in assay buffer
CMV Pr stock	temperature, oxidation	4 C	24 hrs at 4C yields less than 50% reduction in activity	2.25 μ M in assay buffer
P6R substrate (Ac-PVYtLQA(rho110)dP)	light	RT	> 1 month	37.5 μ M in assay buffer + 10% DMSO
MilliQ Water				
Triton X-100		RT	> 2 yrs	2% in MilliQ water

Reagent Recipes

Mixture Name	Reagent Name	Preparation Instructions
10X buffer stock	Potassium phosphate monobasic	2.041 g per L (15 mM, MW: 136.086)
	Potassium phosphate dibasic	40.937 g per L (235 mM, MW: 174.2)
	Potassium chloride	111.827 g per L (1.5 M, MW: 74.5513)
	Ethylenediaminetetraacetic acid tetrasodium salt dihydrate	0.4162 g per L (1 mM, MW: 416.2)
	MilliQ water	925.6 mL or to final volume of 1 L (ensure pH is 8 prior to bringing to final 1L volume)
Assay buffer		100 mL 10X buffer stock per L
	MilliQ water	885 mL at 4 °C per L
	Prionex	10 mL 10% solution (final conc. 0.1%) per L
	DL-Dithiothreitol (DTT)	5 mL 1M stock per L
		Store at 4 °C or on ice to slow oxidation of DTT (prepare fresh daily)

Protein working solution		993.59 mL assay buffer at 4 °C
	CMV Pr stock	6.41 mL per L
		Note: 156 mL working solution per 1 mL aliquot (~20 plates)
		Note: 2.25 μ M enzyme pre-substrate addition (working solution); 1.5 μ M enzyme post-substrate addition in plate
Substrate DMSO stock	P6R substrate (Ac-PVYtLQA(rho110)dP)	Allow solid substrate powder to reach RT prior to opening bag and bottle
		Mass 11.423 mg per mL using metal scoopula (and static gun if necessary). Do not use plastic scoopula.
		Bring up to total 1 mL in fresh DMSO
		Store remaining solid substrate in airtight black ziplock bag, pressing air out of bag prior to sealing to whatever extent possible.
		Note: Stock should be prepared in 50-100 mg (4.377-8.754 mL) batches and remaining powder stored at -20° C in a sealed bag. DMSO stock should be aliquoted and stored at -20 °C. Limit freeze-thaw cycles as much as possible. DMSO stock is stable at RT for > 1 month. Parafilm container to prevent DMSO from taking on water. Wrap container in foil and/or store in dark.

		Note: 50 mg (4.377 mL stock) is enough for 1.1672 L working substrate solution (303.96 plates w/o dead volume)
Substrate working solution	P6R substrate (Ac-PVYtLQA(rho110)dP)	Add 3.75 mL 10 mM DMSO stock to 96.25 mL DMSO (per 1L)
		Add 900 mL assay buffer (with Prionex and DTT)
		Mix well and cover with foil. Allow solution to reach RT before use.
		Note: order of addition matters here, dilute 10 mM stock in DMSO prior to adding aqueous buffer
Triton X quench solution (2%)	Triton X-100	Add 20 mL Triton X-100 to 980 mL MilliQ water
		Incubate in a 37 °C water bath or apply mild heat with stirring until solution is homogenous. Once homogeneous, detergent should stay in solution at RT.

CHAPTER 4: Fragment-based protein-protein interaction antagonists of a dimeric protease

Jonathan E. Gable^{1,2}, Gregory M. Lee¹, Patrick Schweigler³, Samu Melkko³, Christopher J. Farady³, and Charles S. Craik¹

¹ Department of Pharmaceutical Chemistry
University of California
San Francisco, CA 94158-2280
USA

² Biophysics Graduate Group
University of California
San Francisco, CA, 94158-2280
USA

³ Novartis Institutes for BioMedical Research,
Forum 1, Novartis Campus CH-4002
Basel, Switzerland

Abstract

Fragment-based drug discovery has shown promise as an approach for challenging targets such as protein-protein interfaces. We developed and applied a fragment-based screen against dimeric Kaposi's sarcoma-associated herpesvirus protease using an optimized fluorogenic substrate. Dose response determination was performed as a confirmation screen and NMR was used to map fragment inhibitor binding. Despite the use of an unbiased library of commercially available library, more than 80% of confirmed hits disrupted dimerization and bound to a previously reported pocket at the dimer interface. SAR by catalogue improved inhibitors more than 100-fold arriving at multiple 7 μ M inhibitors with improved ligand efficiency and LipE values relative to previously reported inhibitors binding this pocket. This study illustrates the power of fragment-based screening for these challenging targets and highlights the potential for pockets at protein-protein interfaces that are in fact more druggable than active sites.

Fragment-based drug discovery shows great promise for the development of inhibitors, particularly of challenging targets such as protein-protein interfaces.¹ Scientists have reported numerous examples of fragment-based drug discovery leading to clinical candidates.² Over 80% of proteins are predicted to function in complexes giving rise to an estimated 650,000 protein-protein interactions in the human interactome, and still more arising from host-pathogen interactions. The ability to modulate even a fraction of these with small molecules derived from fragments holds enormous potential to better understand biology and address clinical unmet need.^{3, 4} The human herpesviruses provide a case study for this approach.

Human herpesvirus infection is exceptionally prevalent, with greater than 90% of the world's population harboring at least one of the nine human herpesviruses.^{5, 6} The effect of these infections ranges from asymptomatic to life threatening and where disease is severe, treatment options are often lacking. All human herpesviruses rely on an essential conserved dimeric protease for capsid maturation and viral replication.⁷⁻⁹ Since the discovery of these proteases and their genetic validation as potential drug targets, numerous attempts have been made to develop active-site inhibitors.⁹⁻²⁸ Despite considerable efforts and some in vitro success—both academic and industrial—no inhibitors targeting the human herpesvirus proteases have advanced to the clinic.⁵

Protease activity requires dimerization due to an allosteric link between the dimer interface and the active site of each monomer.²⁹ From an inhibitor development perspective this opens up the possibility of developing dimer disruptors—protein-protein interaction antagonists that prevent dimerization thereby allosterically inhibiting the enzyme active site. In order to identify new scaffolds for the development of herpesvirus protease dimer disruptors we adapted a high-throughput screen for cytomegalovirus (CMV) protease (see CHAPTER 3) for use in

fragment-based screening of Kaposi's sarcoma associated herpesvirus (KSHV) protease. We utilized cleavage of a fluorogenic substrate (measuring fluorescence intensity) as our primary screen. By enabling direct measurement of enzyme inhibition results were not restricted to any specific mode of binding or inhibition (e.g. competitive, uncompetitive, dimer disruptor). This is in contrast to methods that measure binding (e.g. NMR, SPR) or dimerization (e.g. fluorescence polarization, AlphaScreen, or FRET). Our confirmation screen consisted of dose response curves based on enzymatic activity. Automated medium-throughput NMR spectroscopy was then used to determine whether the dimer is disrupted in the presence of the fragment. Further NMR analysis allowed mapping fragment binding to a specific part of the protein. Remarkably, all but one of the confirmed hits from an unbiased library disrupted dimerization and did so by binding to a cryptic binding site at the dimer interface.

We screened 15,000 unbiased commercially available fragments at 500 μM against KSHV protease. This resulted in 121 hits (0.8% hit rate), defined as fragments that resulted in >50% inhibition (based on endpoint fluorescence intensity). Of these, 67 fragments were repurchased based on availability and diversity of chemical structure and tested for dose response and mode of binding. Across these, 17 out of 67 (~27%) had mean IC_{50} values less than or approximately equal to 500 μM while the other 49 (73%) were deemed false positives. IC_{50} values ranged from 51.2 μM to 557 μM with ligand efficiencies from 0.21 to 0.45 (**Table 4-1**).

Fifteen of seventeen fragment inhibitors were shown to disrupt dimerization, not target the active site. This was determined by $^{13}\text{C}/^1\text{H}$ Met HSQC acquisition on selectively labeled wild type enzyme in the presence and absence of fragment. The monomer-dimer equilibrium exhibits slow exchange on the NMR time scale resulting in a distinct monomer peak and a distinct dimer

peak for interfacial Met197.³⁰⁻³² All but two fragments resulted in a diminution of the dimer peak relative to the monomer peak, indicating the loss of dimer (Figure 4-1a,c). Fragments **14** and **17** did not significantly disrupt dimerization. Fragment **17** crashed out of solution at 500 μ M and thus was deemed a false positive. It is unclear how fragment **14** inhibits. The result that the overwhelming majority of fragments cause dimer disruption is, at face value, highly counterintuitive. Typically protein-protein interfaces are considered less “druggable” than enzyme active sites due to their large relatively featureless surfaces.^{33, 34} It was previously shown that substrates and active site competitive inhibitors promote dimerization, suggesting that in order for the identified fragments to disrupt dimerization they must either bind at the interface or a site distant from the interface that allosterically causes dimer disruption.^{35, 36} Attempts to co-crystallize fragments with the enzyme did not yield crystals. Soaking DD2-analog crystals in high concentrations of fragment dissolved the crystal, resulted in poor diffraction, or diffracted but failed to displace the DD2-analog. In cases where crystallography is not feasible, NMR provides a powerful way to determine where a fragment is binding. To further investigate the mode of binding we used $^{13}\text{C}/^1\text{H}$ Ile and uniform $^{15}\text{N}/^1\text{H}$ HSQC spectra to map fragment binding on an obligate monomeric construct of the enzyme (KSHV Δ 196). These data revealed that all of the dimer disruptor fragments bind near an aromatic hotspot at the dimer interface, Trp109, as indicated by chemical shift perturbations of neighboring isoleucines 44 and 105 in the $^{13}\text{C}/^1\text{H}$ spectra (Fig 4-1b,d). This pattern of chemical shift perturbations is similar to that previously reported for helical mimetics that disrupt KSHV protease dimerization, suggesting these fragments are binding the same site.³⁰⁻³² $^{15}\text{N}/^1\text{H}$ HSQC spectra strongly support this conclusion, with the most significantly perturbed residues being a part of the surface of the previously

reported cryptic binding site at the dimer interface as illustrated for **8** in Figure 2. Protein-observed NMR spectra for all fragments can be found in the supplemental material.

The pocket into which these fragments bind is formed when tryptophan changes rotameric state; instead of being flush against the core of the protein it is in an “open” confirmation.³¹ This may be the basis for enrichment in indole-containing fragments (**7-9**) and other multi-cyclic aromatic planar structures (**11-13**). These fragments, such as 5-bromo-3-methyl-1H-indole highlighted in figures 1 and 2, could stack on top of the tryptophan indole or take its place in the “closed” position, flush against the protein core, consistent with ¹⁵N/¹H-HSQC mapping of fragment **8** binding (fig. 4-2). These data suggest binding to the lower part of the pocket (fig. 4-2b) and allosteric conformational changes between the interfacial pocket and the active site that perturb buried residues of the enzyme monomer (fig. 4-2a). These results not only provide novel chemical entities from which to develop dimer disruptors, they suggest that the dimer interface is more “druggable” than the active site. Out of a library of 15K unbiased fragments, not one was identified as a competitive active site-directed inhibitor. These results lend credence to the importance of hotspots (in this case Trp109) in binding to protein-protein interfaces and suggest fragments may preferentially bind these regions and thus provide efficient starting points for development of protein-protein interaction antagonists.

We next pursued SAR by catalogue based on these novel dimer disruptor fragments. Indole-containing compounds showed limited clear SAR and little improvement from the original bromoindole hit, **7** (Table 4-2). The only indole-containing compound that showed improvement was 5,6-dichloro-1H,2H,3H,4H-cyclopenta[b]indole with an IC₅₀ of 76 μM, a 4.4-fold improvement over **7**. Addition of a methyl group or an aldehyde to the 3-position of **7** resulted in increased IC₅₀ (decreased potency). Despite this reduction in potency NMR clearly

indicates binding to the same pocket at the dimer interface and disruption of dimerization. Of the 117 fragments tested in our confirmation screen and SAR by catalogue efforts the bromoindole scaffold gave rise to the best compounds in terms of ligand efficiency and LipE, despite high micromolar IC₅₀ values. Given this and the aforementioned structural rationale for inhibition by indoles further exploration of this scaffold is warranted.

The aminothiazoles showed the most promising SAR by catalogue with IC₅₀ values ranging from >1000 μ M to 6.75 μ M and exhibiting clear chemical trends (Table 4-3). Previous publications have expressed concern about the aminothiazole scaffold, particularly as a common hit in fragment-based screening.³⁷ Specifically, aminothiazoles have been reported as promiscuous binders and have exhibited flat SAR against other diverse targets. While these properties are cause for concern, the data herein provide evidence of specific binding as well as clear chemical trends that allow for optimization of the 2-aminothiazole scaffold as inhibitors of KSHV protease dimerization and activity. The unsubstituted 2-aminothiazole has an IC₅₀ greater than 1 mM. A bromo substitution in the para position did not improve potency, while a para methyl exhibited a modest increase in potency (IC₅₀ ~ 770 μ M). However, a para chloro substitution resulted in an inhibitor with an IC₅₀ of 51 μ M, more than an order of magnitude improvement over para methyl. Interestingly, a single meta bromo substitution resulted in an IC₅₀ of 151 μ M. Overall this trend suggests a steric constraint where large substitutions in the para position are not tolerated. Chloro substitution in the para and meta position further improved inhibition yielding an IC₅₀ of 38.4 μ M (table 4-1).

Substitution off the amine also improved potency. Addition of 1,3-benzodioxole off the amine of the para bromo substituted compound improved potency to 350 μ M, while 3-methoxyphenyl improved potency to 211 μ M (Table 4-3). 3- or 4-benzoic acid substitutions

inhibited with 20 μ M IC₅₀s. We therefore hypothesized that the substitution off the amine changes the binding mode, allowing for the bulky bromo substitution in the para position. Indeed, use of Glide (Schrödinger Inc.) to perform docking suggests a reversed binding pose in the presence of either of these substitutions off the amine (data not shown). Consistent with the trend observed without substitution of the amine, the 4-{[4-(3,4-dichlorophenyl)-1,3-thiazol-2-yl]amino}benzoic acid was an even better inhibitor, with an IC₅₀ of 6.8 μ M. The position of the acid on the benzoic acid moiety did not change the degree of inhibition, also consistent with the docking model that predicts that the acid is solvent exposed in both the 3- and 4-positions. While substitution off the amine improved IC₅₀, the ligand efficiency for did not significantly improve. LipE improved with addition of the benzoic acid due to the reduction in cLogD. R-group decomposition for the aminothiazoles is shown in full in Table 4-3.

With an unbiased screen of commercially available compounds and SAR by catalogue we have identified low micromolar inhibitors of the KSHV protease monomer-monomer interaction as well as less potent scaffolds that, with further chemistry, may yield additional potent inhibitors. The ligand efficiency and LipE values for our top aminothiazole inhibitors offer considerable improvement over DD2 analogs and can be readily further derivatized. This approach proved far more efficient and effective than typical HTS screening, in which 250K compounds were screened and no low-micromolar inhibitors of dimerization were identified.

Materials and methods

Materials

Protein expression and purification was performed as previously described (chapter 6). SAR by catalogue purchases were made from ChemBridge (Hit2lead.com) or www.emolecules.com. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Kinetic assays and NMR

All manual kinetic assays were carried out using a Synergy H4 multimode plate reader (Biotek) with excitation/emission wavelengths of 485/530 nm. IC₅₀ determination was carried out as previously described (see chapter 6) adjusting the range of inhibitor concentrations used as necessary to record a full dose response curve. NMR assays were carried out as previously described (see chapter 6).

R-group analysis

R-group analysis and chemical database preparation and manipulation were carried out using Instant JChem (Chemaxon). LogP and LogD were computed using Instant JChem assuming a pH of 7.4. Ligand efficiency (LE) was computed as $-\log(\text{IC}_{50})/N$ where IC₅₀ is in molar and N is the heavy atom count.

FBS primary screen

A biochemical fluorescence intensity based assay was used to screen 16,225 fragments at 500 μM . 0.25 μL compound (50 mM stock solution in DMSO) was incubated with 12.5 μL of KSHV protease in 384-well black microtiter plates (Cat No. 95040020 Thermo Electron Oy, Finland) and incubated for 1 hour. 12.5 μL Ac-Pro-Val-Tyr-tBG-Leu-Gln-Ala-(rhodamine110)-dPro (Biosyntan, Berlin, Germany) was added to each plate to initiate the reaction. Fluorescence intensity was measured after 1 hour using a Tecan Safir2 monochromator-based fluorescence reader (ex/em: 485 nm /535 nm). Final assay conditions were 500 μM compound, 1.5 μM KSHV protease, and 2 μM substrate in 25 mM potassium phosphate pH 8.0, 150 mM KCl, 0.1 mM EDTA, 0.9 % DMSO, 0.1% prionex, 1 mM DTT. Z' values were between 0.6-0.8 across

the 46 plates, and a control IC₅₀ plate was run after every 15 plates to assure assay robustness with KSHV inhibitor DD2. Assay was run in duplicate; correlation between the two replicates was good and the percent inhibition is reported as an average of n1 and n2.

- [1] Surade, S., and Blundell, T. L. (2012) Structural biology and drug discovery of difficult targets: the limits of ligandability, *Chem Biol* 19, 42-50.
- [2] Murray, C. W., Verdonk, M. L., and Rees, D. C. (2012) Experiences in fragment-based drug discovery, *Trends Pharmacol Sci* 33, 224-232.
- [3] Jeong, H., Mason, S. P., Barabasi, A. L., and Oltvai, Z. N. (2001) Lethality and centrality in protein networks, *Nature* 411, 41-42.
- [4] Stumpf, M. P., Thorne, T., de Silva, E., Stewart, R., An, H. J., Lappe, M., and Wiuf, C. (2008) Estimating the size of the human interactome, *Proc Natl Acad Sci U S A* 105, 6959-6964.
- [5] Gable, J. E., Acker, T. M., and Craik, C. S. (2014) Current and Potential Treatments for Ubiquitous but Neglected Herpesvirus Infections, *Chemical reviews*.
- [6] Wald, A., and Corey, L. (2007) Persistence in the population: epidemiology, transmission, In *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (Arvin, A., Campadelli-Fiume, G., Mocarski, E., and al., e., Eds.), Cambridge University Press, Cambridge, Cambridge.
- [7] Tong, L. (2002) Viral proteases, *Chemical reviews* 102, 4609-4626.
- [8] Gao, M., Matusick-Kumar, L., Hurlburt, W., DiTusa, S. F., Newcomb, W. W., Brown, J. C., McCann, P. J., 3rd, Deckman, I., and Colonno, R. J. (1994) The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth, *J Virol* 68, 3702-3712.
- [9] Jiang, X., Gong, H., Chen, Y. C., Vu, G. P., Trang, P., Zhang, C. Y., Lu, S., and Liu, F. (2012) Effective inhibition of cytomegalovirus infection by external guide sequences in mice, *Proc Natl Acad Sci U S A* 109, 13070-13075.

- [10] Abood, N. A., Schretzman, L. A., Flynn, D. L., Houseman, K. A., Wittwer, A. J., Dilworth, V. M., Hippenmeyer, P. J., and Holwerda, B. C. (1997) Inhibition of human cytomegalovirus protease by benzoxazinones and evidence of antiviral activity in cell culture, *Bioorg Med Chem Lett* 7, 2105-2108.
- [11] Bonneau, P. R., Hasani, F., Plouffe, C., Malenfant, E., LaPlante, S. R., Guse, I., Ogilvie, W. W., Plante, R., Davidson, W. C., Hopkins, J. L., Morelock, M. M., Cordingley, M. G., and Deziel, R. (1999) Inhibition of human cytomegalovirus protease by monocyclic beta-lactam derivatives: Kinetic characterization using a fluorescent probe, *J Am Chem Soc* 121, 2965-2973.
- [12] Borthwick, A. D. (2005) Design of translactam HCMV protease inhibitors as potent antivirals, *Med Res Rev* 25, 427-452.
- [13] Borthwick, A. D., Davies, D. E., Ertl, P. F., Exall, A. M., Haley, T. M., Hart, G. J., Jackson, D. L., Parry, N. R., Patikis, A., Trivedi, N., Weingarten, G. G., and Woolven, J. M. (2003) Design and synthesis of pyrrolidine-5,5'-trans-lactams (5-oxo-hexahydropyrrolo[3,2-b]pyrroles) as novel mechanism-based inhibitors of human cytomegalovirus protease. 4. Antiviral activity and plasma stability, *J Med Chem* 46, 4428-4449.
- [14] Borthwick, A. D., Exall, A. M., Haley, T. M., Jackson, D. L., Mason, A. M., and Weingarten, G. G. (2002) Pyrrolidine-5,5-trans-lactams as novel mechanism-based inhibitors of human cytomegalovirus protease. Part 3: potency and plasma stability, *Bioorg Med Chem Lett* 12, 1719-1722.
- [15] Deziel, R., and Malenfant, E. (1998) Inhibition of human cytomegalovirus protease N(o) with monocyclic beta-lactams, *Bioorg Med Chem Lett* 8, 1437-1442.

- [16] Gerona-Navarro, G., Perez de Vega, M. J., Garcia-Lopez, M. T., Andrei, G., Snoeck, R., Balzarini, J., De Clercq, E., and Gonzalez-Muniz, R. (2004) Synthesis and anti-HCMV activity of 1-acyl-beta-lactams and 1-acylazetidines derived from phenylalanine, *Bioorg Med Chem Lett* 14, 2253-2256.
- [17] Gerona-Navarro, G., Perez de Vega, M. J., Garcia-Lopez, M. T., Andrei, G., Snoeck, R., De Clercq, E., Balzarini, J., and Gonzalez-Muniz, R. (2005) From 1-acyl-beta-lactam human cytomegalovirus protease inhibitors to 1-benzyloxycarbonylazetidines with improved antiviral activity. A straightforward approach to convert covalent to noncovalent inhibitors, *J Med Chem* 48, 2612-2621.
- [18] Holwerda, B. C. (1997) Herpesvirus proteases: targets for novel antiviral drugs, *Antiviral Res* 35, 1-21.
- [19] Jarvest, R. L., Connor, S. C., Gorniak, J. G., Jennings, L. J., Serafinowska, H. T., and West, A. (1997) Potent selective thienoxazinone inhibitors of herpes proteases, *Bioorg Med Chem Lett* 7, 1733-1738.
- [20] Jarvest, R. L., Parratt, M. J., Debouck, C. M., Gorniak, J. G., Jennings, L. J., Serafinowska, H. T., and Strickler, J. E. (1996) Inhibition of HSV-1 protease by benzoxazinones, *Bioorg Med Chem Lett* 6, 2463-2466.
- [21] Jarvest, R. L., Pinto, I. L., Ashman, S. M., Dabrowski, C. E., Fernandez, A. V., Jennings, L. J., Lavery, P., and Tew, D. G. (1999) Inhibition of herpes proteases and antiviral activity of 2-substituted thieno[2,3-d]oxazinones, *Bioorg Med Chem Lett* 9, 443-448.
- [22] Ogilvie, W., Bailey, M., Poupart, M. A., Abraham, A., Bhavsar, A., Bonneau, P., Bordeleau, J., Bousquet, Y., Chabot, C., Duceppe, J. S., Fazal, G., Goulet, S., Grand-Maitre, C., Guse, I., Halmos, T., Lavallee, P., Leach, M., Malenfant, E., O'Meara, J., Plante, R.,

- Plouffe, C., Poirier, M., Soucy, F., Yoakim, C., and Deziel, R. (1997) Peptidomimetic inhibitors of the human cytomegalovirus protease, *J Med Chem* 40, 4113-4135.
- [23] Perez-Faginas, P., Aranda, M. T., Garcia-Lopez, M. T., Snoeck, R., Andrei, G., Balzarini, J., and Gonzalez-Muniz, R. (2011) Synthesis and SAR studies on azetidine-containing dipeptides as HCMV inhibitors, *Bioorg Med Chem* 19, 1155-1161.
- [24] Pinto, I. L., West, A., Debouck, C. M., DiLella, A. G., Gorniak, J. G., O'Donnell, K. C., O'Shannessy, D. J., Patel, A., and Jarvest, R. L. (1996) Novel, selective mechanism-based inhibitors of the herpes proteases, *Bioorg Med Chem Lett* 6, 2467-2472.
- [25] Schepetkin, I. A., Khlebnikov, A. I., and Quinn, M. T. (2007) N-benzoylpyrazoles are novel small-molecule inhibitors of human neutrophil elastase, *J Med Chem* 50, 4928-4938.
- [26] Teshima, T., Griffin, J. C., and Powers, J. C. (1982) A new class of heterocyclic serine protease inhibitors. Inhibition of human leukocyte elastase, porcine pancreatic elastase, cathepsin G, and bovine chymotrypsin A alpha with substituted benzoxazinones, quinazolines, and anthranilates, *J Biol Chem* 257, 5085-5091.
- [27] Waxman, L., and Darke, P. L. (2000) The herpesvirus proteases as targets for antiviral chemotherapy, *Antivir Chem Chemother* 11, 1-22.
- [28] Yoakim, C., Ogilvie, W. W., Cameron, D. R., Chabot, C., Guse, I., Hache, B., Naud, J., O'Meara, J. A., Plante, R., and Deziel, R. (1998) beta-Lactam derivatives as inhibitors of human cytomegalovirus protease, *J Med Chem* 41, 2882-2891.
- [29] Reiling, K. K., Pray, T. R., Craik, C. S., and Stroud, R. M. (2000) Functional consequences of the Kaposi's sarcoma-associated herpesvirus protease structure: regulation of activity and dimerization by conserved structural elements, *Biochemistry* 39, 12796-12803.

- [30] Gable, J. E., Lee, G. M., Jaishankar, P., Hearn, B. R., Waddling, C. A., Renslo, A. R., and Craik, C. S. (2014) Broad-spectrum allosteric inhibition of herpesvirus proteases, *Biochemistry* 53, 4648-4660.
- [31] Lee, G. M., Shahian, T., Baharuddin, A., Gable, J. E., and Craik, C. S. (2011) Enzyme inhibition by allosteric capture of an inactive conformation, *J Mol Biol* 411, 999-1016.
- [32] Shahian, T., Lee, G. M., Lazic, A., Arnold, L. A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a viral enzyme by a small-molecule dimer disruptor, *Nat Chem Biol* 5, 640-646.
- [33] Arkin, M. R., Tang, Y., and Wells, J. A. (2014) Small-molecule inhibitors of protein-protein interactions: progressing toward the reality, *Chem Biol* 21, 1102-1114.
- [34] Wells, J. A., and McClendon, C. L. (2007) Reaching for high-hanging fruit in drug discovery at protein-protein interfaces, *Nature* 450, 1001-1009.
- [35] Lazic, A., Goetz, D. H., Nomura, A. M., Marnett, A. B., and Craik, C. S. (2007) Substrate modulation of enzyme activity in the herpesvirus protease family, *J Mol Biol* 373, 913-923.
- [36] Marnett, A. B., Nomura, A. M., Shimba, N., Ortiz de Montellano, P. R., and Craik, C. S. (2004) Communication between the active sites and dimer interface of a herpesvirus protease revealed by a transition-state inhibitor, *Proc Natl Acad Sci U S A* 101, 6870-6875.
- [37] Devine, S. M., Mulcair, M. D., Debono, C. O., Leung, E. W., Nissink, J. W., Lim, S. S., Chandrashekar, I. R., Vazirani, M., Mohanty, B., Simpson, J. S., Baell, J. B., Scammells, P. J., Norton, R. S., and Scanlon, M. J. (2015) Promiscuous 2-aminothiazoles (PrATs): a frequent hitting scaffold, *J Med Chem* 58, 1205-1214.

Table 4-1 Confirmed primary screen hits

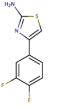
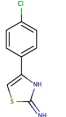
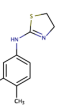
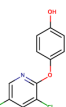
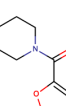
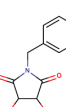
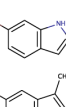
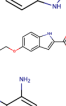
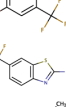
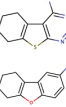
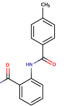
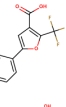
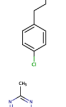
	ID	IC50 (μM)	LE	DD
	1 (NF2/23)	404	0.31	Y
	2 (NF33)	51.2	0.43	Y
	3 (NF26)	187	0.34	Y
	4 (NF1)	337	0.28	Y
	5 (NF32)	405	0.34	Y
	6 (NF34)	367	0.28	Y
	7 (NF28)	337	0.45	Y
	8 (NF19)	421	0.39	Y
	9 (NF24)	557	0.21	Y
	10 (NF20)	235	0.36	Y
	11 (NF17)	495	0.31	Y
	12 (NF38)	89.5	0.33	W
	13 (NF48)	81.7	0.38	Y
	14 (NF6)	197	0.25	?
	15 (NF25)	445	0.23	Y
	16 (NF44)	387	0.44	Y
	17 (NF57)	338	0.24	W

Table 4-2. Indole R-group table

Structure	R1	R2	R3	R4	R5	Mol Weight	IC50	LogD	LogP	LE	LipE (pIC50-logP)	LipE (pIC50-logD)
						226.10	76.00	4.05	4.05	0.41	0.07	0.07
						196.05	337.00	2.84	2.84	0.49	0.63	0.63
						370.84	412.00	2.53	2.53	0.18	0.86	0.86
						210.07	421.00	3.35	3.35	0.43	0.02	0.02
						291.78	466.00	1.73	1.87	0.23	1.47	1.60
						254.08	536.00	-0.88	2.54	0.33	0.73	4.15
						254.08	542.00	2.76	2.76	0.33	0.50	0.50
						267.28	557.00	-0.13	3.22	0.23	0.04	3.38
						336.23	729.00	1.89	2.03	0.22	1.11	1.24
						224.06	746.00	2.55	2.55	0.36	0.57	0.57
						268.11	941.00	-0.53	2.78	0.28	0.25	3.55
						287.33	1,000.00	2.15	2.15	0.21	0.85	0.85

Table 4-3. Aminothiazole R-group table

Structure	R1	R2	R3	IC50	LogD	LogP	TPSA	Molecular weight	LE	LipE (pIC50-logP)	LipE (pIC50-logD)
				6.72	3.05	5.65	62.22	365.23	0.31	-0.48	2.12
				6.75	2.93	5.65	62.22	365.23	0.31	-0.48	2.24
				12.40	2.32	5.05	62.22	330.79	0.31	-0.14	2.58
				20.80	2.49	5.22	62.22	375.24	0.30	-0.53	2.19
				21.50	2.61	5.22	62.22	456.15	0.28	-0.55	2.06
				29.90	1.68	4.29	71.45	326.37	0.28	0.24	2.84
				38.40	3.73	3.73	38.91	245.12	0.44	0.69	0.69
				50.00	2.45	5.05	62.22	330.79	0.27	-0.75	1.86
				51.20	3.12	3.12	38.91	210.68	0.46	1.17	1.17
				151.00	3.29	3.29	38.91	255.13	0.41	0.53	0.53
				211.00	5.40	5.40	34.15	442.17	0.23	-1.72	-1.72
				265.00	1.72	4.45	62.22	296.34	0.24	-0.87	1.86
				350.00	5.18	5.18	43.38	456.15	0.21	-1.73	-1.73
				404.00	2.81	2.81	38.91	212.22	0.34	0.59	0.59
				777.00	3.03	3.03	38.91	190.26	0.33	0.08	0.08
				1,170.00	3.29	3.29	38.91	336.05	0.29	-0.36	-0.36
				1,200.00	2.52	2.52	38.91	176.24	0.34	0.40	0.40
					1.98	4.59	62.22	314.33			
					3.18	3.18	38.91	208.25			

Table 4-4. Trifluorobenzene R-group table

Structure	R1	R2	R3	R4	IC50	LogD	LogP	Molecular weight	LE	LipE (pIC50-logP)	LipE (pIC50-logD)
					221.00	3.51	3.51	310.11	0.30	0.15	0.15
					235.00	1.96	1.96	255.04	0.39	1.67	1.67
					336.00	2.79	2.79	240.02	0.41	0.68	0.68
					618.00	3.03	3.03	273.27	0.25	0.18	0.18
					642.00	2.69	2.94	268.24	0.24	0.25	0.50
					669.00	3.22	3.22	240.57	0.30	-0.04	-0.04
					696.00	2.79	2.79	240.02	0.37	0.37	0.37
					1,000.00	3.11	3.11	280.25	0.21	-0.11	-0.11
					1,000.00	2.48	2.49	295.27	0.20	0.51	0.52
					1,950.00	4.13	4.13	239.04	0.32	-1.42	-1.42
					2,170.00	1.19	1.19	176.14	0.31	1.47	1.47
					4,340.00	3.62	3.62	225.01	0.30	-1.26	-1.26
					5,320.00	4.13	4.13	239.04	0.27	-1.86	-1.86
					8,850.00	3.39	3.39	251.63	0.18	-1.34	-1.34

Table 4-5. Sulfur tricycle R-group table

Structure	R1	R2	R3	IC50	LogD	LogP	Molecular weight	LE	LipE (pIC50-logP)	LipE (pIC50-logD)
				71.30	4.09	4.09	248.34	0.34	0.06	0.06
				85.10	3.85	3.85	379.43	0.22	0.22	0.22
				89.50	3.58	3.58	233.33	0.35	0.47	0.47
				136.00	5.20	5.38	459.01	0.17	-1.51	-1.33
				621.00	3.17	3.17	206.26	0.32	0.04	0.04
				8,830.00	3.46	3.46	220.29	0.19	-1.40	-1.40
					3.31	3.31	220.29			
					3.04	3.43	378.45			

Table 4-6

Structure	R1	R2	IC50	LogD	LogP	Molecular weight	LE	LipE (pIC50-logP)	LipE (pIC50-logD)
			81.70	2.52	2.52	187.24	0.41	1.57	1.57
			637.00	-0.43	2.67	246.26	0.25	0.53	3.63
			1,190.00	2.23	2.24	279.35	0.22	0.69	0.69
				3.04	3.05	188.23			

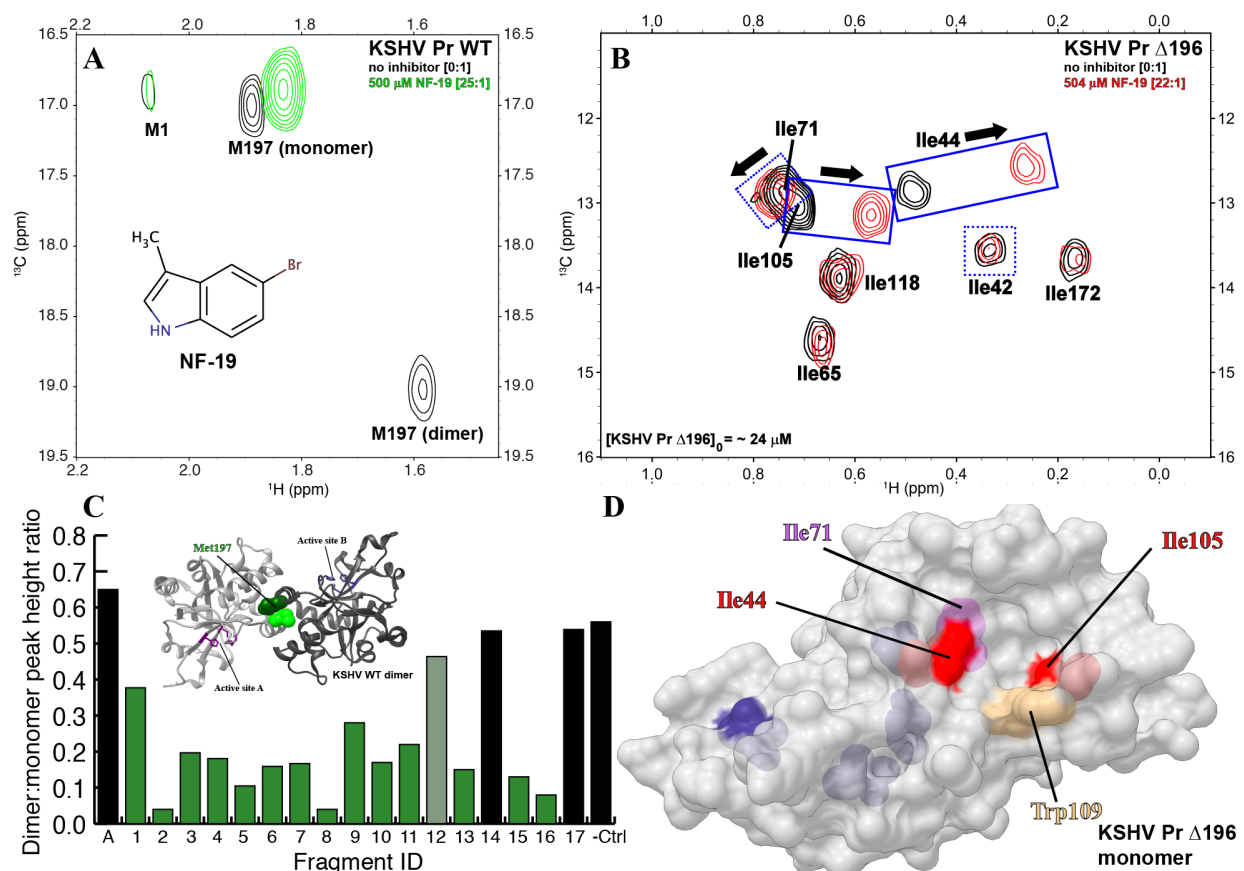


Figure 4-1. NMR mapping of fragment binding and determination of mechanism of action.

$^{13}\text{C}/^1\text{H}$ -HSQC spectra of ^{13}C -Met labeled wildtype (a) and ^{13}C -Ile labeled obligate monomeric KSHV $\Delta 196$ (b) in the presence and absence of fragment 8. The ratio of dimer to monomer peak height is plotted by fragment including apo and a negative control fragment. Lower values (green) indicate a loss of dimer. Black bars indicate no dimer observed disruption. Pastel green indicates small but significant loss of dimer. (c) The structure of WT KSHV protease (PDB: 2PBK) with interfacial Met197 and each monomer's active site is indicated (c, inset). The structure of monomer KSHV $\Delta 196$ is shown in (d). Surface exposed Ile residues within the putative pocket are shown in red (Ile44, 105). Perturbed but buried Ile 71 is shown in magenta. All other Ile residues are shown in dark blue. The aromatic hotspot in its open confirmation (Trp109) is shown in tan.

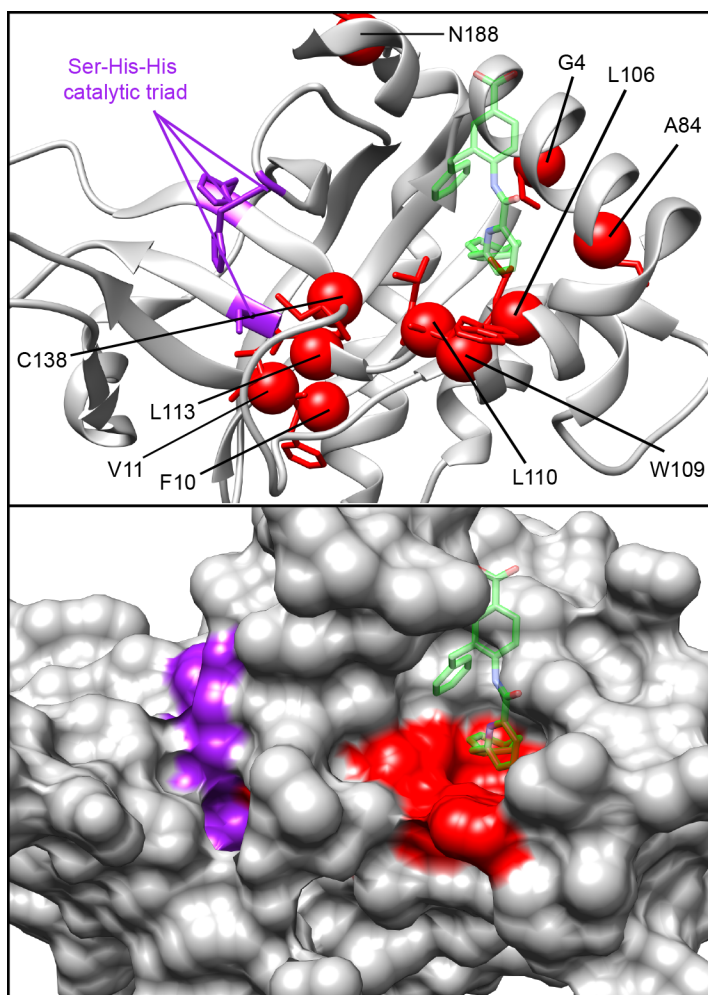
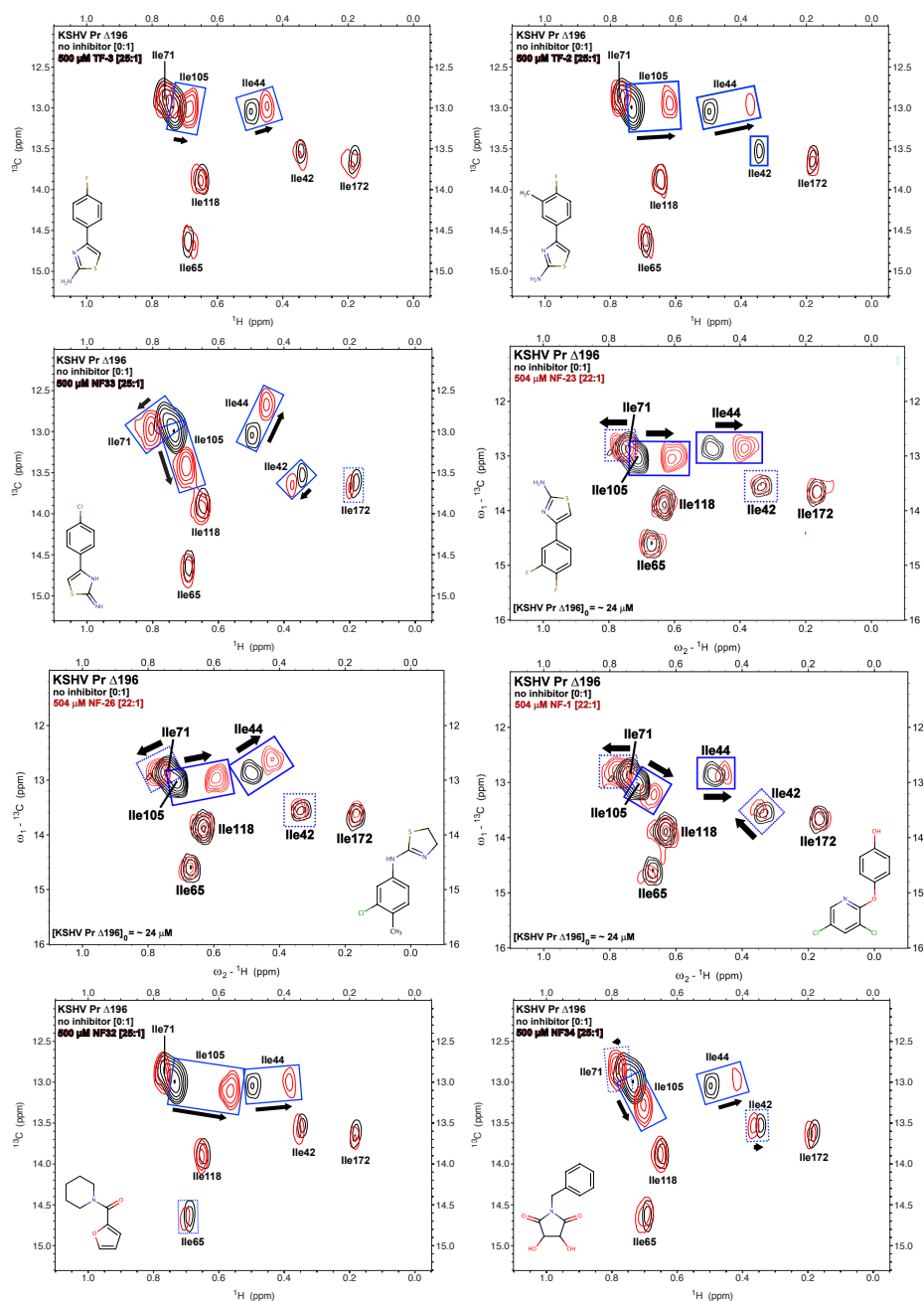
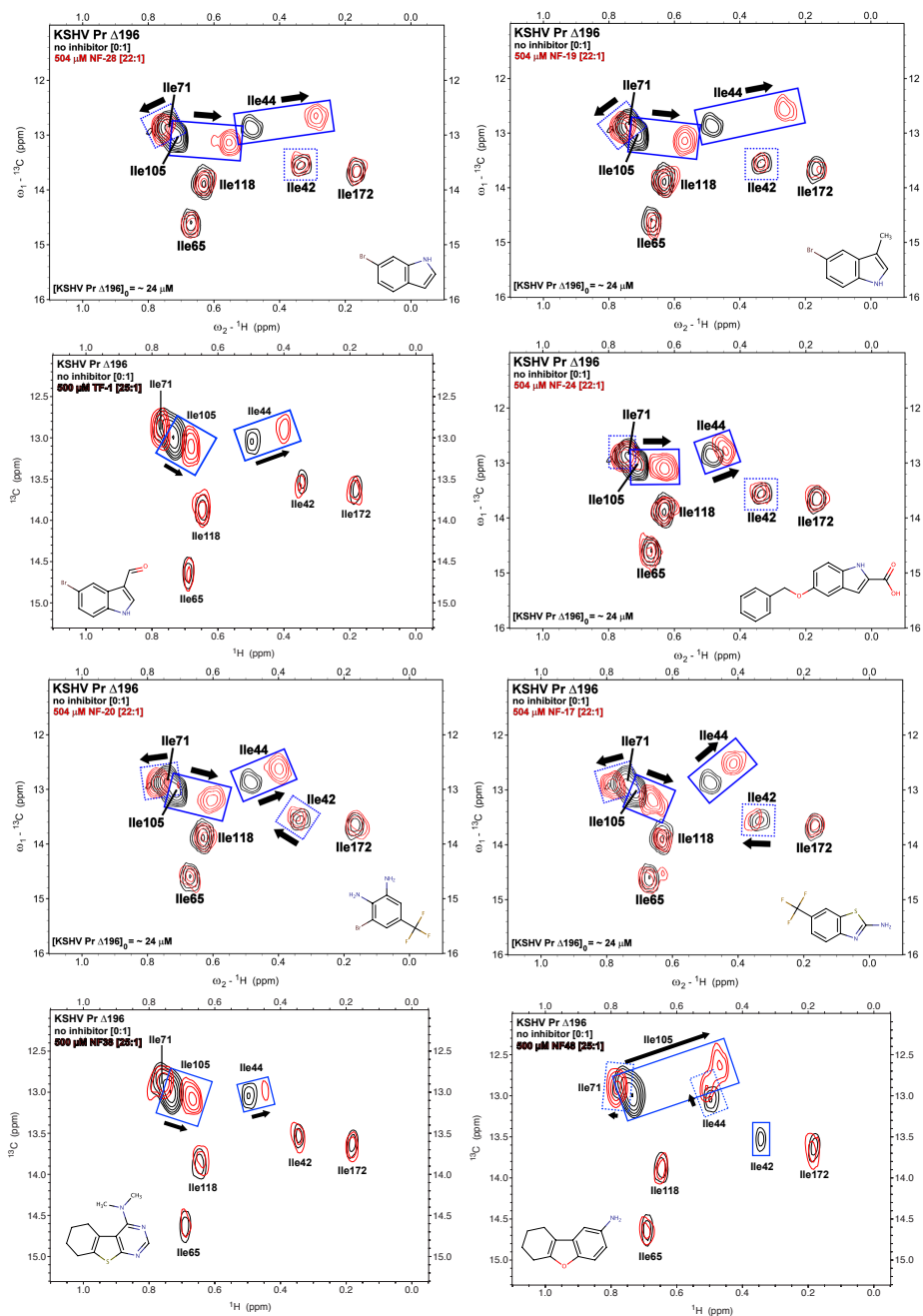
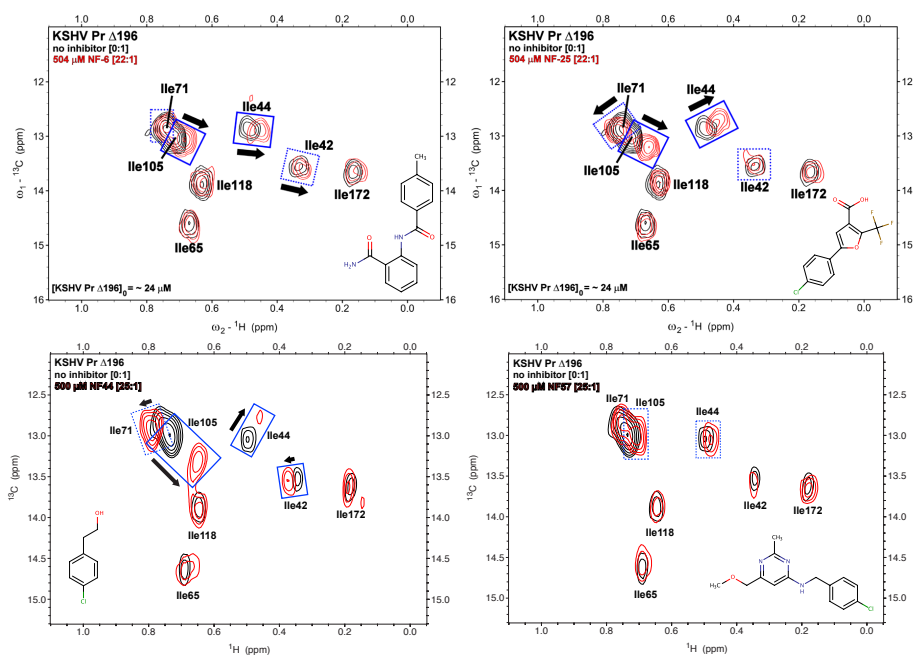


Figure 4-2: The structure of monomeric KSHV $\Delta 196$ (PDB: 3NJQ) with the top 10 $^{15}\text{N}/\text{H}^1$ -HSQC perturbations mapped in red without (a) and with (b) a surface model. Amide backbone nitrogen atoms are shown as spheres. The Ser-His-His catalytic triad is highlighted in magenta. The binding mode of the previously reported helical mimetic dimer disruptor is shown in faded green.

Supplemental material







CHAPTER 5: Activity-based disulfide tethering for the identification of novel human cytomegalovirus protease dimer disruptors

Jonathan E. Gable, Alex J. Martinko, Charles S. Craik

Abstract

Protein-protein interactions are notoriously difficult drug targets. Previously, micromolar protein-protein interaction inhibitors (DD2 and their analogs) of cytomegalovirus (CMV) protease were reported but no crystallographic data has been obtained for these inhibitors. Here we report the development and application of a disulfide-tethering screen against CMV protease to identify novel starting points for protein-protein interaction inhibitor development and to facilitate structure determination in the presence of dimer disruptors. To facilitate this we developed an enzymatically active single-cysteine mutant where the only cysteine is positioned such that tethered fragments are predicted to occupy the putative DD2 binding pocket at the dimer interface. This allowed us to perform the primary tethering screen using enzymatic activity as a readout, dramatically improving throughput compared to the typical approach using mass spectrometry. We identified 42 tethering fragment hits and will use these in future studies to further structure-based drug discovery against this challenging target.

Introduction

Identification of small molecule protein-protein interaction antagonists remains a significant challenge in both chemical biology and drug discovery.¹⁻³ Standard high-throughput screening approaches often do not contain ideal chemical matter for targeting protein-protein interactions and extensive follow-up is required to demonstrate that hit compounds are definitively targeting the protein-protein interaction of interest, either directly or allosterically.¹ Fragment-based screening offers a potential solution to the biases inherent in many screening libraries, however follow-up can be even more challenging given the typically weak potencies associated with fragment hits.⁴ Disulfide tethering offers a way to target thiol-containing fragments to a specific site, for instance at a protein-protein interface. Furthermore, the identified covalent fragment inhibitors can often aid in crystallography enabling subsequent structure-based drug design efforts.⁵⁻⁸ This is especially relevant when targeting a protein-protein interaction as a single monomer of a complex may be inherently unstable and thus difficult to crystalize when not complexed with its partner(s).

Human cytomegalovirus protease is homodimeric, requires dimerization for activity, and is essential for viral replication.^{9, 10} While numerous attempts have been made to inhibit CMV protease through active site directed inhibition they have not resulted in clinical candidates.⁹ Currently all CMV treatments target the viral polymerase. As a result, resistance is on the rise and toxicity can be severe and dose limiting.¹¹ As an alternative to active site inhibitors, we have reported some success with small molecules that inhibit dimerization.¹²⁻¹⁴ While these molecules represent a potential starting point, no crystallographic information is available for these inhibitors bound to CMV protease and the utility of these compounds is limited by poor cell

permeability. These reports show that protein-protein interaction inhibition is a feasible approach for CMV protease inhibition but that new chemical scaffolds may be necessary.

We hypothesized that disulfide tethering could be a productive way to identify small molecules that inhibit cytomegalovirus protease dimerization by binding to a putative pocket analogous to that observed at the KSHV protease dimer interface. In order to identify reversible covalent inhibitors of CMV protease dimerization we developed an enzymatically active construct of CMV protease with a single cysteine positioned near the predicted interfacial pocket. Enzymatic activity was used to rapidly screen thiol-containing fragments and mass spectrometry was used in medium-throughput to confirm one-to-one adduct formation. This is one of few instances where enzymatic activity was used to perform a tethering screen and the only reported tethering screen against CMV protease.

Results

CMV protease has five native cysteines (PDB: 1CMV). Treatment with iodoacetamide followed by protein liquid chromatography-mass spectrometry (LC-MS) revealed that four of the five cysteines are solvent accessible, one more than would be predicted from the static crystal structure (PDB: 1CMV). Cys84 and Cys161 are in close proximity to the active site and thus chemical modification at these sites is likely to disrupt enzymatic activity (**Figure 5-1b**).

Consistent with this observation multiple thiol-reactive compounds have been reported to inhibit CMV protease.^{15, 16} Mutation of all cysteines to serine resulted in a catalytically inactive mutant. However, mutating all cysteines to alanine maintained robust activity (41% that of wildtype enzyme, **Table 5-1**). Previous studies of related Kaposi's sarcoma associated herpesvirus protease revealed a pocket at the dimer interface near a conserved aromatic residue, Tyr128 in CMV protease.¹²⁻¹⁴ Inhibitors that bound to this KSHV pocket also inhibited dimerization of

CMV protease. We therefore hypothesized that a similar pocket may form at the dimer interface of CMV protease in close proximity to Tyr128. Without crystallographic evidence for this pocket we turned to homology modeling to generate a model of CMV protease in the inhibitor-bound monomeric state. The cysteines in this model are predicted to largely stay in the same conformation as those in wildtype CMV protease and none are predicted to be facing into the putative inhibitor pocket (**Figure 5-1**). Based on the homology model A100, L124, S125, S127, and S162 appeared to be residues that could be mutated to cysteine with the resulting thiol pointing into the pocket and with relatively little structural perturbation (**Figure 5-2**). A100C, L124C, S125C, and S127C mutations of the cysteine to alanine construct resulted in little or no enzymatic activity (**Table 5-1**). Mutation of S162 to cysteine resulted in a 75% decrease in activity relative to the all alanine mutant, but had sufficient activity to enable screening and was predicted from our structural model to position the cysteine thiol in the putative dimer interface pocket (**Figure 5-3**). This mutant was carried forward for screening of the library.

We screened 960 thiol-containing fragments at four different reducing agent (β -mercaptoethanol) concentrations and identified 42 hit compounds (4.3% hit rate). Hits were defined as fragments that yield >50% inhibition averaged across all reducing agent concentrations. The average percent inhibition correlates well with BME50, the concentration of BME necessary to yield 50% inhibition by the inhibitor (**Figure 5-S1**). Unlike BME50, average inhibition can be computed for all inhibitors, even those that show little or no inhibition. At the lowest reducing agent concentration used, 0.2 mM β -mercaptoethanol, all 42 hits resulted in >80% inhibition of enzymatic activity. From percent inhibition at four different β -mercaptoethanol concentrations we estimated BME50. Lower values of BME50 indicate weaker inhibitors. Within the 42 hits BME50 values range from 1.0 to 3.7 mM. An inhibitor with an

average percent inhibition of 31% had a BME50 of 0.33 mM (**Figure 5-4**). BME50 could not be reliably estimated for inhibitors weaker than this. **Figure 5-5** shows a plot of percent inhibition versus rank order for all BME concentrations. A boxplot of the percent inhibition for each reducing agent concentration reveals that 5 mM and 10 mM BME share very similar distributions of inhibition across all compounds (**Figure 5-6**). This suggests that the majority of specific inhibitors are fully displaced at both of these concentrations. From this interpretation, the distribution of percent inhibition observed at 10 mM BME indicates the variance due to non-specific effects and random noise/error such as aggregation and dispense errors. Further analysis of the distribution of percent inhibition observed across all compounds screened shows that the data are well approximated by a normal distribution (**Figures 5-S2 and 5-S3**). Summary statistics are presented in **Table 5-2**.

All 42 hits were shown by mass spectrometry to form one-to-one covalent adducts with the screened CMV protease variant. These mass spectrometry experiments also confirmed displacement of fragment with increasing inhibitor concentration. A representative set of spectra is shown in **Figure 5-7**. Twenty-one fragments cause a >5% increase in catalytic activity, however this apparent activation did not titrate with reducing agent. Fourteen representative fragment inhibitors are shown in **Table 5-3** with their estimated BME50 values.

Discussion

We have identified 42 promising thiol-containing fragments that bind to an engineered cysteine at the dimer interface, inhibiting CMV protease enzymatic activity. The design of a CMV protease construct that retained catalytic activity allowed for extremely rapid screening of the tethering library; all 960 fragments were screened at all four reducing agent concentrations in less than a single day in 384-well format. This is a far more efficient approach than utilizing

mass spectrometry as a primary screen. Furthermore, mass spectrometry verified the results of the activity-based screen for all 42 hit fragments. Fluorescence polarization has been successfully used for screening of a tethering library to identify inhibitors of a protein-protein interaction and is similarly rapid. This approach however requires a known fluorescent ligand that can be displaced by fragment binding, something that can be bypassed when enzymatic activity is dependent on the protein-protein interaction.¹⁷

Numerous experiments can be performed to follow-up on the reported data. Size exclusion chromatography should be used to verify that dimerization is indeed blocked by all 42 hit fragments, as opposed to some alternative mechanism for instance where binding causes a conformational change that prevents catalysis but still allows for dimerization. This is especially critical given the proximity of the engineered Cys162 to the active site, though its predicted rotamer points the cysteine thiol away from the active site residues. Cheminformatic analysis would reveal if specific scaffolds or chemical moieties were enriched in the 42 hits relative to the entire 960-member library. This could inform pharmacophore models and elucidate properties of the putative pocket, consistent or inconsistent with the homology model used in this study. If some hits disrupt dimerization and others do not chemical structure analysis may reveal characteristics associated with each type of inhibitor. By visual inspection alone, it is striking that some of the fragments identified in this screen closely resemble hits from a non-tethering fragment-based screen against KSHV protease (**Figure 5-8**), further supporting the notion that there are shared features between KSHV and CMV protease dimer interfaces. Further improvement of the homology model could be achieved by using the results of this tethering screen. Thousands of homology models can be rapidly built using MODELLER and covalent docking of the entire library can be performed against all homology models. The model that best

recapitulates the experimental results is likely to be most accurate. Such a model could inform not only attempts to elaborate the fragment hits identified in this study, but would also offer guidance for structure-based drug design using the previously reported small molecule dimer disruptors. Finally crystallography can be pursued with these fragments. To date there are no structures of monomeric CMV protease in its apo state or bound to an inhibitor. Elucidation of the atomic details of small molecule binding to the dimer interface would have a dramatic impact on inhibitor development against this challenging target.

Materials and Methods

Protein expression and purification

Protein expression, purification, and mutagenesis were performed as previously reported (see chapter 6).

Alkylation with iodoacetamide

Protein was added to 0.2M Tris pH 8 to a final protein concentration of 6.8 μ M. Iodoacetamide was added from a 0.5 M stock to a final concentration of 15 μ M. The total reaction volume per desired timepoint was 25.8 μ L. To quench the reaction 5 μ L 185 μ M DTT was added to a final concentration of about 30 μ M. Quenching was allowed to proceed at room temperature for 30 minutes. As a control, CMV protease was denatured at 82 degrees C for 10 minutes and alkylated. This heat denaturation completely inactivated the protein.

Homology modeling

A homology model was built using MODELLER 9v7 with the KSHV structure PDB 3NJQ and the CMV protease sequence aligned to KSHV protease as input. PROMALS3D web server generated the alignment. One hundred models were built and the model with the best score (normalized DOPE z-score of -1.12449) was used for analysis.

Tethering screen

A WellMate bulk dispenser (Thermo Scientific) was used to dispense 20 μL of 9.375 μM all Ala CMVS162C protease in assay buffer with varying amounts of 2-mercaptoethanol to all wells on each 384-well plate. Fifty nanoliters of compounds were added by 384 slotted pin-tool (VP Scientific) to pre-dispensed assay plates, using a Biomek FXP liquid handler (Beckman Coulter) in 15-minute intervals. Protease reaction was initiated in the first assay plate after 60 minutes through the of addition of 5 μl 375 μM labeled peptide substrate (P6) from an EL406 bulk dispenser (BioTek Instruments) and plate was immediately read for 10 minutes. This process was repeated for all plates.

- [1] Wells, J. A., and McClendon, C. L. (2007) Reaching for high-hanging fruit in drug discovery at protein-protein interfaces, *Nature* 450, 1001-1009.
- [2] Arkin, M. R., Tang, Y., and Wells, J. A. (2014) Small-molecule inhibitors of protein-protein interactions: progressing toward the reality, *Chemistry & biology* 21, 1102-1114.
- [3] Thompson, A. D., Dugan, A., Gestwicki, J. E., and Mapp, A. K. (2012) Fine-tuning multiprotein complexes using small molecules, *ACS chemical biology* 7, 1311-1320.
- [4] Scott, D. E., Ehebauer, M. T., Pukala, T., Marsh, M., Blundell, T. L., Venkitaraman, A. R., Abell, C., and Hyvonen, M. (2013) Using a fragment-based approach to target protein-protein interactions, *Chembiochem : a European journal of chemical biology* 14, 332-342.
- [5] Braisted, A. C., Oslob, J. D., Delano, W. L., Hyde, J., McDowell, R. S., Waal, N., Yu, C., Arkin, M. R., and Raimundo, B. C. (2003) Discovery of a potent small molecule IL-2 inhibitor through fragment assembly, *Journal of the American Chemical Society* 125, 3714-3715.
- [6] Erlanson, D. A., Braisted, A. C., Raphael, D. R., Randal, M., Stroud, R. M., Gordon, E. M., and Wells, J. A. (2000) Site-directed ligand discovery, *Proceedings of the National Academy of Sciences of the United States of America* 97, 9367-9372.
- [7] Erlanson, D. A., Wells, J. A., and Braisted, A. C. (2004) Tethering: fragment-based drug discovery, *Annual review of biophysics and biomolecular structure* 33, 199-223.
- [8] Sadowsky, J. D., Burlingame, M. A., Wolan, D. W., McClendon, C. L., Jacobson, M. P., and Wells, J. A. (2011) Turning a protein kinase on or off from a single allosteric site via disulfide trapping, *Proceedings of the National Academy of Sciences of the United States of America* 108, 6056-6061.

- [9] Gable, J. E., Acker, T. M., and Craik, C. S. (2014) Current and Potential Treatments for Ubiquitous but Neglected Herpesvirus Infections, *Chemical reviews*.
- [10] Tong, L. (2002) Viral proteases, *Chemical reviews* 102, 4609-4626.
- [11] Torres-Madriz, G., and Boucher, H. W. (2008) Immunocompromised hosts: perspectives in the treatment and prophylaxis of cytomegalovirus disease in solid-organ transplant recipients, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 47, 702-711.
- [12] Shahian, T., Lee, G. M., Lazic, A., Arnold, L. A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a viral enzyme by a small-molecule dimer disruptor, *Nat Chem Biol* 5, 640-646.
- [13] Lee, G. M., Shahian, T., Baharuddin, A., Gable, J. E., and Craik, C. S. (2011) Enzyme inhibition by allosteric capture of an inactive conformation, *J Mol Biol* 411, 999-1016.
- [14] Gable, J. E., Lee, G. M., Jaishankar, P., Hearn, B. R., Waddling, C. A., Renslo, A. R., and Craik, C. S. (2014) Broad-spectrum allosteric inhibition of herpesvirus proteases, *Biochemistry* 53, 4648-4660.
- [15] Baum, E. Z., Ding, W. D., Siegel, M. M., Hulmes, J., Bebernitz, G. A., Sridharan, L., Tabei, K., Krishnamurthy, G., Carofiglio, T., Groves, J. T., Bloom, J. D., DiGrandi, M., Bradley, M., Ellestad, G., Seddon, A. P., and Gluzman, Y. (1996) Flavins inhibit human cytomegalovirus UL80 protease via disulfide bond formation, *Biochemistry* 35, 5847-5855.
- [16] Baum, E. Z., Siegel, M. M., Bebernitz, G. A., Hulmes, J. D., Sridharan, L., Sun, L., Tabei, K., Johnston, S. H., Wildey, M. J., Nygaard, J., Jones, T. R., and Gluzman, Y. (1996)

Inhibition of human cytomegalovirus UL80 protease by specific intramolecular disulfide bond formation, *Biochemistry* 35, 5838-5846.

- [17] Lodge, J. M., Rettenmaier, T. J., Wells, J. A., Pomerantz, W. C., and Mapp, A. K. (2014) FP Tethering: a screening technique to rapidly identify compounds that disrupt protein-protein interactions, *MedChemComm* 5, 370-375.

Figure 5-1:

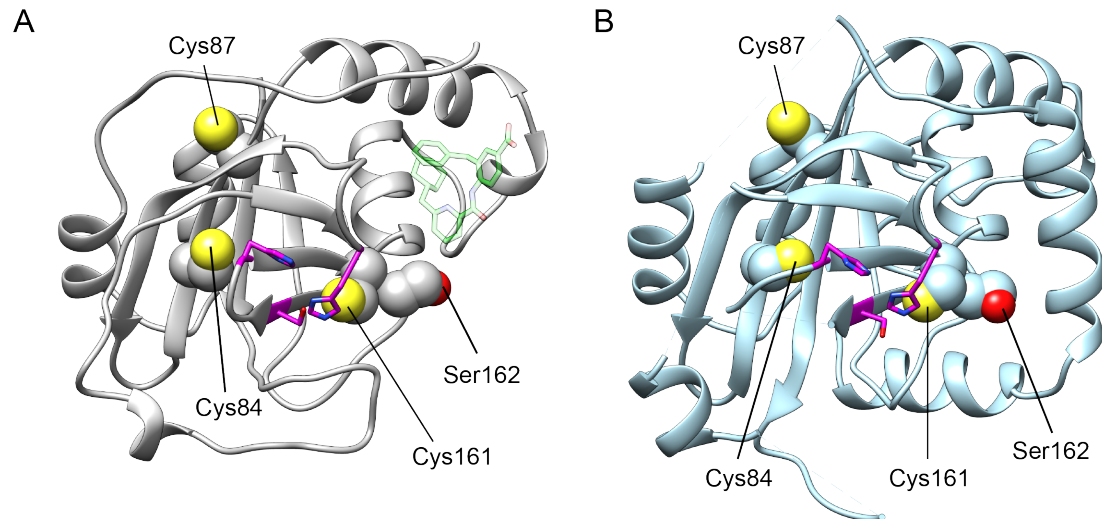


Figure 5-1: CMV protease cysteines in crystal structure and model. The position of the three resolved cysteine residues in the homology model (left) and crystal structure (right) are in comparable positions. Cysteine thiols are shown in yellow. The Ser-His-His catalytic triad is shown in magenta. A green transparent instance of DD2 is shown in the homology model to indicate the predicted binding pocket.

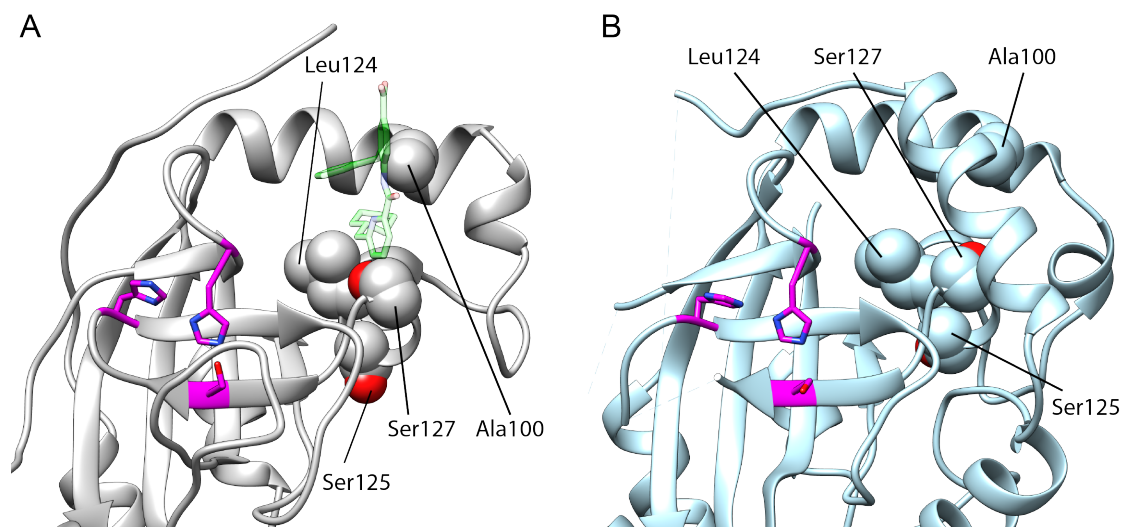


Figure 5-2: Potential cysteine mutations. Residues that, if mutated to cysteine, were predicted to place the cysteine thiol in the putative interfacial pocket are shown space filling representation and labeled. Both the homology model (left) and wildtype crystal structure (right) are shown. The Ser-His-His catalytic triad is shown in magenta. A green transparent instance of DD2 is shown in the homology model to indicate the predicted binding pocket.

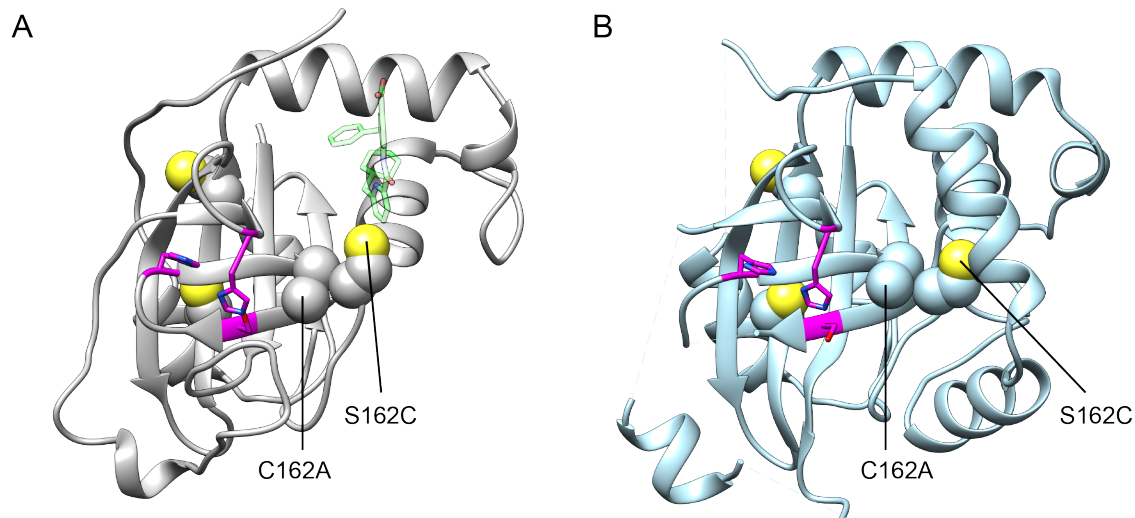


Figure 5-3. Final CMV protease construct. In the final construct used for screening all cysteines were mutated to alanine and serine 162 was mutated to cysteine. The position of the S162C mutation is shown for both the homology model (left) and the crystal structure (right). The Ser-His-His catalytic triad is shown in magenta. A green transparent instance of DD2 is shown in the homology model to indicate the predicted binding pocket.

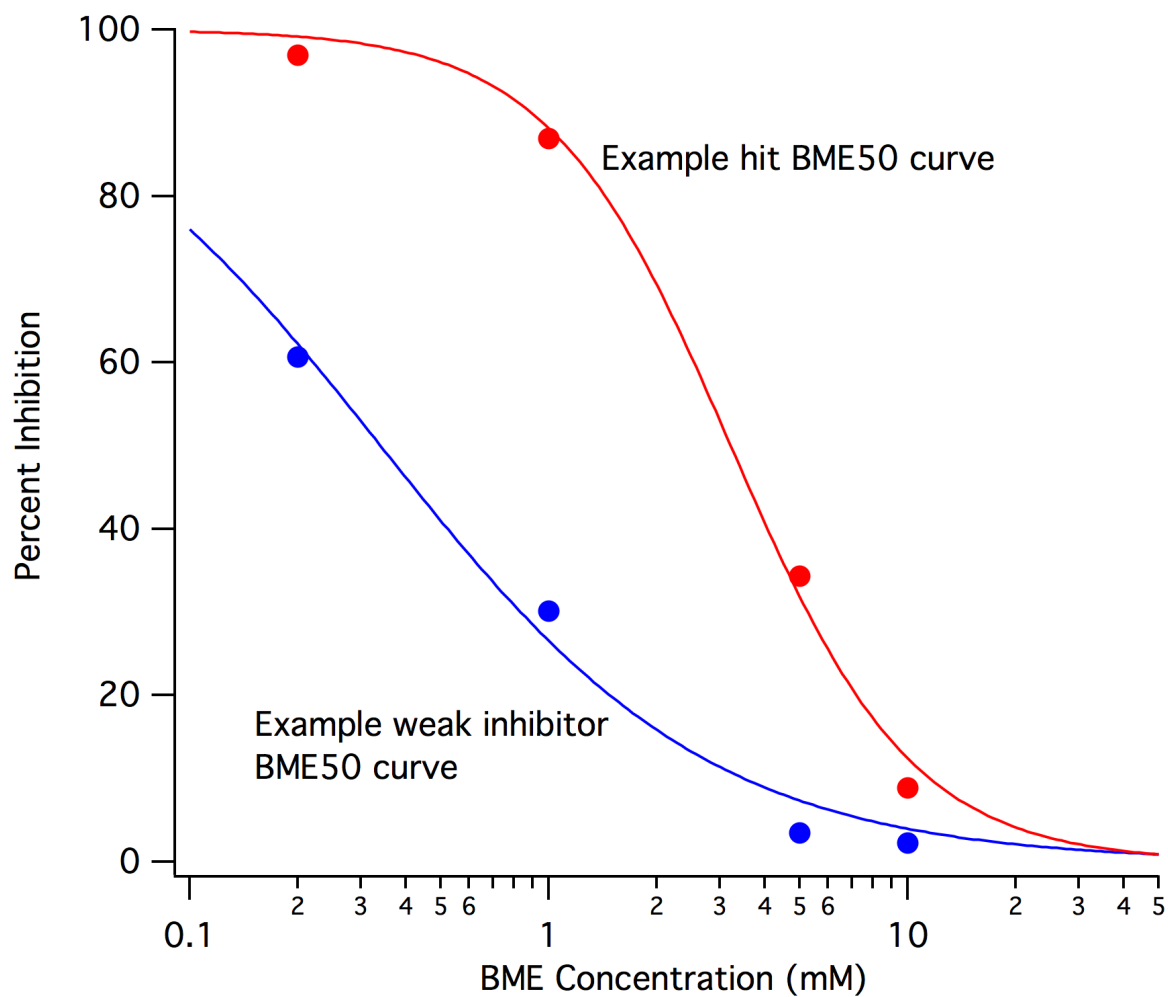


Figure 5-4: Example BME₅₀ curves. Two example BME₅₀ curves are shown using data from the primary screen. A hit compound is shown in red while a weak inhibitor is shown in blue.

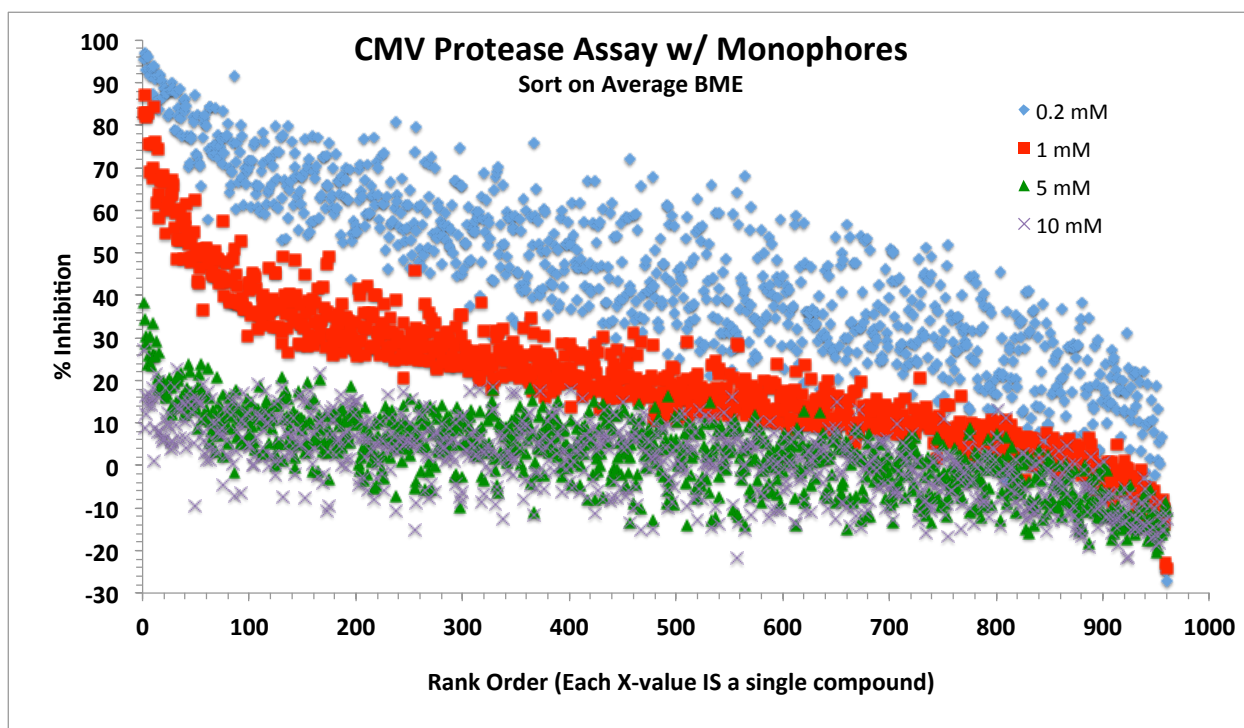


Figure 5-5. Primary screen scatter plot. A scatter plot of percent inhibition versus compound rank order is shown for 0.2 mM (blue diamond), 1 mM (red square), 5 mM (green triangle), and 10 mM (purple X) BME.

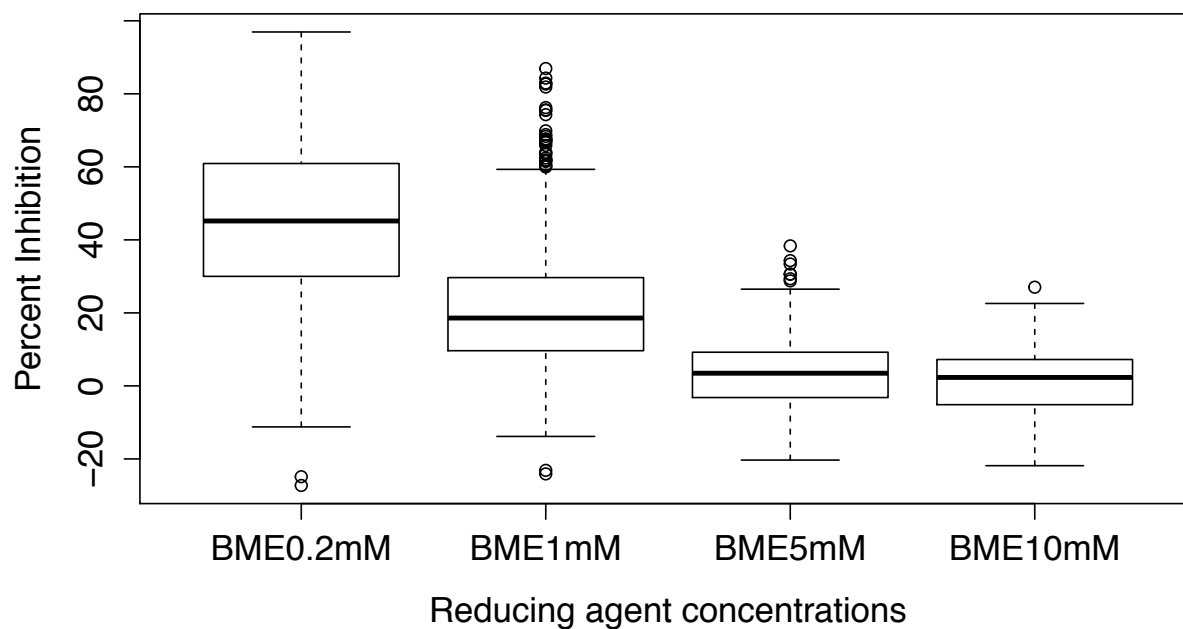


Figure 5-6. Distribution of inhibition at varying BME concentrations. This boxplot summarizes the distribution of percent inhibition observed for each reducing agent concentration across all 960 screened compounds.

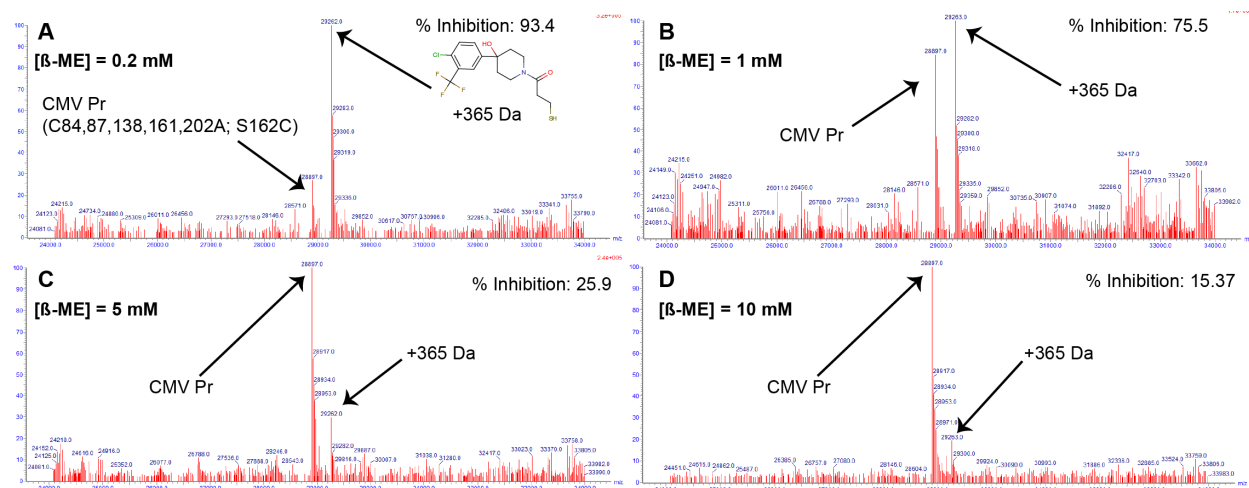


Figure 5-7. Example MS data confirming covalent binding.. Mass-spectrometry was used to confirm covalent modification for the top fragment hits at multiple reducing agent concentrations.

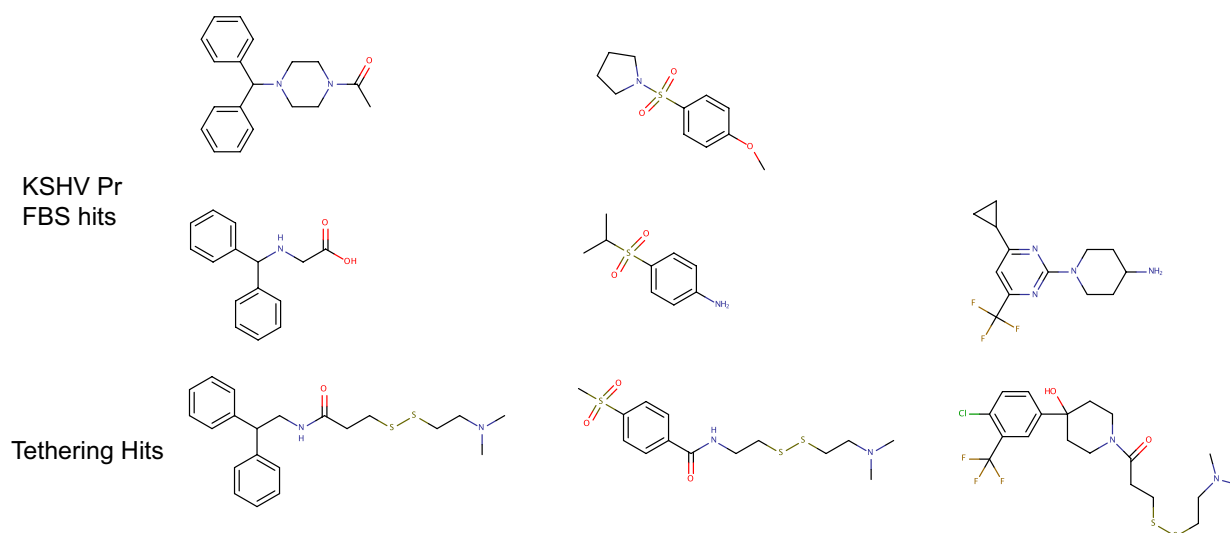


Figure 5-8. Comparison of tethering and KSHV Pr FBS hits. Each column shows similar compounds arising from both tethering against CMV protease and fragment-based screening against KSHV protease.

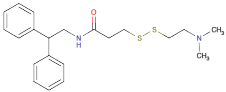
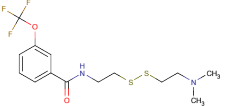
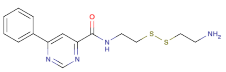
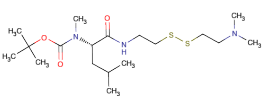
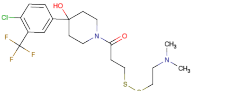
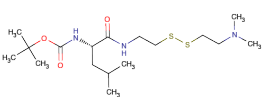
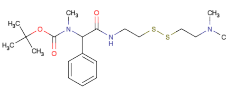
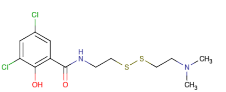
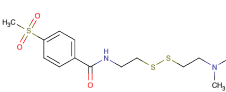
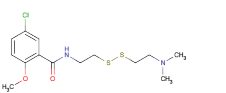
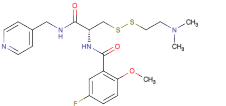
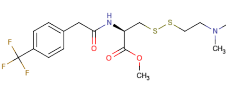
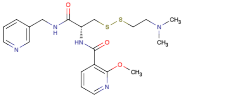
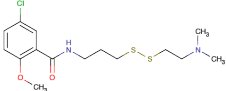
Table 5-1: Enzyme construct activity comparison

	%WT Activity	%Cys to Ala activity
WT	100 ± 5.9	244.3 ± 14.5
Cys to Ala	40.9 ± 1.0	100 ± 2.4
Cys to Ala, S127C	0.07 ± 0.01	0.18 ± 0.02
Cys to Ala, S125C	0.03 ± 0.0004	0.07 ± 0.0009
Cys to Ala, A100C	0.35 ± 0.04	0.87 ± 0.1
Cys to Ala, L124C	0.05 ± 0.008	0.11 ± 0.02
Cys to Ala, S162C	10.4 ± 2.7	25.5 ± 6.5

Table 5-2: Summary statistics for distributions of inhibition

	0.2 mM BME	1 mM BME	5 mM BME	10 mM BME	Averaged over concentrations
Mean	45.73	20.84	2.95	1.42	17.74
Standard Deviation	21.29	16.75	9.13	8.56	12.05
Minimum	-27.27	-24.1	-20.32	-21.85	-18.60
1st Quartile	30.01	9.652	-3.18	-5.14	10.12
Median	45.17	18.59	3.48	2.316	17.05
3rd Quartile	60.89	29.65	9.221	7.228	25.26
Maximum	96.94	86.92	38.35	27.03	60.91

Table 5-3. Representative hit compounds

Fragment	β -ME ₅₀
	3.6 mM
	3.2 mM
	2.8 mM
	2.8 mM
	2.6 mM
	2.3 mM
	2.2 mM
	2.2 mM
	2.2 mM
	2.0 mM
	1.6 mM
	1.5 mM
	1.4 mM
	1.4 mM

Supplemental Material

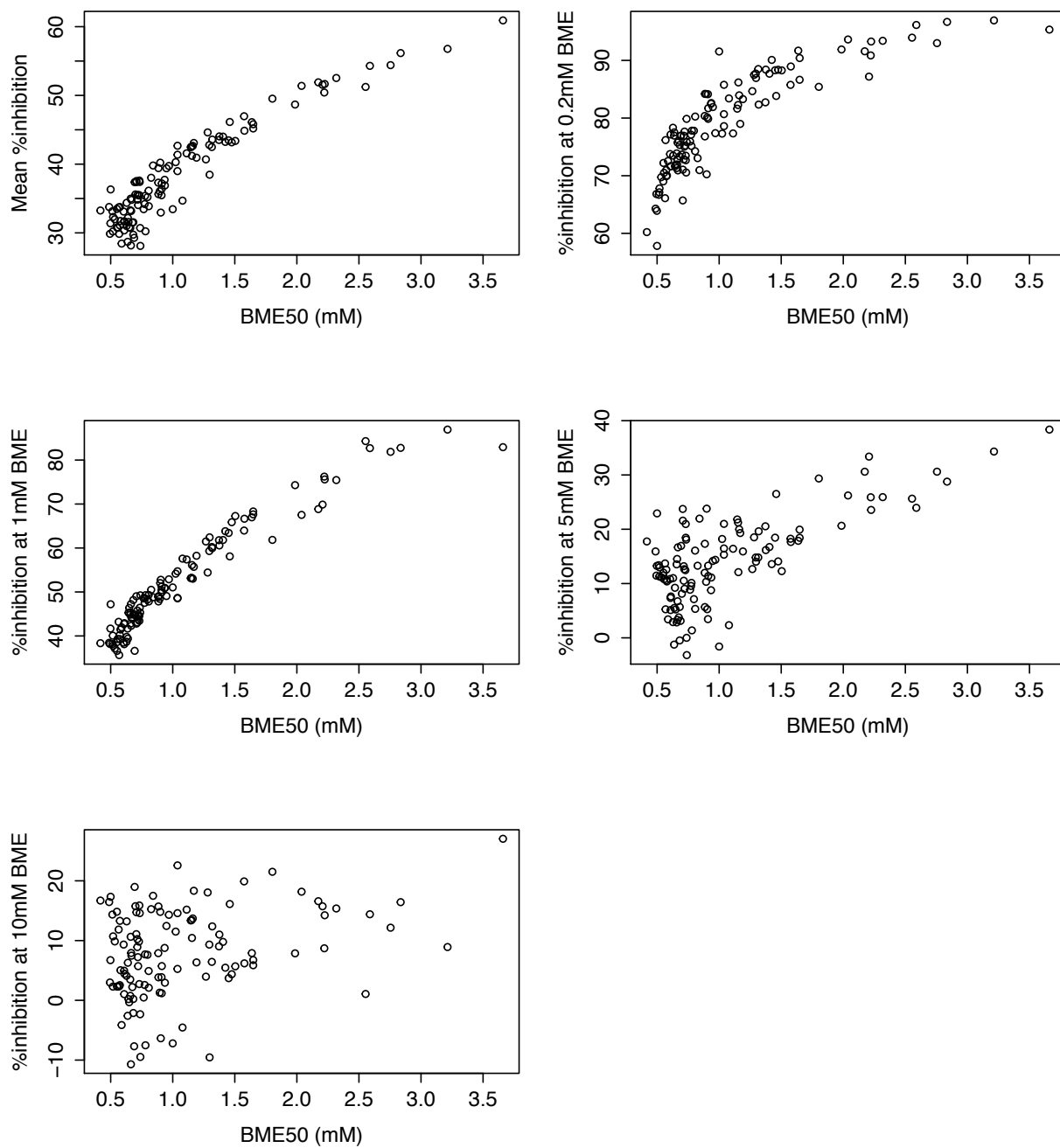


Figure 5-S1. Correlation between mean percent inhibition and BME50.

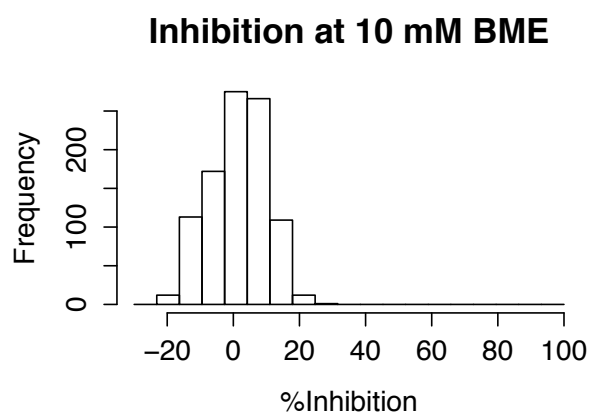
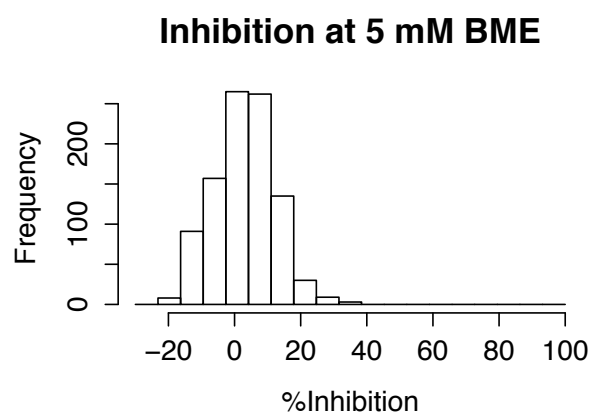
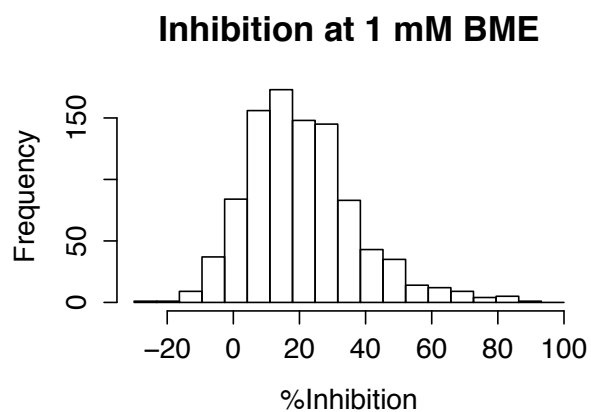
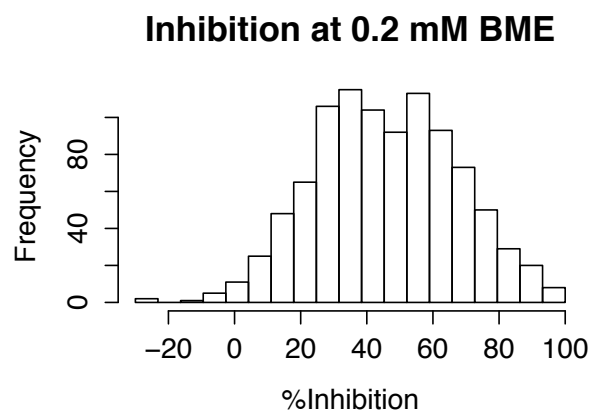


Figure 5-S2. Histograms of percent inhibition.

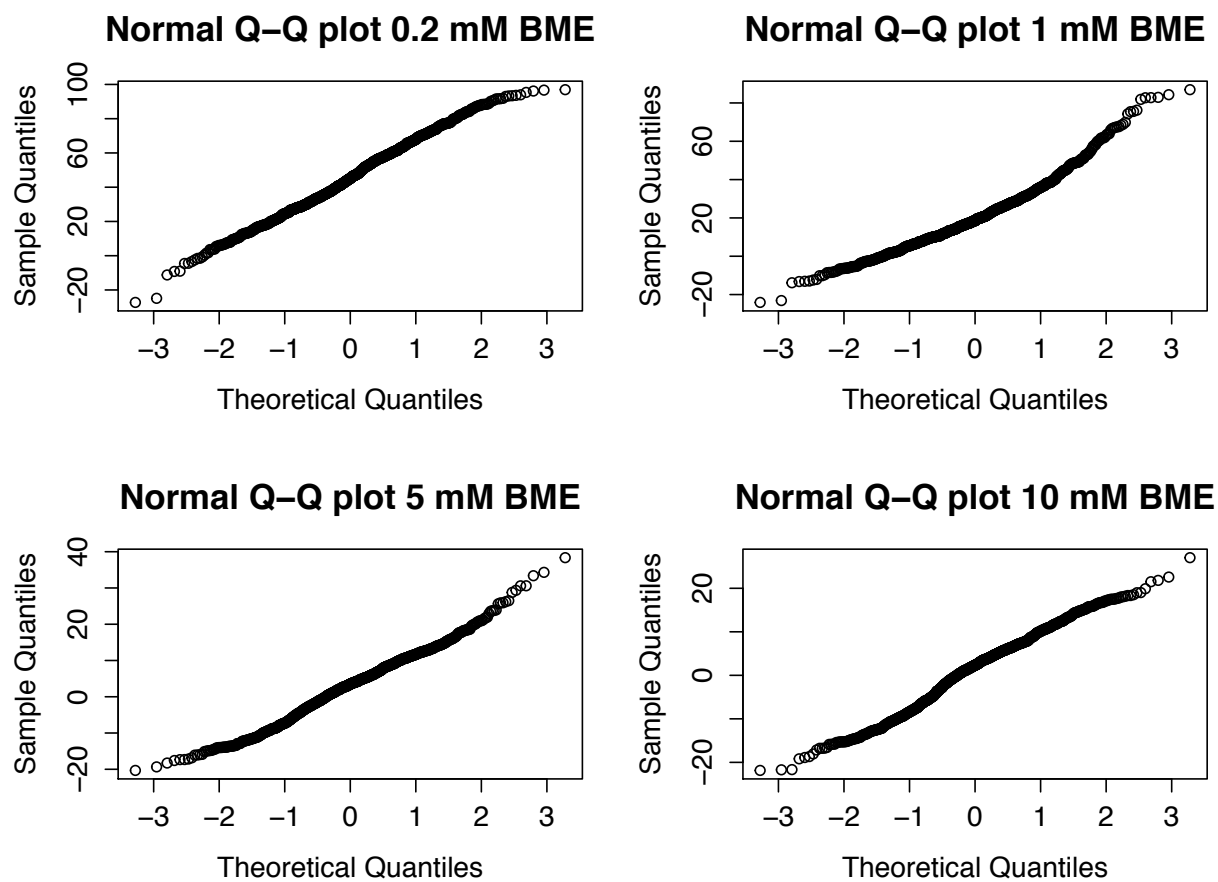


Figure 5-S3. Normal quantile-quantile plots for percent inhibition.

CHAPTER 6: Broad-spectrum allosteric inhibition of herpesvirus proteases

Jonathan E. Gable, Gregory M. Lee, Priyadarshini Jaishankar, Brian R. Hearn, Christopher A. Waddling, Adam R. Renslo, and Charles S. Craik

This chapter contains previously published material from:

Gable, J. E., Lee, G. M., Jaishankar, P., Hearn, B. R., Waddling, C. A., Renslo, A. R., & Craik, C. S. (2014). Broad-spectrum allosteric inhibition of herpesvirus proteases. *Biochemistry*, 53(28), 4648-4660.

Copyright 2014 American Chemical Society.

Abstract

Herpesviruses rely on a homodimeric protease for viral capsid maturation. A small molecule, DD2, previously shown to disrupt dimerization of Kaposi's sarcoma-associated herpesvirus protease (KSHV Pr) by trapping an inactive monomeric conformation and two analogues generated through carboxylate bioisosteric replacement (compounds 2 and 3) were shown to inhibit the associated proteases of all three human herpesvirus (HHV) subfamilies (α , β , and γ). Inhibition data reveal that compound 2 has potency comparable to or better than that of DD2 against the tested proteases. Nuclear magnetic resonance spectroscopy and a new application of the kinetic analysis developed by Zhang and Poorman [Zhang, Z. Y., Poorman, R. A., et al. (1991) *J. Biol. Chem.* 266, 15591–15594] show DD2, compound 2, and compound 3 inhibit HHV proteases by dimer disruption. All three compounds bind the dimer interface of other HHV proteases in a manner analogous to binding of DD2 to KSHV protease. The determination and analysis of cocrystal structures of both analogues with the KSHV Pr monomer verify and elaborate on the mode of binding for this chemical scaffold, explaining a newly observed critical structure–activity relationship. These results reveal a prototypical chemical scaffold for broad-spectrum allosteric inhibition of human herpesvirus proteases and an approach for the identification of small molecules that allosterically regulate protein activity by targeting protein–protein interactions.

Herpesviruses make up one of the most prevalent viral families, including nine human types that cause a variety of severe illnesses and are classified into three subfamilies.⁽¹⁾ The α subfamily of human herpesviruses (HHVs) includes herpes simplex viruses 1 and 2 (HSV-1 and HSV-2, respectively), as well as Varicella Zoster virus (VZV). The β subfamily includes human cytomegalovirus (HCMV) and human herpesviruses 6a, 6b, and 7 (HHV-6a, -6b, and -7, respectively). Lastly, the γ subfamily includes Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). All currently approved treatments for herpesvirus infection target viral DNA replication. However, these drugs suffer from poor efficacy because of viral resistance mutations, the requirement of frequent intravenous injection, or severe dose limiting side effects such as myelosuppression or nephrotoxicity. For these reasons, there has been a prevailing interest in alternative potential therapeutic targets for herpesvirus infection.⁽²⁾

All human herpesviruses share a structurally and functionally conserved serine protease (Pr) that is critical in the formation of the mature capsid and is allosterically activated through dimerization.⁽¹⁾ Genetic deletion of this viral Pr in HSV-1 precluded capsid maturation, confirming that the protease is necessary for successful viral replication and validating the enzyme as a potential therapeutic target.⁽³⁾ Similarly, knockdown of the maturational Pr in murine cytomegalovirus (MCMV), a model of β herpesvirus infection, causes a significantly reduced viral load.⁽⁴⁾ The critical role that these proteases play in the viral replication cycle and their conservation across the HHVs make them potential therapeutic targets.

Initial attempts to exploit HHV proteases as therapeutic targets were directed at the active site. These relied heavily on chemical “warheads” for covalent inhibition and/or peptidomimetic scaffolds.^(5, 6) Despite some *in vitro* success, efforts to target the active site of these essential serine proteases have yet to yield inhibitors ready to advance into the clinic.^(5, 7-11) Structural

evidence helps explain the need for covalent inhibitors and the lack of pharmacologically viable lead compounds. All HHV proteases have a relatively shallow substrate binding pocket with a strict preference for alanine at P1 and serine at P1'. In addition, substrate binding is reported to occur through an induced-fit mechanism.[\(12-15\)](#) Being both shallow and dynamic, this active site is particularly challenging to inhibit.

Through studying the structure–function relationships of these enzymes, researchers built up an understanding of their allosteric regulation.[\(6, 12, 13, 16-26\)](#) Each monomer has an independent active site.[\(1\)](#) In the monomeric state, the enzyme is inactive and partially disordered. As the dimer, the enzyme is active, and the disordered C-terminal residues of the monomer form two helices, one that functions as a major contact surface at the dimer interface and one that interacts with the catalytic site. This disorder-to-order transition links the dimer interface to the catalytic site.[\(16, 27\)](#) Given the evidence supporting an allosteric link between Pr dimerization and activation, we have focused our efforts on identifying molecules that target the dimer interface.[\(6, 12, 16, 22, 23, 28\)](#) In doing so, we previously identified a small molecule inhibitor of KSHV Pr designated DD2 [compound **1** (Table [1](#))].[\(29, 30\)](#)

DD2, a benzyl-substituted 4-(pyridine-2-amido)benzoic acid, is a helical peptide mimetic and allosteric inhibitor that prevents the disorder-to-order transition that activates KSHV Pr, thus trapping an inactive monomeric state.[\(27, 30\)](#) The primary DD2 binding pocket, ~15 Å from the active site, is formed by conformational changes that occur only in the partially disordered monomer. The pocket forms when Trp109, an aromatic hot spot in the core of the protein, changes rotameric state.[\(27\)](#) The presence of a conserved aromatic hot spot in all nine human herpesvirus proteases suggests the potential for the development of broadly antiherpetic small

molecules that allosterically inhibit HHV Pr enzyme activity by disrupting protein–protein interactions.

We set out to determine whether DD2 or analogues thereof could be pan allosteric inhibitors of herpesvirus proteases. To accomplish this, we generated a series of compounds in which the carboxylate of DD2 was replaced with polar nonionic or polar anionic functional groups (Table [1](#)) and assessed the inhibitory activity of the compounds. These new analogues and DD2 were evaluated with respect to their potency and mechanism of action against a panel of representative HHV proteases spanning all HHV subfamilies: HSV-2 (α), HCMV (β), EBV (γ), and KSHV (γ) proteases. Binding of an inhibitor to KSHV Pr was characterized using our repertoire of nuclear magnetic resonance (NMR) assays as well as X-ray crystallographic studies, which established the mechanism of action and binding site at atomic resolution.[\(27\)](#) To facilitate more rapid determination of the mechanism of inhibition, particularly where NMR and crystallographic approaches are not readily available, we applied a kinetic analysis that distinguishes between dissociative (i.e., dimer disruption) and nondissociative inhibitors of obligate dimeric enzymes. This analysis was first described and conducted for dimer disruptors of HIV-1 Pr by Zhang and Poorman.[\(31\)](#) Cumulatively, this approach allowed the development of improved inhibitors and detailed analysis of the inhibition of this highly dynamic protein–protein interface.

Materials and methods

Materials

Buffer and solvent components were purchased from VWR or Fisher Scientific at >99% purity. The P6 peptide substrate (PVYtBuQA-ACC) was purchased crude (AnaSpec, Inc.) and purified

via reverse-phase high-performance liquid chromatography on a C¹⁸ column as described previously.⁽³²⁾ The P4 peptide substrate, YtBuQA-ACC, was synthesized and purified as previously described, but using the Symphony Quartet multiple synthesizer (Protein Technologies, Inc.) for the addition of the last three amino acids.⁽³²⁾

Protein Expression and Purification

Expression and purification of the KSHV, HCMV, HSV-2, and EBV proteases and their respective isoleucine-to-valine and truncated variants were conducted as previously described.^(27, 33) Primer sequences are listed in the [Supporting Information](#).

Acquisition and Analysis of NMR Data

All protein NMR data were acquired at 27 °C on a Bruker Avance 500 MHz spectrometer equipped with a QCI CyroProbe and a B-ACS 60-slot autosampler. Protease sample concentrations, buffer conditions, data acquisition, and data processing were as previously described.^(27, 30) NMR characterization of the small molecule inhibitors is described in the [Supporting Information](#) under Analog Synthesis.

Determination of Kinetic IC₅₀ Values

IC₅₀ values were determined as previously described with the following modifications.⁽³⁰⁾ A 2-fold dilution series of the compound was prepared in 100% DMSO from 10 to 0.156 mM and/or from 5 to 0.078 mM. The P6 substrate concentrations were 5, 65, 150, and 30 μM for KSHV, EBV, HCMV, and HSV-2 proteases, respectively. For KSHV, EBV, and HCMV proteases, the final enzyme concentration was 1 μM in assay buffer [25 mM potassium phosphate (pH 8), 150 mM KCl, 0.1 mM EDTA, and 1 mM dithiothreitol (DTT)] with a final DMSO concentration of 2%. HSV-2 protease assays were conducted at a final enzyme concentration of 10 μM in the assay buffer described above, supplemented with 10% glycerol and 500 mM sodium citrate.

HSV-2 Pr has very low activity and requires large amounts of an antichaotropic agent to induce dimerization and activation.(26) Data were acquired from a Spectra MAX Gemini EM fluorescence microplate reader (Molecular Devices) using excitation and emission wavelengths of 380 and 460 nm, respectively. The initial velocity was used to calculate percent activity, which was plotted versus inhibitor concentration and fit to eq 1 using IGOR Pro (WaveMetrics, Inc.). Y , U , L , C , and H correspond to the percent activity, maximal percent activity, minimal percent activity, inhibitor concentration, and Hill slope, respectively. All IC_{50} values are reported as the mean $\pm$ the standard deviation ($n = 3$).

INSERT EQUATION

Previously reported IC_{50} values for DD2 against KSHV Pr were determined with the concentration of substrate (S_0) used for screening, a value approximately 10-fold greater than the K_M (P6 substrate $K_M = 11 \pm 3 \mu M$; $S_{0,prev} = 100 \mu M$). (30) Herein, reported IC_{50} values were obtained at $S_0 = 0.5-1 \times K_M$ for all enzymes unless explicitly stated otherwise. Substrate-induced dimerization has been reported for KSHV Pr whereby an excess of substrate drives the monomer-dimer equilibrium toward the dimeric state, decreasing the apparent potency of dimer disruptors.(6) This explains why the KSHV IC_{50} value for DD2 reported herein is somewhat lower than that previously reported.

Zhang-Poorman Analysis

The analysis of Zhang and Poorman is based on Scheme 1 and eqs S1 and S2 of the [Supporting Information](#), which describe the linear relationship between $E_0/\sqrt{k_{exp}}$ and $\sqrt{k_{exp}}$ under first-order kinetics for inhibited and apo conditions, where E_0 is the total enzyme concentration and k_{exp} is the experimental first-order rate constant.(31) The enzyme concentration is varied while the initial substrate concentration, S_0 , is held constant with or without inhibitor.

Data collection was conducted in black round-bottom polystyrene 96-well plates (Corning) with reaction volumes of 100 μL in assay buffer (described above). The substrate concentration was optimized for each enzyme to achieve first-order reaction kinetics. For EBV and HCMV proteases, the initial concentration of the P6 substrate was 15 μM . The K_M for KSHV Pr and P6 substrate is approximately 11 μM .[\(30\)](#) This relatively low K_M value prevented us from achieving $S_0 \ll K_M$ while still having substrate in large excess of enzyme. To achieve the conditions described above, we synthesized a shorter substrate, P4 (YtBuQA-ACC), with a K_M of 80 μM , allowing us to satisfy the constraints imposed by Zhang–Poorman analysis. For KSHV Pr, 15 μM P4 substrate was used. Concentrated enzyme stocks were diluted into assay buffer in siliconized Eppendorf tubes to reach concentrations of 1–4.6 μM for all proteases, with final concentrations determined by a Hewlett-Packard 8453 UV–vis spectrometer (1 cm path length). Inhibitor was added from a stock in 100% DMSO, with a final assay DMSO concentration of 2%. Enzyme and inhibitor were incubated at room temperature for 45 min and then dispensed into the 96-well plate. Addition of 5 μL of a substrate stock in 10% DMSO was used to start the reaction, resulting in a final DMSO concentration of 2.5%. Reactions were conducted at room temperature, and data were acquired as described above. Full kinetic curves were recorded for the majority of reactions and fit to a single exponential using IGOR Pro (Wavemetrics, Inc.) to determine k_{exp} . For high inhibitor concentrations, with low enzyme concentrations the signal remained linear and a full curve could not be recorded, even over a 7 h time course. In these cases of low activity, we utilized the relationship $k_{\text{exp}} = v_0/S$ to calculate k_{exp} from the initial velocity, where v_0 is the initial velocity and S is the substrate concentration determined from total hydrolysis. The use of v_0 as a substitute for k_{exp} was noted by Zhang and Poorman and has been used successfully elsewhere.[\(31, 34\)](#) The dependent variable ($E_0/\sqrt{k_{\text{exp}}}$) is plotted as a function of

$\sqrt{k_{\text{exp}}}$ for each inhibitor concentration and fit to a line in accordance with eqs S1 and S2 of the [Supporting Information](#).⁽³¹⁾

Acquisition of X-ray Crystallography Data and Determination of Structure

Stock KSHV Pr Δ 196 solutions for crystallography were prepared at a concentration of 7 mg/mL in 100 mM KCl, 0.07 mM EDTA, 16.5 mM KPi (pH 8.0), and 0.66 mM DTT. Inhibitor was added to the stock protein solution to a final concentration of 1 mM (3.4-fold molar excess, final DMSO concentration of 9%) and incubated at 30 °C for 30 min. The protein/inhibitor solution was added in a 1:1 ratio to the reservoir solution, and crystals were grown at 17 °C with the hanging drop vapor diffusion method. Initial crystallization hits were identified using the MCSG2 sparse matrix screen (Microlytics) and subsequently optimized by grid screening. The final reservoir solution for compound **2** cocrystallization consisted of 0.1 M sodium acetate (pH 7.8), 0.88 M NaH_2PO_4 , 1.32 M K_2HPO_4 , and 0.2 M KCl. For compound **3**, the reservoir solution contained 0.1 M imidazole (pH 8), 0.4 M NaH_2PO_4 , 1.6 M K_2HPO_4 , and 0.1 M NaCl. Crystals appeared after 14–30 days as small rectangular prisms. For compound **2**, a 1.45 Å resolution data set was collected from a 0.2 mm × 0.05 mm × 0.05 mm crystal. In the case of compound **3**, a 2.15 Å resolution data set was collected from a smaller crystal measuring 0.1 mm × 0.03 mm × 0.03 mm. Data were collected at Lawrence Berkeley National Laboratory Advanced Light Source beamline 8.3.1 using a crystal flash-cooled to 100 K in mother liquor with 12% glycerol as the cryoprotectant. Further details of the X-ray diffraction data processing and analysis are described in the [Supporting Information](#). Resulting structures of compounds **2** and **3** in complex with truncated KSHV protease were deposited in the Protein Data Bank (PDB) as entries [4P3H](#) and [4P2T](#), respectively.

Structural figures within this paper and the [Supporting Information](#) were created using UCSF Chimera version 1.8.1. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by National Institute of General Medical Sciences Grant P41-GM103311).

Modeling Using the Protein Local Optimization Program (PLOP)

MarvinSketch version 5.11.3 (ChemAxon) was used to generate mol2 files for compounds **2** and **3** based on the PDB coordinates determined from their cocrystal structures. PLOP (M. Jacobson, <http://www.jacobsonlab.org>) was used to optimize the entire monomer–ligand complex and structural waters within 10 Å of the ligand using the opt boolean followed by the minim side and minim res commands. [\(35–37\)](#) OPLS-AA 2005 force field parameters for compounds **2** and **3** were generated using the hetgrp_ffgen command from Schrodinger with mol2 files from MarvinSketch as input.

Results

A Scaffold for Broad-Spectrum Allosteric Inhibition of Human Herpesvirus Proteases

DD2, compound **2**, and compound **3** inhibit HCMV (β), EBV (γ), HSV-2 (α), and KSHV (γ) proteases with micromolar potencies (Figure 1). KSHV Pr, the HHV Pr used in the discovery of DD2, was the most potently inhibited enzyme with IC_{50} values of $1.0 \pm 0.1 \mu\text{M}$ for compound **2**, $1.5 \pm 0.3 \mu\text{M}$ for DD2, and $3.6 \pm 0.7 \mu\text{M}$ for compound **3** (Figure 1). All compounds bearing a polar but nonionic substitution for the carboxylate of DD2 [compounds **4–7** (Table 1)] showed no inhibition of KSHV Pr, although some of these analogues exhibit poor solubility at low micromolar concentrations. For example, compounds **5** and **7** were found by dynamic light scattering to form large aggregates (>80 nm radii) at $1 \mu\text{M}$. These results suggest limited

concentrations of the free compound are available in solution under such conditions, precluding conclusions about the SAR. Compounds **4** and **6**, however, did not form large aggregates but were inactive (Figure S1 and methods of the [Supporting Information](#)). Overall, the data for compounds **2–7** suggest the importance of an anionic group at the position of the carboxylate in DD2 for inhibition.

The compounds developed here had the lowest potency against HSV-2 Pr, with IC_{50} values ranging from ~37 to 94 μ M (Figure [1](#)). This may be in part due to the high concentration of enzyme and antichaotropic agents necessary for activity with this enzyme ([Materials and Methods](#)). While DD2 inhibits HCMV and EBV proteases with similar potencies (IC_{50} values of 7.4 ± 2 and 7.7 ± 0.6 μ M, respectively), compound **3** inhibits HCMV Pr with an IC_{50} of 39 ± 8 μ M and EBV with an IC_{50} of 19 ± 1 μ M. Compound **2** is more potent than either DD2 or compound **3** against HCMV and EBV proteases (IC_{50} values of 4.7 ± 0.4 and 4.0 ± 0.1 μ M, respectively). It is noteworthy that the IC_{50} values for these enzymes are similar to the total enzyme concentration used. This may indicate tight binding; however, analysis of tight-binding inhibition is confounded by the monomer–dimer equilibrium in our system. The IC_{50} value for DD2 against KSHV protease is similar at 395 and 1300 nM total enzyme, suggesting stoichiometric inhibitor binding is not the only determinant of IC_{50} . For all enzymes, compound **2** was most potent, followed by DD2 and finally compound **3**. While broad-spectrum inhibition of herpesvirus proteases is apparent from these data, the mechanism of inhibition cannot be inferred from IC_{50} data. We therefore applied Zhang–Poorman analysis to determine whether a consistent mechanism of inhibition was operating across Pr family members. For this analysis, we used our most potent inhibitor, compound **2**, as a representative of the inhibitor class.

Application of Zhang–Poorman Analysis in Investigating Dimer Disruption of Human Herpesvirus Proteases

Zhang and Poorman developed a kinetic approach for determining whether an obligate dimeric enzyme, HIV-1 Pr, is inhibited by a dissociative mechanism (dimer disruption) or a nondissociative mechanism (Scheme 1 and eq 1 of the [Supporting Information](#)) using a plot of $E_0/\sqrt{k_{\text{exp}}}$ (y-axis) versus $\sqrt{k_{\text{exp}}}$ (x-axis) with and without inhibitor. This plot is generated by measuring the experimental first-order rate constant (k_{exp}) with varied enzyme (E_0) and inhibitor concentrations as described in [Materials and Methods](#).⁽³¹⁾ When dimer disruption is the dominant mode of inhibition, an increasing inhibitor concentration results in an increasing y-intercept that scales as $1 + [\text{I}]/K_i$, where $[\text{I}]$ is the total concentration of the inhibitor and K_i is the dissociation constant of the inhibitor–monomer complex (eq 1 of the [Supporting Information](#)).⁽³¹⁾ Zhang–Poorman analysis provides orthogonal mechanistic data based solely on enzyme kinetics, complementing our NMR-based assays.

Application of this analysis to KSHV Pr and our known dimer disruptor, DD2, provided a positive control and illustrates the effectiveness of this analysis for HHV proteases. As expected, increasing concentrations of DD2 resulted in increasing y-intercepts, indicating higher apparent dissociation constants for the dimer [$K_{\text{d,app}}$ (Figure 2b)]. For strictly dissociative inhibition where K_i is relatively small and on the order of the inhibitor concentration, the competitive (K_c) and noncompetitive (K_c') dissociation constants are very large, and the noncompetitive rate constant (k_{cat}') is small, the slope of these plots simplifies to $(k_{\text{cat}}/K_M)^{-1}$ and should be constant across all inhibitor concentrations (Scheme 1 and eq 2 of the [Supporting Information](#)).⁽³¹⁾ The slope we observe decreases slightly with an increasing DD2 concentration. This observation is

consistent with mixed-type inhibition previously reported for this inhibitor.⁽³⁰⁾ A decrease in slope with an increasing inhibitor concentration indicates $K_c/K_c' > 1$ and $K_c > K_c'$, suggesting the dimer–substrate–inhibitor complex is more dominant than the dimer–inhibitor complex in contributing to mixed inhibition (derivation in the [Supporting Information](#)). This illustrates how Zhang–Poorman analysis can reveal both the more subtle noncompetitive inhibition (K_c') and the diagnostic increase in the y-intercept indicative of dimer disruption (dissociative inhibition).

We applied this analysis to our new inhibitor compound **2** against our panel of HHV proteases. HSV-2 Pr had too little activity to reliably perform the kinetic analysis under the required first-order conditions and so was excluded from analysis. Zhang–Poorman analysis of compound **2** for the other three proteases (KSHV, EBV, and HCMV) is fully consistent with a mechanism of dimer disruption. In each case, increasing concentrations of compound **2** result in increased y-intercepts and $K_{d,app}$ values (Figure [2a,c,d](#)). Mixed inhibition was also observed for compound **2**, indicated by a decreasing slope with an increasing inhibitor concentration.

Compounds 2 and 3 Disrupt Dimerization by Binding the Dimer Interface Core

Analogously across All HHV Protease Subfamilies

Application of ^{13}C – ^1H HSQC spectroscopy using selectively [^{13}C - ϵ -*methyl*]methionine-labeled KSHV Pr allowed for more direct structural confirmation that compounds **2** and **3** disrupt dimerization. Met197 in helix 5 of KSHV Pr is directly involved in monomer–monomer interaction (Figure [3a,c](#)). The monomer–dimer equilibrium of KSHV Pr exhibits slow exchange on the NMR time scale, giving rise to distinct Met197 monomer and Met197 dimer resonances in the HSQC spectrum. It was previously shown that the addition of DD2 results in the loss of the Met197 dimer peak and a chemical shift perturbation in the Met197 monomer peak, diagnostic

of dimer disruption.[\(30\)](#) Addition of equimolar amounts of compound **2** or **3** has a similar effect with a complete loss of the dimer peak and a shift in the Met197 monomer peak [$\Delta\delta_{\text{total(compound 2)}} = 0.095$ ppm, and $\Delta\delta_{\text{total(compound 3)}} = 0.049$ ppm (Figure [3b](#))]. These data provide further support for the conclusion drawn from Zhang–Poorman analysis though fall short of defining the site of binding for these inhibitors.

To determine whether our new inhibitors **2** and **3** bind at the dimer interface, we first performed selective [^{13}C - $\delta 1$ -*methyl*]isoleucine labeling for analysis by ^{13}C – ^1H HSQC spectroscopy. Like we did for KSHV Pr–DD2 interactions, we hypothesized that binding of compounds **2** and **3** would take place at the conserved putative aromatic hot spot of each enzyme and be largely independent of the two C-terminal helices. This led us to design a truncated obligate monomeric construct of HSV-2 Pr analogous to those previously reported for KSHV and HCMV proteases [KSHV Pr $\Delta 196$ and HCMV Pr $\Delta 221$, respectively (Figures [3a](#) and [4b,d](#), pink)]. For HSV-2 Pr, a stop codon was introduced at residue 214, leaving two turns of helix 5 and completely removing helix 6 to generate HSV-2 Pr $\Delta 213$ (Figures [3a](#) and [4f](#), pink). Selective [^{13}C - $\delta 1$ -*methyl*]isoleucine labeling provided a set of NMR probes for monitoring binding at the dimer interface near the aromatic hot spot. In addition to selective labeling, the large majority of ^{15}N – ^1H HSQC resonances for KSHV Pr have been reported previously, including that of the side chain indole ring of Trp109.[\(28\)](#) Perturbation of the dimer interface residue Trp109 resonance provides additional support for binding at the dimer interface hot spot.

Addition of a >5-fold molar excess of compound **2** or **3** to KSHV Pr $\Delta 196$ yielded resonance perturbations in both the ^{13}C – ^1H and ^{15}N – ^1H HSQC spectra indicative of binding at the dimer interface in the same pocket bound by DD2 (Figure [4a](#) and Figure S3 of the [Supporting](#)

[Information](#)). All seven isoleucines of KSHV Pr Δ 196 are resolved in the ^{13}C - ^1H HSQC spectrum and were previously assigned.[\(27\)](#) The crystal structure of DD2 bound to KSHV Pr Δ 196 shows that Ile44 and Ile105 contribute hydrophobic surface area to the DD2 pocket and are closest to the aromatic hot spot residue Trp109 [≤ 5 Å (Figure [4b](#))]. Therefore, binding of a small molecule to the DD2 pocket is expected to most significantly perturb resonances corresponding to Ile44 and Ile105 while leaving the five remaining isoleucine peaks largely unperturbed. Indeed, in the presence of compound **2**, Ile44 and Ile105 exhibit significant peak shifts [for Ile44, $\Delta\delta_{\text{total}} = 0.078$ ppm; for Ile105, $\Delta\delta_{\text{total}} = 0.094$ ppm (Figure S2 of the [Supporting Information](#))]. Compound **3** binding and positive control DD2 exhibit similar effects [for Ile44, $\Delta\delta_{\text{total(compound 3)}} = 0.063$ ppm and $\Delta\delta_{\text{total(DD2)}} = 0.060$ ppm; for Ile105, $\Delta\delta_{\text{total(compound 3)}} = 0.120$ ppm and $\Delta\delta_{\text{total(DD2)}} = 0.087$ ppm (Figure [5](#) and Figure S2 of the [Supporting Information](#))]. Ile44 also exhibits substantial peak broadening for all compounds. Addition of DD2, compound **2**, or compound **3** causes extensive peak broadening of the Trp109 indole HN ^{15}N - ^1H HSQC resonance, further supporting the conclusion that DD2, compound **2**, and compound **3** bind the same transient allosteric pocket at the dimer interface (Figure S3 of the [Supporting Information](#)). NMR was utilized to map the binding of a small molecule to truncated HCMV Pr (HCMV Pr Δ 221). HCMV Pr Δ 221 contains only two isoleucines, Ile61 and Ile96, assigned by isoleucine-to-valine mutations (Figure [4d](#) and Figure S5 of the [Supporting Information](#)). In the wild-type dimer crystal structure, Ile61 and Ile96 are located ~ 5 and ~ 9 Å from the putative aromatic hot spot Tyr128, respectively (Figure [4d](#), PDB entry [1CMV](#)). Addition of an approximately 10-fold molar excess of compound **2** or **3** to HCMV Pr Δ 221 resulted in peak broadening of both the Ile61 and Ile96 probes (Figures [4c](#) and [5d](#)). A comparable effect was observed for the addition of DD2 at the same relative concentration (Figure [5c](#)). The decrease in peak volume was greater for

compound **2** than for compound **3** with both isoleucine probes. For all three inhibitors, exchange peak broadening is consistent with binding to HCMV Pr.

^{13}C – ^1H HSQC experiments suggest compounds **2** and **3** also bind at the dimer interface of HSV-2 Pr. The truncated HSV-2 Pr construct (HSV-2 Δ 213) contains nine isoleucine residues (Figure 4f), which were assigned by isoleucine-to-valine mutations (Figure S4 of the [Supporting Information](#)). Addition of DD2, compound **2**, or compound **3** causes substantial peak broadening for the Ile21 and Ile57, and to a lesser extent Ile86 and Ile121, peak resonances (Figures 4e and 5e,f). Ile21, Ile86, and Ile121 are predicted to be in the proximity of the putative hot spot residue Tyr124 (PDB entry [1AT3](#)). The observation that the resonance of Ile57 is also perturbed indicates a change in the electronic environment farther from the predicted binding site, consistent with changes in conformational equilibria in the presence of a compound or binding to multiple sites. In total, the NMR experiments described here suggest that DD2, compound **2**, and compound **3** bind the dimer interface across representative members of all HHV Pr subfamilies. For KSHV Pr, X-ray crystallographic analysis of KSHV Pr Δ 196 cocrystallized with compound **2** or **3** confirms at atomic resolution that these compounds bind the same pocket as DD2 and adopt similar conformations (see below).

Cocrystals with Compounds 2 and 3 Confirm the Mode of Binding and Explain Structure–Activity Relationships

The discovery that an anionic group is required for inhibitory activity is not clearly explained by the DD2–KSHV Pr Δ 196 cocrystal structure or other prior studies.[\(27, 30\)](#) To improve our understanding of the SAR for this compound series and to establish the effect of bioisosteric replacement on binding to KSHV protease at an atomic level, we pursued cocrystallization studies of KSHV Pr Δ 196 with compounds **2** and **3**.

Compounds **2** and **3** cocrystallized with KSHV Pr Δ 196 to yield 1.45 and 2.15 Å resolution structures, respectively (PDB entry [4P3H](#) for compound **2** and PDB entry [4P2T](#) for compound **3**). The two structures overlay with a C α rmsd of 0.25 Å. Comparison to the KSHV Pr Δ 196–DD2 cocrystal structure (PDB entry [3NJQ](#)) gives a C α rmsd of 0.3 Å for both. Structures with compounds **2** and **3** have an asymmetric unit containing two truncated monomers, each with largely the same conformation [C α rmsd $\sim$ 0.8 Å (Figure 6)]. A noteworthy difference between the two monomers is the position of the C-terminal residues, which are in the proximity of the inhibitor. Residues Val190 and onward diverge in backbone position (C α rmsd for residues 190–193 of 3 Å), with monomers A and B contacting opposite faces of the small molecule inhibitor (Figure S6 of the [Supporting Information](#)). Those same residues from a given monomer compared across structures overlay well [e.g., residues 190–193 of monomer B from PDB entries [4P3H](#) and [4P2T](#) (Figure S6 of the [Supporting Information](#))]. The difference in the observed conformation of the C-terminal residues between monomers, but not between the same monomer across structures, is likely dictated by differences in crystal packing interactions experienced by the two monomers. The interface between monomers is largely hydrophilic and contains structural waters. The buried solvent-accessible surface areas of the monomer interfaces for the cocrystal structures of compounds **2** and **3** are 1550 and 1400 Å², respectively, while the solvent-excluded surface areas are only 670 and 615 Å², respectively. This strongly suggests the interface between monomers is purely crystallographic, consistent with NMR data showing this construct is monomeric in solution. The overall architecture of the unit cell resembled the configuration and had the same space group (*I*222) as the previously published DD2–KSHV Pr Δ 196 structure (PDB entry [3NJQ](#)) despite the use of different crystallization conditions.⁽²⁷⁾ Both monomer chains have a small molecule bound in the DD2

pocket at aromatic hot spot Trp109. Chain B contains an additional instance of the small molecule that acts as a crystal contact and bridges asymmetric units, as previously observed (Figure 6).⁽²⁷⁾ All three instances of the inhibitor for both structures have an occupancy of 1 and average *B* factors in the range of 20–25, consistent with the *B* factor of surrounding residues. Examination of monomer A for both cocrystal structures shows a hydrogen bond between the carboxylate bioisostere and a structural water molecule, as well as van der Waals interactions with Pro192 and the Leu193 side chain (Figure 7a,c). The partial positive charge of proline and the negative π electron density of aromatic groups such as tetrazoles have been reported to interact specifically.⁽³⁸⁾ The methyl of the sulfonamide in compound **3** of monomer A contacts the Leu193 side chain (Figure 7a). The larger acylsulfonamide moiety appears to help order the C-terminal residues in monomer A through van der Waals interactions between Leu193 and the methyl of the sulfonamide, as well as interactions with the Leu196 backbone. Density extending to the final residue, Leu196, is present in the cocrystal structure with compound **3**, whereas density for residues Glu194, Thr195, and Leu196 was not present in the structure of monomer A with the smaller tetrazole moiety of compound **2**. The structure of monomer B shows hydrogen bonding from the carboxylate bioisostere to the Ser191 hydroxyl for both the tetrazole and sulfonamide substituents (Figure 7b,d). In monomer A, Ser191 was positioned away from the small molecule binding pocket (Figure 7a,c). This interaction with Ser191 may be responsible for ordering the C-terminus, which could be resolved in both cocrystal structures, contrary to monomer A where it was only resolved for the compound **3** cocrystal structure. Hydrogen bonding to structural waters is also observed for the tetrazole of compound **2** in monomer B. Interactions between the remainder of the inhibitor and the pocket for both monomers mirror those previously reported for DD2 and are comprised primarily of interactions between the

benzyl and cyclohexyl moieties of the small molecules and aliphatic and hydrophobic residues of the pocket.⁽²⁷⁾ Data collection and refinement statistics are listed in Table S1 of the [Supporting Information](#).

Given that the small molecule inhibitors are at the interface between asymmetric units and that three distinct conformations are observed, crystal packing was analyzed to determine if one instance of the inhibitor might be more representative of solution-state binding than others. The bridging molecule (associated with monomer B) is oriented such that in the absence of the crystal lattice the benzyl and cyclohexyl rings would be largely solvent-exposed, with a total solvent-excluded surface area of just 70 Å². This would be highly unfavorable and is not likely to exist in solution. In contrast, the inhibitor bound at Trp109 in monomer B buries 288 Å² of solvent-excluded surface area. In monomer A, the symmetry-mate bridging inhibitor molecule interacts extensively with the monomer A inhibitor and appears to severely perturb the small molecule binding pose (Figure S7 of the [Supporting Information](#)). Approximately 30% of the total surface area of the monomer A inhibitor molecule is buried by other instances of the inhibitor in the lattice, in comparison to only half of that (~15%) for the monomer B inhibitor. For these reasons, we hypothesize that the monomer B inhibitor bound in the Trp109 pocket is most representative of the solution state, not that of monomer A or the bridging molecule. It was not immediately apparent how these structures, or the previous DD2–KSHV Pr Δ196 structure, explain the observation that only anionic replacements of the carboxylate in DD2 inhibit enzyme activity, while polar nonionic groups such as the carboxamide and ester (compounds **4** and **6**, respectively) show no inhibition. To improve our understanding of solution-state binding and the observed structure–activity relationships (SARs) for DD2 and its analogues, the inhibitor-bound monomer was minimized using the Protein Local Optimization

Program (PLOP) with the OPLS2005 force field ([Materials and Methods](#)). Minimization of the monomer–ligand complex alone is intended to help remove artifacts introduced by crystal packing. This modeling of monomer B predicts an interaction between cationic Arg82 and the anionic substituent of these inhibitors, with very little change in backbone or side chain positions in the pocket (Figure [8b,e](#) and Figure S8 of the [Supporting Information](#)). Arg82–inhibitor interaction was not observed in either crystal structure or in the previous KSHV Pr Δ 196–DD2 structure. Hypothesizing that this interaction could take place in solution, we examined the packing of neighboring symmetry-mate molecules in the crystal lattice and revealed how crystal packing could prevent this interaction from taking place. In monomer B, the symmetry-mate Leu140 side chain (from chain A of a neighboring asymmetric unit) sterically occludes an Arg82–compound interaction (Figure [8a,d](#)). For monomer A, the symmetry-mate bridging inhibitor molecule interacts with Arg82 and sterically occludes interaction with the monomer A inhibitor molecule (Figure S7 of the [Supporting Information](#)). Crystallographic data for all three structures, however, suggest the Arg82 side chain is dynamic with a high average *B* factor (relative to the structure average) and density consistent with at least one alternate conformation. To test the prediction that Arg82 interacts with the anionic substituent of DD2 and its analogues, IC₅₀ values were determined for DD2, compound **2**, and compound **3** with the R82Q mutant of KSHV Pr. Even with this conservative mutation, which retained potential polar interaction and the overall side chain shape, a 3–4-fold decrease in potency was observed, indicating weaker binding of KSHV Pr R82Q compared to that of the wild-type protease (Figure [8c,f](#) and Figure S9 of the [Supporting Information](#)).

Discussion

Small molecules that allosterically regulate protein function have been increasingly sought-after as an alternative to classical active site inhibitors.[\(39-41\)](#) The homodimeric human herpesvirus proteases illustrate one such case in which small molecule allosteric modulators of activity could find extensive use in both improving our understanding of herpesvirus biology and treating infection. Because these proteases have thus far been recalcitrant to noncovalent active site inhibition, likely because of their shallow and conformationally dynamic substrate binding site, we pursued allosteric inhibitors.[\(12, 13\)](#) Targeting protein–protein interactions with small molecules, while still a major challenge in chemical biology and drug discovery, represented an opportunity and alternative approach to regulating HHV Pr activity. The conserved allosteric link between the dimer interface and each monomer’s active site allowed us to pursue a small molecule that allosterically inhibits HHV proteases from all subfamilies by targeting their dimer interfaces.

Our data support a model in which compounds **2** and **3** allosterically inhibit representatives of all three subfamilies of HHV proteases in a manner analogous to DD2 inhibition of KSHV Pr. In this model, they bind a pocket at the dimer interface, >10 Å from the active site. Binding to this site prevents C-terminal helices 5 and 6 from folding against the hydrophobic dimer interface. While these two helices are in a disordered state, the oxyanion hole of the active site, normally formed in part by the two conserved arginines in contact with helix 6, cannot adopt the correct conformation that allows efficient proteolysis of peptide substrates to occur. Additionally, substrate binding may be prevented by conformational changes that occlude the substrate binding pocket, such as movement of loop residues 14–27 in KSHV Pr (Figure [9](#)).[\(27\)](#) In this model, binding of compound **2** or **3** results in trapping of the inactive monomer, preventing homodimerization and thus allosterically inhibiting proteolysis across all HHV Pr subfamilies.

Via alteration of the carboxylate moiety of the parent compound, DD2, compound **2** was identified and demonstrated to have comparable or improved potency against all HHV proteases. This improvement could be caused by the larger van der Waals surface area of the tetrazole compared to the carboxylate functional group of DD2, without additional rotatable bonds, as found in compound **3**. Compound **3** may exhibit IC₅₀ values higher than those of DD2 due to steric or entropic effects deriving from the larger acylsulfonamide moiety.

The application of Zhang–Poorman analysis to HHV Pr family members provides a new tool for mechanism of action studies with potential inhibitors and suggests utility for the approach with other dimeric enzymes. This strictly kinetic approach is more rapid and cost-effective than NMR-based assays, with potential for use as a secondary screen in high-throughput screening campaigns for dimer disruptors. The data obtained from the kinetic analysis complement direct structural methods when they are available, as showcased here. Zhang–Poorman analysis shows that compound **2** inhibits primarily by dimer disruption of KSHV (γ), EBV (γ), and HCMV (β) proteases. Selective ^{13}C – ^1H [ϵ -*methyl*]Met labeling of KSHV Pr confirms dimer disruption, wherein slow exchange allows for the observation of distinct dimer and monomer Met197 peaks and addition of compound **2** or **3** results in a loss of the dimer resonance. Across representative proteases from all three subfamilies, selective ^{13}C / ^1H [δ 1-*methyl*]Ile data indicate compounds **2** and **3** bind at the dimer interface in the core of the Pr monomer >10 Å from the active site.

Interestingly, nascent structure–activity relationships (SARs) for this class of inhibitors suggest a role for carboxylate or carboxylate bioisosteres in inhibiting HHV proteases. This prompted us to pursue cocrystallization studies with compounds **2** and **3** and to re-examine the DD2-KSHV Δ 196 structure. Initial crystallographic results did not reveal an obvious structural explanation

for this observation. After careful analysis, it became apparent that crystallographic contacts obstructed the key interaction driving the observed SAR, namely, formation of a salt bridge between Arg82 and the anionic substituent of this class of inhibitors. This interaction was predicted from computational modeling of the asymmetric unit outside of the context of the crystal lattice. A conservative mutation of Arg82 to glutamine supported the conclusion that Arg82 interacts with members of this inhibitor series. The IC_{50} of DD2 for the R82Q mutant was roughly 3–4-fold higher than that for the wild-type enzyme, suggesting a significantly weaker binding to the DD2 pocket. In this case, minimization of the structure without surrounding symmetry-mate molecules likely revealed a more accurate solution-state structure. This approach may be particularly important for protein–protein interaction inhibitor studies in which an exposed protein-binding interface may be involved in crystallographic contacts that are not representative of conformations in solution.

Consistent with other examples of small molecules binding at large protein surfaces, compounds **2** and **3** rely on the malleability of protein–protein interfaces, binding a cryptic pocket not apparent from apo structures.[\(42, 43\)](#) Strikingly, our model suggests that some conformations sampled in the dynamic and inactive monomeric states of HHV proteases are shared across subfamilies and are thus available to be trapped by compounds such as DD2, compound **2**, and compound **3**. This has broad implications for the discovery of allosteric inhibitors, indicating that highly dynamic proteins of the same family may share pockets present in states with diminished or enhanced activity that could be exploited for modulation by small molecules. This observation is consistent with and expands on prior reports using sequence conservation in large protein families to infer residue networks involved in allostery.[\(44, 45\)](#) Many examples of both homo- and heterodimeric complexes that undergo conformational

changes upon dimerization are detailed in the literature and could be subject to allosteric regulation, especially those in which one or both partners are in part intrinsically unfolded.[\(46-49\)](#)

In summary, we have established kinetic and structural methods for analysis of representative members of all three subfamilies of the HHV proteases. In doing so, we showed that compound **2** has improved potency and allosterically inhibits HHV proteases broadly by disrupting dimerization. At 1.45 Å resolution, the cocrystal structure with compound **2** bound provides the highest-resolution structure to date of the KSHV Pr monomer bound to a protein–protein interaction inhibitor. SAR data and modeling inform our interpretation of cocrystallization studies as well as previously published data for this class of inhibitors and will aid in future screening and design against this family of targets. These analyses and discoveries provide an approach for the identification of small molecules that allosterically regulate protein activity by targeting protein–protein interactions.

1. Tong, L. (2002) Viral proteases Chem. Rev. 102, 4609– 4626
2. Coen, D. M. and Schaffer, P. A. (2003) Antiherpesvirus drugs: A promising spectrum of new drugs and drug targets Nat. Rev. Drug Discovery 2, 278– 288
3. Gao, M., Matusick-Kumar, L., Hurlburt, W., DiTusa, S. F., Newcomb, W. W., Brown, J. C., McCann, P. J., III, Deckman, I., and Colonno, R. J. (1994) The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth J. Virol. 68, 3702– 3712,
4. Jiang, X. H., Gong, H., Chen, Y. C., Vu, G. P., Trang, P., Zhang, C. Y., Lu, S. W., and Liu, F. Y. (2012) Effective inhibition of cytomegalovirus infection by external guide sequences in mice Proc. Natl. Acad. Sci. U.S.A. 109, 13070– 13075

5. Waxman, L. and Darke, P. L. (2000) The herpesvirus proteases as targets for antiviral chemotherapy *Antiviral Chem. Chemother.* 11, 1– 22,
6. Marnett, A. B., Nomura, A. M., Shimba, N., Ortiz de Montellano, P. R., and Craik, C. S. (2004) Communication between the active sites and dimer interface of a herpesvirus protease revealed by a transition-state inhibitor *Proc. Natl. Acad. Sci. U.S.A.* 101, 6870– 6875
7. Borthwick, A. D., Crame, A. J., Ertl, P. F., Exall, A. M., Haley, T. M., Hart, G. J., Mason, A. M., Pennell, A. M., Singh, O. M., Weingarten, G. G., and Woolven, J. M. (2002) Design and synthesis of pyrrolidine-5,5-trans-lactams (5-oxohexahydropyrrolo[3,2-b]pyrroles) as novel mechanism-based inhibitors of human cytomegalovirus protease. 2. Potency and chirality *J. Med. Chem.* 45, 1– 18[ACS Full Text ACS Full Text]
8. Borthwick, A. D., Exall, A. M., Haley, T. M., Jackson, D. L., Mason, A. M., and Weingarten, G. G. (2002) Pyrrolidine-5,5-trans-lactams as novel mechanism-based inhibitors of human cytomegalovirus protease. Part 3: Potency and plasma stability *Bioorg. Med. Chem. Lett.* 12, 1719– 1722
9. Borthwick, A. D., Weingarten, G., Haley, T. M., Tomaszewski, M., Wang, W., Hu, Z., Bedard, J., Jin, H., Yuen, L., and Mansour, T. S. (1998) Design and synthesis of monocyclic β -lactams as mechanism-based inhibitors of human cytomegalovirus protease *Bioorg. Med. Chem. Lett.* 8, 365– 370
10. Gopalsamy, A., Lim, K., Ellingboe, J. W., Mitsner, B., Nikitenko, A., Upeslacis, J., Mansour, T. S., Olson, M. W., Bebernitz, G. A., Grinberg, D., Feld, B., Moy, F. J., and O'Connell, J. (2004) Design and syntheses of 1,6-naphthalene derivatives as selective HCMV protease inhibitors *J. Med. Chem.* 47, 1893– 1899[ACS Full Text ACS Full Text]

11. Ogilvie, W., Bailey, M., Poupart, M. A., Abraham, A., Bhavsar, A., Bonneau, P., Bordeleau, J., Bousquet, Y., Chabot, C., Duceppe, J. S., Fazal, G., Goulet, S., Grand-Maitre, C., Guse, I., Halmos, T., Lavallee, P., Leach, M., Malenfant, E., O'Meara, J., Plante, R., Plouffe, C., Poirier, M., Soucy, F., Yoakim, C., and Deziel, R. (1997) Peptidomimetic inhibitors of the human cytomegalovirus protease *J. Med. Chem.* 40, 4113– 4135[ACS Full Text ACS Full Text]
12. Lazic, A., Goetz, D. H., Nomura, A. M., Marnett, A. B., and Craik, C. S. (2007) Substrate modulation of enzyme activity in the herpesvirus protease family *J. Mol. Biol.* 373, 913– 923
13. Reiling, K. K., Pray, T. R., Craik, C. S., and Stroud, R. M. (2000) Functional consequences of the Kaposi's sarcoma-associated herpesvirus protease structure: Regulation of activity and dimerization by conserved structural elements *Biochemistry* 39, 12796– 12803[ACS Full Text ACS Full Text]
14. Batra, R., Khayat, R., and Tong, L. (2001) Molecular mechanism for dimerization to regulate the catalytic activity of human cytomegalovirus protease *Nat. Struct. Biol.* 8, 810– 817
15. LaPlante, S. R., Bonneau, P. R., Aubry, N., Cameron, D. R., Deziel, R., Grand-Maitre, E., Plouffe, C., Tong, L., and Kawai, S. H. (1999) Characterization of the human cytomegalovirus protease as an induced-fit serine protease and the implications to the design of mechanism-based inhibitors *J. Am. Chem. Soc.* 121, 2974– 2986
16. Nomura, A. M., Marnett, A. B., Shimba, N., Dotsch, V., and Craik, C. S. (2005) Induced structure of a helical switch as a mechanism to regulate enzymatic activity *Nat. Struct. Mol. Biol.* 12, 1019– 1020
17. Qiu, X., Janson, C. A., Culp, J. S., Richardson, S. B., Debouck, C., Smith, W. W., and Abdel-Meguid, S. S. (1997) Crystal structure of varicella-zoster virus protease *Proc. Natl. Acad. Sci. U.S.A.* 94, 2874– 2879

18. Buisson, M., Hernandez, J. F., Lascoux, D., Schoehn, G., Forest, E., Arlaud, G., Seigneurin, J. M., Ruigrok, R. W., and Burmeister, W. P. (2002) The crystal structure of the Epstein-Barr virus protease shows rearrangement of the processed C terminus *J. Mol. Biol.* 324, 89– 103
19. Chen, P., Tsuge, H., Almassy, R. J., Gribskov, C. L., Katoh, S., Vanderpool, D. L., Margosiak, S. A., Pinko, C., Matthews, D. A., and Kan, C. C. (1996) Structure of the human cytomegalovirus protease catalytic domain reveals a novel serine protease fold and catalytic triad *Cell* 86, 835– 843
20. Tong, L., Qian, C., Massariol, M. J., Bonneau, P. R., Cordingley, M. G., and Lagace, L. (1996) A new serine-protease fold revealed by the crystal structure of human cytomegalovirus protease *Nature* 383, 272– 275
21. Shieh, H. S., Kurumbail, R. G., Stevens, A. M., Stegeman, R. A., Sturman, E. J., Pak, J. Y., Wittwer, A. J., Palmier, M. O., Wiegand, R. C., Holwerda, B. C., and Stallings, W. C. (1996) Three-dimensional structure of human cytomegalovirus protease *Nature* 383, 279– 282
22. Pray, T. R., Nomura, A. M., Pennington, M. W., and Craik, C. S. (1999) Auto-inactivation by cleavage within the dimer interface of Kaposi's sarcoma-associated herpesvirus protease *J. Mol. Biol.* 289, 197– 203
23. Pray, T. R., Reiling, K. K., Demirjian, B. G., and Craik, C. S. (2002) Conformational change coupling the dimerization and activation of KSHV protease *Biochemistry* 41, 1474– 1482[ACS Full Text ACS Full Text]
24. Shimba, N., Nomura, A. M., Marnett, A. B., and Craik, C. S. (2004) Herpesvirus protease inhibition by dimer disruption *J. Virol.* 78, 6657– 6665
25. Hoog, S. S., Smith, W. W., Qiu, X., Janson, C. A., Hellmig, B., McQueney, M. S., O'Donnell, K., O'Shannessy, D., DiLella, A. G., Debouck, C., and Abdel-Meguid, S. S. (1997)

- Active site cavity of herpesvirus proteases revealed by the crystal structure of herpes simplex virus protease/inhibitor complex *Biochemistry* 36, 14023– 14029[ACS Full Text ACS Full Text]
26. Schmidt, U. and Darke, P. L. (1997) Dimerization and activation of the herpes simplex virus type 1 protease *J. Biol. Chem.* 272, 7732– 7735
27. Lee, G. M., Shahian, T., Baharuddin, A., Gable, J. E., and Craik, C. S. (2011) Enzyme Inhibition by Allosteric Capture of an Inactive Conformation *J. Mol. Biol.* 411, 999– 1016
28. Nomura, A. M., Marnett, A. B., Shimba, N., Dotsch, V., and Craik, C. S. (2006) One functional switch mediates reversible and irreversible inactivation of a herpesvirus protease *Biochemistry* 45, 3572– 3579[ACS Full Text ACS Full Text]
29. Craik, C. S. and Shahian, T. (2012) A screening strategy for trapping the inactive conformer of a dimeric enzyme with a small molecule inhibitor *Methods Mol. Biol.* 928, 119– 131,
30. Shahian, T., Lee, G., Lazic, A., Arnold, A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a Viral Enzyme by a Small Molecule Dimer Disruptor *Nat. Chem. Biol.* 9, 640– 646
31. Zhang, Z. Y., Poorman, R. A., Maggiora, L. L., Heinrikson, R. L., and Kezdy, F. J. (1991) Dissociative Inhibition of Dimeric Enzymes: Kinetic Characterization of the Inhibition of Hiv-1 Protease by Its COOH-Terminal Tetrapeptide *J. Biol. Chem.* 266, 15591– 15594,
32. Harris, J. L., Backes, B. J., Leonetti, F., Mahrus, S., Ellman, J. A., and Craik, C. S. (2000) Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries *Proc. Natl. Acad. Sci. U.S.A.* 97, 7754– 7759
33. Buisson, M., Valette, E., Hernandez, J. F., Baudin, F., Ebel, C., Morand, P., Seigneurin, J. M., Arlaud, G. J., and Ruigrok, R. W. (2001) Functional determinants of the Epstein-Barr virus protease *J. Mol. Biol.* 311, 217– 228

34. Bowman, M. J., Byrne, S., and Chmielewski, J. (2005) Switching between allosteric and dimerization inhibition of HIV-1 protease *Chem. Biol.* 12, 439– 444
35. Jacobson, M. P., Friesner, R. A., Xiang, Z. X., and Honig, B. (2002) On the role of the crystal environment in determining protein side-chain conformations *J. Mol. Biol.* 320, 597– 608
36. Jacobson, M. P., Kaminski, G. A., Friesner, R. A., and Rapp, C. S. (2002) Force field validation using protein side chain prediction *J. Phys. Chem. B* 106, 11673– 11680
37. Li, X., Jacobson, M. P., and Friesner, R. A. (2004) High-resolution prediction of protein helix positions and orientations *Proteins* 55, 368– 382
38. Zondlo, N. J. (2013) Aromatic-Proline Interactions: Electronically Tunable CH/ π Interactions *Acc. Chem. Res.* 46, 1039– 1049[ACS Full Text ACS Full Text]
39. Wells, J. A. and McClendon, C. L. (2007) Reaching for high-hanging fruit in drug discovery at protein-protein interfaces *Nature* 450, 1001– 1009
40. Kar, G., Keskin, O., Gursoy, A., and Nussinov, R. (2010) Allostery and population shift in drug discovery *Curr. Opin. Pharmacol.* 10, 715– 722
41. Lee, G. M. and Craik, C. S. (2009) Trapping moving targets with small molecules *Science* 324, 213– 215
42. Sundberg, E. J. and Mariuzza, R. A. (2000) Luxury accommodations: The expanding role of structural plasticity in protein-protein interactions *Structure* 8, R137– R142
43. Teague, S. J. (2003) Implications of protein flexibility for drug discovery *Nat. Rev. Drug Discovery* 2, 527– 541
44. Suel, G. M., Lockless, S. W., Wall, M. A., and Ranganathan, R. (2003) Evolutionarily conserved networks of residues mediate allosteric communication in proteins (vol 10, pg 59, 2003) *Nat. Struct. Biol.* 10, 232

45. Reynolds, K. A., McLaughlin, R. N., and Ranganathan, R. (2011) Hot Spots for Allosteric Regulation on Protein Surfaces *Cell* 147, 1564– 1575
46. Yuan, Y., Zhao, W., Wang, X., Gao, Y., Niu, L., and Teng, M. (2012) Dimeric Sfh3 has structural changes in its binding pocket that are associated with a dimer-monomer state transformation induced by substrate binding *Acta Crystallogr. D* 69, 313– 323
47. McDonald, C. B., Seldeen, K. L., Deegan, B. J., Lewis, M. S., and Farooq, A. (2008) Grb2 adaptor undergoes conformational change upon dimerization *Arch. Biochem. Biophys.* 475, 25– 35
48. Demchenko, A. P. (2001) Recognition between flexible protein molecules: Induced and assisted folding *J. Mol. Recognit.* 14, 42– 61
49. Dyson, H. J. and Wright, P. E. (2002) Coupling of folding and binding for unstructured proteins *Curr. Opin. Struct. Biol.* 12, 54– 60

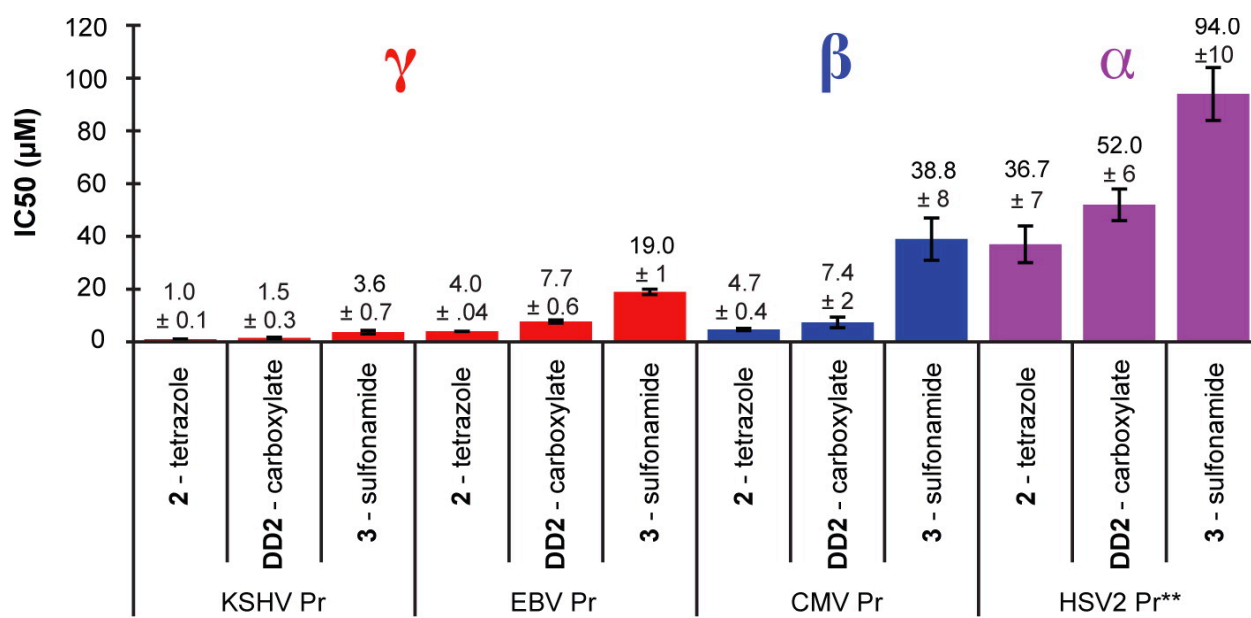


Figure 6-1. IC₅₀ values across multiple HHV proteases. IC₅₀ values for DD2 and compounds 2 and 3 against KSHV, EBV, HCMV, and HSV-2 proteases. Two asterisks for HSV-2 indicate values were determined with a buffer and enzyme concentration different from those used for all other enzymes (Materials and Methods).

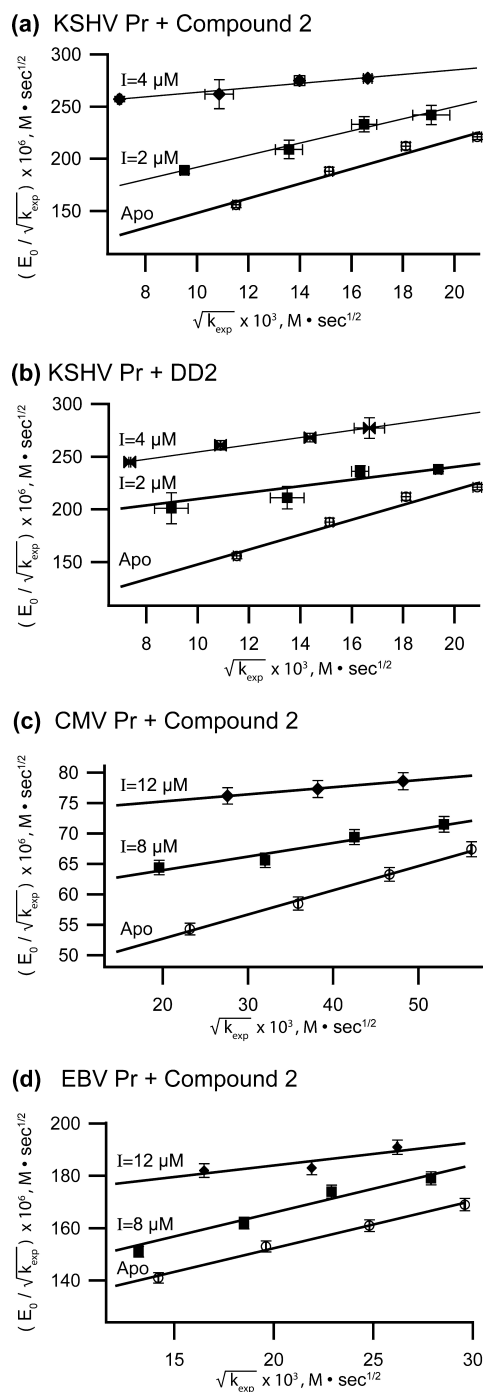


Figure 6-2. Zhang–Poorman analysis of DD2 and compound 2. Against KSHV protease, both compound 2 (a) and DD2 (b) show an increasing intercept with an increasing inhibitor concentration, indicative of dimer disruption. The same trend is observed for compound 2 with HCMV (c) and EBV (d) proteases.

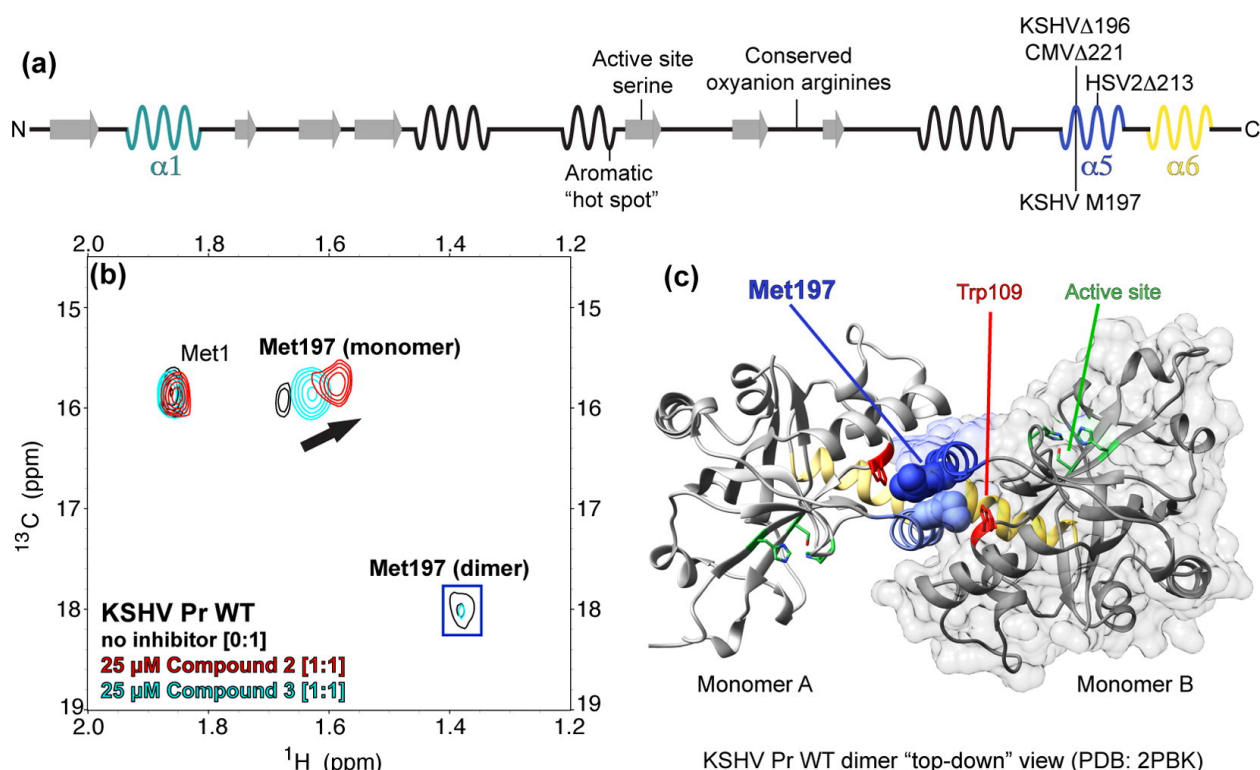


Figure 6-3. HHV domain diagram and ^{13}C – ^1H HSQC spectrum of KSHV protease with selectively labeled Met197. HHVs share a common structure consisting of seven β strands and six major helices. Helices 1 (cyan) and 5 (blue) are major components of the dimer interface. Helices 5 and 6 (yellow) undergo a disorder-to-order transition upon dimerization. The locations of the aromatic hot spot residues (KSHV W109, HCMV Y128, EBV W111, and HSV2 Y124), active site serines (KSHV S114, HCMV S132, EBV S116, and HSV2 S129), conserved oxyanion arginines (KSHV R142 and R143, HCMV R165 and R166, EBV R147 and R148, and HSV2 R156 and R157), and sites of truncation are indicated. (a) Met197 exhibits distinct resonances for the dimeric and monomeric states of KSHV Pr. The spectral overlay of the apo form (black), 1 molar equiv of compound 3 (cyan), and 1 molar equiv of compound 2 (b) shows addition of either compound results in a reduction in the Met197 dimer peak intensity as well as a chemical shift perturbation and an increase in the intensity of the Met197 monomer peak. The Met1 peak remains unperturbed. The location of Met197 at the dimer interface (c) is shown on the wild-type dimeric KSHV protease crystal structure (PDB entry 2PBK) in the proximity of the aromatic hot spot residue Trp109.

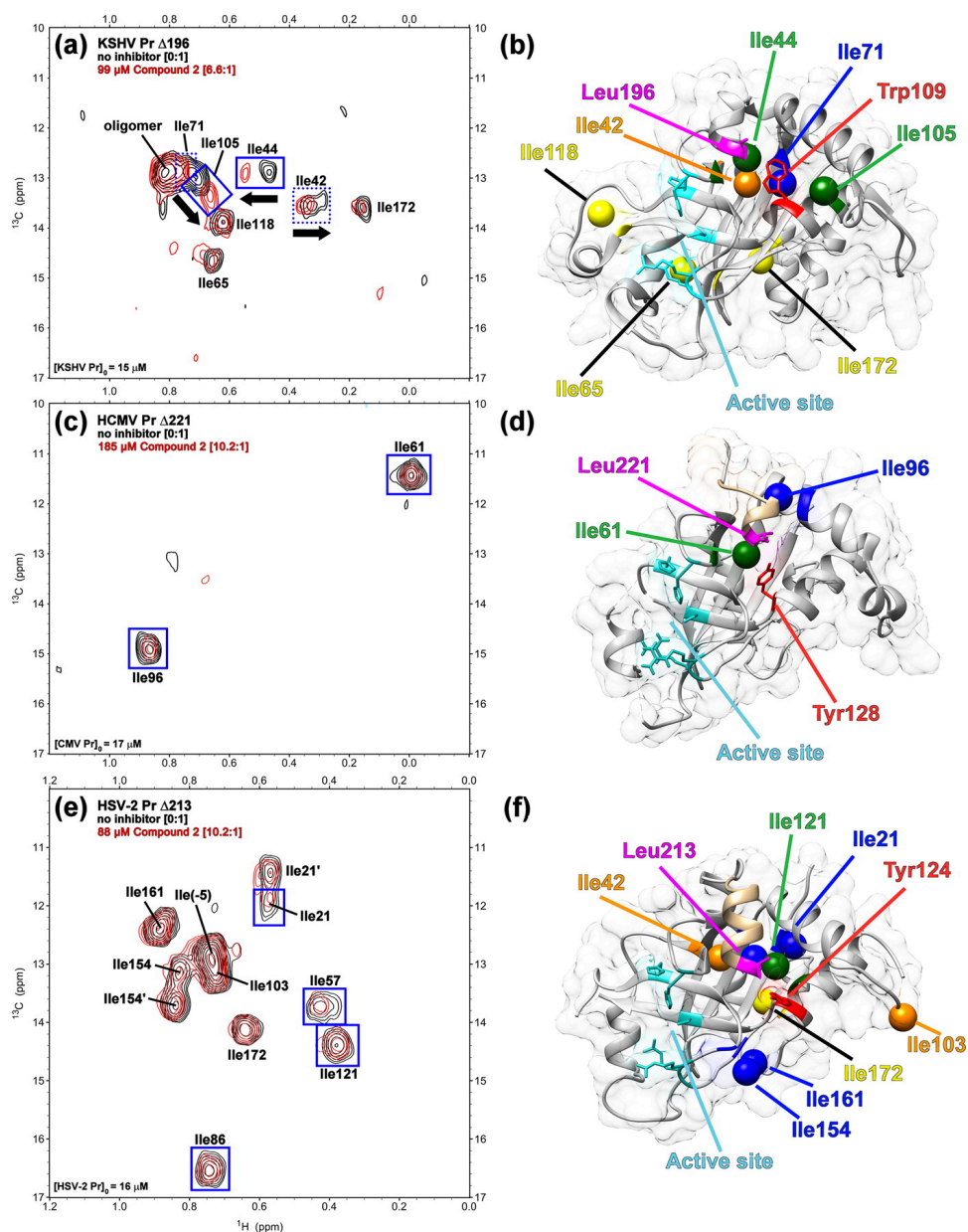


Figure 6-4. ^{13}C - ^1H HSQC Ile spectra in the presence of compound 2. The ^{13}C - ^1H HSQC spectra of selectively ^{13}C - ^1H [$\delta 1$ -methyl]isoleucine-labeled truncated constructs KSHV Pr $\Delta 196$ (a), HCMV Pr $\Delta 221$ (c), and HSV-2 Pr $\Delta 213$ (e) in the presence of 0 (black) and 5–10 molar equiv of compound 2 (red) indicate compound 2 binds at the dimer interface across representative members of all three herpesvirus subfamilies. Prime signs denote minor conformer peak resonances. The locations of isoleucine $\delta 1$ -methyl groups in the KSHV (b), HCMV (d), and HSV-2 (f) truncated constructs are shown at the dimer interface, and color-coded with respect to the distance to the putative aromatic hot spot (red). Isoleucine residues within 5 Å (green), 5–10 Å (blue), 10–15 Å (orange), and >15 Å (yellow) are indicated. Helix 5 (tan), the active site (cyan), and the point of truncation (pink) are also denoted.

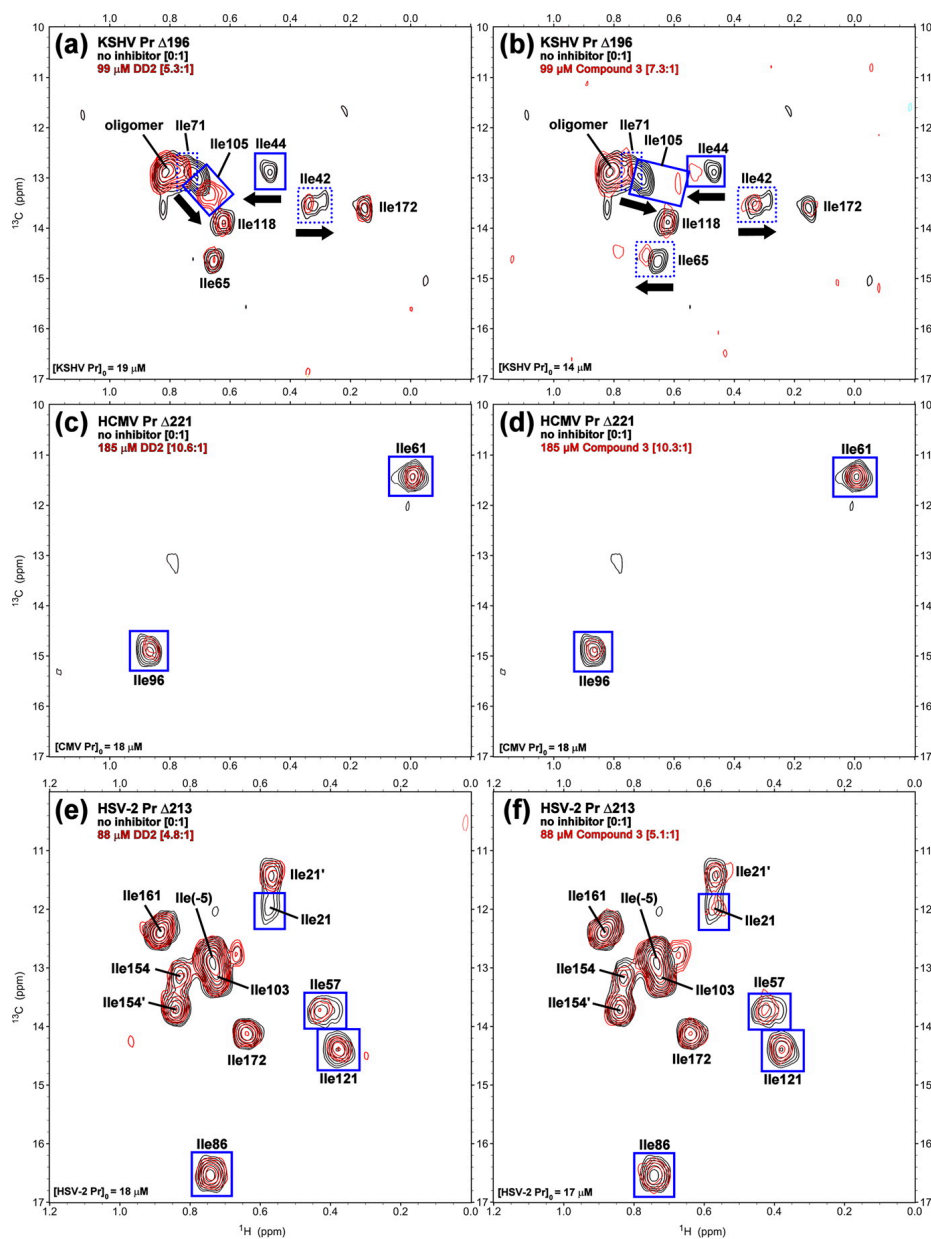


Figure 6-5. ^{13}C – ^1H HSQC Ile spectra in the presence of compound 3. The ^{13}C – ^1H HSQC spectra of selectively ^{13}C – ^1H [δ 1-methyl]isoleucine-labeled truncated constructs KSHV Pr Δ 196 (a and b), HCMV Pr Δ 221 (c and d), and HSV-2 Pr Δ 213 (e and f) in the presence of 0 (black) and 5–10 molar equiv of compound 3 (red; a, c, and f) or DD2 (red; b, d, and e) indicate compound 3 and DD2 bind at the dimer interface across representative members of all three herpesvirus subfamilies.

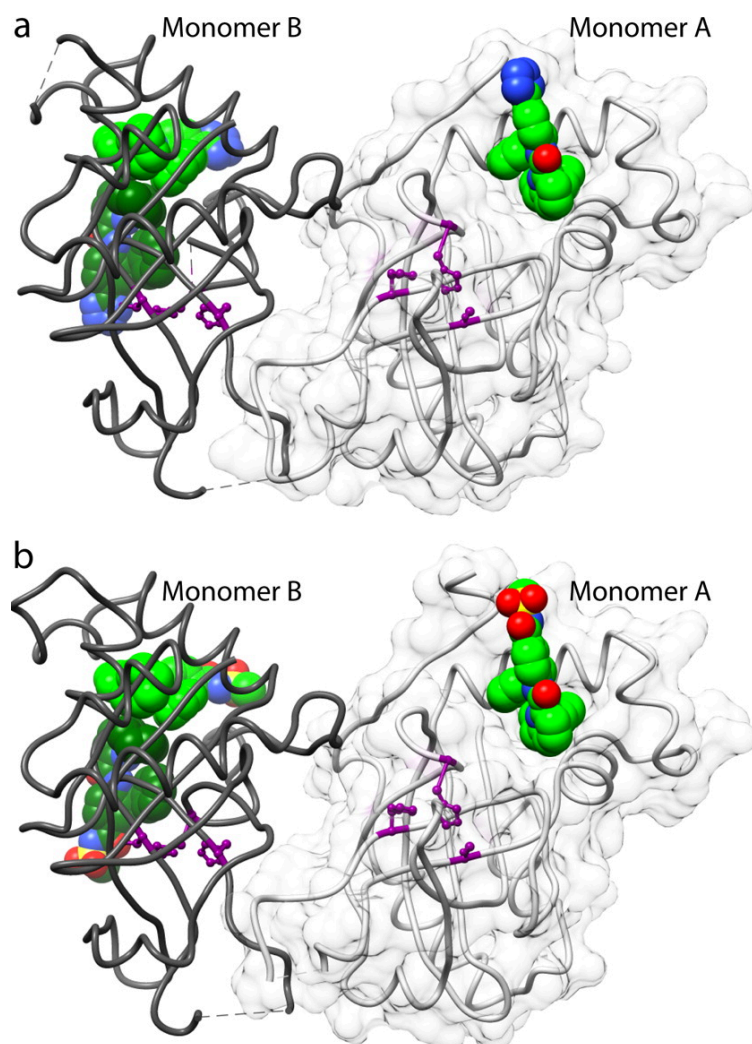


Figure 6-6. Asymmetric unit with the inhibitor bound. The asymmetric unit contains a crystallographic dimer of inhibited monomers (monomers A and B). The compound 2-bound structure (a) and compound 3-bound structure (b) show the major inhibitor molecules (bright green) and the bridging inhibitor molecule (dark green). The active site Ser-His-His catalytic triad is colored purple, distant from the major inhibitor molecules.

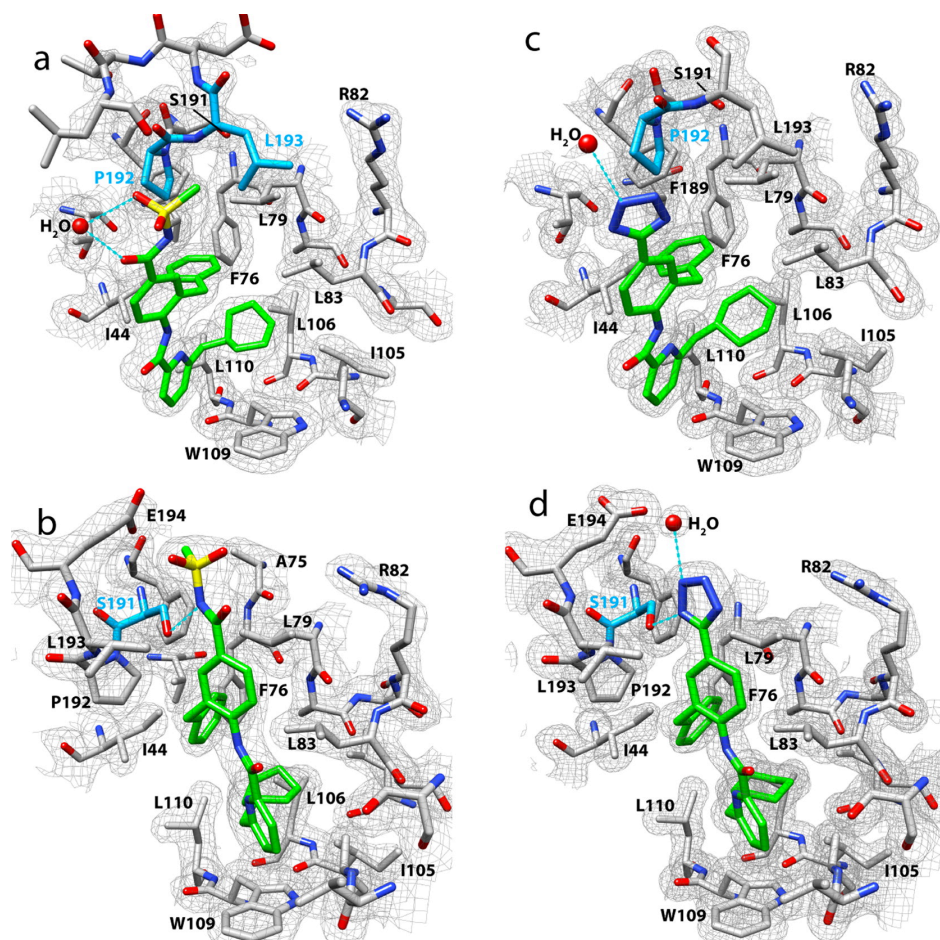


Figure 6-7. Compound 2 and 3 binding pockets. Compound 2 (green) in monomers A (a) and B (b) and compound 3 (green) in both monomers (c and d) bind the same largely hydrophobic pocket. The pocket is comprised primarily of aliphatic residues where binding is driven by hydrophobic interactions with the cyclohexyl and benzyl substituents of the compound. Trp109 adopts an open rotameric conformation relative to the dimeric KSHV Pr structure (PDB entry 2PBK). In monomer A, both compounds interact with Pro192 (cyan) and a structural water molecule. Compound 3 interacts extensively with Leu193 (cyan). In monomer B, both compounds hydrogen bond to the Ser191 hydroxyl, while in monomer A, Ser191 faces away from the small molecule. The mesh displays the $2F_o - F_c$ density map contoured to 1σ .

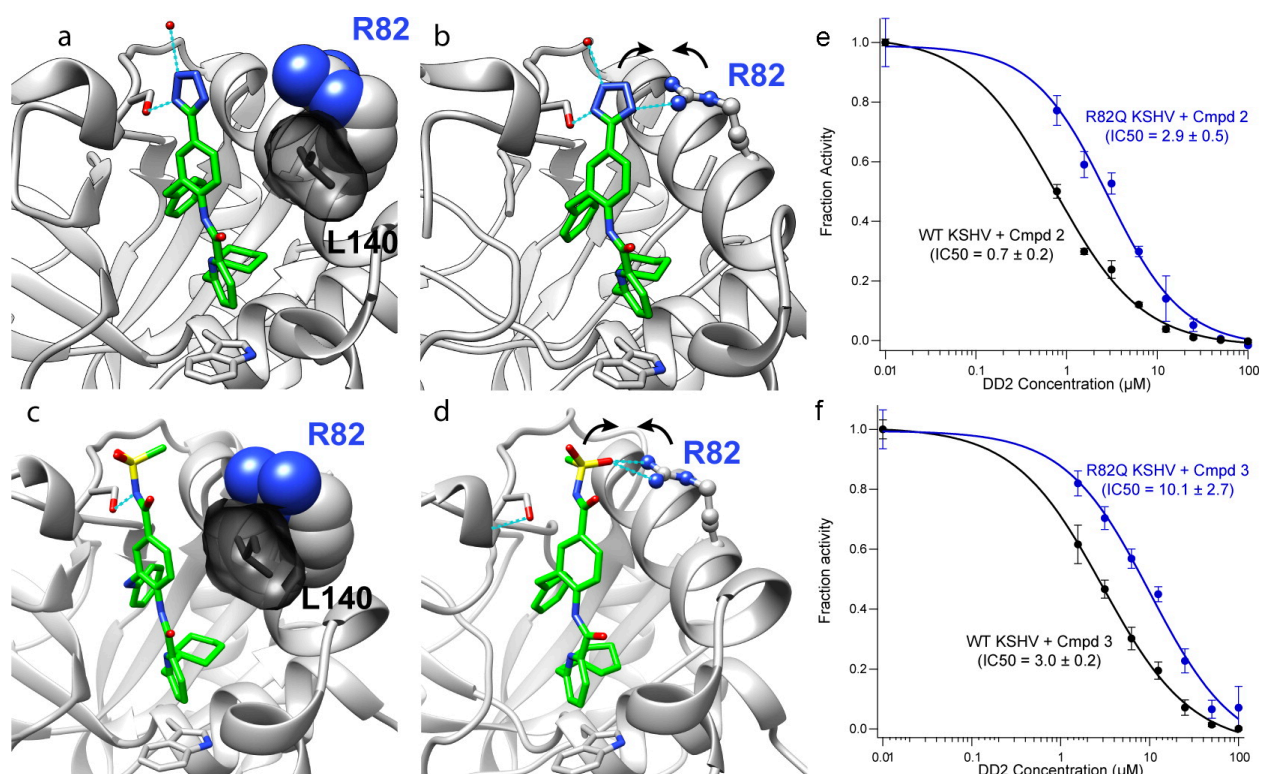


Figure 6-8. Compound binding mode with and without crystal packing. Models of compounds 2 and 3 were generated by minimizing the compound–monomer complex using PLOP (b and e). Arg82 is predicted to interact with the anionic carboxylate bioisostere, in contrast to the crystal structure in which a symmetry-mate Leu140 side chain prevents such interaction (a and d). IC₅₀ values determined for KSHV Pr R82Q are substantially greater (reduced potency) than those for WT KSHV Pr (c and f).

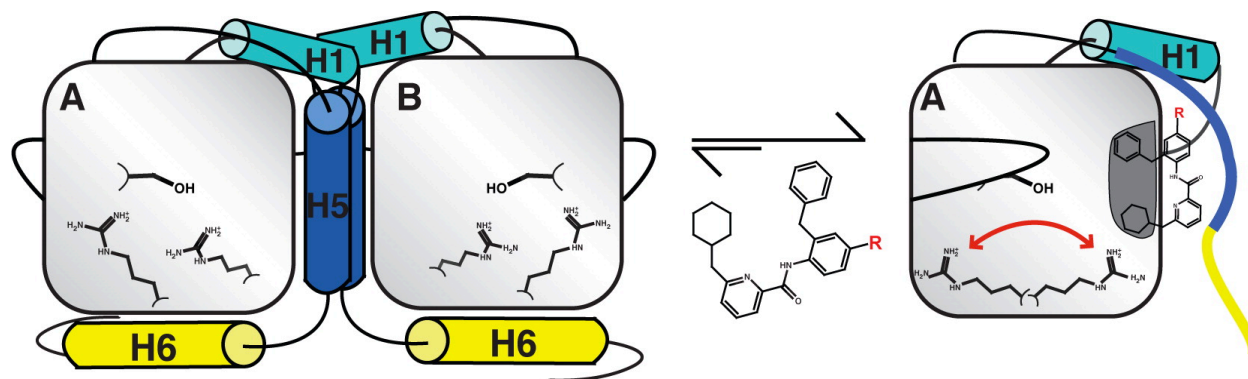
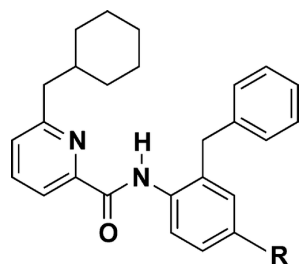


Figure 6-9. Model of the conserved mechanism of inhibition. Compounds 2 and 3 and DD2 bind an allosteric pocket in the core of the protein preventing folding of helix 5 (H5, blue) and helix 6 (H6, yellow), “trapping” the inactive monomer (right). In contrast to the active dimer (left), when H6 is unfolded, two conserved arginines no longer stabilize the oxyanion hole (red arrow), thus preventing proteolytic activity. The active site serine is largely unperturbed; however, a loop (right) partially occludes the substrate binding cleft. R in the chemical structure represents anionic carboxylate bioisosteres.

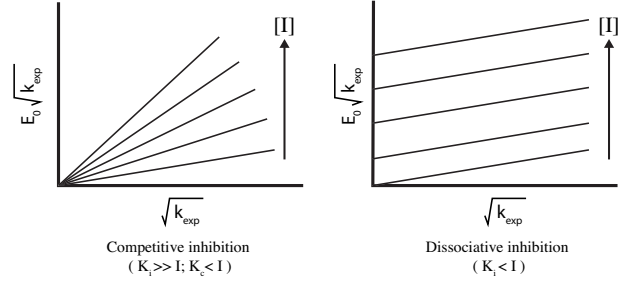
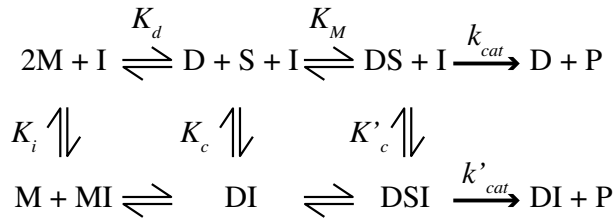
Table 6-1. DD2 analogs



R	KSHV Pr IC ₅₀ (μM)
 DD2	1.5 ± 0.3
 Compound 2	1.0 ± 0.1
 Compound 3	3.7 ± 0.7
 Compound 4	No inhibition
 Compound 5	No inhibition
 Compound 6	No inhibition
 Compound 7	No inhibition

Supplemental Information:

Scheme 1:



Supplemental equations:

Equation 1 describes the linear relationship between dependent variable $\frac{E_0}{\sqrt{k_{exp}}}$ and independent variable $\sqrt{k_{exp}}$ in the presence of inhibitor used for Zhang-Poorman analysis.¹

$$\frac{E_0}{\sqrt{k_{exp}}} = \sqrt{k_{exp}} \frac{K_M \left(1 + \frac{I}{K_c}\right)}{k_{cat} + k'_{cat} \frac{I}{K'_c}} + \frac{\sqrt{\frac{K_d K_M}{4}}}{\sqrt{k_{cat} + k'_{cat} \frac{I}{K'_c}}} \left(1 + \frac{I}{K_i}\right) \quad (1)$$

In the absence of inhibitor equation 1 simplifies to equation 2 below.

$$\frac{E_0}{\sqrt{k_{exp}}} = \sqrt{\frac{K_d K_M}{4 k_{cat}}} + \frac{K_M}{k_{cat}} \times \sqrt{k_{exp}} \quad (2)$$

The slope of each line is equal to $\frac{K_M \left(1 + \frac{I}{K_c}\right)}{k_{cat} + k'_{cat} \frac{I}{K'_c}}$ where $K_c = D \cdot I / DI$ is the competitive inhibition

equilibrium constant and $K'_c = DS \cdot I / DSI$ is the noncompetitive equilibrium constant (D , I , DI , DS , and DSI are the molar concentrations of dimer, inhibitor, dimer-inhibitor, dimer-substrate, and dimer-substrate-inhibitor, respectively). The noncompetitive rate constant, k'_{cat} , is the rate of conversion of dimer-substrate-inhibitor complex to dimer-inhibitor plus product. Dissociative inhibition (dimer disruption) results in an increasing intercept with increasing inhibitor concentration. Purely dissociative inhibition results in a constant slope while mixed-type inhibition results in variable slope with varying inhibitor concentration.

Supplemental derivation 1:

In our Zhang-Poorman analysis with both DD2 and compound **2**, increasing inhibitor concentration resulted in decreasing slope. This indicates that the derivative of the slope term with respect to inhibitor concentration (a) must be less than zero (b). To investigate whether this could inform mode of binding we solved this inequality.

$$\frac{\partial}{\partial I} \left(\frac{K_M \left(1 + \frac{I}{K_c} \right)}{k_{cat} + k'_{cat} \frac{I}{K'_c}} \right) = \frac{K_M K'_c (k_{cat} K'_c - K_c k'_{cat})}{K_c (I k'_{cat} + k_{cat} K'_c)^2} \quad (\text{a})$$

$$\frac{K_M K'_c (k_{cat} K'_c - K_c k'_{cat})}{K_c (I k'_{cat} + k_{cat} K'_c)^2} < 0 \quad (\text{b})$$

Solving (b) while assuming all constants greater than 0, it follows that:

$$0 < K'_c < \frac{K_c k'_{cat}}{k_{cat}} \text{ and } \frac{K'_c}{K_c} < \frac{k'_{cat}}{k_{cat}} \quad (\text{c})$$

Since inhibition takes place we assume $k'_{cat} < k_{cat}$, therefore in combination with (c):

$$\frac{K'_c}{K_c} < \frac{k'_{cat}}{k_{cat}} < 1 \text{ and } K'_c < K_c \quad (\text{d})$$

Since K'_c is less than K_c , apparent binding of inhibitor to the dimer-substrate-inhibitor complex is tighter than binding of inhibitor to the dimer alone. Without additional knowledge of k'_{cat} , K_c , or K'_c the inequality $(K'_c / K_c) < (k'_{cat} / k_{cat})$ is uninformative, though necessarily true for the above interpretation.

Supplemental Methods:**Site-Directed Mutagenesis**

All DNA plasmids were produced by mutagenesis of the template sequences using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent). The resulting PCR-reaction mixtures were first transformed into XL-10 Gold Ultracompetent Cells (Agilent). Cultures

containing the XL-10 transformed cells were grown overnight in Luria Broth containing carbenicillin (100 mg/mL) and miniprep using Qiagen Miniprep Kits. DNA sequences of the mutated plasmids were verified via DNA sequencing prior to re-transformation into Rosetta2 (DE3) pLysS Competent Cells (Novagen/EMD Millipore). Primers (Integrated DNA Technologies, Inc.) encoding the isoleucine to valine mutations are listed in Table S1, below.

Table 6-S1: Ile-to-Val site-directed mutagenesis primers

CMV Pr D221		
Ile61Val	5'-CGCTCCCGCTCAACGTCAACCACGACGAC-3'	FWD
	5'-GTCGTTCGTGGTTGACGTTGAGCGGGAGCG-3'	REV
Ile96Val	5'-CCAGGTTTCTGGAGGTTGTACGCCGCGCT-3'	FWD
	5'-AGCGCGGCGTACAACCTCCAGAAACCTGG-3'	REV

HSV-2 Pr D213		
Ile21Val	5'-GGGCGGTGCCCCGTCTACGTGGCC-3'	FWD
	5'-GGCCACGTAGACGGGCACCGCCC-3'	REV
Ile57Val	5'-AGAACCCCTGCCGGTCAACGTAGACCAC-3'	FWD
	5'-GTGGTCTACGTTGACCGGCAGGGGGTTCT-3'	REV
Ile86Val	5'-TTTTTGTGGGGCTGGTCGCGTGCGTGCG-3'	FWD
	5'-CTGCACGCACGCGACCGAGCCCCACAAAA-3'	REV
Ile103Val	5'-CGCCAGCGCCGCTGTTTTTGAGCGCCG-3'	FWD
	5'-CGGCGCTCAAAAACAGCGGCGCTGGCG-3'	REV
Ile121Val	5'-CGTCTGCTGTACCTGGTCACCAACTACCTGC-3'	FWD
	5'-GCAGGTAGTTGGTGACCAGGTACAGCAGACG-3'	REV
Ile154Val	5'-CCCTGTGCGCCGTCGGGCGGCGC-3'	FWD
	5'-GCGCCGCCCCGACGGCGCACAGGG-3'	REV
Ile161Val	5'-GGCGCCTTGGAACCGTCGTCACCTACGAC-3'	FWD
	5'-GTCGTAGGTGACGACGGTTCCAAGGCGCC-3'	REV
Ile172Val	5'-CCTAGACGCGGCCGTCGCTCCGTTTCG-3'	FWD
	5'-CGAAACGGAGCGACGGCCGCGTCTAGG-3'	REV

Crystallographic data processing

Diffraction images were processed using MOSFLM² and the CCP4 suite³ (specifically SCALA/Truncate), operated through the Elves scripts.⁴ The resulting structure was solved by molecular replacement with Phaser⁵ using PDB 3NJQ as the template search model. The resulting structure model was a dimer in an asymmetric unit and was subjected to multiple rounds of restrained refinement and isotropic *B*-factor minimization with Phenix⁶ and Coot.⁷ “Riding” hydrogens were included during refinement.

Analog synthesis

¹H NMR spectra were recorded on a Varian INOVA-400 400 MHz spectrometer. Chemical shifts are reported in δ units (ppm) relative to TMS as an internal standard. Coupling constants (*J*) are reported in hertz (Hz). ¹³C NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer equipped with a QCI CyroProbe. All other reagents and solvents were purchased from Sigma-Aldrich and used as received. Air and/or moisture sensitive reactions were carried out under an argon atmosphere in oven-dried glassware using anhydrous solvents from commercial suppliers. Air and/or moisture sensitive reagents were transferred via syringe or cannula and were introduced into reaction vessels through rubber septa. Solvent removal was accomplished with a rotary evaporator at ca. 10-50 Torr. Column chromatography was carried out using a Biotage SP1 flash chromatography system and silica gel cartridges from Biotage. Analytical TLC plates from EM Science (Silica Gel 60 F254) were employed for TLC analyses. Hydrogenation reactions were carried out with a ThalesNano H-Cube hydrogenator.

All synthesized analogs were judged to be of 95% or higher purity based on analytical LC/MS analysis. LC/MS analyses were performed on a Waters Micromass ZQ/Waters 2795 Separation Module/Waters 2996 Photodiode Array Detector system controlled by MassLynx 4.0

software. Separations were carried out on an XTerra® MS C₁₈ 5 μ m 4.6x50mm column at ambient temperature using a mobile phase of water-acetonitrile containing 0.05% trifluoroacetic acid. Gradient elution was employed wherein the acetonitrile-water ratio was increased linearly from 5 to 95% acetonitrile over 2.5 minutes, then maintained at 95% acetonitrile for 1.5 min., and then decreased to 5% acetonitrile over 0.5 min, and maintained at 5% acetonitrile for 0.5 min. Compound purity was determined by integrating peak areas of the liquid chromatogram, monitored at 254 nm. Compound **2** was a cream-colored powder. Compounds **1** and **3-7** were white powders.

N-(2-Benzyl-4-carbamoyl-phenyl)-6-(cyclohexylmethyl)pyridine-2-carboxamide (4) 3-Benzyl-4-[[6-(cyclohexylmethyl)pyridine-2-carbonyl]amino]benzoic acid (**DD2**, 16 mg, 0.037 mmol), di-*tert*-butyl dicarbonate (11 mg, 0.0048 mmol), ammonium bicarbonate (4 mg, 0.048 mmol) and pyridine (0.5 ml, 0.0048 mmol) in acetone (1 mL) were stirred at room temperature for 18 h. The reaction mixture was concentrated *under reduced pressure*, diluted with water and extracted with ethyl acetate. The ethyl acetate layer was washed with brine, dried over magnesium sulfate and concentrated under reduced pressure. The crude material thus obtained was purified by flash column chromatography (30% ethyl acetate-hexanes and 5% methanol-dichloromethane) to obtain the title compound in 63% yield. ¹H NMR (CDCl₃) δ 10.35 (s, 1H), 8.53 (d, *J* = 6 Hz, 1H), 8.06 (d, *J* = 6 Hz, 1H), 7.83 (s, 1H), 7.73-7.76 (m, 2H), 7.20-7.28 (m, 6H), 4.18 (s, 2H), 2.64 (d, *J* = 6 Hz, 2H), 1.60-1.689 (m, 5H), 1.16-1.24 (m, 4H), 0.93-0.98 (m, 2H); LCMS (ESI) *m/z* 428 (MH⁺). ¹³C NMR (125.73 MHz, DMSO- *d*₆) δ 164.7, 159.1, 157.3, 145.6, 136.1, 135.8, 135.5, 127.8, 127.7, 127.5, 125.9, 125.6, 124.5, 124.0, 123.8, 118.2, 116.9, 29.8, 23.3, 23.0

N-[2-Benzyl-4-(4H-1,2,4-triazol-3-yl)phenyl]-6-(cyclohexylmethyl)pyridine-2-carboxamide

(5) N-(2-Benzyl-4-carbamoyl-phenyl)-6-(cyclohexylmethyl)pyridine-2-carboxamide (**4**, 10 mg, 0.023 mmol) and acetic acid (0.01 mL) in N,N-dimethylformamide dimethyl acetal (0.100 mL) were heated to 80 °C for 30 min. The reaction mixture was concentrated under reduced pressure and diluted with AcOH (0.1 mL). Hydrazine hydrate (0.01 mL) was added and the mixture was heated to 110 °C for 45 min. The reaction mixture was then diluted with ethyl acetate and washed with saturated sodium bicarbonate, water and brine. The ethyl acetate layer was dried over magnesium sulfate and concentrated under reduced pressure. The crude material thus obtained was purified by flash column chromatography (40% ethyl acetate-hexanes) to obtain the title compound in 85% yield. ¹H NMR (CDCl₃) δ 10.39 (s, 1H), 8.55 (d, *J* = 6 Hz, 1H), 8.13-8.23 (m, 4H), 7.82 (t, *J* = 6 Hz, 1H), 7.17-7.29 (m, 6H), 4.19 (s, 2H), 2.65 (d, *J* = 6 Hz, 2H), 1.61-1.68 (m, 5H), 1.16-1.24 (m, 4H), 0.93-1.02 (m, 2H); LCMS (ESI) *m/z* 452 (MH⁺). ¹³C NMR (125.73 MHz, DMSO- *d*₆) δ 161.7, 160.6, 159.9, 151.9, 148.4, 144.5, 139.0, 138.4, 131.7, 131.4, 128.6, 128.3, 128.2, 127.7, 127.0, 126.6, 126.4, 125.1, 124.8, 123.5, 122.0, 119.4, 44.8, 37.8, 37.0, 32.5, 26.0, 25.6

N-(2-Benzyl-4-cyanophenyl)-6-(cyclohexylmethyl)pyridine-2-carboxamide (7) N-(2-Benzyl-

4-carbamoyl-phenyl)-6-(cyclohexylmethyl)pyridine-2-carboxamide (**4**, 20 mg, 0.047 mmol), trifluoroacetic anhydride (0.026 mL, 0.19 mmol) and pyridine (0.022 mL, 0.28 mmol) in 1,4-dioxane (1 mL) were stirred at room temperature for 18 h. The reaction mixture was diluted with ethyl acetate and washed with water and brine. The organic extract was dried over magnesium sulfate and concentrated under reduced pressure. The crude material thus obtained was purified by flash column chromatography (20% ethyl acetate-hexanes) to afford the title compound in

89% yield. ¹H NMR (CDCl₃) δ 10.43 (s, 1H), 8.63 (d, *J* = 6 Hz, 1H), 8.06 (d, *J* = 6 Hz, 1H), 7.77 (t, *J* = 6 Hz, 1H), 7.63 (dd, *J* = 6, 3 Hz, 1H), 7.50 (s, 1H), 7.23-7.34 (m, 6H), 4.14 (s, 2H), 2.64 (d, *J* = 6 Hz, 2H), 1.63-1.70 (m, 5H), 1.15-1.20 (m, 4H), 0.95-0.98 (m, 2H); LCMS (ESI) *m/z* 410 (MH⁺). ¹³C NMR (125.73 MHz, DMSO- *d*₆) δ 161.9, 160.0, 147.8, 140.0, 138.5, 137.9, 134.6, 131.8, 131.7, 128.7, 128.2, 127.4, 126.7, 121.6, 119.7, 118.9, 106.4, 44.8, 37.8, 36.4, 32.5, 26.0, 25.6

N-[2-Benzyl-4-(1H-1,2,3,4-tetrazol-5-yl)phenyl]-6-(cyclohexylmethyl)pyridine-2-carboxamide (2) N-(2-Benzyl-4-cyanophenyl)-6-(cyclohexylmethyl)pyridine-2-carboxamide (**7**, 14 mg, 0.034 mmol), sodium azide (9 mg, 0.13 mmol), zinc bromide (8 mg, 0.034 mmol) in a 1:2 mixture of 2-propanol/water (0.9 mL) were heated to reflux for 60 h. The reaction mixture was cooled, concentrated under reduced pressure, diluted with water and extracted with ethyl acetate. The organic extract was dried over magnesium sulfate and concentrated under reduced pressure. The crude material thus obtained was purified by flash column chromatography to afford the title compound in 60% yield. ¹H NMR (DMSO-*d*₆) δ 10.34 (s, 1H), 8.37 (d, *J* = 9 Hz, 1H), 8.05 (s, 1H), 7.93-7.99 (m, 3H), 7.48-7.50 (m, 1H), 7.19-7.27 (m, 5H), 4.20 (s, 2H), 2.67 (d, *J* = 6 Hz, 2H), 1.71-1.75 (m, 1H), 1.54-1.61 (m, 4H), 1.11-1.44 (m, 4H), 0.94-0.96 (m, 2H); LCMS (ESI) *m/z* 453 (MH⁺). ¹³C NMR (125.73 MHz, DMSO- *d*₆) δ 162.3, 160.3, 148.6, 139.0, 138.8, 132.2, 129.8, 129.1, 128.7, 127.6, 127.0, 126.4, 122.64, 119.9, 45.2, 38.2, 37.3, 32.9, 26.4, 26.0.

N-[2-Benzyl-4-(methylsulfonylcarbamoyl)phenyl]-6-(cyclohexylmethyl)pyridine-2-carboxamide (3) 3-benzyl-4-[[6-(cyclohexylmethyl)pyridine-2-carbonyl]amino]benzoic acid (**DD2**, 15mg, 0.035 mmol) and 1,1'-carbonyldiimidazole (11 mg, 0.07 mmol) in tetrahydrofuran (1.0 mL) were stirred at room temperature for an hour. Methanesulfonamide (5 mg, 0.053 mmol)

and 1,8-diazabicyclo[5.4.0]undec-7-ene (0.008 mL, 0.053 mmol) were added and the mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with ethyl acetate and washed with 1 N HCl, water and brine. The organic extract was dried over magnesium sulfate and concentrated under reduced pressure. The crude material thus obtained was purified by flash column chromatography (40% ethyl acetate-hexanes) to afford the title compound in 34% yield. ^1H NMR (CDCl_3) δ 10.45 (s, 1H), 8.64 (d, $J = 6$ Hz, 1H), 8.55 (s, 1H), 8.07 (d, $J = 6$ Hz, 1H), 7.75-7.82 (m, 4H), 7.20-7.31 (m, 5H), 4.18 (s, 2H), 3.43 (s, 3H), 2.64 (d, $J = 6$ Hz, 2H), 1.91-1.95 (m, 1H), 1.55-1.72 (m, 4H), 1.16-1.24 (m, 4H), 0.96-0.98 (m, 2H); LCMS (ESI) m/z 506 (MH $^+$).

Methyl 3-benzyl-4-[6-(cyclohexylmethyl)pyridine-2-amido]benzoate (6) Oxalyl chloride (0.040 mL) was added to 3-benzyl-4-[[6-(cyclohexylmethyl)pyridine-2-carbonyl]amino]benzoic acid (**DD2**, 5.2 mg, 0.012 mmol) in anhydrous methanol (0.4 mL) and stirred at room temperature for 20 h. The reaction mixture was concentrated under reduced pressure and the residue thus obtained was purified by flash column chromatography (5% ethyl acetate-hexanes) to obtain the title compound in 57% yield. ^1H NMR (CDCl_3) δ 10.35 (s, 1H), 8.55 (d, $J = 6$ Hz, 1H), 7.98-8.06 (m, 2H), 7.75 (t, $J = 6$ Hz, 1H), 7.20-7.30 (m, 7H), 4.17 (s, 2H), 3.90 (s, 3H), 2.63 (d, $J = 6$ Hz, 2H), 1.56-1.70 (m, 6H), 1.16-1.24 (m, 3H), 0.95-0.98 (m, 2H); LCMS (ESI) m/z 443 (MH $^+$). ^{13}C NMR (125.73 MHz, $\text{DMSO}-d_6$) δ 166.4, 162.9, 162.5, 160.5, 148.3, 140.4, 138.9, 132.2, 131.4, 129.1, 128.6, 127.8, 127.1, 125.7, 121.5, 120.0, 52.6, 45.2, 38.3, 37.1, 32.9, 26.4, 26.0.

Dynamic light scattering

Inhibitors were diluted from concentrated DMSO stocks to buffer with a final DMSO concentration of 2%. Measurements were made on a DynaPro MS/X equipped with a 55 mM

laser at 826.6 nm. The integration time was 100 s (10x10 sec) and laser power was 100% unless reduction in laser power was necessary to avoid saturating the detector. The detector angle was 90 degrees. Measurements were performed at room temperature (25 degrees Celsius).

SUPPLEMENTARY REFERENCES

1. Zhang, Z. Y., Poorman, R. A., Maggiora, L. L., Heinrikson, R. L. and Kezdy, F. J. (1991) Dissociative Inhibition of Dimeric Enzymes - Kinetic Characterization of the Inhibition of Hiv-1 Protease by Its CooH-Terminal Tetrapeptide, *Journal of Biological Chemistry* 266, 15591-15594.
2. Leslie, A. G. W. and Powell, H. R. (2007) Processing diffraction data with MOSFLM, *Nato Sci Ser II Math* 245, 41-51.
3. Winn, M. D., Ballard, C. C., Cowtan, K. D., Dodson, E. J., Emsley, P., Evans, P. R., Keegan, R. M., Krissinel, E. B., Leslie, A. G. W., McCoy, A., McNicholas, S. J., Murshudov, G. N., Pannu, N. S., Potterton, E. A., Powell, H. R., Read, R. J., Vagin, A. and Wilson, K. S. (2011) Overview of the CCP4 suite and current developments, *Acta Crystallogr D* 67, 235-242.
4. Holton, J. and Alber, T. (2004) Automated protein crystal structure determination using ELVES, *P Natl Acad Sci USA* 101, 1537-1542.
5. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. and Read, R. J. (2007) Phaser crystallographic software, *J Appl Crystallogr* 40, 658-674.
6. Adams, P. D., Afonine, P. V., Bunkoczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C. and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution, *Acta Crystallogr D Biol Crystallogr* 66, 213-221.
7. Emsley, P., Lohkamp, B., Scott, W. G. and Cowtan, K. (2010) Features and development of Coot, *Acta Crystallogr D Biol Crystallogr* 66, 486-501.

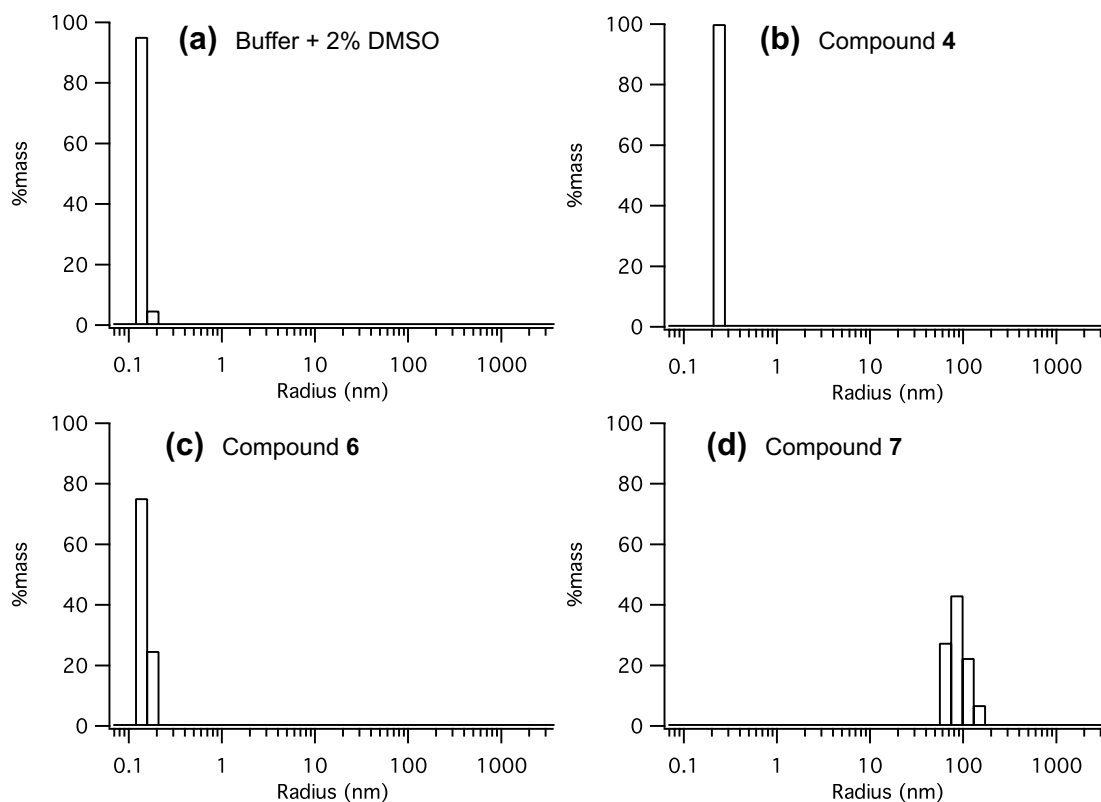


Figure 6-S1. Dynamic light scattering of non-ionic compounds. Buffer and DMSO alone (a), compound 4 (b), and compound 6 (c) show no aggregation. Compound 7 (d) exhibits large ~ 100 nm radius aggregates. All compounds were at 1.56 μ M concentration in 2% DMSO.

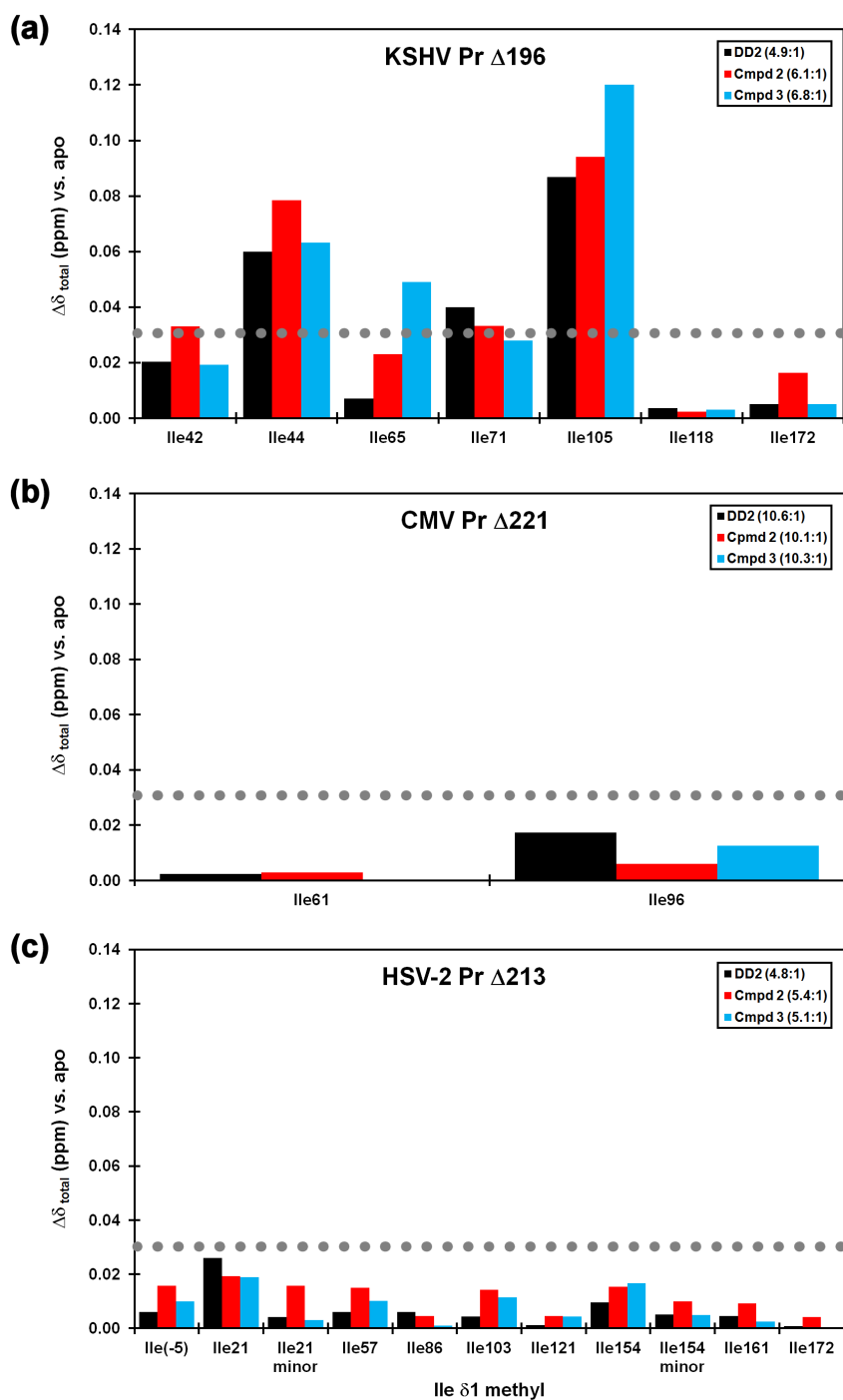


Figure 6-S2. Combined chemical shift perturbations for ^{13}C - ^1H HSQC Ile spectra. The combined chemical shift perturbations for KSHV (a), CMV (b), and HSV-2 (c) proteases for all isoleucines in their respective truncated constructs. The dotted line indicates a cutoff for significant perturbation.

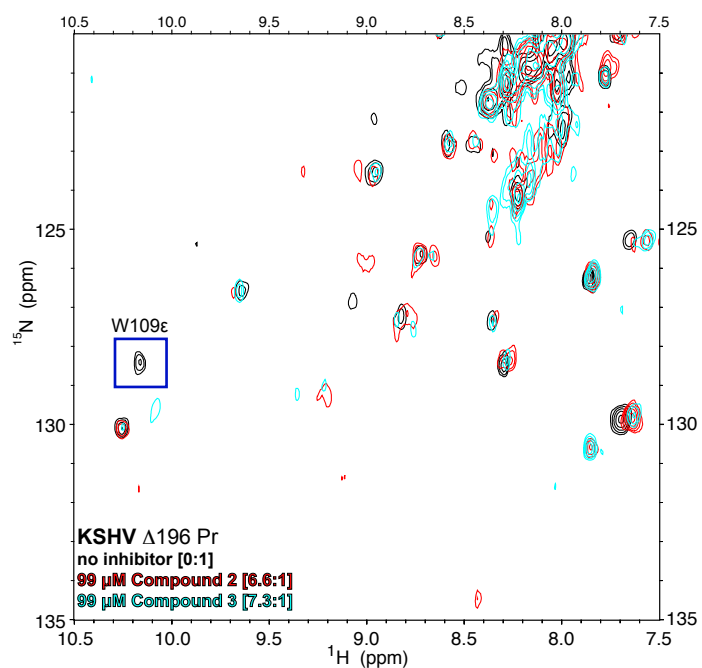


Figure 6-S3. ^{15}N -HSQC spectra for truncated KSHV protease with inhibitors. Spectra for KSHVD196 protease in the absence of inhibitor (black), presence of compound 2 (red), or presence of compound 3 (cyan) are shown. The aromatic hot spot residue tryptophan 109 (W109) is highlighted.

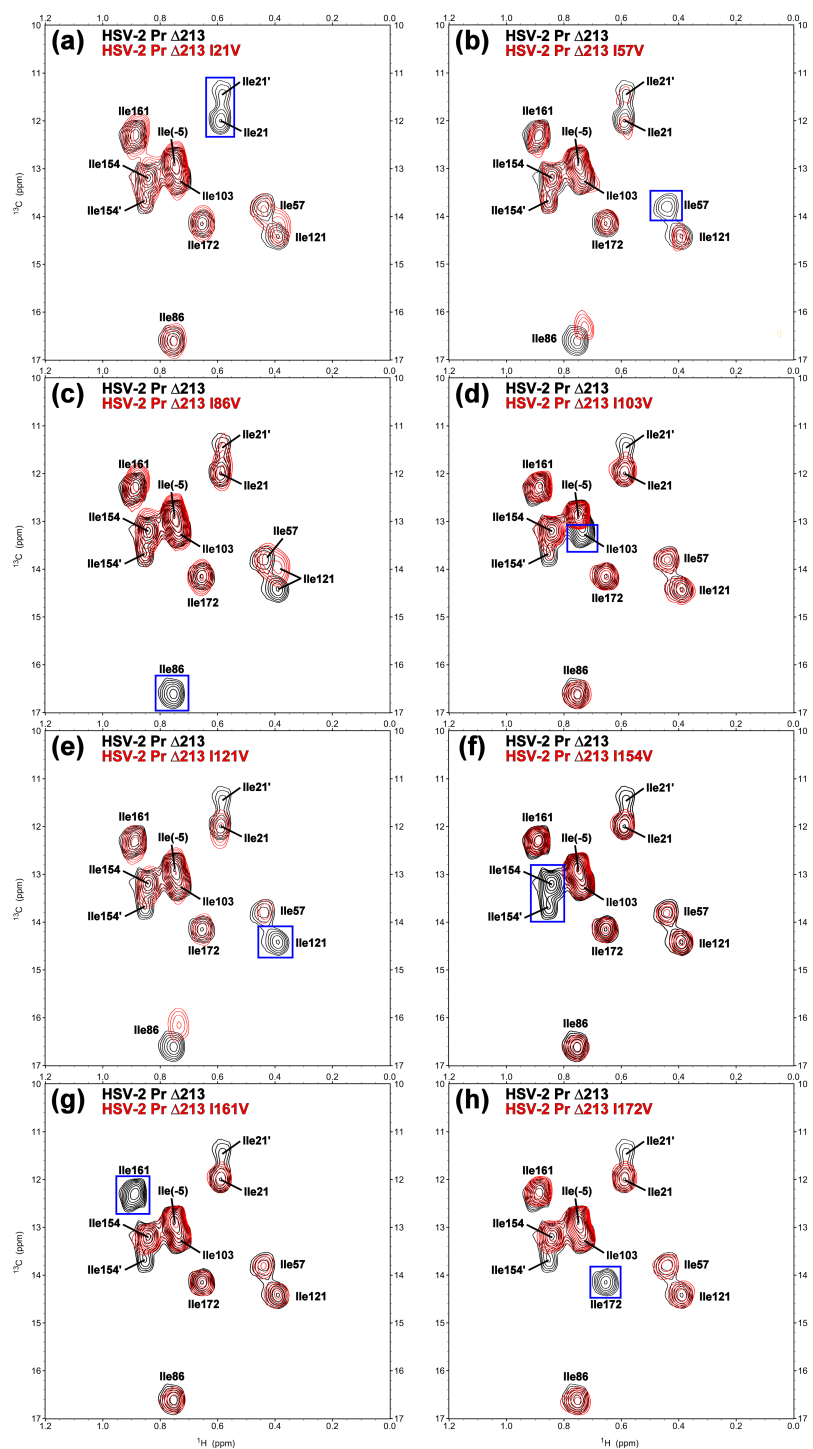


Figure 6-S4. HSV-2 Pr D213 ^{13}C - ^1H HSQC isoleucine assignments. Single point mutations from isoleucine to valine for each isoleucine in the truncated HSV-2 protease construct were made and their spectra recorded (a-h), allowing assignment of all peaks. Resonances labeled with a prime sign indicate minor conformations.

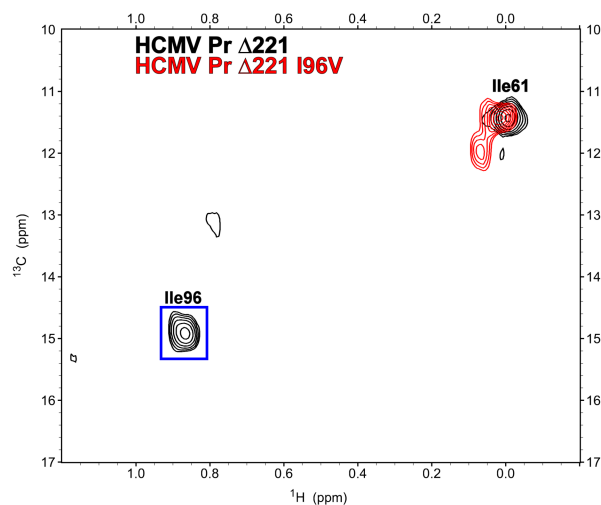


Figure 6-S5. CMV Pr D221 ^{13}C - ^1H HSQC isoleucine assignments. A single point mutation, I96V, was introduced the spectrum recorded, allowing assignment of both I61 and I96 peak resonances.

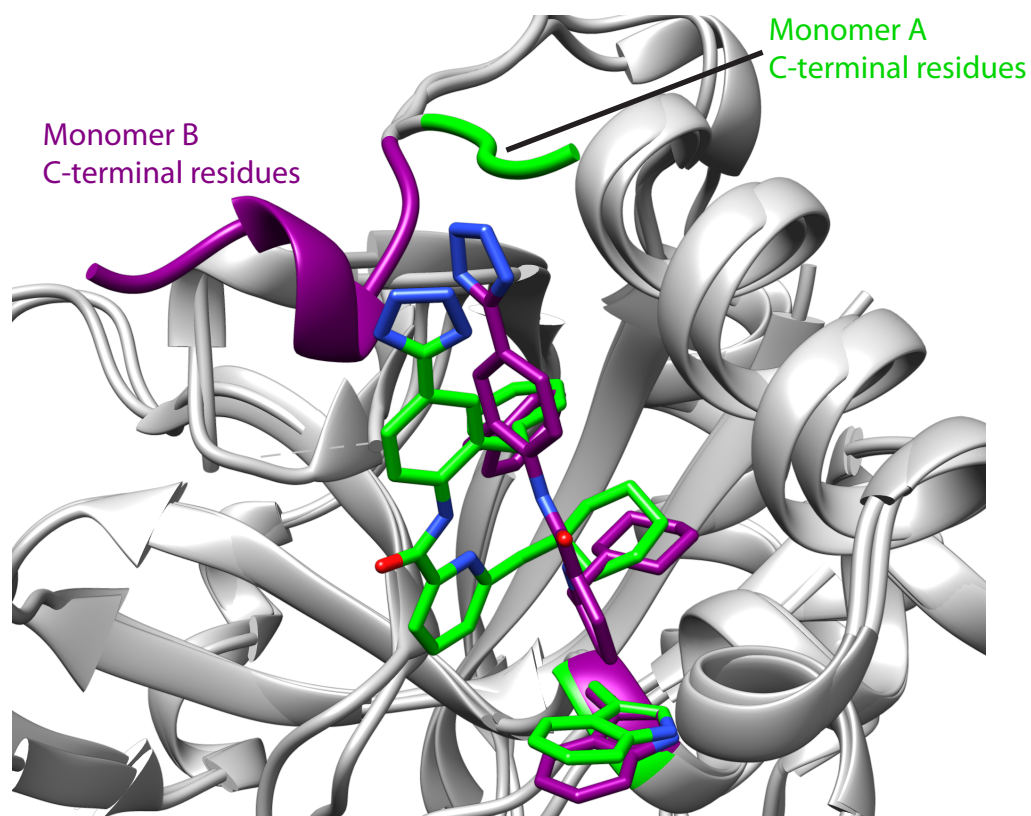


Figure 6-S6. Overlay of monomer A and monomer B. Monomer A and B largely overlap, however the C-terminal residues of monomer A (green) differ in position from those of monomer B (purple), resulting in distinct contact surfaces with the small molecule inhibitor.

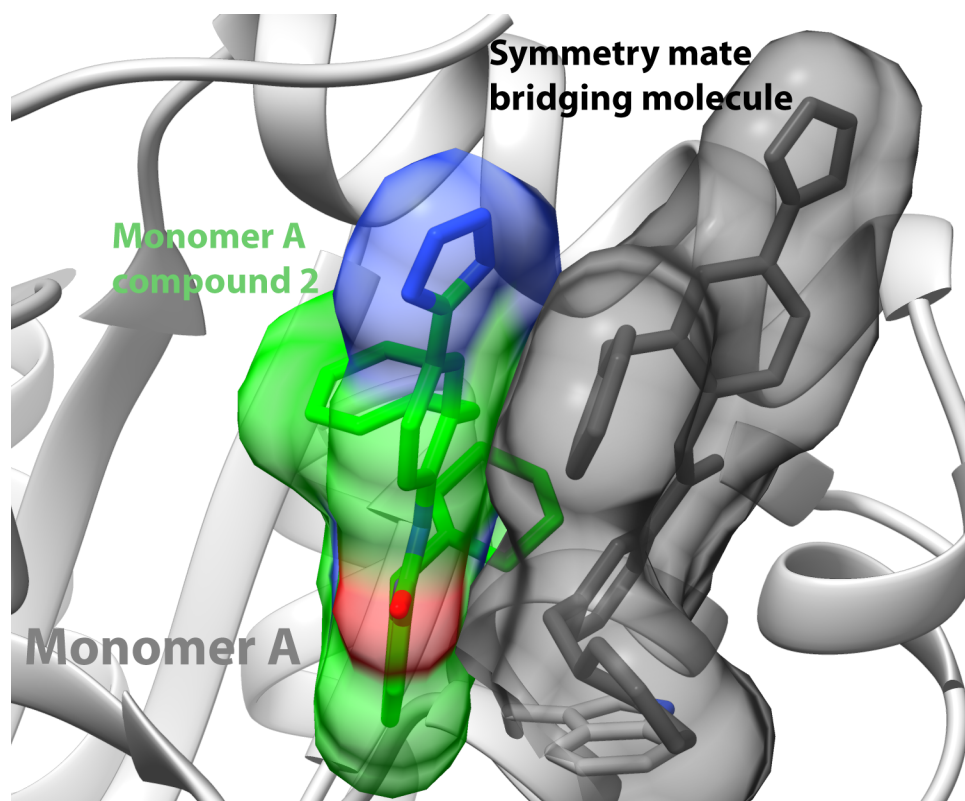


Figure 6-S7. Symmetry-mate bridging molecule perturbs inhibitor binding to monomer A. The crystal structure of compound 2 bound to monomer A (green) is shown in the presence of a symmetry-mate bridging molecule (dark grey).

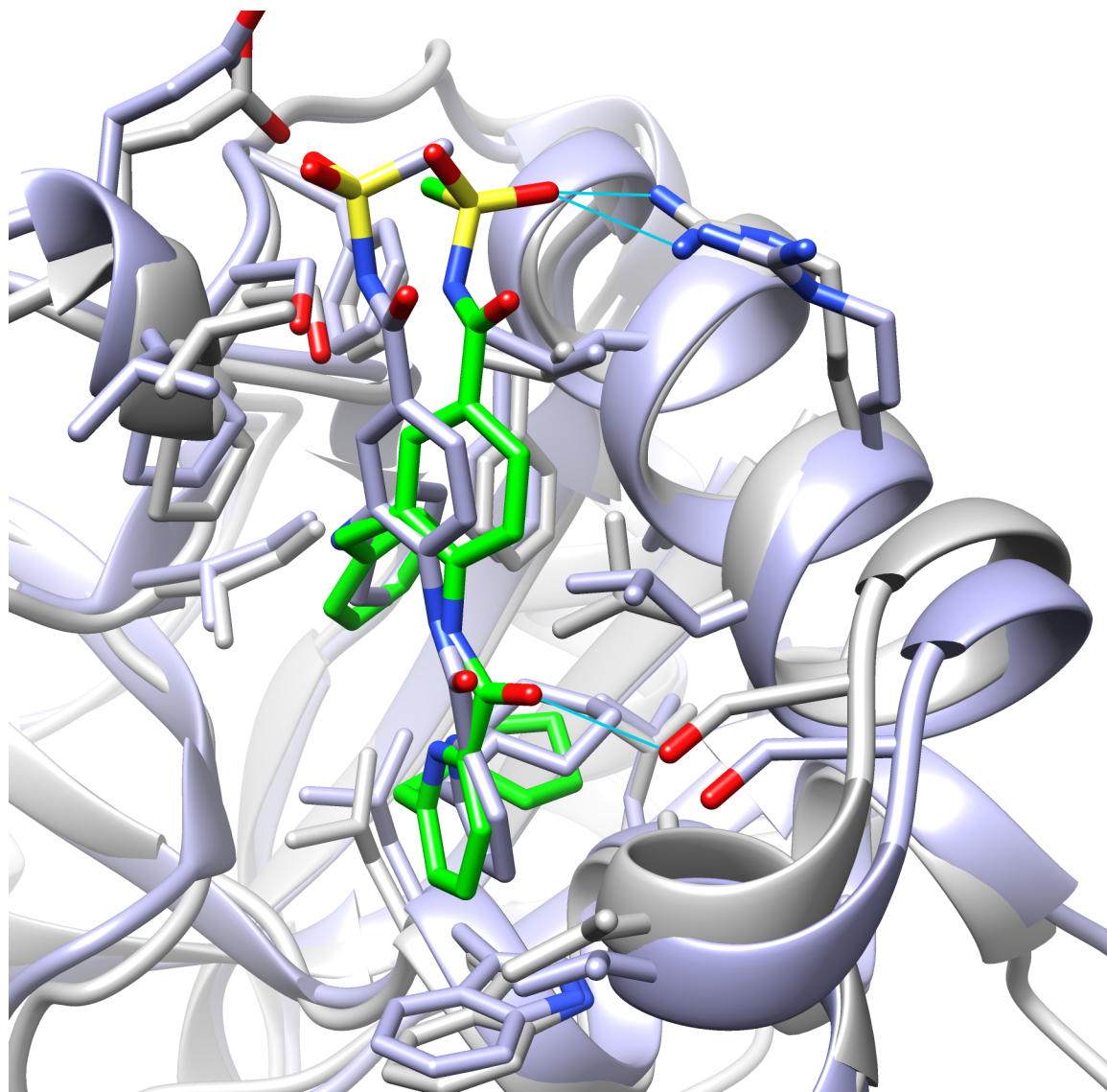


Figure 6-S8. Overlay of PLOP-minimized model and crystal structure. The PLOP-minimized model (grey and green) is shown overlayed with the crystal structure (blue-grey). Relatively little structural change occurs with minimization, however a critical interaction between inhibitor and R82 is observed.

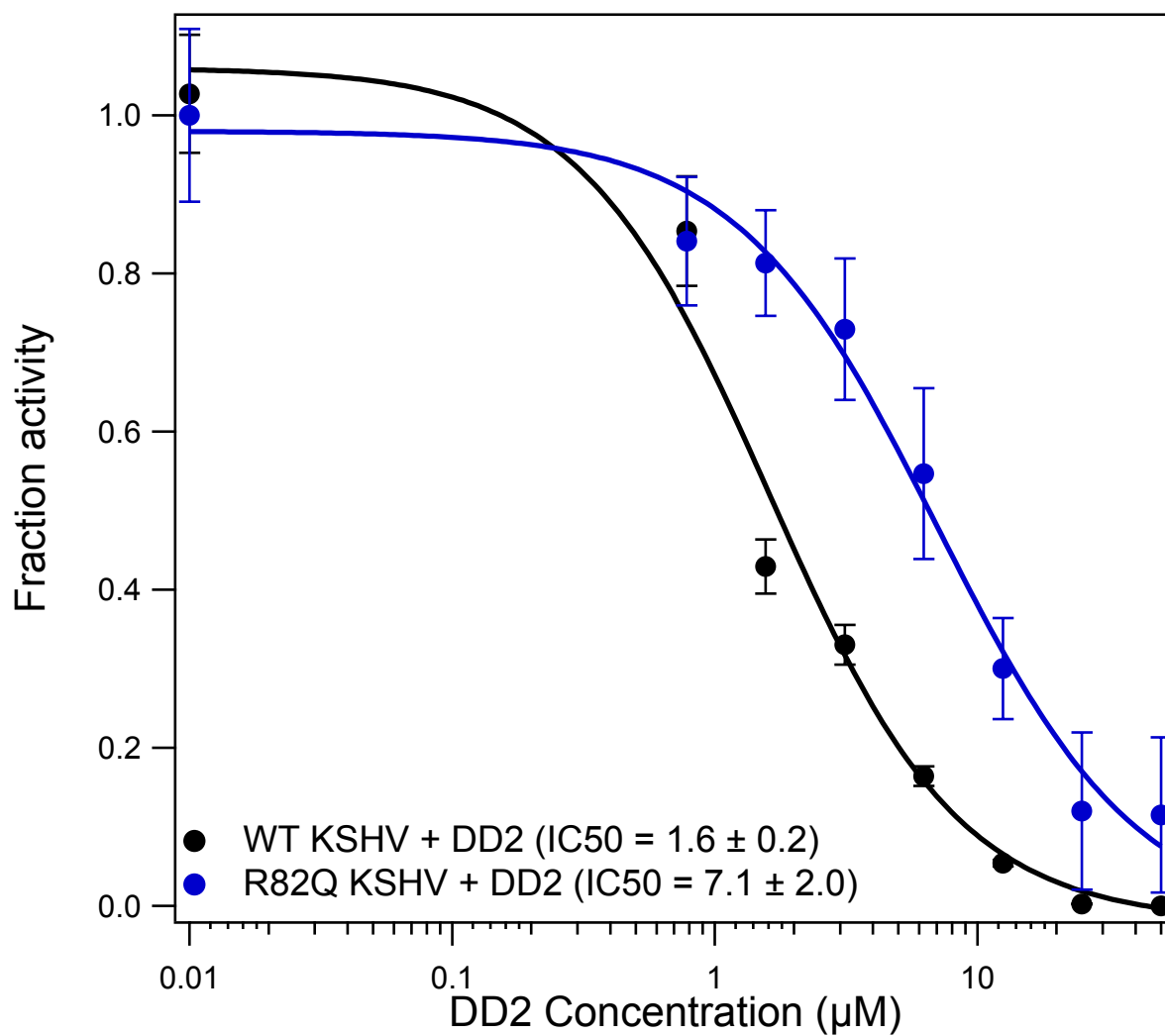


Figure 6-S9. DD2 inhibition of KSHV Pr R82Q. The potency of DD2 is reduced against KSHV Pr R82Q relative to WT. This supports a model where DD2 interacts with R82.

Table 6-S2. Data collection and refinement statistics compound 2

	Compound 2-KSHV Pr Δ196
Wavelength (Å)	1
Resolution range (Å)	74.75 - 1.45 (1.481 - 1.43)
Space group	I 2 2 2
Unit cell	69.12 95.892 119.342 90 90 90
Total reflections	748120 (84478)
Unique reflections	73379 (10614)
Multiplicity	10.2 (8.0)
Completeness (%)	100.0 (100.0)
Mean I/sigma(I)	13.1 (2.1)
Wilson B-factor	20.23
R-merge	0.096 (0.919)
R-meas	0.104
CC1/2	0.998 (0.857)
CC*	0.824 (0.917)
R-work	0.1729 (0.3457)
R-free	0.1978 (0.3466)
Number of non-hydrogen atoms	3404
macromolecules	2973
ligands	112
water	319
Protein residues	375
RMS(bonds)	0.012
RMS(angles)	1.42
Ramachandran favored (%)	98
Ramachandran outliers (%)	0
Clashscore	3.75
Average B-factor	38.30
macromolecules	38.40
ligands	23.80
solvent	42.00

Statistics for the highest-resolution shell are shown in parentheses.

Table 6-S3. Data collection and refinement statistics for compound 3

	Compound 3-KSHV Pr Δ196
Wavelength (Å...)	1
Resolution range (Å...)	74.75 - 2.15 (2.165 - 2.09)
Space group	I 2 2 2
Unit cell	69.737 95.947 119.245 90 90 90
Total reflections	150488 (11776)
Unique reflections	22808 (2491)
Multiplicity	6.6 (4.7)
Completeness (%)	93.7 (72.6)
Mean I/sigma(I)	18.1 (1.7)
Wilson B-factor	29.3
R-merge	0.118 (0.708)
R-meas	0.140 (0.903)
CC1/2	0.665 (0.577)
CC*	0.894 (0.855)
R-work	0.1843 (0.2683)
R-free	0.2460 (0.3552)
Number of non-hydrogen atoms	3336
 macromolecules	3033
 ligands	112
 water	191
Protein residues	382
RMS(bonds)	0.003
RMS(angles)	0.79
Ramachandran favored (%)	97
Ramachandran outliers (%)	0.26
Clashscore	1.28
Average B-factor	42.70
 macromolecules	43.50
 ligands	28.60
 solvent	38.10

Statistics for the highest-resolution shell are shown in parentheses.

CHAPTER 7: Kinetic and crystallographic analysis of DD2 analogs

Jonathan E. Gable, Timothy M. Acker, Priyadarshini Jaishankar, Adam R. Renslo, Charles S. Craik

Abstract

We previously reported the discovery of DD2, a small molecule protein-protein interaction antagonist that blocks dimerization of Kaposi's sarcoma-associated herpesvirus (KSHV), cytomegalovirus, Epstein Barr virus, and herpes simplex virus 2 proteases. For KSHV protease we reported crystallographic data for DD2 and two analogs varying the carboxylate of the original molecule. Here we report kinetic and structural characterization of seven additional DD2 analogs varying the "side chains" of the molecule. We establish that this class of molecules works through a slow time-dependent two-step mechanism involving enzyme isomerization. Furthermore we identify a potential structural explanation for this slow inhibition wherein two distinct conformations of the protein are observed depending on the specific inhibitor. These data allow for improved structure-based drug design and SAR studies while adding to the growing list of slow tight-binding inhibitors of clinically relevant targets. To our knowledge DD2 and its analogs are the first slow tight-binding inhibitors that bind a protein-protein interface and prevent dimerization.

Introduction

Previously we reported the effect of replacing the carboxylic acid in our known small molecule dimer disruptor, DD2, with other carboxylate bioisosteres.¹ Anionic substitutions were well tolerated with the tetrazole being most efficacious (CHAPTER 6). Crystallographic analysis revealed a similar binding mode for all three analogs (carboxylate, tetrazole, and sulfonamide). A combination of kinetic analysis, computational modeling, and crystallography led to a model where arginine 82 plays a role in binding by forming a salt bridge with the anionic portion of the small molecule inhibitor. This previous study was limited to only 3 active DD2 analogs.¹

A logical extension of the previous work involves modification of the cyclohexyl and phenyl “side chains” of the DD2 scaffold, as well as the linker to these “side chain” moieties. We hypothesized that substitutions off the phenyl and cyclohexyl groups of DD2 analogs would improve potency, in large part through additional van der Waals interactions with the relatively hydrophobic interfacial pocket. Here we analyze the kinetics, potency, and crystallographic structure of a number of diverse DD2 analogs.

Results

Substitutions off the phenyl ring had only modest effects on IC₅₀ (Table 7-1). The linker that extends out to the phenyl ring exhibited SAR with a thioether linkage having the lowest IC₅₀ values, followed by an amine linkage, and finally an ether linkage having the highest IC₅₀ values. Replacing the phenyl ring with a cyclohexyl such that both “side chains” are cyclohexyl moieties appeared to improve IC₅₀ slightly, even with the ether linkage to the cyclohexyl (**560** vs. **248**, **Table 7-1**). Substituting a phenyl for the cyclohexyl off the pyridine increased IC₅₀ (reduced apparent potency), despite the potential for π -stacking between the Trp109 and the newly introduced phenyl ring (**89** vs. **29**, **Table 7-1**). An adamantane group in place of the cyclohexyl

off the pyridine reduced IC₅₀ (**67** vs. **72**, Table 7-1), presumably due to further filling the space between the two “side chains”. Introduction of a methyl to the amide nitrogen of the small molecule completely eliminated inhibition (**982**, Table 7-1) but improved permeability as measured by MDCK assay (data not shown).

During the course of kinetic analysis it became apparent that inhibition was time-dependent. This was especially clear for the progress curves with high concentrations of inhibitor; signal plateaued long before substrate was depleted indicating a gradual increase in inhibition over the course of the experiment. Comparison to the apo progress curve ruled out other potential causes of the plateau such as the inner filter effect or product inhibition. **Figure 7-1** highlights the dramatic time-dependent slow inhibition observed for DD2-177 (Table 7-1) where it is clear that IC₅₀ changes with incubation time prior to substrate addition. Fitting to

$$v_t = (v_i - v_s)e^{-k_{obs}t} + v_s \quad (1)$$

estimates the rate of inhibition, k_{obs} , where v_i is the initial fractional velocity, v_s is the steady-state fractional velocity, v_t is the velocity at time t , and t is time. A plot of k_{obs} against inhibitor concentration yields a hyperbolic shape (**Figure 7-2**). This is consistent with an enzyme isomerization model that follows:

$$k_{obs} = k_6 + \frac{k_5[I]}{K_{i,app} + [I]} \quad (2)$$

from which $K_{i,app}$, k_5 , and k_6 are estimated to be $4.48 \pm 3 \mu\text{M}$, $1.05 \times 10^{-3} \pm 2 \times 10^{-4} \text{ s}^{-1}$, and $3.3 \times 10^{-4} \pm 1 \times 10^{-4} \text{ s}^{-1}$, respectively.² With k_6 approaching zero, inhibition can be treated as approximately irreversible. In this case k_5 of equation (2) is replaced with k_{inact} and potency is best reported as $k_{inact}/K_{i,app, irr}$. For DD2-177 k_{inact} , $K_{i,app, irr}$, and $k_{inact}/K_{i,app, irr}$ are $1.05 \times 10^{-3} \pm 9.4 \times 10^{-5} \text{ s}^{-1}$, $1.0 \pm 0.3 \mu\text{M}$, and about 66 ml/min/ μmol , respectively. The fit using this approach is not as good (**Figure**

7-S2), consistent with rapid dilution experiments showing that while k_6 is very small inhibition is in fact reversible. Many other DD2 analogs exhibit slow time-dependent inhibition and k_5 appears to vary among the analogs (data not shown). Dynamic light scattering and centrifugation experiments ruled out aggregation-based inhibition as the cause for the observed time dependence.

Of the 18 DD2 analogs for which crystallization was attempted eleven crystallized and ten diffracted (**Figure 7-S1**). Overlaying the structures of monomer B (the monomer with the bridging molecule) reveals that the C-terminal residues close to the interfacial binding pocket adopt two distinct conformations depending on the substitutions made to the phenyl “side chain” of the DD2 scaffold (**Figure 7-3**). Large substitutions and substitutions in the meta position cause the C-terminal residues to be displaced from the green position to the blue position, making room for groups such as the meta trifluoromethoxy. **Figure 7-4** highlights the most variable regions based on per residue C-alpha RMSD across all monomer B structures. Interestingly the loop comprised of residues 17-23 also adopts two distinct conformations depending on the small molecule identity and correlates exactly with the two conformations of the C-terminal residues, despite being distant from the binding site. This loop occludes the active site and thus is relevant to inhibition and the allosteric link between the dimer interface and the active site. Residues 121-130 also appear to have conformational changes that correlate with compound identity and C-terminal positions. Small substitutions that give rise to the green conformation leave residues 121-130 highly dynamic such that too little density is observed to reliably model the loop. Analogs with larger substitutions result in a well-defined position for residues 121-130. The remaining dynamic loops do not adopt conformations that are correlated the C-termini positions.

DD2-29 is the only analog that was successfully crystalized where the phenyl “side chain” was replaced entirely, in this case with a tetrahydropyran. In monomer B the main DD2-29 molecule adopts a confirmation nearly identical to that of DD2-110 (the tetrazole derivative of the original DD2 scaffold). The bridging molecule of DD2-29 however adopts a dramatically different confirmation in which the tetrahydropyran is turned out away from the protein surface and amide is inverted with the amide oxygen pointing toward to the protein and hydrogen bonding with a structural water not present in the DD2-110 structure (**Figure 7-5a**). The monomer B backbones overlay well with a C-alpha RMSD of 0.454 Å (**Figure 7-5b**). Both adopt the green confirmation, consistent with the lack of substitution on the tetrahydropyran.

In monomer A DD2-29 has a significantly different confirmation than that observed in monomer B or DD2-110 in monomer A. The tetrahydropyran of DD2-29 in monomer A is rotated out of the main pocket instead reaching into an anionic grove comprised in part of Glu49 (**Figure 7-6a**). Despite this difference in small molecule confirmation, the overall backbone confirmation of monomer A for the DD2-29 and DD2-110 structures is very similar with a C-alpha RMSD of 0.509 Å (**Figure 7-6b**).

Discussion

We have identified and characterized a diverse set of novel protein-protein interaction antagonists of the human herpesvirus proteases. From IC₅₀ values alone no strong SAR emerged around alterations to the DD2 scaffold “side chains” contrary to our original hypothesis that these changes would further van der Waals interactions in the hydrophobic pocket resulting in decreased IC₅₀ values. Importantly however in light of the slow time-dependent inhibition kinetics IC₅₀ may fail to capture the true potency of these small molecules.

All of the DD2 analogs tested showed slow time-dependent inhibition consistent with an enzyme isomerization model where reverse isomerization is extremely slow but still irreversible.² This presents both challenges and potential opportunities going forward with this system. Variation in the reverse isomerization rate will be difficult to capture if all values are close to zero, making it difficult to identify trends among the analogs that could drive SAR. The forward isomerization rate is also very slow, but likely to be more readily measured. Analysis of potency is further complicated by the depletion of free inhibitor over time and the requirement for relatively high concentrations of enzyme in order to achieve dimerization and thus activity.³ Part of the apparent lack of SAR with these DD2 analogs may be due to the use of 1 μ M enzyme, making it difficult to observe IC₅₀ values well below 1 μ M. Reduction in the total enzyme concentration can be further pursued and the use of kinetic analysis that accounts for depletion of free inhibitor should enable future studies to overcome these challenges. Already the measurement of forward and reverse isomerization rates as well as apparent K_i may offer a vast improvement over a simple measurement of IC₅₀ after 30 minutes of incubation.

The time-dependent inhibition and slow reverse isomerization rate may be hugely beneficial in the context of treating virally infected cells. The protease is expressed as a monomer in the cytoplasm and is thought to remain in the monomeric state until it reaches the procapsid where the local concentration of protease is much higher.⁴ Overcoming the local concentration of protease in the procapsid is not an issue if the DD2 analogs bind to and nearly irreversibly inhibit the monomer prior to it reaching the procapsid. In addition, if the compound remains bound to the monomer until the reverse isomerization step is complete the compound will have an effectively very long off rate. In this case the compound itself may be translocated to the procapsid and thus achieve local concentrations comparable to that of the protease.

A physical and structural explanation for the observed kinetics remains elusive, though the crystal structures reported here provide some insight. There appears to be correlated motion between the C-terminal residues and residues 17-23 that is compound-dependent, even with a truncated construct. It is intriguing to imagine that these structural rearrangements could explain the time-dependent inhibition observed. Preliminary data suggest that the forward isomerization rate varies with compound identity, evidence of an induced fit model of enzyme inactivation. It will be extremely interesting to see if the forward isomerization falls within two distinct bins that correlate with the green and blue states described here. Further careful analysis of the existing crystal structures is warranted to rule out the possibility that residues 17-23 contact the compounds in the crystal lattice, suggesting that the correlation observed is a crystal artifact. Minimization of these structures should also be performed as described in CHAPTER 6. It also remains unclear whether monomer A or monomer B better represents the solution state of binding. Only monomer B exhibits distinct compound-dependent conformations but this alone is insufficient to support the claim that monomer B more closely resembles what takes place in solution. Molecular dynamics simulations of both monomers could provide some hints or help to arrive at a consensus structure. Additional modeling may also help explain the role, if any, of the residues typically comprising the C-terminal helices that were truncated to enable crystallography.

This report adds to a growing list of interesting time-dependent inhibitors of clinically relevant enzymes.^{2, 5-10} This is one of few if not the only report of such a molecule targeting a protein-protein interface. Crystal structures presented here will guide experiments to elucidate the physical basis for this time dependence and help with compound optimization.

Methods

Materials, protein expression and purification, determination of IC₅₀ values, and acquisition of X-ray crystallography data and structure determination were performed as reported in CHAPTER 6. Time dependent inhibition studies were carried out using the same conditions as those for IC₅₀ determination but instead of incubating for 30 minutes prior to substrate addition the incubation time was varied between 2 minutes and 2 hours. Detailed conditions used for each of the ten reported crystal structures can be found in supplemental **Table 7-S1**.

References

- [1] Gable, J. E., Lee, G. M., Jaishankar, P., Hearn, B. R., Waddling, C. A., Renslo, A. R., and Craik, C. S. (2014) Broad-spectrum allosteric inhibition of herpesvirus proteases, *Biochemistry* 53, 4648-4660.
- [2] Copeland, R. A. (2000) *Enzymes : a practical introduction to structure, mechanism, and data analysis*, 2nd ed., Wiley, New York.
- [3] Shahian, T., Lee, G. M., Lazic, A., Arnold, L. A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a viral enzyme by a small-molecule dimer disruptor, *Nat. Chem. Biol.* 5, 640-646.
- [4] Gable, J. E., Acker, T. M., and Craik, C. S. (2014) Current and Potential Treatments for Ubiquitous but Neglected Herpesvirus Infections, *Chem. Rev.*
- [5] Agafonov, R. V., Wilson, C., Otten, R., Buosi, V., and Kern, D. (2014) Energetic dissection of Gleevec's selectivity toward human tyrosine kinases, *Nat. Struct. Mol. Biol.* 21, 848-853.
- [6] Badet, B., Inagaki, K., Soda, K., and Walsh, C. T. (1986) Time-dependent inhibition of *Bacillus stearothermophilus* alanine racemase by (1-aminoethyl)phosphonate isomers by isomerization to noncovalent slowly dissociating enzyme-(1-aminoethyl)phosphonate complexes, *Biochemistry* 25, 3275-3282.
- [7] Blobaum, A. L., and Marnett, L. J. (2007) Structural and functional basis of cyclooxygenase inhibition, *J. Med. Chem.* 50, 1425-1441.
- [8] Copeland, R. A., Pompliano, D. L., and Meek, T. D. (2006) Drug-target residence time and its implications for lead optimization, *Nat. Rev. Drug Discov.* 5, 730-739.

- [9] Dunford, J. E., Kwaasi, A. A., Rogers, M. J., Barnett, B. L., Ebetino, F. H., Russell, R. G., Oppermann, U., and Kavanagh, K. L. (2008) Structure-activity relationships among the nitrogen containing bisphosphonates in clinical use and other analogues: time-dependent inhibition of human farnesyl pyrophosphate synthase, *J. Med. Chem.* *51*, 2187-2195.
- [10] Selinsky, B. S., Gupta, K., Sharkey, C. T., and Loll, P. J. (2001) Structural analysis of NSAID binding by prostaglandin H2 synthase: time-dependent and time-independent inhibitors elicit identical enzyme conformations, *Biochemistry* *40*, 5172-5180.

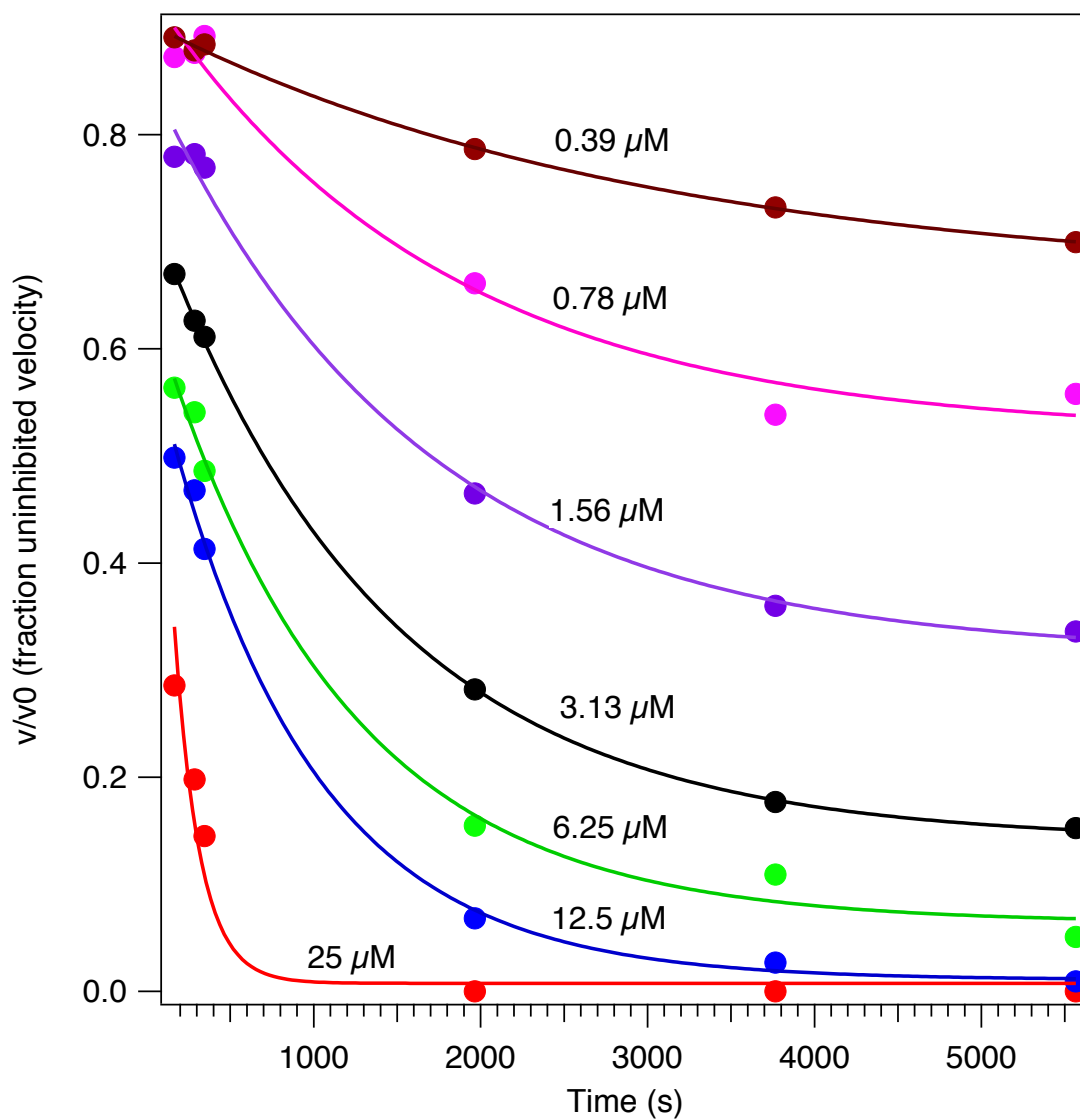


Figure 7-1 Time dependent inhibition with DD2-177. A plot of fractional velocity versus inhibitor incubation time reveals time-dependent inhibition on the minute time scale.

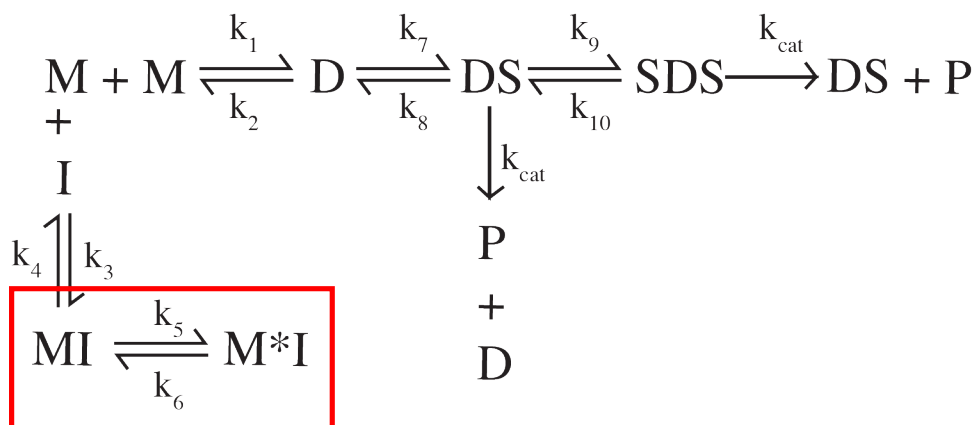
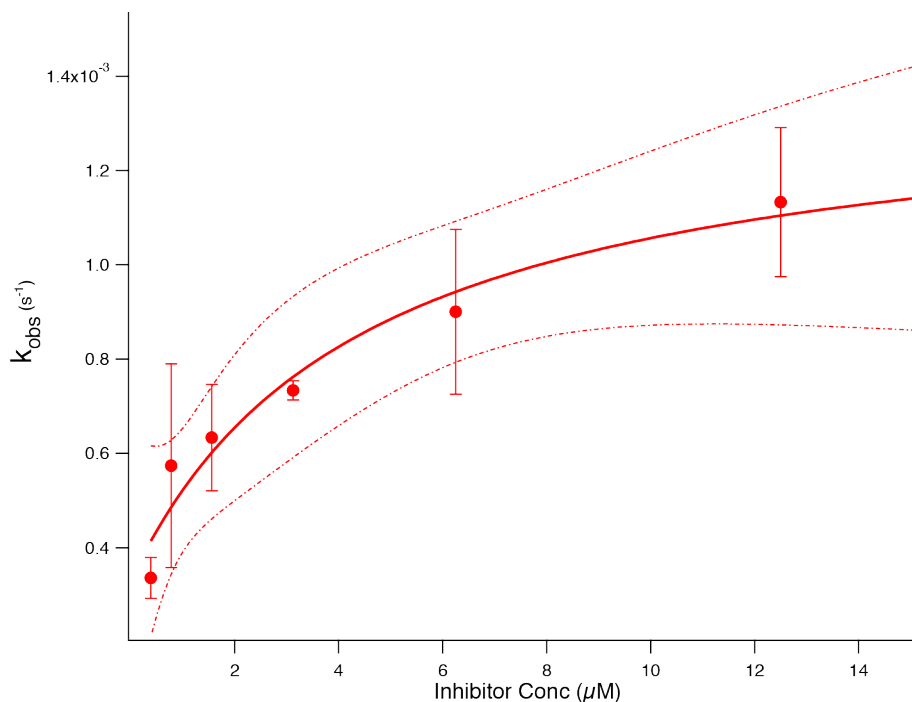


Figure 7-2. Evidence of an enzyme isomerization model. Rate of inhibition (k_{obs} derived from Figure 7-1) is plotted against inhibitor concentration and yields a hyperbolic form. Saturation of inhibition rate at high inhibition concentrations is consistent with the enzyme isomerization model depicted (bottom) where the isomerization step is boxed in red.

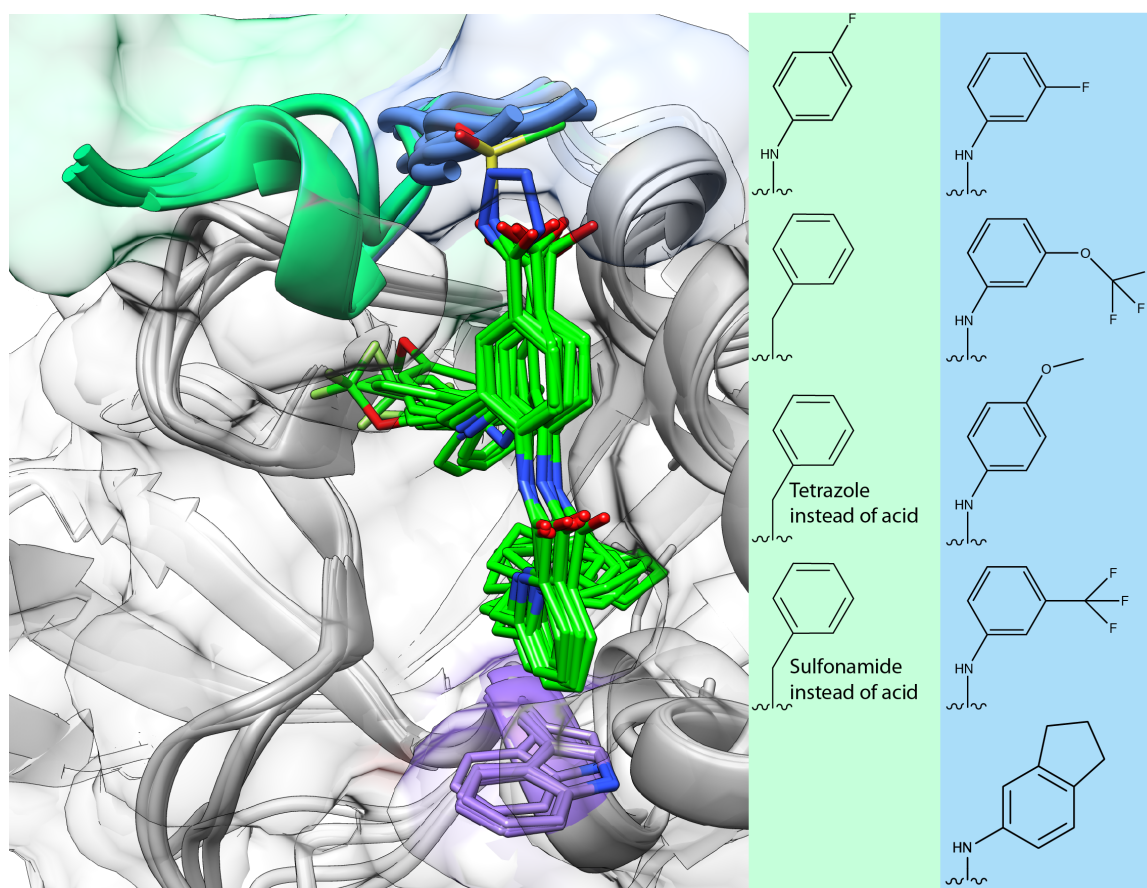


Figure 7-3. Overlay of DD2 analog crystal structure interfacial pockets. The crystal structures for the analog indicated (left) are overlaid. Two distinct conformations (blue and green) of the C-terminal residues are observed. Hotspot Trp109 is shown in purple. DD2 analogs are shown in bright green and colored by heteroatom.

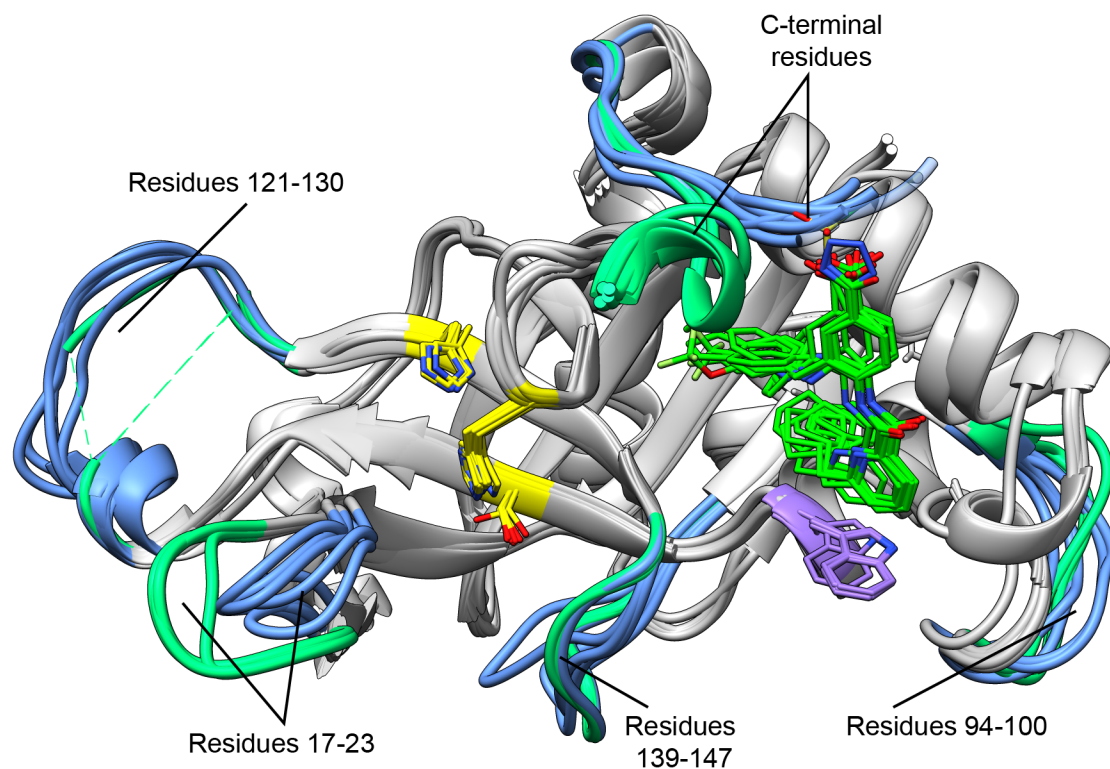


Figure 7-4. DD2 analog crystal structure variable region analysis. Nine DD2 analog crystal structures (as in Figure 7-3) are overlaid. The variable regions as determined by per-residue C-alpha RMSD are highlighted in blue and green based on the either blue or green confirmation adopted by the C-terminal residues.

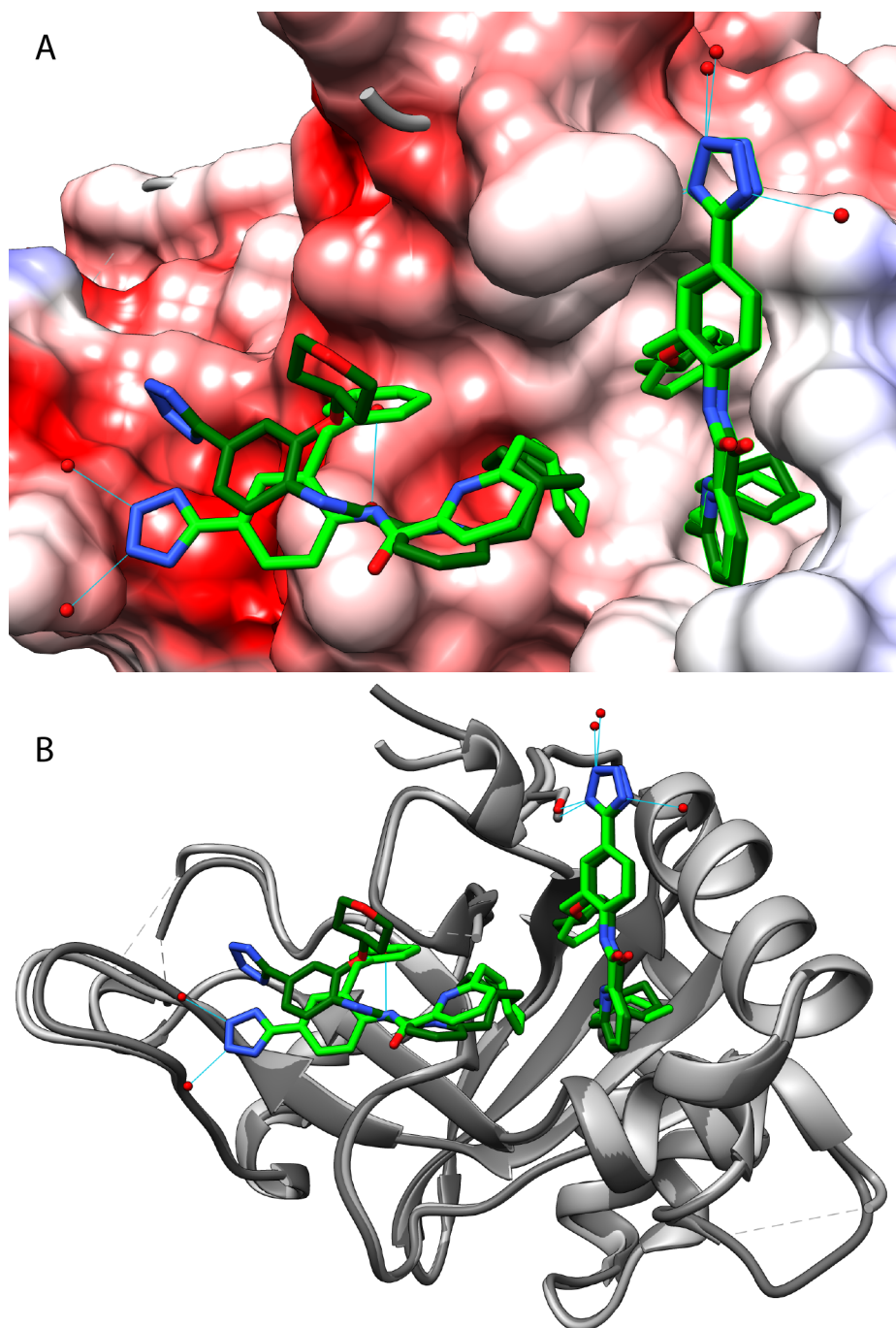


Figure 7-5. DD2-29 monomer B crystal structure. (A) The binding pocket for DD2-29 (dark green) bound to monomer B. Both the major confirmation (right) and the bridging molecule (left) are shown overlaid with the tetrazole analog (bright green) structure. The surface is colored based on coulombic potential (red is negative, blue is positive, white is neutral). (B) Ribbon diagram of DD2-29 structure (dark grey) overlaid with the tetrazole structure (grey).

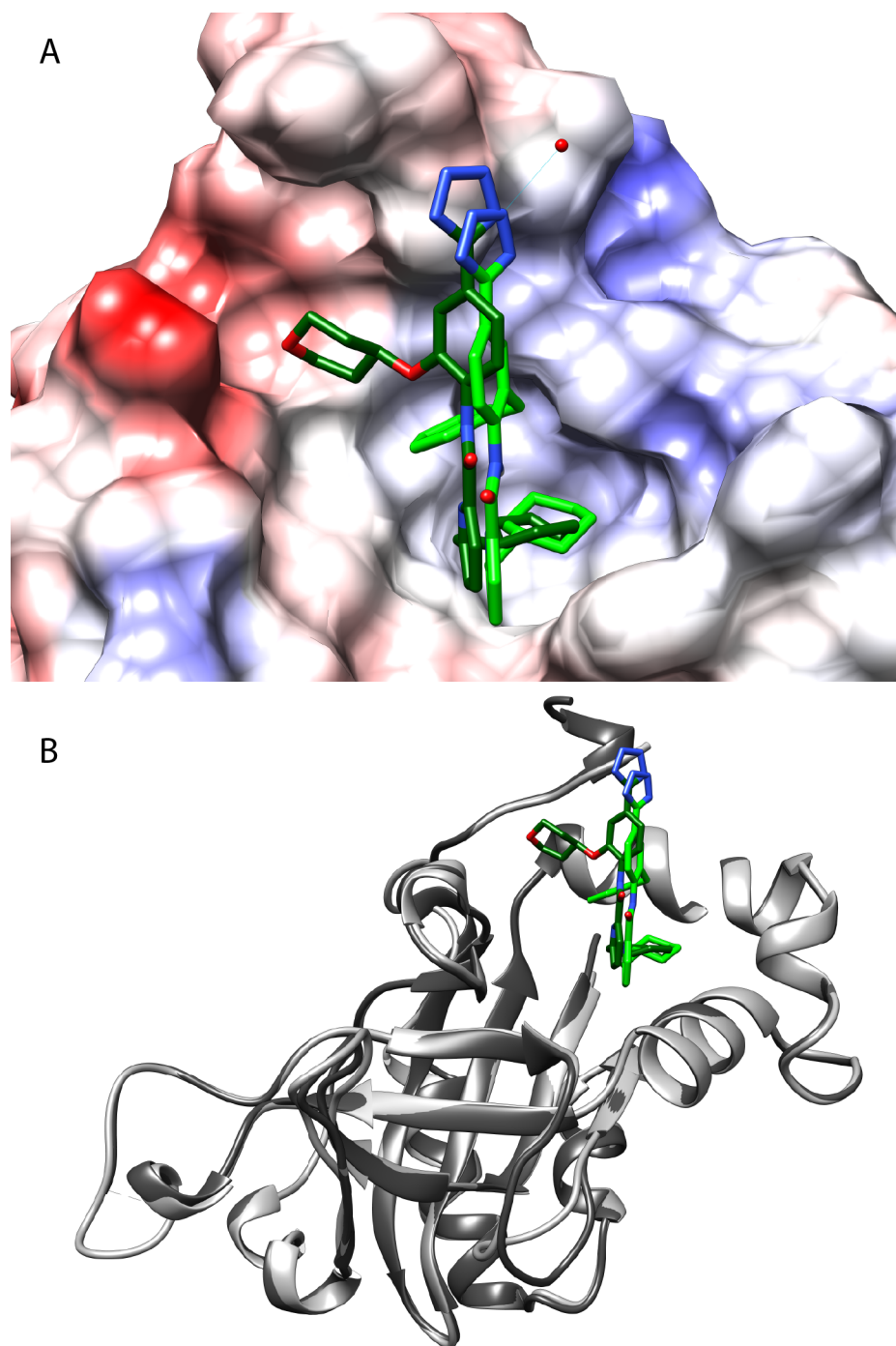


Figure 7-6. DD2-29 monomer A crystal structure. (A) The binding pocket for DD2-29 (dark green) bound to monomer A overlaid with the tetrazole analog (bright green) structure. The surface is colored based on coulombic potential (red is negative, blue is positive, white is neutral). (B) Ribbon diagram of DD2-29 structure (dark grey) overlaid with the tetrazole structure (grey).

Table 7-1. DD2 analogs

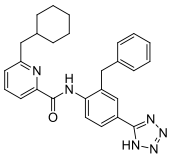
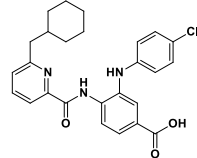
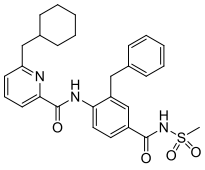
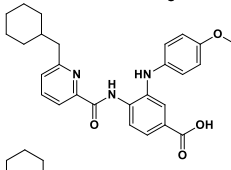
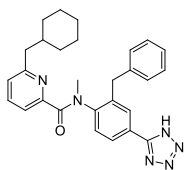
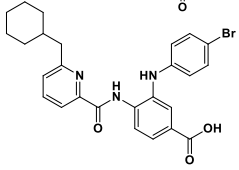
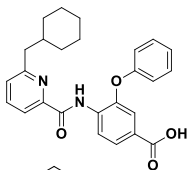
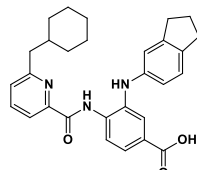
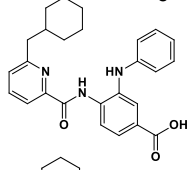
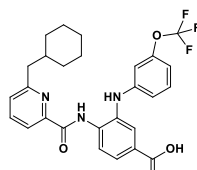
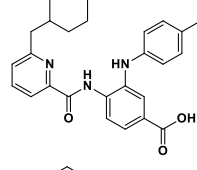
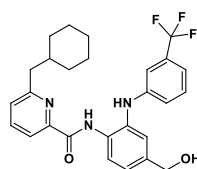
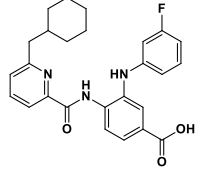
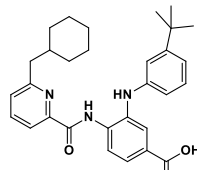
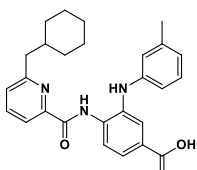
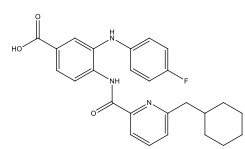
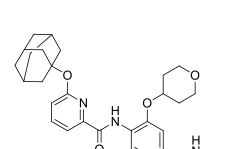
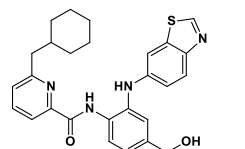
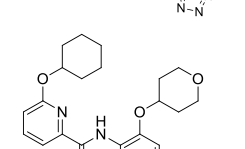
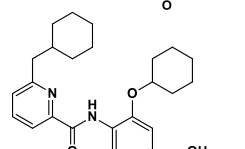
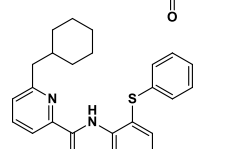
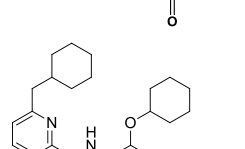
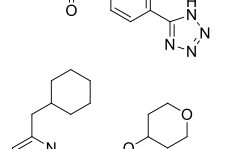
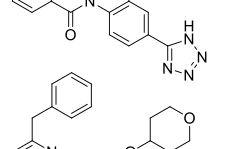
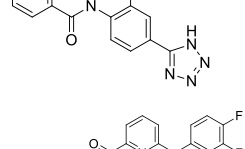
	ID	IC50 (μM)		ID	IC50 (μM)
	tetrazole	1.0		337	2.0
	sulfonamide	3.7		352	4.7
	982	No inh		357	1.9
	560	6.0		732	2.6
	250	5.4		733	2.8
	334	4.0		734	2.5
	335	2.3		753	2.9
	336	2.8			

Table 7-1 cont. DD2 analogs

	ID	IC ₅₀ (μM)		ID	IC ₅₀ (μM)
	754	2.6		67	7.8
	755	10.2		72	25.6
	248	0.8			
	279	0.9			
	26	2.3			
	29	1.9			
	89	22.5			
	177	1.4			

Supplemental Materials

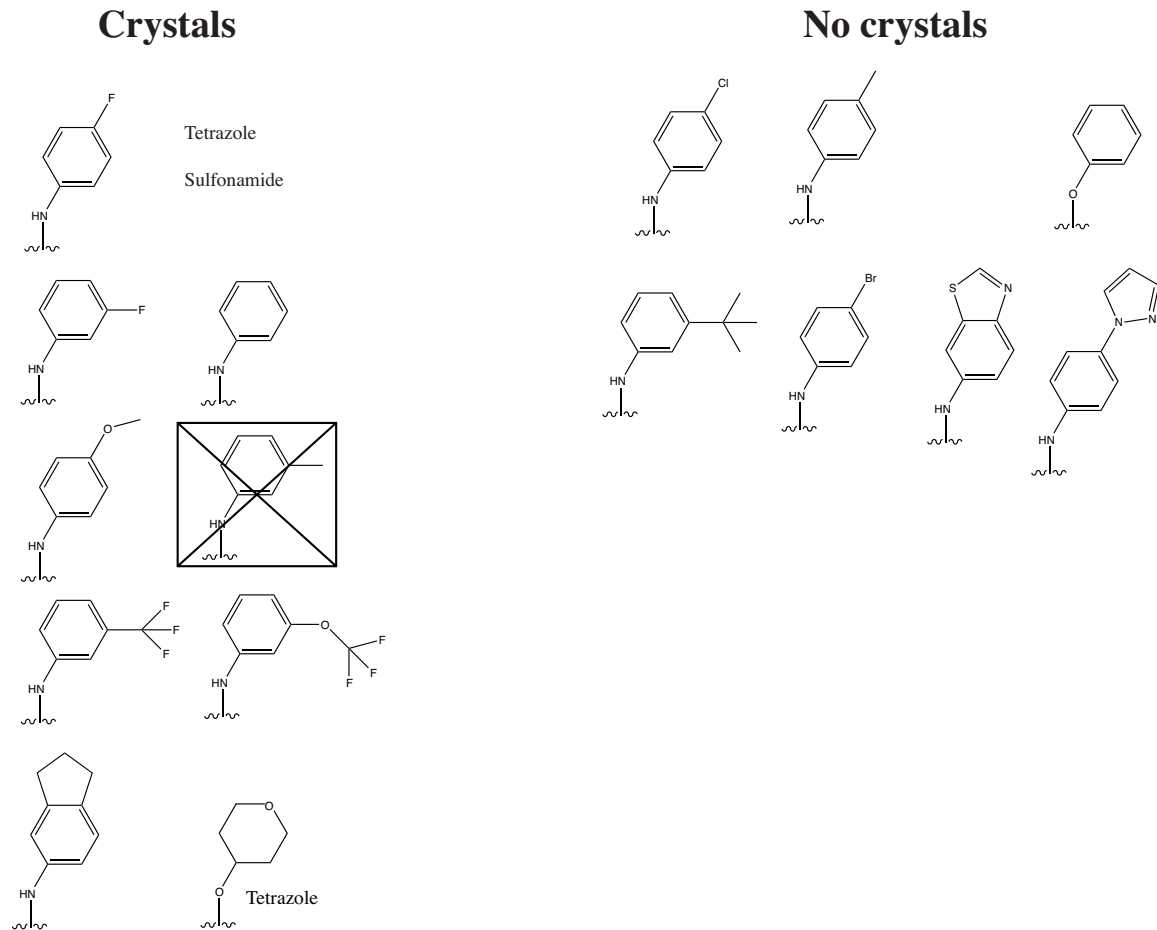


Figure 7-S1. Summary of crystallography trials

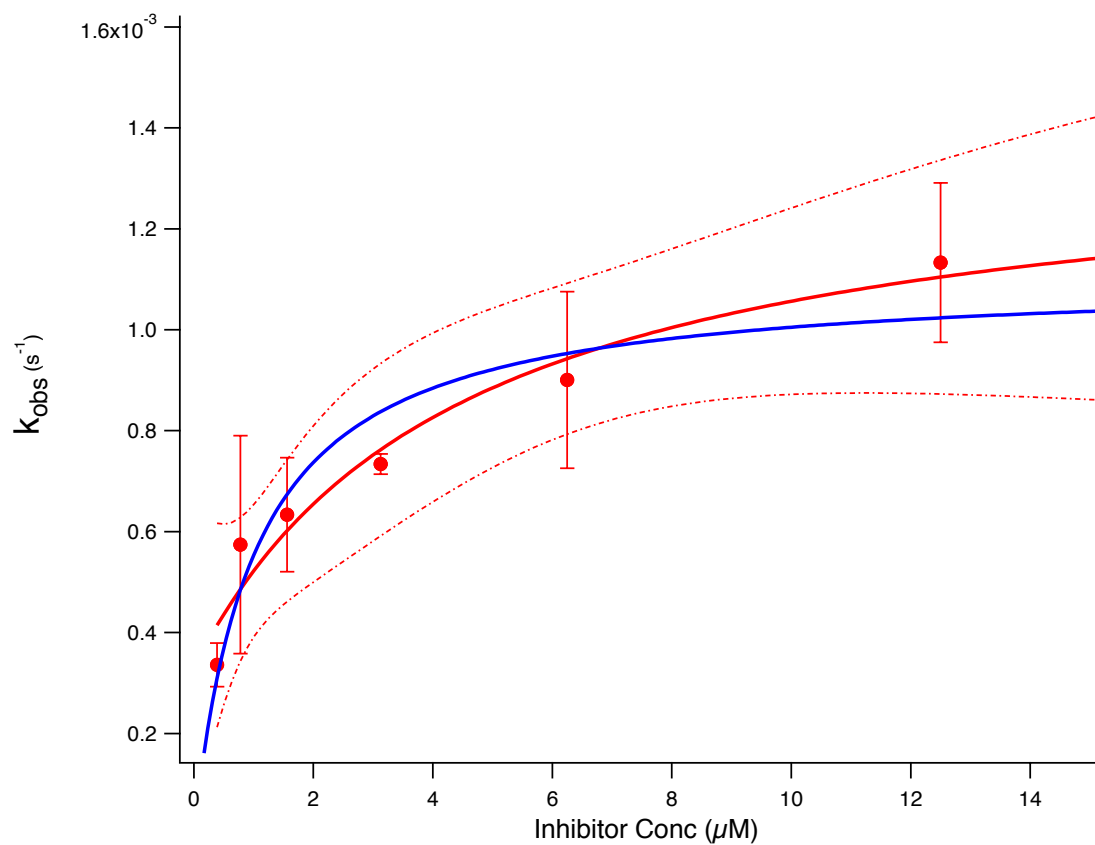


Figure 7-S2. Comparison between enzyme isomerization and irreversible inhibition model fits.

Table 7-S1. Optimized 24-well grid screen

	1	2	3	4	5	6
a	0.1M Sodium acetate pH 5.5, 0.8M NaH ₂ PO ₄ /1.2M K ₂ HPO ₄	0.1M Sodium acetate pH 4.8, 0.8M NaH ₂ PO ₄ /1.2M K ₂ HPO ₄ , 0.02M TCEP	0.1M Sodium acetate pH 4.8, 0.8M NaH ₂ PO ₄ /1.2M K ₂ HPO ₄ , 0.02M TCEP, 0.2M Urea	0.1M Imidazole pH 8, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.1M NaCl	0.1M Imidazole pH 8, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.2M NaCl	0.1M Imidazole pH 8, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.3M NaCl
b	0.1M Sodium acetate pH 7.8, 0.88M NaH ₂ PO ₄ /1.32M K ₂ HPO ₄ , 0.2M KCl	0.3M Imidazole pH 8.0, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.2M NaCl	0.1M Imidazole pH 8.0, 0.44M NaH ₂ PO ₄ /1.76M K ₂ HPO ₄ , 0.2M KCl	0.1M Imidazole pH 8.0, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.3M KCl, 0.2M Urea	0.2M Imidazole pH 8.0, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.2M NaCl	0.1M Imidazole pH 8.0, 0.42M NaH ₂ PO ₄ /1.68M K ₂ HPO ₄ , 0.2M KCl
c	0.1M Imidazole pH 8.0, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.3M KCl, 0.1M Urea	0.1M Imidazole pH 8.0, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.1M KCl	0.1M Imidazole pH 8.0, 0.38M NaH ₂ PO ₄ /1.52M K ₂ HPO ₄ , 0.2M KCl	0.1M Imidazole pH 8.0, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.3M KCl, 0.02M TCEP	0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.2M NaCl	0.1M Imidazole pH 8.0, 0.34M NaH ₂ PO ₄ /1.36M K ₂ HPO ₄ , 0.2M KCl
d	0.1M Imidazole pH 8.0, 0.4M NaH ₂ PO ₄ /1.6M K ₂ HPO ₄ , 0.3M KCl					

Table 7-S2. Conditions for crystal growth

Compound	Well (table 7-S1)
754 (para fluoro)	B1
110 (tetrazole)	B1
335 (meta fluoro)	A4
733 (meta trifluoromethoxy)	B6
827 (sulfonamide)	C1
29 (tetrahydropyran)	A1 (96-well format)
352 (para methoxy)	B4
734 (meta trifluoro)	B3
732 (dihydroindene)	C6
250 (amine linker)	B1

CHAPTER 8: Characterization of a fluorescence lifetime substrate for the human herpesvirus proteases and its apparent activation of KSHV protease

Jonathan E. Gable^{1,2}, Patrick Schweigler³, Samu Melkko³, Christopher J. Farady³, and Charles S. Craik¹

¹ Department of Pharmaceutical Chemistry

University of California

San Francisco, CA 94158-2280

USA

² Biophysics Graduate Group

University of California

San Francisco, CA, 94158-2280

USA

³ Novartis Institutes for BioMedical Research,

Forum 1, Novartis Campus CH-4002

Basel, Switzerland

Abstract

The herpesvirus proteases are a structurally unique group of essential conserved dimeric proteases critical for the viral replication cycle. Many attempts have been made to inhibit their activity as a means of preventing viral replication. Here we report a substrate that exhibits remarkable activation of Kaposi's sarcoma-associated herpesvirus (KSHV) protease and fails to be inhibited by previously reported dimer disruptors of KSHV protease. Exploration of the features required for activation suggests the need for the specific fluorescence lifetime fluorophore PT14. Fragment inhibitors of PT14 substrate turnover but not other substrate turnover were identified. Mass spectrometry and HPLC were used as orthogonal ways to ensure substrate cleavage is taking place and to rule out potential artifacts. Crystallographic experiments thus far have not elucidated the mechanism of activation at atomic level. It will be interesting to further explore how this activation takes place, whether or not it is biologically relevant, and if premature activation of the protease could hold any therapeutic benefit in the course of herpesvirus replication and infection.

During the course of development of a high throughput fragment-based screen for Kaposi's sarcoma-associated herpesvirus (chapter 4) we pursued the use of fluorescence lifetime as a screening modality. Fluorescence lifetime generally has fewer assay artifacts than fluorescence intensity and has been successfully employed in high throughput screening of proteases.¹ We developed substrate **1** (Ac-PVYtBuQASSC(PT14M)-NH₂) for use in our screen (**Figure 8-1**) based on the KSHV protease endogenous cleavage sequence and previously reported ACC substrates.^{2, 3} Interaction between the dye (PT14) and tyrosine results in a short lifetime for intact peptide and a longer fluorescence lifetime for the cleavage product.

Initial testing revealed something very surprising; while typically KSHV protease is only active at high nanomolar concentrations, using the FLT substrate KSHV protease was highly active at 50 nM (FBS study update 2012.12.11). Cleavage at the expected scissile bond, between the alanine and serine, was confirmed by liquid chromatography-mass spectrometry (LC-MS) supporting true cleavage by KSHV protease, not assay artifacts or contaminant protease. In addition to this surprising activity our known KSHV protease inhibitor DD2 failed to inhibit enzymatic activity. Despite this, a handful of fragments did appear to inhibit in our FLT assay but not our fluorescence intensity assay (**Figure 8-2**). Given this result we first hypothesized that the extended substrate, which occupies prime side pockets S1'-S3', binds much more tightly to and promotes dimerization of KSHV protease enabling activity at low enzyme concentrations and precluding dimer disruption by DD2. This however would be in contrast to previous experiments showing that DD2 inhibits enzymatic turnover of an extended FRET substrate.

To begin to understand this unexpected observation we first confirmed that DD2 was active against KSHV protease in the presence of our FRET substrate (**2**), which shares the same

amino acid sequence as **1** except for the functionalized C-terminal lysine in place of a functionalized cysteine (**Figure 8-1**). While the IC_{50} of DD2 with FRET substrate was somewhat higher than that measured for a 6-mer substrate Ac-PVYtBuQA-ACC both values were low-micromolar (**Figure 8-3**). Also, 50 nM KSHV protease showed no detectable cleavage of FRET substrate by MALDI-TOF MS, HPLC, or fluorescence plate reader over a 3-hour time course in contrast to the robust activity observed with the FLT substrate. We then hypothesized that the fluorophore, quencher, or biotin on the FRET substrate abrogates the effect of occupying prime-side pockets and that this could explain the failure of the FRET substrate to activate KSHV protease to the same extent as the FLT substrate. If this hypothesis was true 9-mer substrates with no fluorophore should activate KSHV protease and fail to be inhibited by DD2.

We synthesized two fluorophore-free peptides, **3** and **4**. Each of these peptides share the same P6-P2' residues but vary in their C-terminal residue. Substrate **3** has a phenylalanine in the P3' position to roughly mimic possible interactions made by the maleimide linker of our FLT substrate while substrate **4** simply has an alanine at P3'. HPLC was used to monitor their cleavage in the presence and absence of DD2 at 500 and 50 nM enzyme concentrations. These experiments were performed in the presence and absence of 0.1% Prionex used in initial screening optimization and thought to promote dimerization. Enzymatic activity of 500 nM enzyme was completely inhibited by 100 μ M DD2 for both substrates with and without Prionex (**Figure 8-4**). These results were confirmed with MALDI-TOF mass spectrometry (**Figures 8-S2 and 8-S3**). No cleavage was observed with 50 nM enzyme (data not shown). These results strongly suggest that the effect observed with the FLT substrate is unique and can likely be attributed to the presence of the PT14 dye.

In order to interrogate the structural basis for this apparent activation we turned to crystallography. The active site serine of KSHV protease was mutated to an alanine (S114A mutant), inactivating the enzyme. Hanging drop diffusion was used to screen for conditions that resulted in crystals with KSHV protease S114A in the presence of equimolar FLT substrate. Crystals were obtained and diffracted to 2.8 Å. The structure was solved by molecular replacement using WT KSHV protease structure 2PBK. No density was observed for the FLT substrate and no structural changes were observed relative to the 2PBK monomer (**Figure 8-5**). The only major difference between prior structures and this one is that the asymmetric unit in this case contained a single monomer, however analysis of the unit cell revealed the typical dimer interface observed in the asymmetric unit of 2PBK. In this instance the space group is C121 with unit cell dimensions (54.7, 65.7, 53.7, 90, 90, 90). For 2PBK the space group is P12₁1 and the unit cell dimensions are (45.9, 108.9, 54.1, 90, 104, 90).

The activation observed with this FLT substrate remains a mystery. It is interesting to consider how this activation might take place. Since substrate is turned over and released, one could hypothesize that it is actually the C-terminal product fragment containing PT14 that causes activation. This could begin to explain why no density for the substrate was observed in the crystal structure. Crystallography was also greatly limited by the poor substrate solubility. It would be interesting to use WT KSHV protease for crystallographic studies. Further analysis was hindered by the lack of direct access to an FLT plate reader. Time and instrumentation permitting it would be worthwhile to test the following: 1) does the product peptide activate KSHV protease?, 2) does the PT14 dye alone activate the enzyme, 3) do any of these species potentiate the cleavage of other substrates at low nanomolar enzyme concentrations?, 4) what are the kinetic parameters for FLT substrate turnover, and 5) does FLT substrate appear to activate any

other human herpesvirus proteases. It is also interesting to hypothesize that the fragment inhibitors of FLT substrate turnover but not FI substrate turnover (**Figure 8-2**) bind a putative “exosite” occupied by the PT14 dye. These fragments would then exhibit competitive inhibition with FLT substrate, something that can be readily tested. If this was the case, co-crystallization or soak experiments with wildtype KSHV protease and these fragments could allow the elucidation of the putative exosite at atomic resolution.

Identification of a *bona fide* activator of KSHV protease and the structural basis for activation would be extremely informative. If an activating exosite exists on this protease and is accessed by endogenous substrates it would affect the ability of KSHV protease to be targeted therapeutically. Also, premature activation of KSHV protease may well be as problematic as inhibition for the viral replication process opening the door for a novel therapeutic strategy to target these essential dimeric proteases.

References

- [1] Doering, K., Meder, G., Hinnenberger, M., Woelcke, J., Mayr, L. M., and Hassiepen, U. (2009) A fluorescence lifetime-based assay for protease inhibitor profiling on human kallikrein 7, *J Biomol Screen* 14, 1-9.
- [2] Harris, J. L., Backes, B. J., Leonetti, F., Mahrus, S., Ellman, J. A., and Craik, C. S. (2000) Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries, *Proc. Natl. Acad. Sci. U.S.A.* 97, 7754-7759.
- [3] Shahian, T., Lee, G. M., Lazic, A., Arnold, L. A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a viral enzyme by a small-molecule dimer disruptor, *Nat. Chem. Biol.* 5, 640-646.

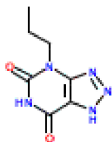
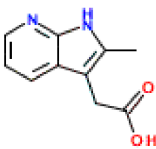
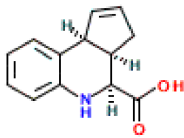
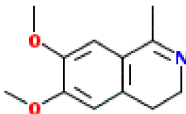
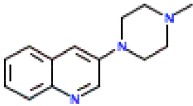
PKF211-217-IX-1  % Inhibition @FLT 121.11 % Inhibition @FI 5.99	ZPS007-793-IX-1  % Inhibition @FLT 118.54 % Inhibition @FI -11.09	IIVP-LDQ735-IX-2  % Inhibition @FLT 73.27 % Inhibition @FI -16.36	IIVP-AGI516-AA-1  % Inhibition @FLT 52.52 % Inhibition @FI 20.40
PKF205-902-AF-1  % Inhibition @FLT 31.38 % Inhibition @FI 10.48			

Figure 8-2 Hits from fluorescence lifetime pilot screen

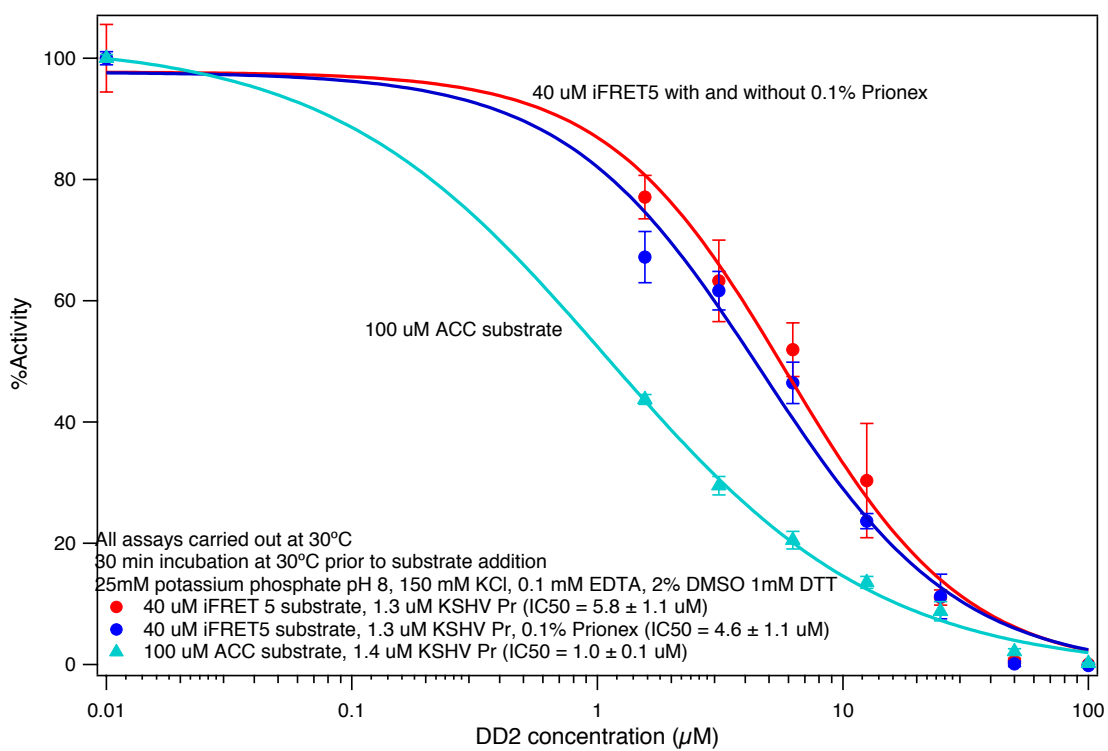


Figure 8-3 FRET vs ACC substrate IC_{50} curves

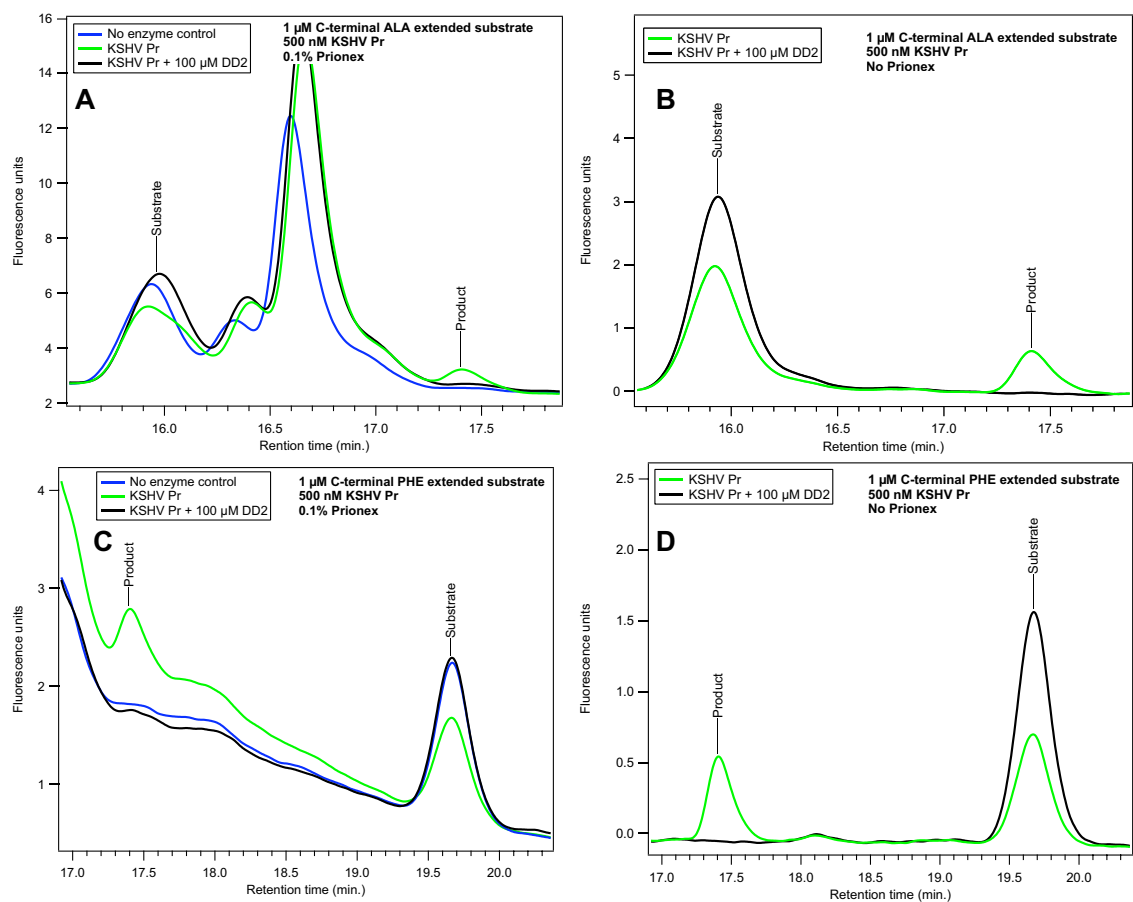


Figure 8-4. HPLC traces of unlabeled substrate turnover and inhibition

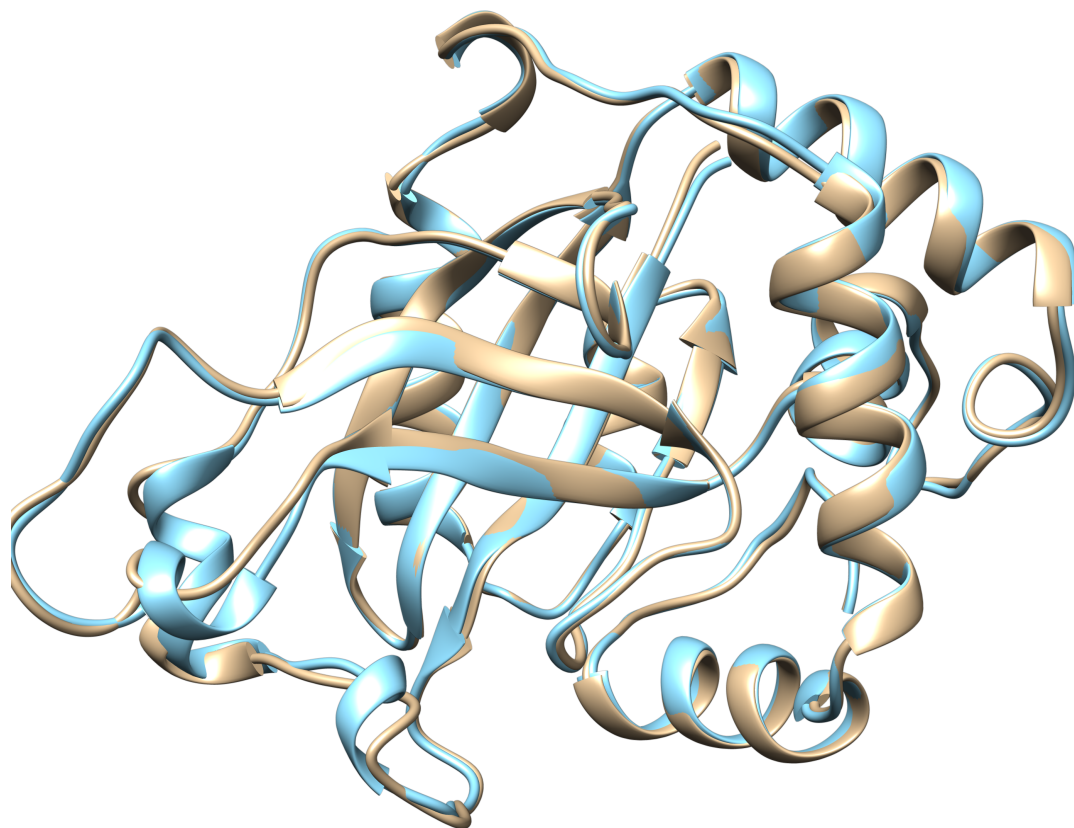


Figure 8-5 Overlay of WT KSHV protease and KSHV S114A protease in the presence of FLT substrate crystal structures

Supplemental Material

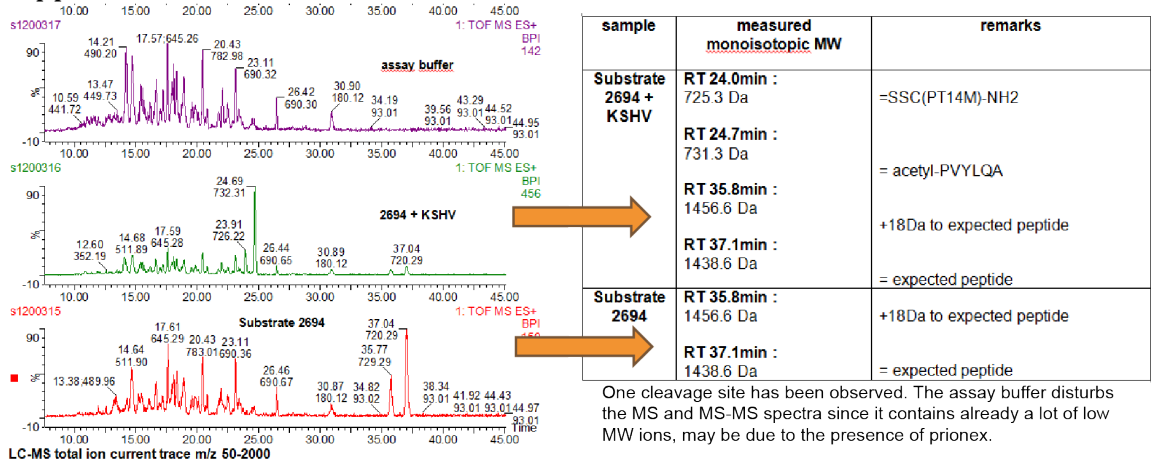


Figure 8-S1. LC-MS confirmation of FLT substrate cleavage

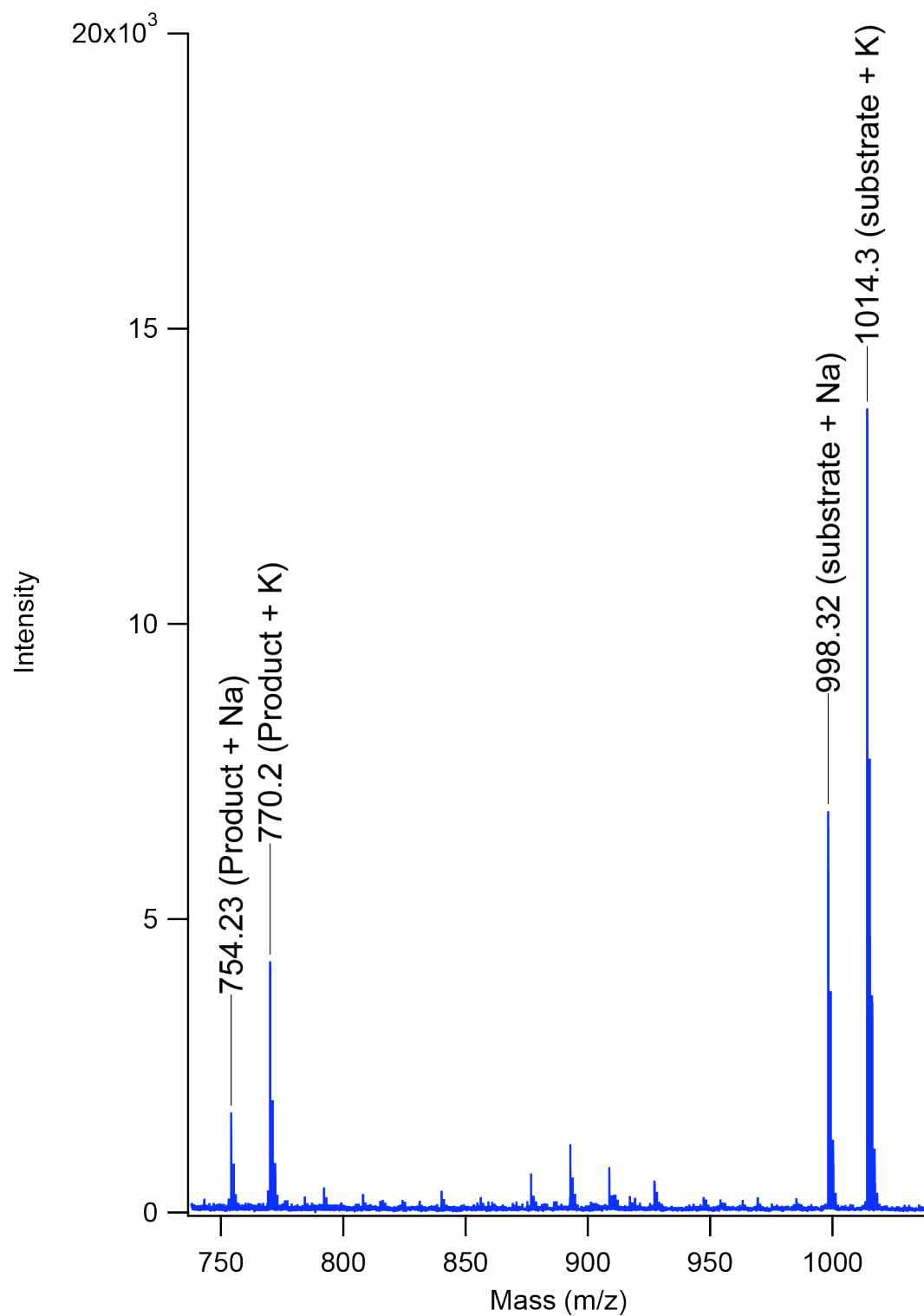
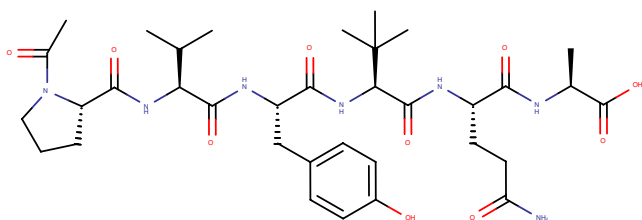
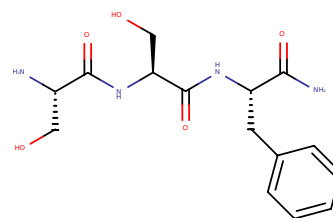


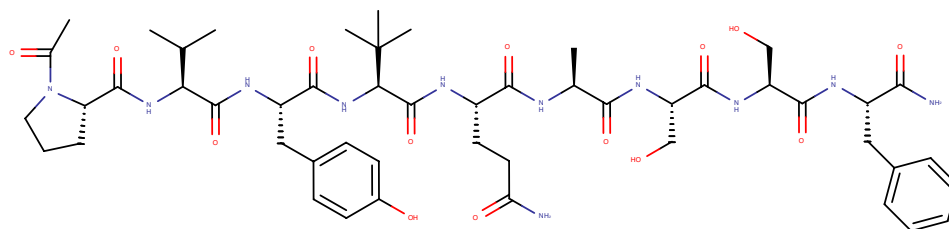
Figure 8-S2. MALDI-TOF MS confirmation of unlabeled substrate cleavage



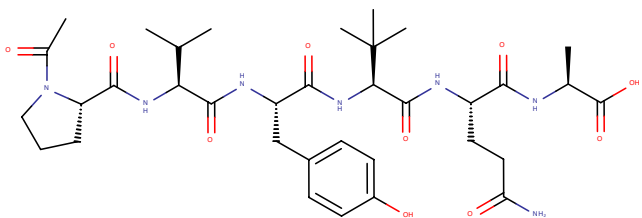
Exact Mass: 731.39
Molecular Weight: 731.84
m/z: 731.39 (100.0%), 732.39 (38.8%), 733.39 (10.2%), 732.38 (2.6%), 734.39 (1.0%)



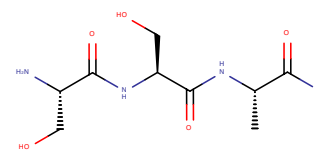
Exact Mass: 338.16
Molecular Weight: 338.36
m/z: 338.16 (100.0%), 339.16 (17.9%), 340.17 (1.3%), 340.16 (1.3%)



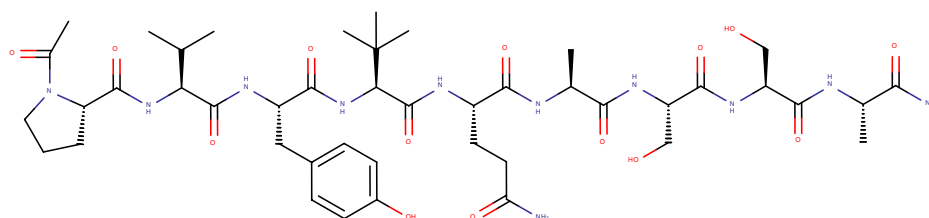
Chemical Formula: $C_{50}H_{73}N_{11}O_{14}$
Molecular Weight: 1052.18
m/z: 1051.53 (100.0%), 1052.54 (55.5%), 1053.54 (18.0%), 1054.54 (4.9%), 1052.53 (4.1%), 1053.53 (2.3%)



Molecular Weight: 731.84
m/z: 731.39 (100.0%), 732.39 (38.8%), 733.39 (10.2%), 732.38 (2.6%), 734.39 (1.0%)



Molecular Weight: 262.26
m/z: 262.13 (100.0%), 263.13 (10.1%), 264.13 (1.6%), 263.12 (1.5%)



Chemical Formula: $C_{44}H_{69}N_{13}O_{14}$
Molecular Weight: 976.08
m/z: 975.50 (100.0%), 976.51 (48.9%), 977.51 (14.6%), 976.50 (4.1%), 978.51 (3.6%), 977.50 (2.0%)

Figure 8-S3. Unlabeled substrates and their cleavage products

CHAPTER 9: Inhibition of viral replication in mammalian cells by a protein-protein interaction antagonist of KSHV protease

Jonathan E. Gable, Timothy M. Acker, Charles S. Craik

Abstract

DD2 was previously shown to inhibit KSHV protease in biochemical assays but due to limited cell permeability had never been shown to inhibit viral replication in mammalian cell culture. Here we report cytotoxicity and viral reinfection data for a host of permeable DD2 analogs. Our most promising analog, DD2-29, exhibited dose-dependent inhibition of viral reinfection and was shown via western blot to perturb the amount of viral assembly protein observed. While we were unable to conclusively show inhibition of the viral protease in the infected cells, this report lays the groundwork for establishing DD2-29 mechanism of action and importantly rules out a variety of off-target effects. If we are able to show that DD2-29 inhibits viral replication by targeting the protease it would be a significant milestone in pharmacological validation of the human herpesvirus proteases as drug targets and the use of protein-protein interaction antagonists in general, as well as specifically for an infectious disease target.

Introduction

Numerous small molecules have been shown biochemically to inhibit herpesvirus protease yet none have shown robust efficacy in a cellular model.¹⁻¹² The herpesvirus proteases have been genetically validated through both knockout and knockdown experiments in both cellular and animal models.^{13, 14} Despite this validation, even potent active site inhibitors have failed to show an effect on viral replication. Initial concerns that near complete inhibition of the protease, like that achieved through genetic knockout, is required for reduction in viral replication were quelled by partial knockdown experiments in a mouse model of KSHV that showed reduced viral burden.¹³ A different challenge not captured by typical biochemical assays may be the culprit for lack of pharmacological prior validation.

Active site inhibitors of the herpesvirus proteases suffer from having to compete with the extremely high local concentration of substrate in the procapsid.¹⁵ Furthermore, the active site is not formed until dimerization takes place in the procapsid, leaving no opportunity for active site-directed inhibitors to bind the protease before local substrate concentration becomes high.¹⁶⁻¹⁹ Given these challenges to active site inhibition we have pursued dimer disruptors—small molecules that antagonize herpesvirus dimerization by binding to the dimer interface thus allosterically inhibiting enzymatic activity. We have identified multiple such molecules and have shown their ability to trap the inactive monomer in biochemical assays.^{12, 20, 21} The previously reported dimer disruptors have exhibited poor cell permeability and therefore were not tested for their ability to inhibit KSHV replication in mammalian cell culture.

In chapter seven we introduced DD2-29, a DD2 analog wherein a phenyltetrazole replaces the benzoic acid of DD2 and the phenyl ring off the benzoic acid of DD2 is replaced with an ether linkage to a tetrahydropyran (Figure 9-5). We hypothesized that DD2-29 would

have improved cellular permeability and would be able to inhibit KSHV protease in a cellular assay, preventing viral replication. This would provide the first clear pharmacological validation of a herpesvirus protease and one of few examples of protein-protein interaction antagonists against intracellular infectious disease targets. To test this we implemented a yield reduction assay for KSHV using a construct that expresses GFP during latent infection and RFP during lytic infection off the viral episome (Figure 9-1).²² We tested a host of DD2 analogs including DD2-29 and evaluated potential off-target effects with these compounds. Western blot analysis was performed to monitor the endogenous protease substrate and product as a means of confirming molecular mechanism of action.

Results

DD2-29 exhibited dose-dependent inhibition of viral reinfection in a mammalian KSHV-infected cell line (Figures 9-3 and 9-4). DD2-29 differs from the tetrazole analog of DD2 in that it has an ether linkage out to a tetrahydropyran in place of the carbon linker to a phenyl ring off the phenyltetrazole (Figure 9-5). This substitution dramatically improved cell culture efficacy, despite a modest decrease in biochemical potency (see chapter 7). The tetrazole and sulfonamide derivatives of DD2 showed modest effect, with the tetrazole being more efficacious than the sulfonamide (Figure 9-4). The biochemically inactive negative control, DD2-73, resulted in an apparent increase in reinfection relative to DMSO. Cidofovir, an FDA-approved viral polymerase inhibitor, completely blocked reinfection at a concentration of 5 μ M in media.

DD2 analogs are known to be heavily serum bound (personal communication with Prof. R. Kip Guy). Unfortunately, the SLK cell line used for reinfection assays does not tolerate serum-free media for prolonged periods of time. Given this limitation we tested whether pre-treatment with compound in serum-free media for 2 hours improved cellular efficacy. For all

compounds tested there was no significant difference between the two treatment strategies (Figure 9-6). DD2-29 had not been synthesized at the time this experiment was carried out and therefore was not tested. We were concerned that the carboxylate of DD2 might prevent cell entry. We therefore synthesized a DD2 prodrug where the carboxylate was converted to an ester that could then be acted on by cellular esterases to recover the original DD2 molecule. This prodrug showed a modest but statistically significant effect (serum free: p-val 0.03; serum-containing: p-val 0.006, paired two-tailed t-test). The tetrazole, sulfonamide, and N-methyl tetrazole also showed modest but significant effects. The N-methyl tetrazole has no biochemical activity suggesting either off-target effects or that the modest difference in reinfection across all of the compounds, while statistically significant, is not scientifically significant (Figure 9-6). It may be more appropriate to use ANOVA and subsequent post hoc analysis rather than multiple t-tests.

Cytotoxicity is arguably the most obvious “off-target” effect. Two assays were performed to assess cytotoxicity of DD2 analogs. An MTS assay that measures metabolic rate showed no appreciable toxicity (Figure 9-S1). Sytox Red (Life Technologies) is a DNA-intercalating dye, where the extent of labeling is a measure of cell permeabilization. Flow cytometry revealed minimal labeling with Sytox Red after 48 hrs treatment with 40 μ M DD2 analog and no statistically significant difference between the DMSO control and compound (Figure 9-S2).

Some DD2 analogs showed potent inhibition of reinfection that was seemingly discordant with biochemical IC_{50} against KSHV protease (Figure 9-S3), for instance DD2-201 which showed full inhibition of viral reinfection at 20 μ M despite having a biochemical IC_{50} of 4 μ M. Fluorescence microscopy in the presence of DD2-201 showed that lytic induction was blocked, observed as a lack of RFP production (data not shown). As a control we tested whether DD2-201

blocked doxycycline induction of GFP production in a completely unrelated HEK293 cell line. DD2-201 showed dose-dependent inhibition of GFP induction. Centrifugation and order-of-addition experiments ruled out the possibility that DD2-201 aggregates with doxycycline thus preventing it from entering cells (Figures 9-S4 and 9-S5). Addition of compound to cells at low confluence revealed a halt in cell growth. The cells remained visually healthy and did not show signs of cell lysis or significant morphological change. In general treatment with DD2 analogs containing a BOC group resulted in this effect. DD2-29 did not prevent induction of GFP or the lytic cycle.

Western blot analysis can directly monitor the endogenous protease substrate and products (Figure 9-7). KSHV protease is expressed as a fusion to the assembly protein (AP-Pr). Upon activation it cleaves itself away from the assembly protein (AP), and cleaves AP away from the major capsid protein. KSHV protease can also cleave itself as a means of inactivation.²³⁻²⁵ A rabbit bleed derived from injection of recombinant KSHV protease was purified over a Protein A column and used to detect AP-protease fusion, mature protease, and cleaved protease. Optimization of the western blot conditions allowed for detection of as little as 375 pg recombinant KSHV protease (Figure 9-8). Blotting against cell lysate failed to identify a reliable band for mature protease (Figure 9-9). Induction-dependent bands at ~80 kDa and ~65 kDa were observed, but neither clearly corresponds with Pr-AP, which has a molecular weight of 58 kDa. The 80 kDa band is significantly diminished for cell lysate from DD2-29-treated cells at the 70-hour time point, but not negative controls DMSO and DD2-73. The ~65 kDa band is diminished for all treatment conditions at the 70 hour time point relative to the 48 hour time point (Figure 9-9).

Western blots using an anti-AP rabbit bleed successfully identified AP and AP-Pr fusion in cell lysates (Figure 9-10). Consistent with previous reports AP is expressed in large excess of AP-Pr fusion and the AP-Pr fusion is not observed until greater than 24 hours post induction.²⁶ In the 70-hour time point AP is lost in lysates from DD2-29-treated cells but not with either negative control (DMSO or DD2-73). Only a slight diminution of AP is observed at the 48-hour time point for cells treated with DD2-29. DD2-73 appears to result in an increase in both AP and AP-Pr fusion. A similar loss of AP was observed with cells treated for 96 hours. At this later time point AP-Pr fusion was also lost in lysates from DD2-29-treated cells but not those treated with DMSO. AP but not AP-Pr fusion was detected in virions isolated from the 96-hour treatment protocol. The AP band was weak and distorted, likely due to the large amount of DNA loaded on the gel as part of the virion preparation. No significant loss of AP with DD2-29 treatment was observed for isolated virions. Similar patterns were observed in samples induced with only doxycycline or doxycycline and sodium butyrate, however the effect of compound was more apparent in the doxycycline and sodium butyrate-induced sample (Figure 9-11).

Discussion

We found that DD2-29 inhibits KSHV reinfection in a dose-dependent manner. These results are consistent with a reduction in viral replication. None of the DD2 analogs tested caused metabolic toxicity or any form of toxicity that would result in cell permeabilization. Some DD2 analogs containing a BOC group halted cell growth and inhibited doxycycline induction. This gave rise to false positives in our viral reinfection assay. The cause of this unexpected off-target effect is unknown. When treated with these compounds the cells halt growth but do not die,

suggesting a quiescent or senescent state. Given that the DD2 scaffold was originally designed as a p53-MDM2 interaction inhibitor it is tempting to hypothesize that modification of the scaffold resulted in an inhibitor of this interaction, however we have no evidence to support this claim.²⁷ It is interesting to note that this effect occurs in both SLK cells and HEK293 cells, two fairly different immortalized cell lines, suggesting the off-target(s) is conserved. Importantly, DD2-29 did not halt cell growth in this way supporting potential protease inhibition as the source of reduced viral reinfection.

Our western blot analysis was not able to clearly detect the mature protease or the AP-Pr fusion. No reliable band was observed at 25 kDa, the molecular weight of the mature protease. Higher molecular weight induction-specific bands were observed but they were above the expected molecular weight for the AP-Pr fusion (58 kDa). It is possible that the band at approximately 65 kDa is in fact the AP-Pr fusion. If the protease is inhibited we should observe accumulation of AP-Pr relative to the DMSO control. At the 70 hour time point there is some indication this is the case, but the quality of the data preclude a definitive determination (Figure 9-10). Furthermore, additional experiments in the presence of proteasome inhibitors may be necessary to rule out the possibility that AP-Pr is being degraded. Anti-AP western blots showed a loss of AP in the presence of DD2-29 at later time points. At face value protease inhibition would be expected to result in an accumulation of full-length AP, though we were not able to resolve the cleaved and full-length forms of AP. In the normal replication cycle AP functions as a scaffold for capsid formation but is processed and expelled from the capsid before egress.¹⁵ It is possible that the apparent loss of AP is due to export of procapsids still containing AP that could not be processed due to protease inhibition. While this may be happening, one would expect continued expression of AP so the extent of AP loss is surprising and poorly explained by

the data currently available. A blot of isolated virus does show the presence of AP supporting the hypothesis that immature capsids are released into the media, however the data are insufficient to explain the loss of the AP with treatment in cell lysate samples (Figure 9-11). It is interesting to note that the extent of AP loss appears to be dependent on progression through the viral replication cycle, not simply time dependent. At the same time point more AP is lost when sodium butyrate and doxycycline are used for induction than when doxycycline alone is used (Figure 9-11). This provides another clue for building a model of DD2-29 viral reinfection inhibition. Overall the complexity and quality of the data obtained to date do not allow us to definitively state that the protease is inhibited by DD2-29 in virally infected cells, though they do not rule out this possibility.

A number of key experiments may help clarify the mechanism of inhibition. Antibodies purified over a column with immobilized recombinant KSHV protease should be used for future anti-Pr western blots to eliminate background and non-specific bands. Using these antibodies, western blotting should be performed against isolated virions to determine if protease and AP-Pr fusion are present in extracellular capsids. Todd Pray reported in his dissertation that “KSHV Pr could not be detected by western immunoblotting in whole lysates... [however] in released capsids and/or viral particles not only was the presence of KSHV Pr confirmed, D-site cleavage was evident.” These results are consistent with our inability to detect the protease in cell lysates. Pursuit of immunoblotting against released capsids and virions from DD2-29-treated cells should be extremely high priority in furthering the work presented here. Further variation of treatment time courses should also be performed to rule out inhibition of other steps of the viral replication cycle.

We demonstrate for the first time a protein-protein interaction antagonist targeted at the conserved dimeric herpesviral protease that successfully reduces viral reinfection in mammalian cell culture. If we are able to show conclusively that our small molecule functions in cells as it does in biochemical assays it will be a significant milestone in the use of small molecules to target protein interfaces generally and specifically in the context of infectious disease.

Methods

Viral reinfection assay

Media refers to DMEM High glucose + 10% FBS + 100 µg/mL Streptomycin sulfate + 100 units/mL Penicillin ‘G’ unless otherwise stated. Both iSLK (uninfected) and iSLK-219 (latently infected) cells were plated in 24-well sterile tissue culture-treated plates with lids (Costar 3526) at 1×10^4 cells/well and 4.5×10^4 cells/well, respectively, in 0.5 mL media per well. Cells were allowed to adhere overnight in a temperature, humidity, and atmosphere controlled incubator at 37 degrees Celsius, 5% CO₂, relative humidity >60%. After overnight incubation iSLK-219 cells should be approximately 80% confluent. Induction media was prepared by the addition of 3 µg/mL doxycycline and 1 mM sodium butyrate (no puromycin is included in this media). Test compounds were added to induction media to a final concentration of 0.4% DMSO for all compound concentrations tested. This concentration of DMSO is non-cytotoxic. Induction of the lytic cycle in the presence or absence of compound was allowed to take place for 48 hours. Four hundred microliters of spent media from iSLK-219 cells was collected and filtered using a sterile 0.45 µm spin filter (Spin-X Centrifuge Tube Filter 0.45 µm Cellulose Acetate RNase/DNase free; Costar 8162) to remove cells while maintaining virus. Spent media was diluted 1:1 into media supplemented with 12 µg/mL Polybrene transfection reagent (Millipore) to arrive at 0.8 mL virus-containing media with 6 µg/mL Polybrene. Media from the iSLK

(uninfected) cells was discarded and replaced with virus-containing media. Cells were spinoculated at 2000 rpm for 1 hr at RT. Cells were examined under a microscope before and after spinoculation to identify wells that may have dried during centrifugation (if this occurs, increase the total volume per well while being careful to avoid spillage during centrifugation). Immediately after centrifugation 1.0 mL warm media + 6 µg/mL Polybrene is added to each well and plate is allowed to incubate for an additional hour at 37 degrees Celsius. Media is then replaced with 0.5 mL fresh warm media. Newly infected cells were allowed to incubate for 48 hours to allow GFP signal to develop. After 48 hours, these cells are trypsinized, spun down, and resuspended in flow cytometry media (PBS, Ca²⁺ and Mg²⁺ free with 0.04% EDTA, 2% FBS). GFP positive cells were counted using a FACScalibur (BD Biosciences) with 10,000 cells counted per sample. Percent reinfection was computed as the number of GFP positives cells in a given sample divided by the number of GFP positive cells in the DMSO control times 100.

Western immunoblotting

Cells were lysed in RIPA buffer and run on a denaturing gel. Lysate protein concentration was determined via BCA assay (Cell Signaling Technology) as per the manufacturers instructions, allowing for normalized of protein across wells. Virus was isolated from spent media by centrifugation at 16000g for one hour at 4 degrees Celsius and lysed and boiled in 4X LDS Sample Buffer (Invitrogen). After wet transfer the blot was washed in TBST (50 mM Tris pH7.5, 150 mM NaCl, 0.05% Tween 20) and blocked with either 1% Clear Milk Blocking Buffer (Pierce/Life Technologies) or 5% milk in TBST for at least 60 minutes at room temperature on a orbital shaker. Blocking reagent was removed by at least 3X TBST washes, 1 minute each. Primary was added either in sterile 2% BSA in TBST or 1% Clear Milk Blocking buffer and incubated overnight at 4 degrees Celsius on an orbital shaker. Primary was removed

and blot was washed with TBST for 1 hour at room temperature with shaking followed by two brief TBST washes. Goat anti-rabbit (H+L) HRP conjugate (Bio-Rad) was diluted 10000-fold in TBST + 2% milk (for anti-AP antibody) or 1% Clear Milk Blocking buffer (protein-A purified anti-Pr antibody) and applied as a secondary for one hour at room temperature with shaking. Secondary was removed and blot was washed with TBST three times over the course of an hour. ECL reagent was added according to the manufacturers instructions and western blots were visualized on a ChemiDoc MP imaging system (Bio-Rad).

Sytox red assay

Cells were treated with compound as described for the viral replication assay but in the absence of any induction reagents (doxycycline and sodium butyrate). After 48 hours treatment cells were removed from the plate using Enzyme Free Cell Dissociation Buffer (Gibco), pelleted, and resuspended in flow cytometry buffer (PBS, Ca^{2+} and Mg^{2+} free with 0.04% EDTA, 2% FBS) supplemented with Sytox red dye. The remainder of the assay was performed according to the manufacturer instructions and measured were made using a FACScalibur (BD Biosciences) instrument.

MTS assay

MTS assays were performed in the same way that Sytox red assay was performed except in 96-well plates and according to the MTS instructions (Promega). Cells per well was optimized (Figure 9-S6) and 1000 cells per well was found to be optimal.

- [1] Abood, N. A., Schretzman, L. A., Flynn, D. L., Houseman, K. A., Wittwer, A. J., Dilworth, V. M., Hippenmeyer, P. J., and Holwerda, B. C. (1997) Inhibition of human cytomegalovirus protease by benzoxazinones and evidence of antiviral activity in cell culture, *Bioorganic & Medicinal Chemistry Letters* 7, 2105-2108.
- [2] Bonneau, P. R., Hasani, F., Plouffe, C., Malenfant, E., LaPlante, S. R., Guse, I., Ogilvie, W. W., Plante, R., Davidson, W. C., Hopkins, J. L., Morelock, M. M., Cordingley, M. G., and Deziel, R. (1999) Inhibition of human cytomegalovirus protease by monocyclic beta-lactam derivatives: Kinetic characterization using a fluorescent probe, *J. Am. Chem. Soc.* 121, 2965-2973.
- [3] Borthwick, A. D. (2005) Design of translactam HCMV protease inhibitors as potent antivirals, *Med. Res. Rev.* 25, 427-452.
- [4] Borthwick, A. D., Angier, S. J., Crame, A. J., Exall, A. M., Haley, T. M., Hart, G. J., Mason, A. M., Pennell, A. M. K., and Weingarten, G. G. (2000) Design and synthesis of pyrrolidine-5,5-trans-lactams (5-oxo-hexahydro-pyrrolo[3,2-b]pyrroles) as novel mechanism-based inhibitors of human cytomegalovirus protease. 1. The alpha-methyl-trans-lactam template, *Journal of Medicinal Chemistry* 43, 4452-4464.
- [5] Deziel, R., and Malenfant, E. (1998) Inhibition of human cytomegalovirus protease N(o) with monocyclic beta-lactams, *Bioorg. Med. Chem. Lett.* 8, 1437-1442.
- [6] Finke, P. E., Shah, S. K., Fletcher, D. S., Ashe, B. M., Brause, K. A., Chandler, G. O., Dellea, P. S., Hand, K. M., Maycock, A. L., Osinga, D. G., Underwood, D. J., Weston, H., Davies, P., and Doherty, J. B. (1995) Orally active beta-lactam inhibitors of human

- leukocyte elastase. 3. Stereospecific synthesis and structure-activity relationships for 3,3-dialkylazetidin-2-ones, *J. Med. Chem.* **38**, 2449-2462.
- [7] Gerona-Navarro, G., Perez de Vega, M. J., Garcia-Lopez, M. T., Andrei, G., Snoeck, R., Balzarini, J., De Clercq, E., and Gonzalez-Muniz, R. (2004) Synthesis and anti-HCMV activity of 1-acyl-beta-lactams and 1-acylazetidines derived from phenylalanine, *Bioorg. Med. Chem. Lett.* **14**, 2253-2256.
- [8] Jarvest, R. L., Connor, S. C., Gorniak, J. G., Jennings, L. J., Serafinowska, H. T., and West, A. (1997) Potent selective thienoxazinone inhibitors of herpes proteases, *Bioorganic & Medicinal Chemistry Letters* **7**, 1733-1738.
- [9] Jarvest, R. L., Parratt, M. J., Debouck, C. M., Gorniak, J. G., Jennings, L. J., Serafinowska, H. T., and Strickler, J. E. (1996) Inhibition of HSV-1 protease by benzoxazinones, *Bioorganic & Medicinal Chemistry Letters* **6**, 2463-2466.
- [10] Jarvest, R. L., Pinto, I. L., Ashman, S. M., Dabrowski, C. E., Fernandez, A. V., Jennings, L. J., Lavery, P., and Tew, D. G. (1999) Inhibition of herpes proteases and antiviral activity of 2-substituted thieno[2,3-d]oxazinones, *Bioorganic & Medicinal Chemistry Letters* **9**, 443-448.
- [11] Schepetkin, I. A., Khlebnikov, A. I., and Quinn, M. T. (2007) N-benzoylpyrazoles are novel small-molecule inhibitors of human neutrophil elastase, *J. Med. Chem.* **50**, 4928-4938.
- [12] Gable, J. E., Lee, G. M., Jaishankar, P., Hearn, B. R., Waddling, C. A., Renslo, A. R., and Craik, C. S. (2014) Broad-spectrum allosteric inhibition of herpesvirus proteases, *Biochemistry* **53**, 4648-4660.

- [13] Jiang, X., Gong, H., Chen, Y. C., Vu, G. P., Trang, P., Zhang, C. Y., Lu, S., and Liu, F. (2012) Effective inhibition of cytomegalovirus infection by external guide sequences in mice, *Proc. Natl. Acad. Sci. U.S.A.* *109*, 13070-13075.
- [14] Gao, M., Matusick-Kumar, L., Hurlburt, W., DiTusa, S. F., Newcomb, W. W., Brown, J. C., McCann, P. J., 3rd, Deckman, I., and Colonno, R. J. (1994) The protease of herpes simplex virus type 1 is essential for functional capsid formation and viral growth, *J. Virol.* *68*, 3702-3712.
- [15] Gable, J. E., Acker, T. M., and Craik, C. S. (2014) Current and Potential Treatments for Ubiquitous but Neglected Herpesvirus Infections, *Chem. Rev.*
- [16] Batra, R., Khayat, R., and Tong, L. (2001) Molecular mechanism for dimerization to regulate the catalytic activity of human cytomegalovirus protease, *Nat. Struct. Biol.* *8*, 810-817.
- [17] Marnett, A. B., Nomura, A. M., Shimba, N., Ortiz de Montellano, P. R., and Craik, C. S. (2004) Communication between the active sites and dimer interface of a herpesvirus protease revealed by a transition-state inhibitor, *Proc. Natl. Acad. Sci. U.S.A.* *101*, 6870-6875.
- [18] Nomura, A. M., Marnett, A. B., Shimba, N., Dotsch, V., and Craik, C. S. (2005) Induced structure of a helical switch as a mechanism to regulate enzymatic activity, *Nat. Struct. Mol. Biol.* *12*, 1019-1020.
- [19] Pray, T. R., Reiling, K. K., Demirjian, B. G., and Craik, C. S. (2002) Conformational change coupling the dimerization and activation of KSHV protease, *Biochemistry* *41*, 1474-1482.

- [20] Lee, G. M., Shahian, T., Baharuddin, A., Gable, J. E., and Craik, C. S. (2011) Enzyme inhibition by allosteric capture of an inactive conformation, *J. Mol. Biol.* *411*, 999-1016.
- [21] Shahian, T., Lee, G. M., Lazic, A., Arnold, L. A., Velusamy, P., Roels, C. M., Guy, R. K., and Craik, C. S. (2009) Inhibition of a viral enzyme by a small-molecule dimer disruptor, *Nat. Chem. Biol.* *5*, 640-646.
- [22] Myoung, J., and Ganem, D. (2011) Generation of a doxycycline-inducible KSHV producer cell line of endothelial origin: maintenance of tight latency with efficient reactivation upon induction, *J. Virol. Methods* *174*, 12-21.
- [23] Gibson, W., and Roizman, B. (1972) Proteins specified by herpes simplex virus. 8. Characterization and composition of multiple capsid forms of subtypes 1 and 2, *J. Virol.* *10*, 1044-1052.
- [24] Matusick-Kumar, L., McCann, P. J., 3rd, Robertson, B. J., Newcomb, W. W., Brown, J. C., and Gao, M. (1995) Release of the catalytic domain N(o) from the herpes simplex virus type 1 protease is required for viral growth, *J. Virol.* *69*, 7113-7121.
- [25] Welch, A. R., Woods, A. S., McNally, L. M., Cotter, R. J., and Gibson, W. (1991) A herpesvirus maturational proteinase, assemblin: identification of its gene, putative active site domain, and cleavage site, *Proc. Natl. Acad. Sci. U.S.A.* *88*, 10792-10796.
- [26] Arias, C., Weisburd, B., Stern-Ginossar, N., Mercier, A., Madrid, A. S., Bellare, P., Holdorf, M., Weissman, J. S., and Ganem, D. (2014) KSHV 2.0: a comprehensive annotation of the Kaposi's sarcoma-associated herpesvirus genome using next-generation sequencing reveals novel genomic and functional features, *PLoS Pathog.* *10*, e1003847.

[27] Lu, F., Chi, S. W., Kim, D. H., Han, K. H., Kuntz, I. D., and Guy, R. K. (2006)

Proteomimetic libraries: design, synthesis, and evaluation of p53-MDM2 interaction inhibitors, *Journal of combinatorial chemistry* 8, 315-325.

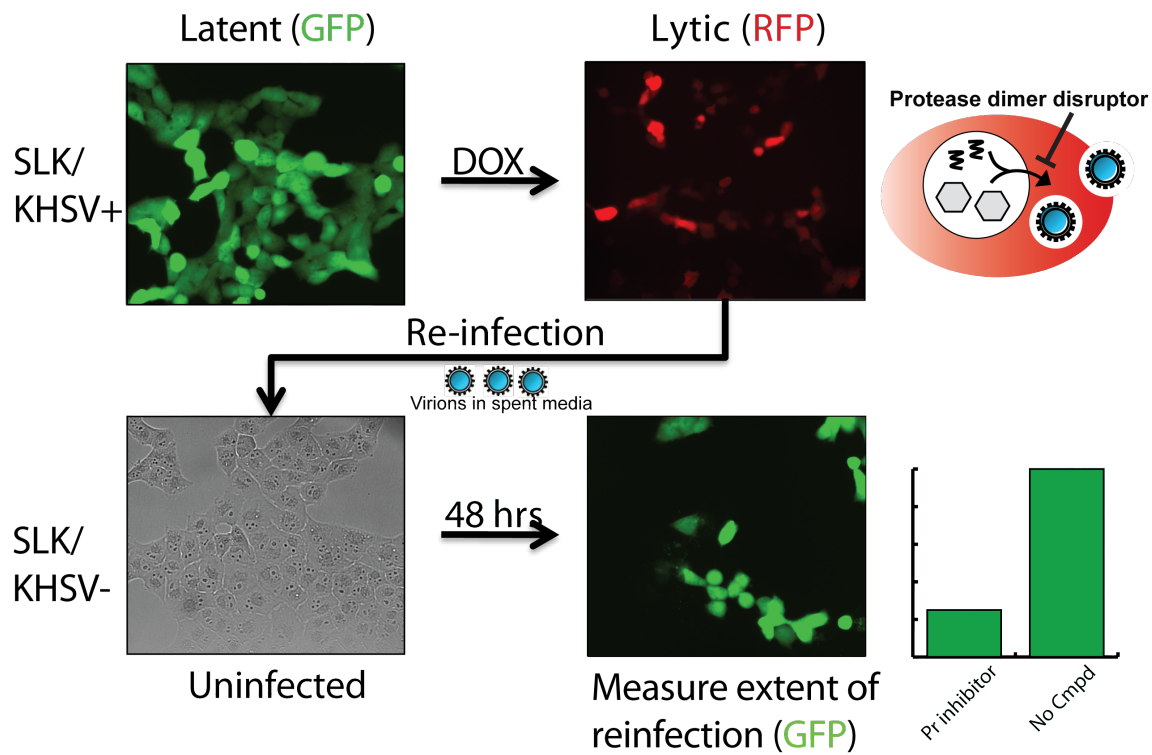


Figure 9-1. Reinfection assay overview. Latently infected cells are induced to enter the lytic cycle with addition of doxycycline. Spent media from lytic cells in the presence or absence of inhibitor is used to reinfect uninfected cells. Newly infected cells will be latent and GFP positive allowing quantification by flow cytometry.

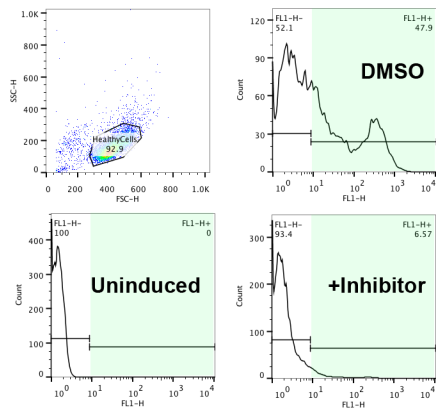


Figure 9-2. Example flow cytometry curves. (Upper left) Example side- and forward-scatter plot highlighting the region for healthy cells. (Upper right) DMSO control treatment results in robust GFP signal indicating reinfection. (Lower left) Uninduced cells show no shift in population to GFP positive. (Lower right) Addition of inhibitor shifts the population toward GFP negative. These data can be used to quantify extent of reinfection. The GFP positive portion of the plot is shown in green.

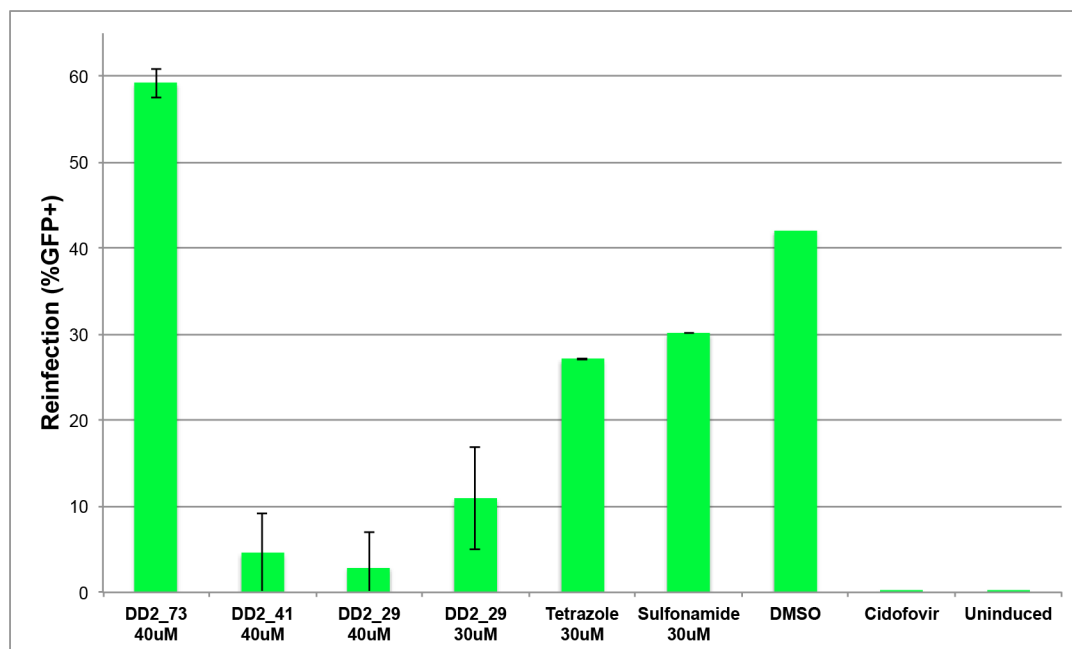


Figure 9-3. Refection bar plot. A bar plot of extent refection measured as %GFP+ cells in the presence of absence of compound. Cidofovir is used as a positive control and DD2-73 as a negative control.

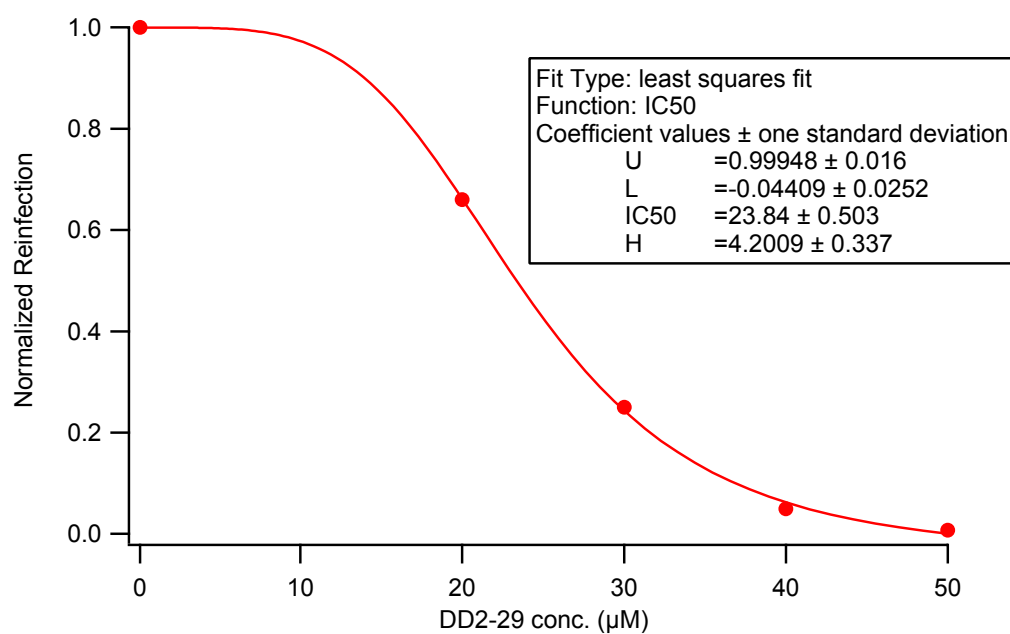


Figure 9-4. DD2-29 reinfection dose response. DD2-29 concentration plotted against normalized reinfection (relative to DMSO control). Least squares fitting estimates an IC₅₀ of 24 μM with a Hill slope of 4.

DD2

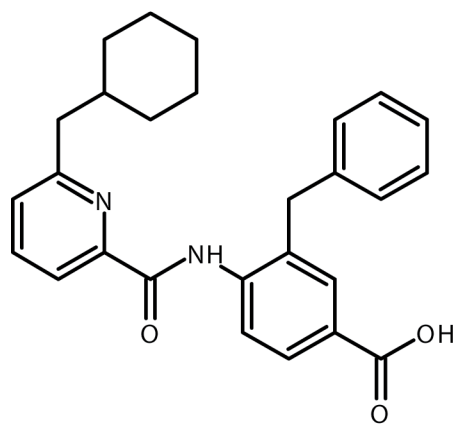
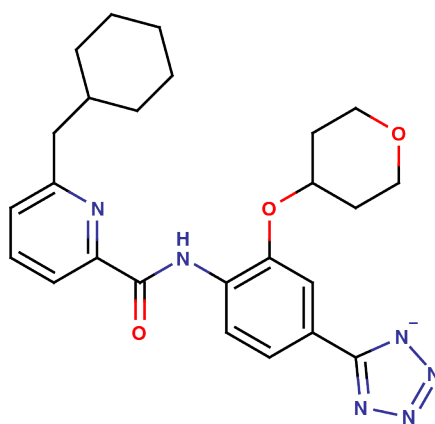


Figure 9-5: DD2 and DD2-29.

DD2-29



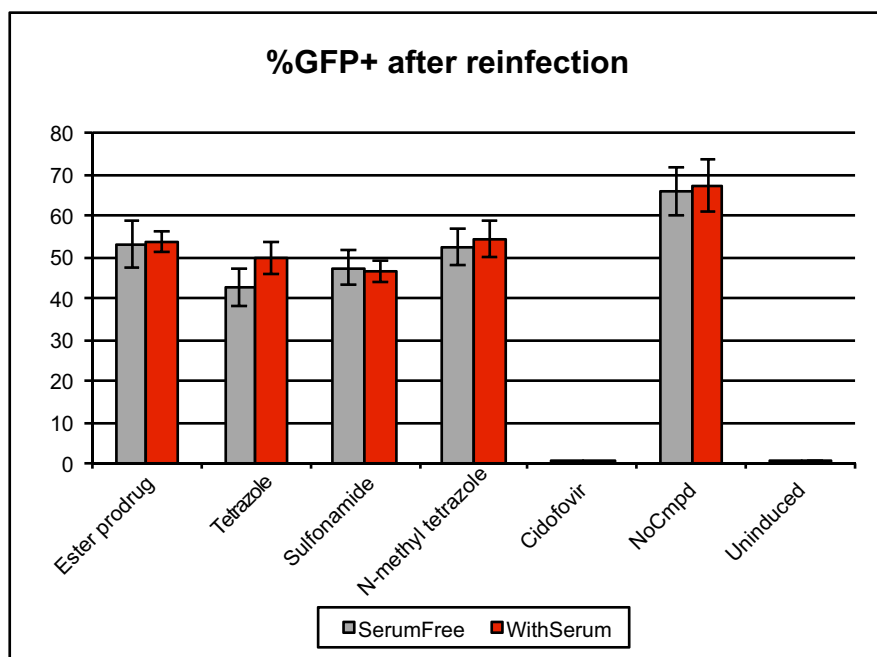


Figure 9-6. Reinfection with and without serum during treatment. This bar plot shows the extent of reinfection after treatment with a number of inhibitors, where the Y-axis is percent GFP+ cells. The serum-free condition involved pre-treatment with compound for 2 hours in the absence of serum followed by an additional 46 hours treatment and induction with serum.

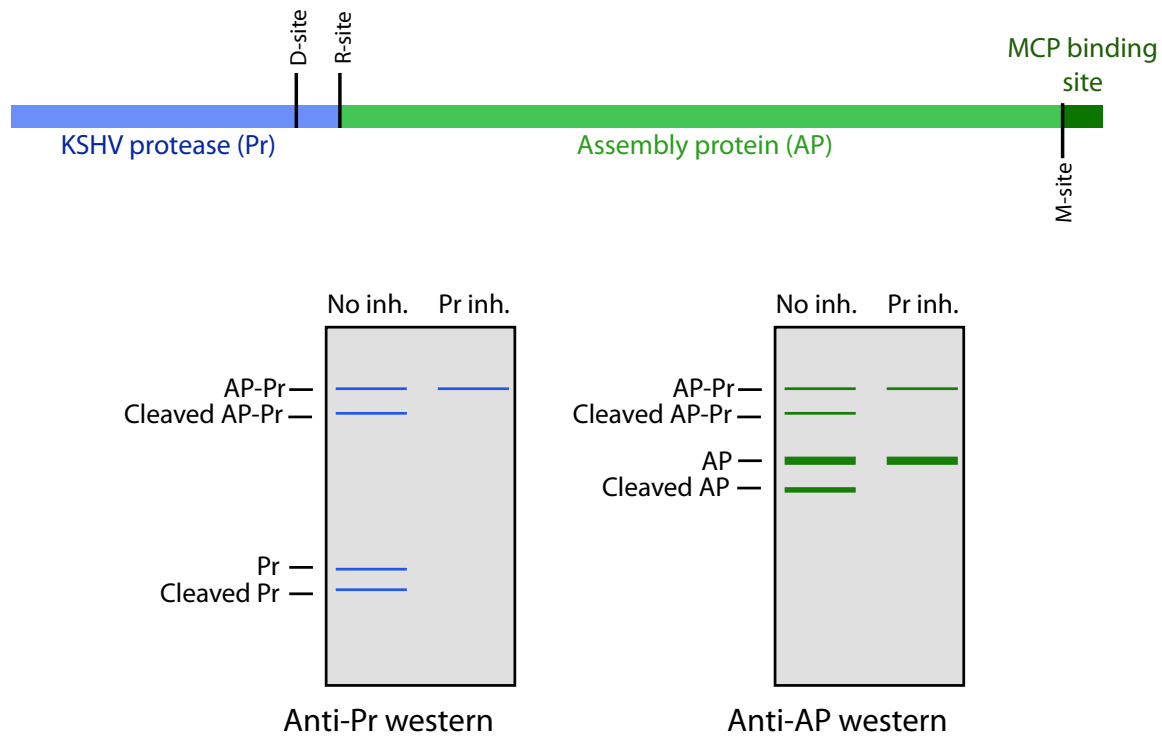


Figure 9-7. Theoretical western blot outcomes. The protease cleaves the AP-Pr fusion at three sites, the D-site, R-site, and M-site (top). Probing with anti-Pr antibody should yield a blot similar to the diagram on the left in the presence and absence of 100% protease inhibition. Probing with anti-AP antibody should yield a blot similar to the diagram on the right.

**Serial dilution of recombinant KSHV Pr probed with
Protein A-purified anti-protease rabbit bleed**

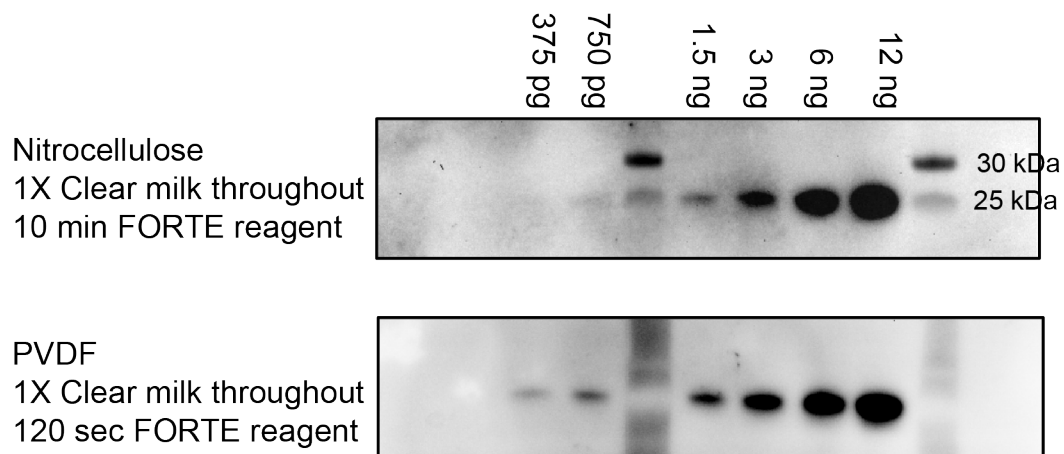


Figure 9-8. Anti-protease western blot optimization.

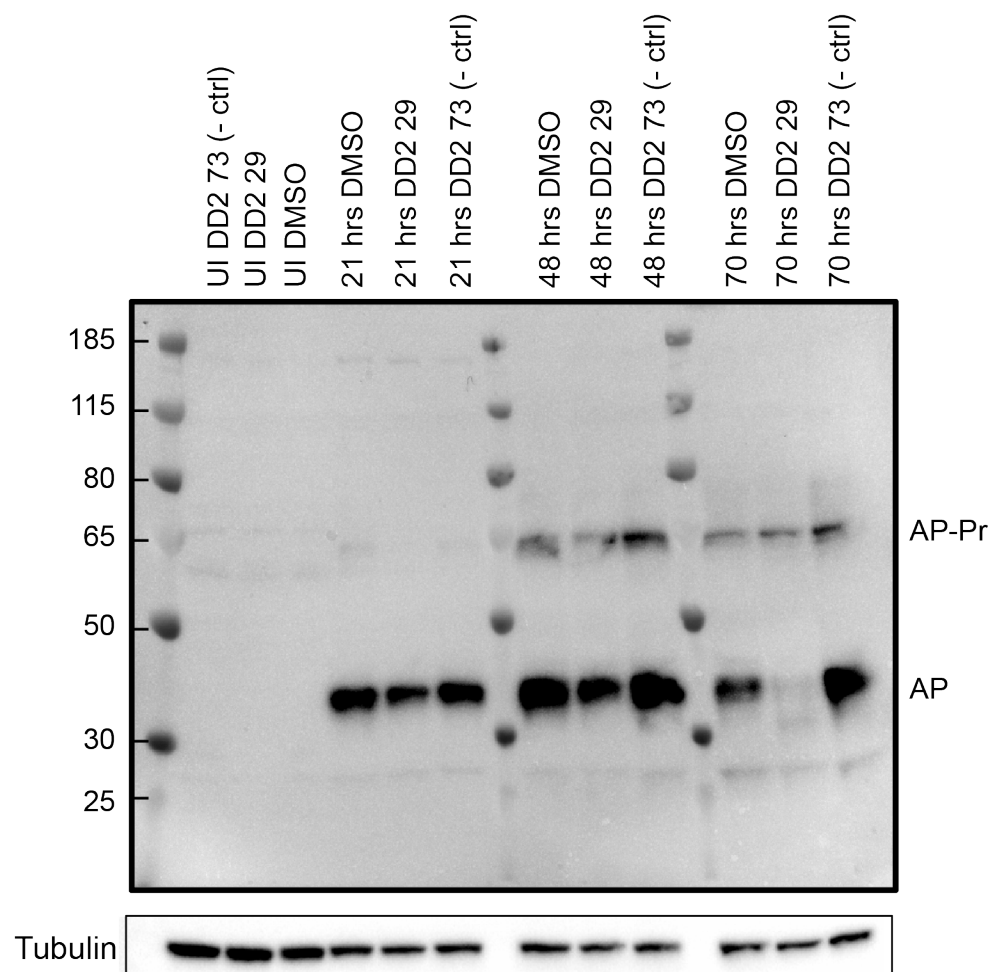


Figure 9-9. Anti-AP western time course.

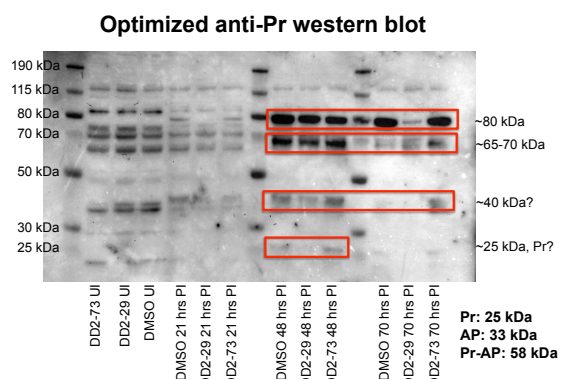


Figure 9-10. Anti-protease western time course.

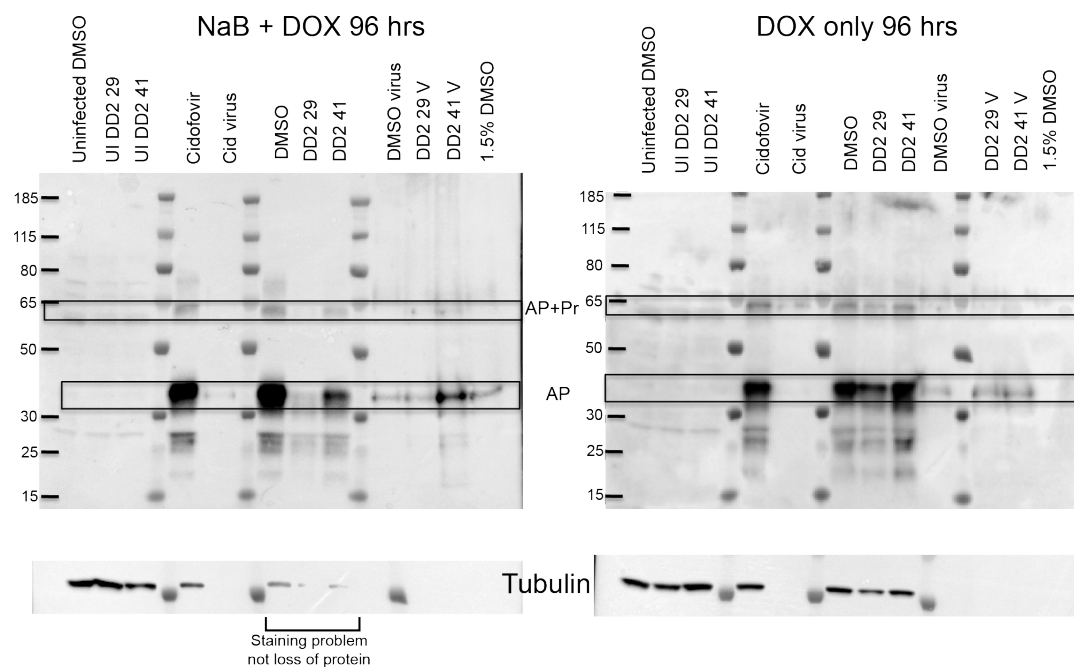


Figure 9-11. Anti-AP western varying treatment and induction strategy.

Supplemental material

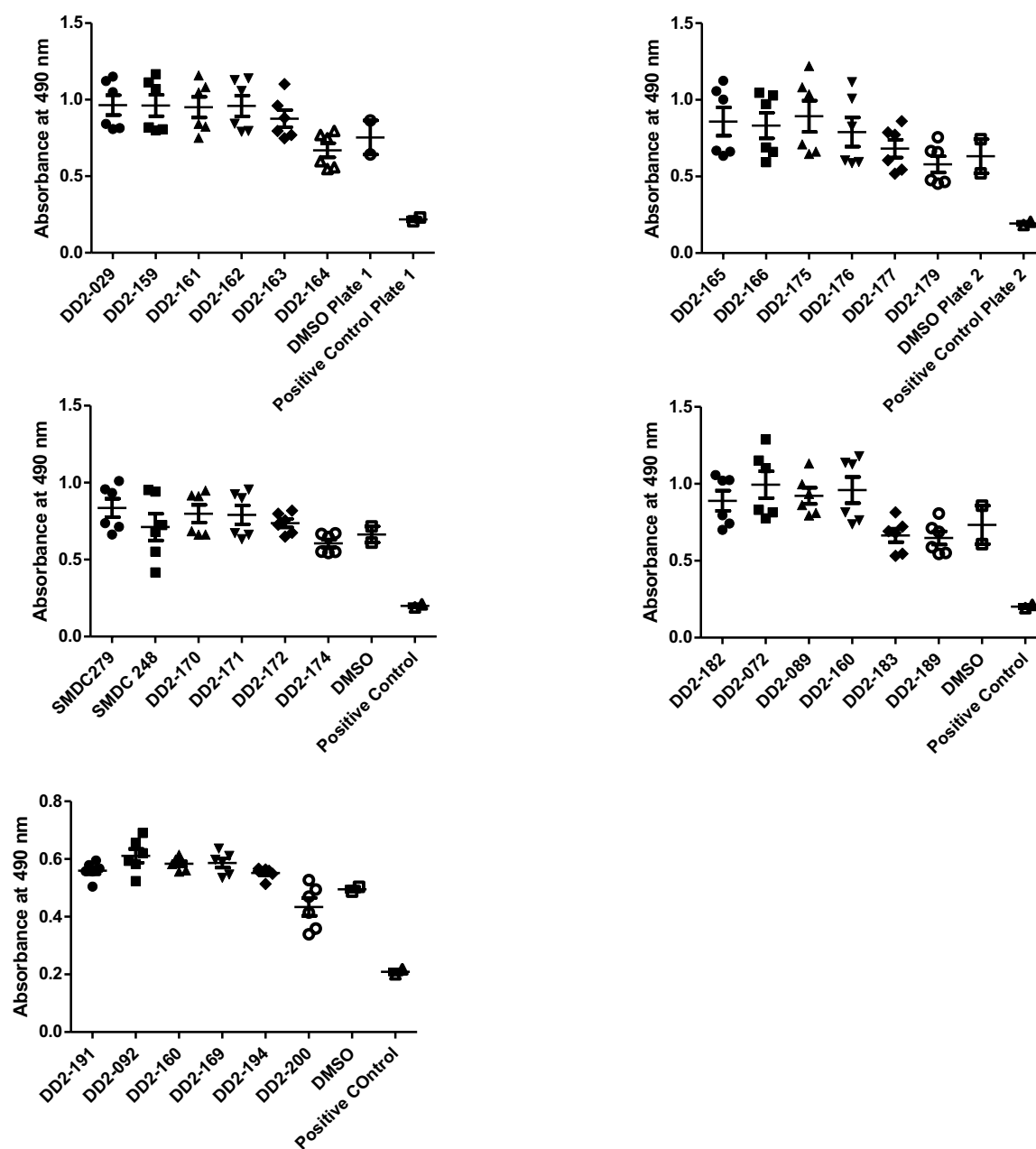


Figure 9-S1. Summary of MTS assay data.

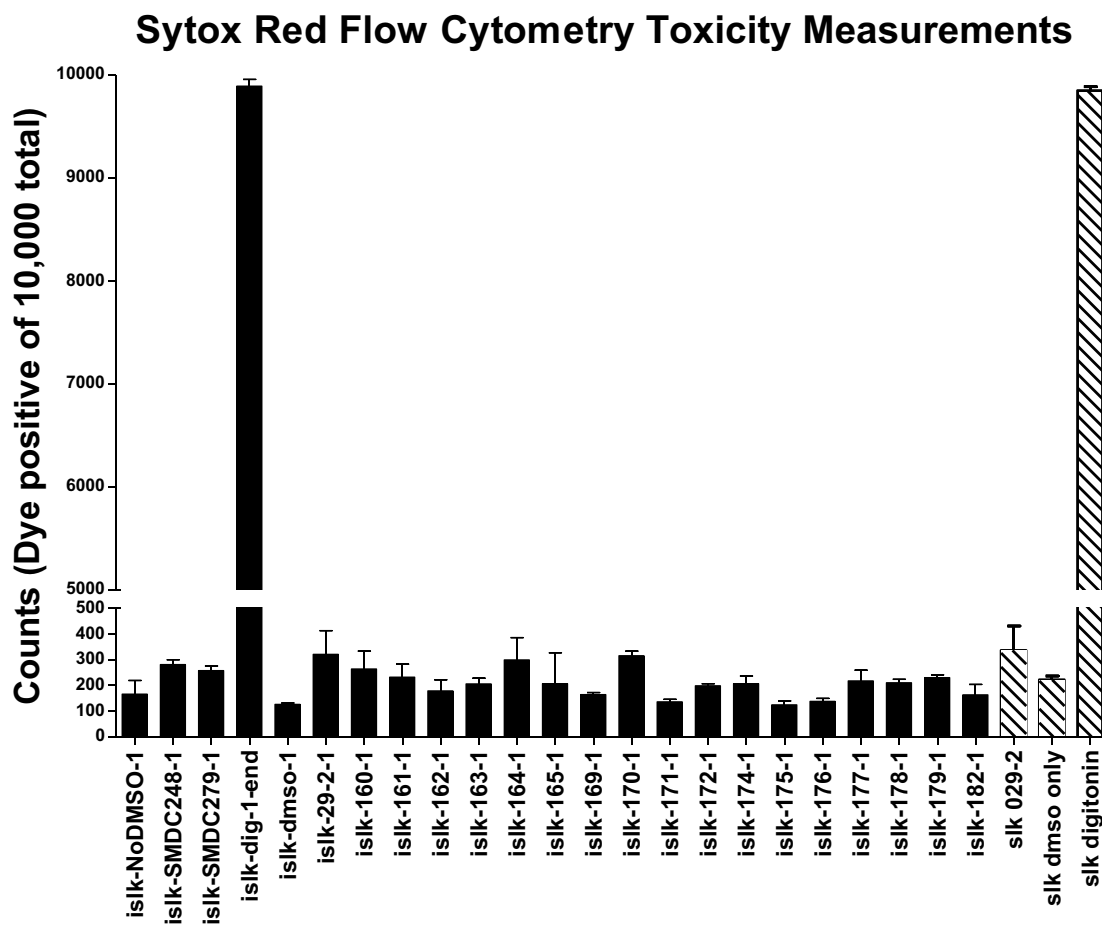


Figure 9-S2. Summary of Sytox red data.

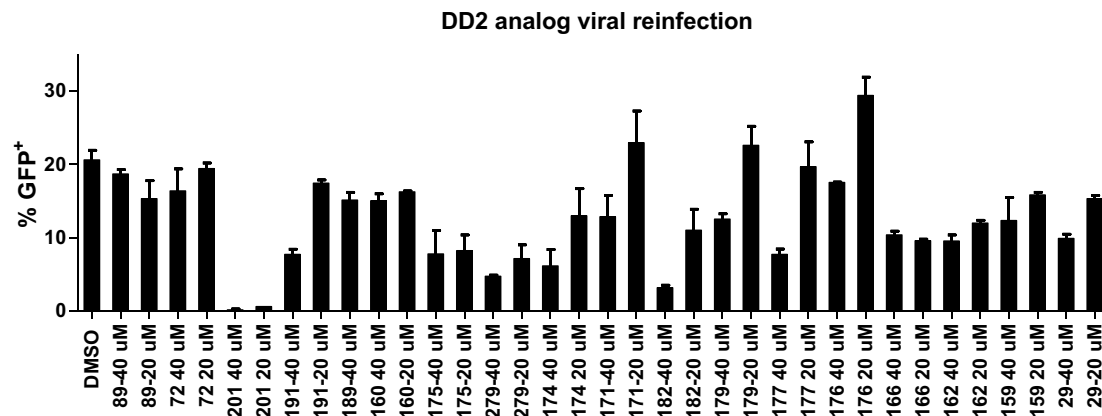


Figure 9-S3. Summary of reinfection data.

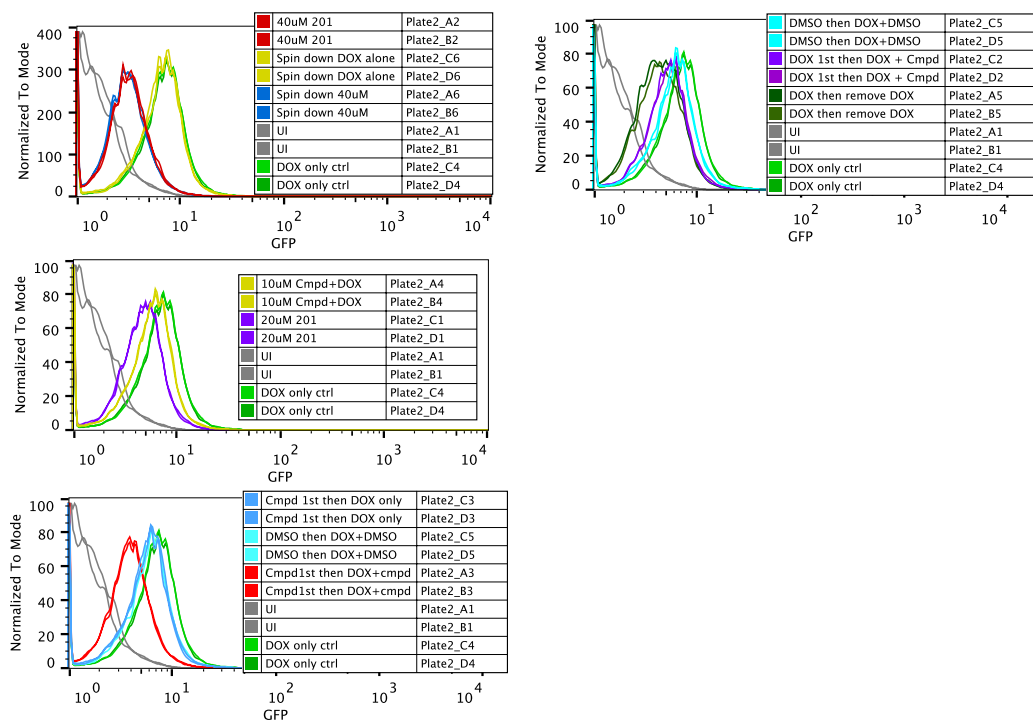


Figure 9-S4. HEK293 dox-inducible GFP flow data.

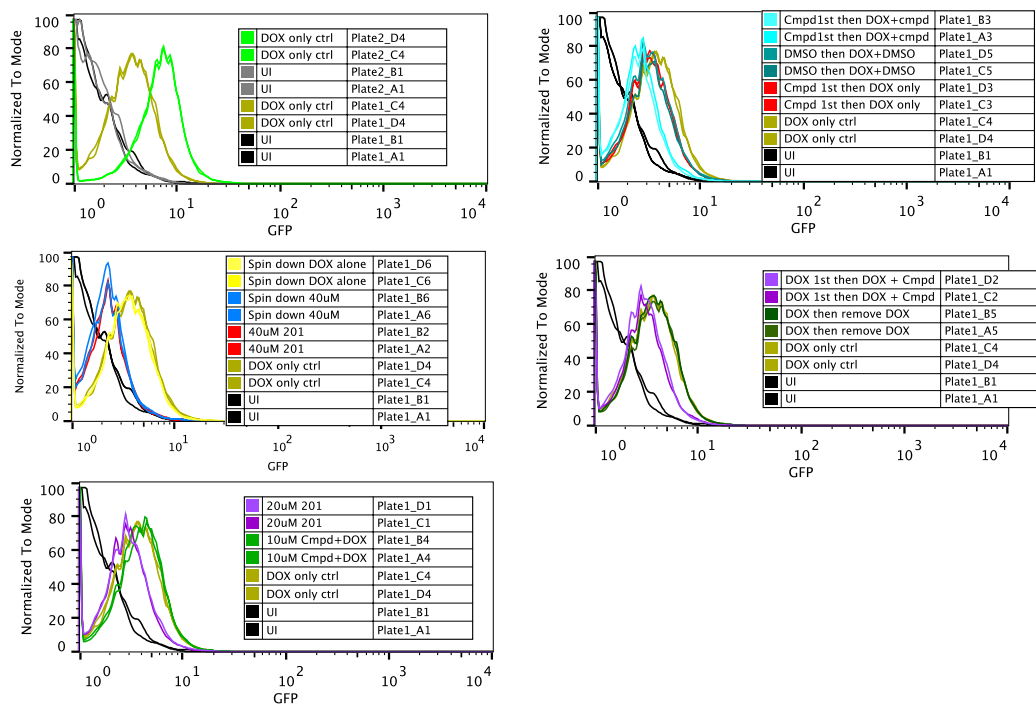


Figure 9-S5. HEK293 dox-inducible GFP flow data continued.

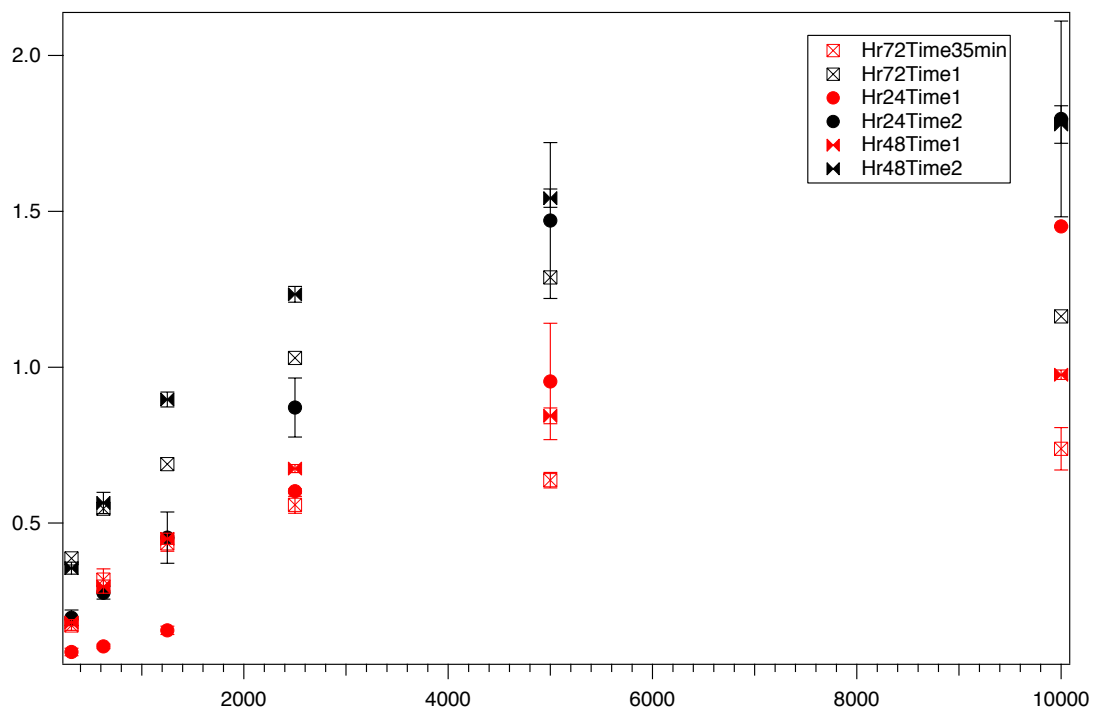


Figure 9-S6. MTS assay cell number optimization.

Concluding remarks

This work was motivated in large part by two factors. First was the goal of better understanding how to develop protein-protein interaction antagonists in general, and for the herpesviruses specifically. Second, was the desire to validate and form the groundwork for allosteric inhibition of the herpesvirus protease as a novel approach to treatment. Both goals start with and are informed by over a decade of prior research on the herpesvirus proteases establishing their unique structure and compelling communication between the dimer interface and the active site of each monomer. This dissertation extends that work, particularly in understanding the DD2 pocket and how binding to this pocket results in inhibition of KSHV protease. Chapters 6 and 7 reveal that DD2 and its analogs are broad-spectrum inhibitors of the herpesvirus proteases, inhibiting enzymes across all three subfamilies. It also reveals with atomic resolution the interactions that are made in the KSHV interfacial pocket and how the pocket is dynamic, allowing it to accommodate a variety of substantial changes to the original DD2 scaffold. Importantly inhibition through binding this pocket is time-dependent and appears to operate through an induced fit model; an initial complex between the protein and small molecule slowly converts to the inactive state on the minute time scale and the rate at which this slow step occurs is compound-dependent. Chapter 4 shows the “druggability” of a protein-protein interface and highlights the utility of fragments in inhibitor development for challenging targets. In a broad sense and addressing the first goal, these results further support the importance of dynamics and cryptic binding sites in targeting protein-protein interfaces. While there may not appear to be a pocket for a small molecule, protein motion may reveal one or binding of a small molecule may induce one. In the case of KSHV and DD2 it appears that DD2 selects for the monomer (arguably a process of conformational selection) and induces either the pocket and/or

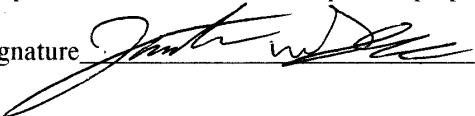
the inactivation of the enzyme. Dynamics of a protein-protein interaction and structural changes between the apo and complexed states should be examined for any protein-protein interaction target. The value of biased libraries is also apparent. A 183-compound library identified DD2 while subsequent screening of over 100,000 compounds failed to identify a comparable inhibitor. Fragments may be an ideal option when no rationally biased library is available. Even with this progress questions still remain. We have only a qualitative coarse-grain idea of where and how DD2 analogs bind to herpesvirus proteases other than KSHV protease. The physical basis for time-dependent inhibition remains elusive. Significant improvement in potency has not yet been achieved, though a clear definition of potency is arguably lacking as well.

The second goal requires a potent cell permeable dimer disruptor of at least one herpesvirus protease to enable pharmacological validation via protein-protein interaction antagonism. In light of the limited permeability of DD2 we established and implemented screens against both KSHV and CMV proteases to identify novel inhibitors (Chapters 2-5). These screens informed our understanding of the enzyme and also identified novel fragment-based dimer disruptors of KSHV protease and a novel mixed inhibitor of CMV protease. In doing so these results inform our first goal while furthering the second. Ultimately it was modification of DD2 that led to our most compelling cellular data to date. For the first time we have shown inhibition of viral reinfection in human cells using a small molecule dimer disruptor of KSHV protease. We are extremely excited about these data, but the story is not yet over. Our data fall short of proving our inhibitor is hitting the protease in cells, though we do rule out a number of off-target effects. Overall this text is an important step forward toward the larger scientific goal of modulating protein-protein interactions with small molecules and the larger medical goal of developing novel treatments for the human herpesviruses.

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.

Author Signature  Date 2015.5.18