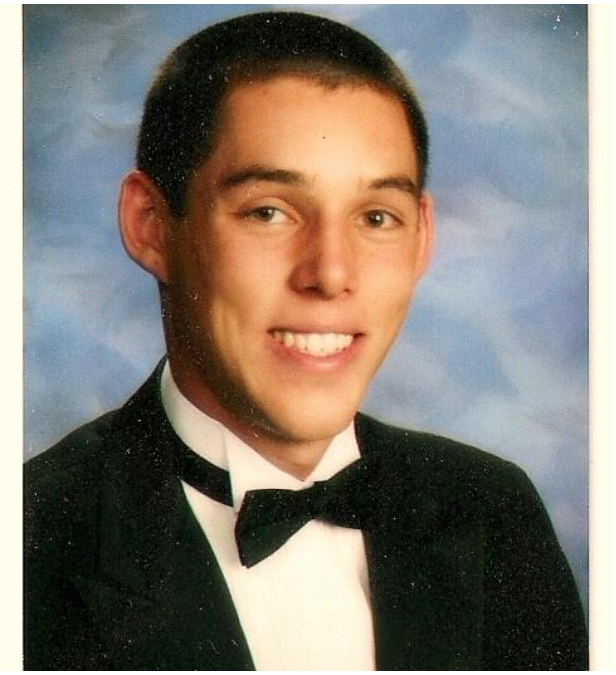

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Research Review: Recent Progress in HIV Entry Inhibition

Abstract:

Research into HIV entry inhibitors has made monumental progress over the past decade particularly in the development of highly active small-molecule fusion inhibitors, receptor antagonists, antibody-based inhibitors, and non-peptide inhibitors. Each of these inhibitor designs marks a different approach to blocking HIV cell entry, yet each has proven to be as effective, or even more effective, at neutralizing multiple strains of HIV-1. In addition, many of the recently designed entry inhibitors have shown excellent inhibitory efficacy against T20-resistant strains of HIV- T20 is one of the three HIV entry inhibitors approved for clinical use. Moreover, extensive research has been completed to study the effects of combining multiple entry inhibitors that target multi-step entry; such linking of inhibitors has generated complexes that demonstrate orders-of-magnitude greater potency in comparison to the individual inhibitor subunits. These results exemplify the rapid improvements being made in the field of HIV research; however, they also emphasize the leaps in knowledge that need to be made for successful synthesis of an escape-proof entry inhibitor.

Introduction:

An impervious, escape-proof HIV inhibitor has yet to be discovered, or engineered; however, many scientific groups continue to expand the possibilities of HIV inhibition through cutting-edge research. Retroviral pathogenesis of HIV can be targeted at multiple stages of viral entry, replication, and release, with one of the most promising points of sequestration being the initial interaction between and envelope-mediate fusion of the HIV virion with the CD4+ Helper T cell. Currently, HIV entry inhibitors range from small lectins found in algae, to antibody based peptides, to elemental nanoparticles, and each targets a specific step, or multiple sequential steps, in the HIV entry process. Currently, only two HIV entry inhibitors, T20 and maraviroc, have been approved for clinical use (1), which exemplifies the incredible opportunity for continued advancement in the field of HIV entry inhibitors. Here, the most recent innovations in the area of HIV entry inhibitors will be discussed and analyzed for trends, gaps, and potential for future exploration.

Small-Molecule Fusion Inhibitors Targeting gp120 and gp41:

The molecular processes involved in HIV cell entry are well understood and widely accepted (1). The process of cell entry involves initial attachment to the surface of the CD4+ Helper T cell through direct interaction with the CD4 cell-surface receptor, and co-receptor, CXCR4 or CCR5. The gp120/CD4 and gp120/CCR5 or CXCR4 interactions facilitate a conformational change of the trimeric gp120 that exposes gp41. The lipophilic terminal region of gp41 inserts into the plasma membrane of the target cell and performs a critical step in viral envelop-membrane fusion. The mechanism is well-understood and involves the formation of a six-helix bundle via intimate interaction between C-terminal and N-terminal heptad repeats (2,7). A number of fusion inhibitors prevent HIV from binding the CD4 receptor and CCR5/CXCR4 co-receptors in a competitive manner through direct interaction with the HIV envelope glycoprotein gp120 (2-6,10), thereby blocking conformational changes necessary for envelope-mediated fusion. Other entry inhibitors bind directly to gp41 (1,8,9,11) and prevent formation of the six-helix bundle required for viral entry through high-affinity interac-

tion with the C-terminal heptad repeat or N-terminal heptad repeat of the trimeric gp41. By binding to random sequences within gp120 and gp41, it is fully understood that HIV will acquire mutations that confer resistance to the inhibitor, thereby enabling HIV to escape inhibition (1). Although the general structure and sequences of gp41 and gp120 are known, it is not understood which sequences within the HIV envelope are absolutely vital for cell entry- meaning HIV cannot mutate these sequences and still maintain infectivity and virulence. If these heavily conserved sequences were identified, entry inhibitors could be designed to bind these sequences, neutralize HIV, and minimize antigenic drift; however, the field does not know what these sequences are, or if they even exist.

Fusion Inhibitors targeting gp120

Five of the most recent and effective gp120-binding inhibitors are the carbohydrate-binding protein Griffithsin (2), Poly (4-styrenesulfonic acid-co-maleic acid) (3), the natural bacterial antibiotic peptide ferglymycin (4), lignosulfonic acid (5), and the platelet-derived chemokine CXCL4 (6). These molecules exhibit high efficacy by binding directly to conserved gp120 amino acid sequences (Griffithsin binds saccharides comprising envelope glycoproteins covalently linked to the conserved amino acid sequences) while also demonstrating inhibition at sub micro-molar to nano-molar levels (2-6). Still, these molecules have not shown the ability to maintain a high affinity for gp120 following single point-mutations within HIV gp120 gene sequence (2-6). As a result, more research needs to be done to develop gp120-binding inhibitors capable of binding gp120 even after multiple mutations have been introduced into the peptide sequence.

Fusion Inhibitors Targeting gp41

The second point in the HIV cell-entry process that is at the center of much of the fusion inhibitor research is gp41-mediated membrane fusion. So far, only one entry inhibitor targeting gp41 has been approved for clinical use- T20 (1); however, second (T1249) and third generation (T2635) peptides derived from T20 have been developed, with the most recent being T2635. T2635 exhibits high affinity for the C-terminal heptad repeat of gp41, yet the most interesting results indicate that, unlike T20 and T1249, T2635 can withstand multiple mutations within the amino acid sequence of gp41 and still bind with high affinity (8). This is an incredible improvement in terms of fusion inhibitors because, through analysis of the peptide interactions between T2635 and the gp41 binding pocket, conserved residues necessary for gp41-mediated fusion have been identified (8). These residues are very difficult for HIV to alter because their chemical characteristics are required for membrane interaction and six-helix bundle formation. As such, mutations within the C-terminal heptad repeat of gp41 meant to escape T2635 also result in a significant decrease in viral fitness and infectivity (8). In addition, mutating around T2635 also requires consequential mutations in gp120 following gp41 sequence alteration. Therefore designing inhibitors that target the residues forming the core of the gp41 deep-

binding pocket is an effective approach to blocking HIV cell entry and successively altering HIV virulence. Continued development of T20-based peptides should prove to be a powerful approach to combating HIV pathogenesis.

A newly designed gp41-binding inhibitor, CP32M, has also been developed and tested experimentally for inhibitory activity. CP32M showed excellent inhibitory activity at low nanomolar concentration (9), but, by far, the most significant findings resulting from the CP32M experiments were of structural nature. CP32M was shown to form inter-helical and intra-helical salt bridges with the N-terminal heptad repeat of gp41, which increased the stability of the inhibitor-gp41 interaction. By increasing stability through formation of salt bridges, the inhibitor becomes much more mutation resistant and makes considerably greater affinity contact with gp41. The aforementioned gp41 residues participating in salt bridge formation were identified through structural analysis, NMR, thus providing the field with additional targets for inhibitor design. Much like sequences targeted by T2635, the CP32M-targeted sequence is evolutionarily conserved as a direct result of its importance to gp41-mediated membrane fusion. Moreover, both CP32M and T2635 exhibit high inhibitory activity against T20-resistant strains of HIV-1 (9), meaning these next-generation HIV entry inhibitors may soon enter clinical trials. Although T2635 and CP32M exemplify outstanding advancements in the understanding of gp41-mediated resistance, the role of each residue within the gp41 primary structure is not fully understood; so, if an escape-proof inhibitor targeting gp41 is to be successfully synthesized, much more information pertaining to gp41 sequence-function relationship needs to be gathered.

Targeting Multiple Steps of Viral Entry

Dual receptor antagonist peptide triazoles (PT) have also exhibited high affinity binding resulting in increased inhibitory activity. PTs bind to a conserved sequence that overlaps with the CD4 binding site of gp120 with very high affinity (10). This interaction allosterically induces a conformational change in the co-receptor binding site, which is located in a region of gp120 distinct from the CD4bs, that inhibits interaction between gp120 and the CCR5 co-receptor. By blocking two-steps of viral entry, CD4 binding and co-receptor binding, peptide triazoles have demonstrated a greater ability to inhibit viral entry than small-molecule inhibitors that target single steps in the viral entry process (10,2,1).

In addition to individual gp120- and gp41-binding inhibitors, experiments utilizing linked fusion inhibitors have shown promise in blocking envelope-mediated fusion between HIV and the cell membrane. Such linked fusion inhibitors target multi-step HIV entry by binding various combinations of gp120, gp41, and co-receptors CCR5 and CXCR4, which generates dramatic increases in potency-- effective at nano-molar to mid-picomolar concentrations (2,11). For instance, four T20 peptides were linked to an IgG antibody, tested for inhibitory efficacy using viral activity assays and cell-cell assays, and compared to the effectiveness of an indi-

vidual T20 peptide. The results indicated the T20 tetramer was 100-fold more powerful at blocking HIV cell entry than its individual subunits (11,1). In another study, the lectin Griffithsin was covalently linked to the C-peptide C37, which showed eight-fold higher potency than Griffithsin or C37 alone (2). These findings, along with the result depicting the outstanding inhibitory activity of linked antibodies, which will be discussed later, illustrate a powerful direction in the future of HIV entry inhibitory design. At the same time, it is not yet known if linking the most effective gp120 inhibitors to the most potent gp41 inhibitors will prove to be the elusive escape-proof fusion inhibitor- for this goal, more research needs to be done to identify residues that HIV cannot mutate in order to maintain virulence, as well as to identify the most effective fusion inhibitor combinations.

Co-Receptor Antagonists:

Co-receptor antagonists act as competitive inhibitors to block HIV interaction with co-receptors CCR5 and CXCR4. Many co-receptor antagonists have been developed, but only one has been approved by the FDA for clinical use- maraviroc (12). As with the fusion inhibitors discussed previously, HIV can acquire mutations that promote resistance to the inhibitor, and, because deep knowledge of heavily-conserved envelope sequences have not been identified, current receptor antagonists become ineffective after single point mutations in HIV envelope glycoproteins (13). Since the structures of CCR5 and CXCR4 are well understood, co-receptor inhibitor designs are making rapid progress and a large number of inhibitors are currently in existence, or being improved to withstand multiple point mutations at the inhibitor binding site. These include: natural chemokine ligands MIP-1a, MIP-1b, and RANTES, monoclonal antibodies, small-molecule quinolone, pyrrole, piperidine, and propane-diamino bridge derivatives (12). These R5-tropic receptor antagonists all demonstrate IC50 values within the low nanomolar range but are susceptible to mutations in the V3 loops of gp120 that enable HIV to interact with CCR5 regardless of the presence of receptor antagonists (13). It is not known if CCR5 receptor antagonists can be developed to withstand HIV evasion or if they can function as small molecule entry inhibitors beyond simply competitive inhibitors- specifically, covalent inhibitors.

While CCR5 receptor antagonists have made substantial progress and many are in the various stages of clinical trials, CXCR4 receptor antagonist development is not as rapidly progressing. HIV-1 strains exhibiting R4-tropism begin to appear at the later stages of HIV-1 infection, while R5-tropic strains are characteristic of early infection. Consequently, by the time R4-tropic viral strains are generated, HIV exists as a mixture of R5- and R4-tropic strains. Inhibition is just the R4 component would not effectively block HIV-1 cell entry and replication, so both CCR5 and CXCR4 receptor antagonists would need to be used to successfully combat HIV in mid-to-late stage infection (12). At this point in time, CCR5 and CXCR4 inhibitors have not been tested in combination, thus research has not yet shown this combination to be safe or effective (12).

Antibody-based inhibitors:

Over the past decade, monoclonal antibodies have been studied for inhibitory efficacy against HIV-1; however, numerous findings indicate that HIV has evolved methods to evade antibody-mediated neutralization, or humoral immunity (14). Accordingly, monoclonal antibodies have the capacity to protect the host during the initial stages of infection, but are not effective when used for therapy of an established infection due to viral evasion. One of the primary reasons full-size human antibodies are unable to bind HIV envelope glycoproteins, gp120 and gp41, results from steric hindrance within close proximity of conserved sequences necessary for HIV cell entry (15). As a result, Chen et al. has conducted fairly extensive research into antibody-based therapeutics with the goal of overcoming the steric effects preventing full-size antibodies from binding envelope glycoproteins. Perhaps the most innovative and potentially useful of these antibody-based inhibitors are the engineered antibody domains (eAds), which are derived from hypervariable Ig domains, or CDR regions, yet lack the large constant Fc and constant Fab regions common to naturally occurring antibodies. The small size of the eAds enables the inhibitor to contact conserved sequences of HIV envelope glycoproteins that are inaccessible to larger, native-form antibodies. Consequently, a drastic improvement in the capacity to block HIV entry through antibody-based inhibitors has been observed in multiple studies (14-19). Due to the specificity, diversity, and massive hypervariability potential of antibody domains, eAds may serve as the principal approach to blocking HIV entry.

Past research (14-19) has concluded that due to the spatial arrangement of epitopes on the surface of the HIV virion, interaction between antibody Fab regions and binding multiple envelope glycoproteins is severely hindered. The inability to maintain high affinity contact causes the antibodies to simply release from the HIV surface, which essentially places the humoral branch of the adaptive immunity in a state of inactivity. To solve the problem of absent polyvalency, a number of polyvalent side chain peptide-synthetic polymers designed to aid in antibody binding at the gp120 CD4 binding site have been developed (18). Upon gp120 binding, polyvalent polymers act as functional antigens capable of interacting with antibodies containing hypervariable regions specific for the side-chains exposed on the surface of the polymer, therefore promoting HIV neutralization. By functioning as both a direct inhibitor of CD4 interaction and as an adaptor for subsequent antibody opsonization, polyvalent synthetic polymers show promise for possible implementation with other HIV entry inhibitors.

Returning to the topic of linked inhibitors, Chen et al. has experimented with various antibody combinations that induce conformational changes within HIV envelope glycoproteins, one of which incorporates two gp120-binding antibodies- CD4 induced (CD4i) Ig and CD4 binding site(CD4bs) Ig. In the study, a primary antibody, CD4i, was designed to make high affinity contact with the CCR5/CXCR4 co-receptor binding site on gp120- the co-receptor binding site is not impacted as greatly by steric effects, which allows for antibody

binding. CD4i binding subsequently induced a conformational change within gp120 that exposed the epitopes of the CD4 binding site (CD4bs) and pointedly decreased steric hindrance at this site, thereby allowing the secondary CD4bs antibody to bind tightly to the newly exposed CD4 binding site. By linking anit-CD4i and anti-CD4bs, the two antibodies work in combination to stimulate conformational changes with gp120, permitting antibody binding to epitopes in the viral envelope that were previously inaccessible (19). This combinatorial approach makes antibody-based entry inhibitors strikingly more applicable than previously understood; however, it is still to be determined whether or not this antibody-induced conformational change is possible at other sites on the viral envelope because steric hindrance and HIV evasion are still major issues yet to be solved completely. Moreover, the utilization of eAds, polyvalent synthetic polymers, and antibodies functioning in combination with one another has not been attempted, but, based on the previously described studies outlining the increased potency of inhibitor permutations (2,10,11), it would be feasible to test the antibody-based inhibitors in combination with one another.

Non-peptide Inhibitors:

Aside from the traditional small-molecule and peptide inhibitors, HIV cell entry can also be inhibited using a novel non-peptide inhibitor, dihydrosphingopeptides (20). Up to this point, HIV entry inhibition research has focused on blocking gp120 and gp41 interaction with cell-surface receptors; however, one group has looked deeper into the fact that envelope-mediated fusion involves the interaction between envelope and plasma membrane lipids, the first of which, is rich in dihydrosphingomyelin. This recent study analyzed the effectiveness of a dihydrosphingosine-based inhibitor at blocking membrane fusion, and suggests that during the process of membrane fusion, the dihydrosphingopeptide is recruited and incorporated into the fusing lipid bilayers, which in turn prevents melting of the two membranes. By targeting a process of membrane fusion that is independent of gp120 and gp41, dihydrosphingopeptides inhibit cell entry of T20-resistant strains of HIV (20), therefore suggesting an alternative approach to avoiding mutation-dependent HIV resistance. The fascinating aspect of the aforementioned sphingopeptides is that the peptides alone have no antiviral activity; however, when complexed with dihydrosphingosine, the sphingosine-peptide complex demonstrates high antiviral activity at low nanomolar levels (20). Numerous other studies (21-24) have also looked into the lipid-mediated mode of inhibition, and all emphasize the importance of viral sphingolipids in membrane fusion. These results suggest that lipid-based HIV entry inhibitors may provide an effective method for future inhibitor design, yet far more research must be done to determine how effective sphingolipid analogs can be, if they can resist HIV mutations, and if they can safely be used in combination with other entry inhibitors.

Conclusion:

Recent progress in the field of HIV entry inhibition yielded vital information explaining, both chemically and mechanistically, how to design increasingly effective entry inhibitors. Two apparent consensuses are developing in the field: 1) fused inhibitors targeting multi-step entry show much higher antiviral activity than individual inhibitors, and 2) the highly-conserved sequences of HIV envelope glycoproteins need to be identified in order to generate escape-proof inhibitors. Nevertheless, major questions remain unanswered. For instance, are non-peptide inhibitors that directly target membrane fusion capable of resisting HIV mutations? Can sphingopeptides work in combination with other entry inhibitors? What are the functional consequences of linking eAds with receptor antagonists and third generation fusion inhibitors such as T2635? Does HIV use non-mutable sequences capable of binding inhibitors? What are these sequences and are they buried determinants? These questions will need to be answered if we are to permanently neutralize HIV at the envelope-membrane interface prior to cell entry.

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    a. Reviews the trends, breakthroughs, and gaps in HIV entry inhibition research up to 2012, which offers the scientific field direction on how and where to focus future research. The author centers the majority of attention on small-molecule/peptide fusion inhibitors targeting gp41 and gp120, while also touching on receptor antagonists. Berkout et al. is a review, so not methods were explained. Berkout et al. provides background information necessary to establish a point of reference and comparison necessary for the explanation of more recent fusion inhibitors, especially those not discussed in this review (eAds and non-peptide inhibitors).

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    a. Primary investigator, Liwang, utilized cell and viral activity assays to provide substantiation suggesting the increased inhibitory activity of fused entry inhibitors targeting multiple steps of HIV cell entry. The fused inhibitors, Griffithsin and C37, demonstrated several-fold greater inhibitory activity than the individual inhibitors. Dr. Liwang's results emphasize a new method of inhibiting envelope-mediated fusion that the rest of the field could also employ when designing entry inhibitors. In combination with other studies, Liwang et al. signifies a trend in inhibitor design that is strongly leaning towards blocking HIV cell entry by means of combinatorial, sequentially-acting, multi-step inhibitors, as is discussed in the review.

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    a. The purpose of the study was to determine the inhibitory efficacy and mechanism of action of Poly (4-styrenesulfonic acid-co-maleic acid) against HIV-1. PSM acts to inhibit HIV cell entry at sub-nanomolar levels by directly interacting with the CD4-binding site located on gp120. PSM shows low cytotoxicity, which means that it has the potentially to be a fairly safe drug that causes limited side effects. Provides an alternative method for HIV entry inhibition that uses a more non-traditional small molecule that blocks CD4 binding, while also contributing to the explanation of gaps in knowledge regarding what other possible molecules could be used to inhibit HIV cell entry.

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a. The study examined the effectiveness of the bacterial antibiotic peptide Feglymycin at inhibiting HIV cell entry. Viral activity assays and cell-cell transfer assays showed that feglymycin maintains function at low micro-molar concentrations, and SPR analysis indicated that the peptide potentially interacts with gp120; however, this is not certain. The discovery that feglymycin shows some anti-viral activity suggests that other antibiotics may also exhibit antiviral activity, which ultimately broadens the range of possible inhibitors available for experimentation. In terms of the review, feglymycin serves as an example of a very recent gp120-binding entry inhibitor that is not fully understood with regards to mechanism and potential.

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    a. The purpose of the study aimed at analyzing the effectiveness of the lignosulfonic acid at inhibiting HIV cell entry. Viral activity assays and cell-cell transfer assays suggested that Lignosulfonic acid exhibits antiviral activity at nanomolar levels, while also showing low cytotoxicity. It was found that lignosulfonic acid acts by blocking both the CD4-binding site and co-receptor binding sites on the gp120 envelope glycoprotein. This inhibitor supports the claim, as stated in the review, that targeting multiple steps of HIV cell entry is much more effective than targeting individual steps. For the remainder of the field, lignosulfonic acid can be incorporated into other inhibitor designs or simply used as a model depicting domains susceptible to inhibition.

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    a. The purpose of the study was to test the inhibitory efficacy of the chemokine CXCL4. CXCL4 inhibits a broad spectrum of HIV-1 isolates at low nanomolar levels and acts as both a competitive inhibitor of the CCR5/CXCR4 co-receptor and as a direct gp120-binding inhibitor. This endogenous inhibitor is not limited to R4- and R5-tropic HIV subtypes, meaning that it exhibits a broad neutralizing capacity that many other inhibitors lack. This may serve as the foundation for future research into cytokine-based HIV inhibitors. This is important to the research review because it presents an idea that is not being closely studied by other parties, which indicates the potential antiviral activity of chemokine-based inhibitors is just recently being uncovered.

7. Huarte, N., Lorizate, M., Perez-Paya, E., & Nieva, J. L. (2011). Membrane-transferring regions of gp41 as targets for HIV-1 fusion inhibition and viral neutralization. *Current Topics in Medicinal Chemistry, 11* (24), 2985-2996.

    a. Review

8. Eggink, D., Bontjer, I., Langedijk, J. P., Berkhout, B., & Sanders, R. W. (2011). Resistance of human immunodeficiency virus type 1 to a third-generation fusion inhibitor requires multiple mutations in gp41 and is accompanied by a dramatic loss of gp41 function. *Journal of Virology*, 85(20), 10785-10797. doi: 10.1128/JVI.05331-11

    a. The purpose of the study was to determine the inhibitory capacity of the third generation fusion peptide T2635, while also comparing the ability of T2635 to withstand point mutations in the gp41 C-terminal heptad repeat. Viral activity assays, in-vitro neutralization assays, and competition assays indicating a dramatic increase in the inhibitory activity of T2635 as compared to first- and second-generation C-peptides. Furthermore, it was found that HIV resistance to T2635 required multiple mutations within gp41, and compensatory mutations in gp120, that resulted in a sharp decrease in infectivity. For the field, these results suggest continued development of next-generation gp41-targeting entry inhibitors has the potential to improve both direct neutralization and viral escape. For the review, Eggink et al. provides indicates advancement in escape-resistance and inhibitor design, which is vital for the future development of HIV entry inhibitors.

9. Yao, X., Chong, H., Zhang, C., Qiu, Z., Qin, B., Han, R., . . . Cui, S. (2012). Structural basis of potent and broad HIV-1 fusion inhibitor CP32M. *The Journal of Biological Chemistry, 287*(32), 26618-26629. doi: 10.1074/jbc.M112.381079

    a. The study set out to deduce the antiviral activity of CP32M as well as its mechanism of action. The researchers found, by means of NMR, that CP32M forms intra- and interhelical salt bridges with conserved residues of the N-terminal heptad repeat of gp41. This promotes stable interactions between the inhibitor and gp41, thereby increasing the inhibitory activity and mutation resistance of CP32M. The determination of specific, conserved gp41 residues that participate in CP32M binding may contribute to future HIV inhibitor design. For the research review, this study supports the claim that targeting conserved gp41 residues comprising the N-terminal heptad repeat generates more potent and escape-resistant entry inhibitors.

10. Tuzer, F., Madani, N., Kamanna, K., Zentner, I., Lalonde, J., Holmes, A., . . . Chaiken, I. (2012). HIV-1 env gp120 structural determinants for peptide triazole dual receptor site antagonism. *Proteins,* doi: 10.1002/prot.24184; 10.1002/prot.24184

    a. The purpose of the study was to analyze the mechanism of action of peptide triazoles and to test the ability of dual receptor antagonist peptide triazoles to inhibit HIV cell entry. Results of ELISA screens, surface plasmon resonance, virus inhibition assays, competition assays, viral capture assays indicated that peptide triazoles bind to conserved residues in a region of gp120 that overlaps the CD4 binding site, which subsequently induces conformational changes in, or act allosterically on, the co-receptor-binding site. Knowledge that inhibitor binding at the CD4bs allosterically affects the co-receptor binding site exposes another point of inhibition in the viral entry process. In the review, the study further supports the consensus that inhibiting multiple steps of HIV entry is more effective than targeting single steps.

11. Chang, C. H., Hinkula, J., Loo, M., Falkeborn, T., Li, R., Cardillo, T. M., . . . Wahren, B. (2012). A novel class of anti-HIV agents with multiple copies of enfuvirtide enhances inhibition of viral replication and cellular transmission in vitro. *PloS One, 7*(7), e41235. doi: 10.1371/journal.pone.0041235

    a. The study aimed to test the inhibitory efficacy of multiple T20 molecules against HIV-1. In-vitro neutralization assays and inhibition of cell-cell HIV transfer assays showed that the T20 tetramer exhibited HIV neutralization at subnanomolar levels, which is 100 fold greater than a single T20 alone, as well as increased stability with mouse models. The study supports the current view, and one of the main ideas of the review, that linking multiple inhibitors together increases the neutralizing capabilities of the HIV entry inhibitor complex. The results also indicate that combining multiple peptides into an inhibitory complex increases the stability of the inhibitor, which is very important when considering progression into clinical trials and clinical use.

12. Chen, W., Zhan, P., De Clercq, E., & Liu, X. (2012). Recent progress in small molecule CCR5 antagonists as potential HIV-1 entry inhibitors. *Current Pharmaceutical Design, 18*(1), 100-112

    a. Review

13. Anastassopoulou, C. G., Ketas, T. J., Sanders, R. W., Klasse, P. J., & Moore, J. P. (2012). Effects of sequence changes in the HIV-1 gp41 fusion peptide on CCR5 inhibitor resistance. *Virology, 428*(2), 86-97. doi: 10.1016/j.virol.2012.03.008; 10.1016/j.virol.2012.03.008

    a. The purpose of the study was to determine the gp41 mutations that conferred HIV resistance to CCR5-Vicriviroc complex. Viral inhibition assays and quantitative models suggested that three mutations are critical for resistance- G516V accompanied by either M518V or F519I. Knowing that these residues are vital for HIV resistance gives a promising direction in future inhibitor design and may potentially lead to the discovery of other gp41 residues critical for CCR5 inhibitor resistance. In terms of the review, the results of the study exemplify progression in the knowledge of resistance to CCR5 inhibitors, and, specifically, the mechanisms by which resistance is conferred; however, this also introduces more gaps and questions that remain to be unanswered.

14. Chen, W., & Dimitrov, D. S. (2009). Human monoclonal antibodies and engineered antibody domains as HIV-1 entry inhibitors. *Current Opinion in HIV and AIDS, 4*(2), 112-117. doi: 10.1097/COH.0b013e328322f95e

    a. Review

15. Chen, W., & Dimitrov, D. S. (2012). Monoclonal antibody-based candidate therapeutics against HIV type 1. *AIDS Research and Human Retroviruses, 28*(5), 425-434. doi: 10.1089/AID.2011.0226

    a. Review

16. Gong, R., Wang, Y., Ying, T., & Dimitrov, D. S. (2012). Bispecific engineered antibody domains (nanoantibodies) that interact noncompetitively with an HIV-1 neutralizing epitope and FcRn. PloS One, 7 (8), e42288. doi: 10.1371/journal.pone.0042288

    a. The purpose of the study focused on generating stable CH2-based engineered antibody domains capable of binding HIV-1 isolates with high affinity. Multi-step PCR was used to recombinantly engineer the CH2 domain with an additional disulfide bond for stability. Viral neutralization assays showed that the eAds exhibited modest antiviral activity; however, the main goal of the study was to determine of a stable CH2 eAd could be successfully engineered, which it was. This finding provides the field with additionally options for eAd design which generates greater diversification of the antibody-based entry inhibitors. For the review, Gong et al. suggests eAds can successfully be made independent of full-size antibodies, thereby indicating the utility and potential of antibody-based therapeutics, but also emphasizes the relative infancy of the field.

17. Gach, J. S., Leaman, D. P., & Zwick, M. B. (2011). Targeting HIV-1 gp41 in close proximity to the membrane using antibody and other molecules. *Current Topics in Medicinal Chemistry, 11*(24), 2997-3021.

    a. Review

18. Chen, W., Feng, Y., Wang, Y., Zhu, Z., & Dimitrov, D. S. (2012). Fusion proteins of HIV-1 envelope glycoprotein gp120 with CD4-induced antibodies showed enhanced binding to CD4 and CD4 binding site antibodies. *Biochemical and Biophysical Research Communications, 425*(4), 931-937. doi: 10.1016/j.bbrc.2012.08.013

    a. The purpose of the study was to analyze the effects of CD4-induced antibody binding on the availability of CD4 binding site on gp120, and CD4-binding-site antibody induced neutralization of HIV. Results of ELISA, Surface Plasmon Pseudovirus Neutralization Assays suggested that CD4i antibody binding facilitates a conformational change in gp120 that exposes the CD4bs. As a result, antibodies specific for the CD4-binding site have increased accessibility to the CD4bs and, thus, are better able to neutralize HIV prior to cell entry. This study supports the consensus that multi-protein fusion inhibitors are much more potent HIV inhibitors than their individual counterparts. This helps to develop the argument that linked HIV inhibitors targeting multi-step entry are excellent candidates for future research and potential clinically-used HIV microbicides, as described in the review.

19. Danial, M., Root, M. J., & Klok, H. A. (2012). Polyvalent side chain peptide-synthetic polymer conjugates as HIV-1 entry inhibitors. *Biomacromolecules, 13*(5), 1438-1447. doi: 10.1021/bm300150q; 10.1021/bm300150q

    a. The study set out to determine the inhibitory efficacy of synthetic polyvalent polymers that bind to the CD4 binding site on gp120, and subsequently promote antibody-mediated neutralization. NMR techniques and infectivity assays indicated that the polyvalent polymer conjugates showed much greater inhibitory activity as compared to the peptide alone and did induce antibody binding. The results of the study present a method to increase the overall neutralization ability of antibodies, which may be assist future inhibitor development. The study is a critical part of the research re-

view because it supports the method of using antibody-based inhibitors to block HIV cell entry, yet, at the same time, clearly introduces gaps in current knowledge and suggests future direction in HIV research.

20. Ashkenazi, A., Viard, M., Unger, L., Blumenthal, R., & Shai, Y. (2012). Sphingopeptides: Dihydrosphingosine-based fusion inhibitors against wild-type and enfuvirtide-resistant HIV-1. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology,* doi: 10.1096/fj.12-215111

    a. The study tested the hypothesis that dihydrosphingopeptides would be incorporated into the viral envelope upon fusion initiation, thereby inhibiting membrane fusion. The results gathered from cell-cell fusion assays and virus neutralization assays showed that the dihydrosphingopeptides were integrated into the viral membrane and blocked viral entry at low nanomolar levels, and inhibited T20-resistant HIV strains. Using sphingolipid-based inhibitors provides the field with another mode of inhibition that greatly differs from previously explored techniques. Based on the results, sphingolipid-based inhibitors may become an increasingly popular choice for inhibitor design. For the review, the study suggests a possible direction for future research while also emphasizing gaps in knowledge.

21. Harrison, A. L., Olsson, M. L., Jones, R. B., Ramkumar, S., Sakac, D., Binnington, B., . . . Branch, D. R. (2010). A synthetic globotriaosylceramide analogue inhibits HIV-1 infection in vitro by two mechanisms. *Glycoconjugate Journal, 27*(5), 515-524. doi: 10.1007/s10719-010-9297-y

    a. The purpose of the study was to determine the effectiveness of a synthetic globotriaosylceramide analogue at inhibiting HIV-1 entry into cells. ELISA, in vivo toxicity assays, and fluorescent antibody cell sorting results showed that the synthetic analog blocked HIV entry through direct neutralization and inhibition of membrane fusion- both at low micromolar levels. These results suggest a potential approach to future inhibitor design that is just recently being analyzed. This may be important for the field as scientists continue to search for escape-proof inhibitors because it would be much more difficult for HIV to alter its envelope lipid composition than to mutate single amino acid residues in envelope glycoproteins.

22. Haughey, N. J., Tovar-y-Romo, L. B., & Bandaru, V. V. (2011). Roles for biological membranes in regulating human immunodeficiency virus replication and progress in the development of HIV therapeutics that target lipid metabolism. *Journal of Neuroimmune Pharmacology : The Official Journal of the Society on NeuroImmune Pharmacology, 6*(2), 284-295. doi: 10.1007/s11481-011-9274-7

    a. Review

23. Lingwood, C. A., & Branch, D. R. (2011). The role of glycosphingolipids in HIV/AIDS. *Discovery Medicine, 11*(59), 303-313.

    a. Review

24. Vieira, C. R., Munoz-Olaya, J. M., Sot, J., Jimenez-Baranda, S., Izquierdo-Useros, N., Abad, J. L., . . . Goni, F. M. (2010). Dihydrosphingomyelin impairs HIV-1 infection by rigidifying liquid-ordered membrane domains. *Chemistry & Biology, 17*(7), 766-775. doi: 10.1016/j.chembiol.2010.05.023

a. The purpose of the study was to build a better understanding of how dihydrosphingomyelin inhibits HIV-1 membrane fusion and cell entry. It was found, using viral activity assays, cell fusion assays, and immunofluorescence analysis, that dihydrosphingomyelin inhibits HIV cell entry by inducing increase membrane rigidity and, thus, impairing gp41 insertion into the cell membrane. In combination with other HIV entry inhibitors, dihydrosphinomyelin-based inhibitors may serve as an excellent route for future design of inhibitors that do not directly interact with gp41 or gp120. In the review, the study functions as an example of an innovative, non-traditional inhibitor that functions as well, or even better, than traditional small-molecule and antibody-based entry inhibitors. Accordingly, the study infers future direction and presents gaps that require further research and support.