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Re-evaluation of 5-fluorouracil Bolus injection as a radiosensitizer for chemoradiotherapy using glioblastoma multiforme cells.

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Re-evaluation of 5-fluorouracil Bolus injection as a radiosensitizer for chemoradiotherapy using glioblastoma multiforme cells.

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor in Philosophy in Biomedical Physics.

by

Gilmer Valdes Diaz

2013

ABSTRACT OF THE DISSERTATION

Re-evaluation of 5-fluorouracil Bolus injection as a radiosensitizer for chemoradiotherapy using glioblastoma multiforme cells.

by

Gilmer Valdes Diaz

Doctor in Philosophy in Biomedical Physics

University of California, Los Angeles, 2013

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During the past decades it has been widely assumed that the anticancer drug 5-fluorouracil should be administrated chronically and in low doses to maximize radiosensitization during chemoradiotherapy. However, optimization of the timing and dose administrated of both agents still remains incomplete, as the efficiency of bolus 5FU delivery has never been properly evaluated. In the present dissertation it is shown that the administration of 5FU in high-dose pulses does radiosensitize glioblastoma multiform cells U87MG-VIII and colon cancer cells HCT-116 through modification of alpha and beta ratios in survival fraction curves. Additionally, it was also shown that this radiosensitization last for at least 24 hours if cells are exposed to 2Gy after the 5FU exposure which suggests possible use of 5FU bolus

injection together with fractionation schemes. This shift in paradigm from today's protocols could not only help improve fractionated radiotherapy outcomes, but it could greatly benefit patients by shortening clinical stays and lowering overall therapeutic costs. On the other hand, due to the fact that 5FU does not cross the blood brain barrier, other ways to deliver the same to brain tumors were studied. The effect of the administration of the prodrug 5FC, that crosses the blood brain barrier, on the radiosensitization status of glioblastoma multifurme cell lines that contain the gene AC3yCD2, a cytosine deaminase gene (CD), was analyzed. It was shown that the integration of AC3yCD2 on the genome of U87MG-VIII cells does not change the sensitivity of these cells to 5FU, radiation or both agents combined. On the other hand, it was also shown that after the integration of AC3yCD2 on the genome of these cells, the sensitivity of the same to 3 hours exposure of 5FC changed dramatically. Moreover, it was also shown that these changes in sensitivity to 5FC also resulted in a huge radiosensitization effect even at low doses of radiation. These results could be of paramount importance if radiation is incorporated in the protocol of a current clinical trial www.clinicaltrials.gov NCT01156584. Finally, a novel way to target produgs, in particular 5FU, to tumors by taking advantage of the high concentrations of glucose and lactate present in the same is discussed. A molecular switch, MBP317-347, is shown to be a low-affinity switch (being activated at tens of millimolar) for glucose. It is proposed that our low-affinity glucose switch could be used as a proof of concept for a new prodrug therapy strategy denominated Metabolically-directed Enzyme Prodrug Therapy (MDEPT) where glucose or lactate serves as the activators. Additionally, considering the typical differential concentrations of lactate found in tumors and in healthy tissues, our data demonstrates for the first time that a lactate-binding switch analogous to MBP317-347 would be an order of

magnitude more active in tumors than in normal tissues and, therefore, would work as a differential activator of anticancer drugs in tumors.

The dissertation of Gilmer Valdes Diaz is approved.

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- Suma Cum Laude in the 2005 Academic Year of the Higher Institute of Applied Science and Technologies (InSTEC)
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- Gauss Award (2003-2004) given to the best scientific work of an undergraduate student, with the paper: "Critical Phenomena in micellar systems induced by organic compounds".
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PUBLICATIONS:

- **G. Valdés-Díaz** S. Rodríguez-Calvo, M. Rapado-Paneque, A. Pérez-Gramatges, F. A. Fernández, E. Frota, C. Ribeiro." Effects of gamma radiation on phase behaviour and critical micelle concentration of Triton X-100 aqueous solutions", *Journal of Colloid and Interface Science 311 (**2007**) 253–261*.
- **G. Valdés-Díaz** and R. Schulte "PROTON-BEAM DIRECTED RELEASE OF DRUGS AND IMAGING MEDIA FROM NANOPARTICLES", Patent Application (Loma Linda Medical University), AN: 60952773. Receipt Date: 30-JUL-2007. Application Type: Provisional.
- **G. Valdés-Díaz**, S. Rodríguez-Calvo, M. Rapado-Paneque, A. Pérez-Gramatges, F. A. Fernández, E. Frota, C. Ribeiro. *"Effect of Gamma Radiation from ^{60}Co in the phase behaviour of a model non-ionic surfactant"* Proceeding of 27th Latin American Congress on Chemistry, La Havana, Cuba, October **2006**, ISBN: 959-282-27-X.

- **G. Valdés-Díaz**, S. Rodríguez-Calvo, M. Rapado-Paneque, A. Pérez-Gramatges *"Effect of gamma radiation on the clouding behaviour of surfactant TRITON X-100"*. Proceeding: Fifth Symposium on Nuclear and Related Technique, Havana, Cuba, April **2006**. ISBN: 959-7136-42-2
- **G. Valdés-Díaz,** A. Pérez-Gramatges, S. Rodríguez-Calvo *"Nanodosimetry: Recent Advances"***,** Proceeding of III National Workshop: Use and Development of Health-Related Industrial Isotope Products, La Habana, Cuba, March **2006**, ISBN: 959-7136-33-3.
- **G. Valdés-Díaz**, Y.Díaz-Fernández, S. Rodríguez-Calvo, and A. Pérez-G*ramatges* "Critical phenomena in micellar systems induced by organic compounds", *Revista Cubana de Química*, vol. 16, No. 3, 2004, ISSN 0258-5595.

CONFERENCES:

1. G.Valdes-Diaz et al. Oral Presentation. "Effect of 5FU bolus injection as a radiosensitizer." RSNA, Chicago, USA, 2012.
2. AAPM Southern California Chapter, 2012. Student Participation.
3. AAPM Southern California Chapter, 2010. Student Participation.
4. Mathematical Problems, Models and Methods in Biomedical Imaging, February 8-10, 2010. Los Angeles, California. Student Participation.
5. AAPM Annual Meeting, 2009. Anaheim. Student Participation.
6. **G. Valdés-Díaz** et al "Mass spectroscopy investigation of the effect of the gamma irradiation on the mean value of the number of ethoxy groups in the Triton X-100". **Nucleu**s (Special edition: XI Workshop on Nuclear Physics), No. 41, **2007**, pg. 50-52.
7. **G. Valdés-Díaz**, S. Rodríguez-Calvo, M. Rapado-Paneque, A. Pérez-Gramatges, F. A. Fernández-Lima, E. Frota da Silveira, C. Ribeiro Ponciano. *"Effect of Gamma Radiation from ^{60}Co in the phase behaviour of a model non-ionic surfactant".* 27th Latin American Congress on Chemistry, La Havana, Cuba, October **2006**.

Introduction.

1. 1 *5FU as a Chemotherapeutic Agent*

Five-fluorouacil (5FU), an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen, was first synthesized and evaluated as an antineoplastic agent more than 50 years ago by Heidelberg, et al.[1]. This study showed that hepatic cancer cells had a higher rate of uracil uptake for RNA synthesis than normal cells, and suggested that 5FU could be used in cancer therapy as an uracil analog to block DNA synthesis. Since then, 5FU has been used as an effective chemotherapeutic drug, which when combined with radiation, have proved to be a successful strategy for treating several human cancers such as gastric, colorectal, and pancreatic [2].

5FU enters cells using the same transport mechanism as uracil and upon entrance it is converted intracellularly to different metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP) as it is shown in Figure 1. Whether 5FU is converted to one metabolite or the other will depend very much on the administration method. If 5FU is administered as continuous infusion or as on oral pill resulting in an steady but low concentration of 5FU (few μM) then FdUMP is the main metabolite obtained [3]. FdUMP binds to the nucleotide-binding site of thymidine synthase (TS), forming a stable ternary complex with this enzyme and reduced folate CH2THF which acts as a cofactor [4, 5]. TS is involved in DNA synthesis by catalyzing the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This reaction constitutes the sole *de novo* source of thymidine, used in DNA replication and repair. However, due to the high affinity of TS for FdUMP, the latest blocks the normal substrate dUMP, inhibiting dTMP synthesis [4, 5]. As it has been shown by Jackson et al., the depletion of

dTMP results in depletion of deoxythymidine triphosphate (dTTP), and further alteration of the levels of other deoxynucleotides (dATP, dGTP and dCTP) [6]. This effect creates an imbalance in the the dATP/dTTP ratio which has been shown to severely disrupt DNA synthesis and repair [7, 8]. On the other hand, TS inhibition results in an accumulation of deoxyuridine triphosphate (dUTP) and FdUTP which could both be misincorporated into DNA [9, 10]. The enzyme responsible for repairing this mismatch has been shown to be uracil-DNA-glycosylase (UDG) [11]. However, this enzyme has also shown to be ineffective in repairing misincorporation of dUTP and FdUTP in presence of high dUTP/dTTP ratios, as it is the case when TS is inhibited [11]. Therefore, these misincorporations, together with inefficient repair mechanisms result in strand breaks accumulation and eventually cell death. Nevertheless, it must be said that when only TS is inhibited, as it is the case when 5FU is administered as a continuous infusion and for some oral pills, thymidine could be obtained through the salvage pathway, being thymidine kinase (TK) the main protein involved. This mechanism has been highlighted to be one of the causes for the 5-FU resistance shown by some tumors [12].

On the other hand, when 5FU is administered as bolus injections, not only TS is inhibited but also TK. In this case, the three initial metabolites product of 5FU catabolism (FdUMP, FdUTP, FUTP) are obtained and the different pathways for the action of 5FU after bolus administration could be observed in Figure 1. From these metabolites, FUTP is the one with the biggest cytotoxic effect in this case [3]. The same is incorporated into RNA which disrupts normal RNA function and provokes cell death [13]. When FUTP is incorporated into the RNA a wide spectrum of proteins and RNAs species are affected as this metabolite acts at different levels. First, it inhibits the processing of pre-rRNA into mature rRNA [14, 15]. Additionally, it also affects the post-transcriptional modification of tRNAs together with the assembly and

activity of snRNA/protein complexes which in turns inhibits the splicing of pre-mRNA [16-19]. Finally, FUTP has also been shown to inhibit the post-trascriptional conversion of uridine to pseudoridine in rRNA, tRNA and snRNA but the effect on the stability of these species is not well known yet [20]. Therefore, the administration of 5FU as a bolus injection will result in damage to a wide spectrum of proteins including TS and Thymidine Kinase (TK), responsible for the novo and salvage pathways of thymidine synthesis. In fact, Nord et al. showed that when 5FU is administered as a bolus, activities of both TS and TK decreased for 96 hours [21].. However, they returned to normal without a second administration which shows the cyclic effect of 5FU administration [21].

1.2 *5FU catabolism and pharmacokinetics*

5FU has been administered clinically by various methods and routes: oral, i.v. bolus or, continuous and protracted infusions [22-24] . In all cases, the plasma concentration achieved will depend on the administration method. Both continuous infusion and oral administration guarantee a steady and rather constant low concentration of 5FU in plasma while bolus is characterized by high concentration peaks follow by a rapid clearance [22-24]. 5FU follows a nonlinear pharmacokinetics where a decreases in the rate of clearance occurs with increasing 5-FUra doses [25].

If administered in-vivo, 5FU has a very short half-life from few minutes to few hours depending on the body fluid being measured. [23, 26, 27]. The concentration of 5FU in physiological fluids could be measured using radiolabeled 5-FU or gas-liquid chromatography combined or not with mass spectroscopy [28-30]. From all these methods, using radiolabeled 5-FU is the only one

that allows identification of all circulating metabolites downstream of the catabolism of 5FU at once. However, if

different metabolites are wished to be determined independently, chromatography together with mass spectroscopy is preferred. On the other hand, 5FU catabolism plays an essential role in its fast clearance as it has been shown by the correlation found between dihydropyrimidine dehydrogenase activity and the systemic clearance of 5FU [31]. The main organ involved in the catabolism of 5FU is the liver which has the highest concentration of dihydrouracil dehydrogenase (DPD) though high concentrations of this enzyme are also found on the kidneys and on the gastrointestinal epithelium, but not in colonic carcinomas, which could explain the susceptibility of colon cancer to 5-FU [32].

DPD converts 5-FU to dihydrofluorouracil (DHFU) which is further converted by the enzyme dihydro–pyrimidase and ureido propianase to 5-fluoro-ureido propionate and F -β3-alanine respectively [33]. Further catabolism of F-β3-alanine (F-BAL) is not unlikely and it could also be converted to acetate [33] On the other hand, it has also been pointed out that F-BAL can be converted to fluoroacetate, which has been related to neurotoxicity [34].

1.3 *5FU as a radiosensitizer*

The timing-dosing patterns of 5FU in chemoradiotherapy remain controversial. Although it has been a consensus that continuous venous infusion (CVI) of 5FU is more efficient than bolus injection (BI) or high dose pulse (HDP), the relative efficacy of each delivery method has not yet been properly determined.

Previous studies have shown that treating HT-29 human colon carcinoma and HeLa human cervical cancer cells with 5FU and radiation leads to a time-dependent enhancement

of cell killing [35]. From a series of acute and chronic exposures, Byfield et al. showed that the administration of 20ug/ml (153uM) of 5FU for 30 min. gives *equivalent* cell killing as 1ug/ml (7.7uM) for 10 days. Accordingly, it was shown that 5FU must be administrated after radiation to have maximum effect, and that radiosensitization required 5FU to be actively available in the blood for at least 24-48 hrs after irradiation. Therefore, they concluded that if bolus and CVI kill by the same mechanism, pulsed 5FU would not be useful given the short serum 5FU half-life of 10-20 minutes [23, 26, 27]. As a result of these experiments and despite the fact that the 5FU BI combined with radiation was never fully examined, CVI became the preferred delivery method for combinational therapy. However, there is evidence that some of these statements are not accurate; i.e. it has been widely discussed that CVI and BI do have different mechanisms of action [3, 36-41]. Chronic delivery of the drug at low concentrations (1 μM) inhibits the enzyme thymidylate synthase (TS), which catalyzes the de-novo synthesis of thymidine monophosphate (dTMP) used in DNA synthesis and repair [3, 36-41]. Alternatively, when cells are exposed to pulses of high concentrations (100 $\mu M - 1 mM$), 5FU incorporates into the RNA impairing the function of a wide variety of molecular species [21, 42]. These two different mechanisms will also result on two different interaction mechanisms between 5FU and radiation.

Specifically for BI, although there are limited studies showing the efficacy of BI 5FU as radiosensitizer, it has been reported that radiosensitization does occur in vitro [43]. Additionally, corroborating Byfield et al. results, this study shows that there is no difference in the sublethal damage repair (SLDR) for BI and CVI. Thus, it suggests that

although there is radiosensitization with both methods, a little impact on the shoulder region of the survival curve is to be expected, and therefore radiosensitization would be less effective with conventional fractionation schemes of radiation therapy [43-46]. Taking this into consideration, the authors suggested that synergistic effect previously described in several clinical trials using the CVI regime could be the result of spatial collaboration, and not radiosensitization *per se* [16]. However, this argument would be valid for BI, where clinical trials have failed to unequivocally prove the 5FU radiosensitizing effect [43, 47-50]. Notwithstanding, it should be pointed out that the BI scheduling used in these trials could have been suboptimal, as the majority of the irradiation fractions where given in between and distant from the 5FU doses. Nord et al. showed that when 5FU is administered as a bolus, although activities of both TS and thymidine kinase (TK) decreased for 96 hours, they returned to normal without a second administration [21]. Therefore, probably most of the irradiation protocols in the clinical trials mentioned above were done without a 5FU radiosensitizing effect when 5FU was administered as a bolus injection. Because of this, we believe that previous clinical trials are not an absolute proof that bolus 5FU is not an effective radiosensitizer, and consider necessary a re-evaluation of this delivery method.

1.4 *Glioblastoma Patients*

Glioblastoma multiforme (GBM) is the most common tumor in the central nervous system and one of the most aggressive [51] In the United States, every year 14,000 new cases are diagnosed [51]. The most common mutations found in GBM patients involve mutations on the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor [52]. In fact, about 50% of new GBM patients show upregulation of EGFR and half of those express a constitutively autophosphorylated variant of EGFR, known as EGFRvIII, that lacks the

extracellular ligand-binding domain (exons 2 through 7) [52, 53]. The standard therapy for newly diagnosed malignant gliomas involves surgical resection when feasible, radiotherapy, and chemotherapy. An International clinical trial lead by the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) showed that concomitant RT together with temozolamide (TMZ) is preferable over radiation alone for newly diagnosed patients [54]. The combination of TMZ and radiation became the standard care of treatment and extended the median survival to 14.6 months which is better but still low [55]. There are several reasons for this low efficacy. For instance, it has been shown that increase in the Methylated-DNA-protein-cysteine methyltransferase (MGMT) expression produces *in vitro* and *in vivo* glioma resistance to TMZ [56-59]. MGMT is able to receive alkyl groups from the DNA which counteract the action produced by alkylating agents [60]. However, during this process the MGMT protein is degraded by the ubiquitin-proteasome pathway and as such the action of this protein will depend in great measurement of the mechanism for its replenishment making it a target for a new agent in combination with TMZ [60]. In fact, it has been argued that because of coactivation of multiple tyrosine kinases, as well as redundant signaling pathways, the action of single agents that focus only in certain pathways have been limited [51]. Therefore, great effort have been devoted to the identification of inhibitors of tyrosine kinases (one of the hallmarks of GBM) such as EGFR, PDGFR, and vascular endothelial growth factor receptor (VEGFR) that could be used in conjunction with TMZ and radiation [61-63]. For instance, Bevacizumab (Avastin, BEV) is a humanized monoclonal antibody that has been proven to inhibit the activity of VEGF [64-66]. The efficacy of the use of Avastin with TMZ was shown in a randomized, noncomparative phase II trial (BRAIN study; AVF3708g) for GBM patients who experienced first or second recurrence [65]. Other strategy could also be to use a drug that has a

wide spectrum of damage to different pathways that could overlap with the action of TMZ at various levels. In that sense it is possible to hypothesize that 5FU bolus injection due to its broad spectrum damage could be a good match for GBM patients. However, the efficacy of 5FU to cross the brain-blood barrier and its toxicity to the central nervous system should be taken into account for such treatment and it will be discussed below. A possible solution to this problem could be the use of Enzyme Prodrug Therapy, a concept that will be introduced on this dissertation.

1. 5 *Enzyme Prodrug Therapy*

One critical complication of chemotherapeutic oncology drugs is that they do not discriminate between diseased and healthy cells, leading to unwanted sequelae and lowering the patient's quality of life upon treatment. Efforts have been made to specifically localize anticancer drugs in tumor cells; one such approach being Enzyme/Prodrug Therapy (EPT). This method is based on administering a prodrug together with an enzyme that specifically activates the drug only in the tumor, sparing the healthy tissue [67]. To target only the cancer cells, the localization of the enzyme is specifically directed to the tumor, and can be accomplished by gene-directed enzyme prodrug therapy (GDEPT) [68, 69], virally-directed enzyme prodrug therapy (VDEPT) [70], antibody-directed enzyme prodrug therapy (ADEPT) [71] or by lectin directed enzyme prodrug therapy (LEAPT) [72]**.** GDEPT and VDEPT are characterized by the physical delivery of a gene, by a vector or a virus, to tumor cells followed by the delivery of the prodrug after the enzyme is expressed. Alternatively, ADEPT uses a tumor-associated monoclonal antibody linked

to a drug-activating enzyme to create a systemically administered conjugate that only targets tumor tissues and LEAPT relies on endogenous carbohydrate-to-lectin binding to localize glycosylated enzymes to cancer cells. In particular for ADEPT and LEAPT, the dissociation constant of the molecules coupled to the enzyme and the target should be as small as possible to guarantee a high-affinity binding to the target.

Despite enormous efforts to improve the effectiveness of these four methods, they still present many limitations [67-72]**.** Among them are the possible reversion of the virus to the wild type form in VDEPT, induction of mutagenesis of the host genome, a limited binding of the antibody/enzyme to the cell due to antigen heterogeneity, specificity of the mutation or insufficient expression of target molecules by tumor cells [67-72].

1.6 *5FU, the blood brain barrier and 5FC*

Some authors have highlighted that 5FU does not cross the blood–brain barrier and should not be indicated for treatment of brain tumors [73]. However, there are several evidences that 5-FU at least partially crosses the blood brain barrier. For instance, some toxicity to 5-FU has been reported which includes cerebella dysfunction, encephalopathy, and peripheral neuropathy [74, 75]. However, other authors have reported that infusion or controlled release of 5-FU in the brain has been tolerated in humans [76-78]. On the other hand, regression of brain metastases in patients that have been treated with 5FU has been reported [79, 80]. Nevertheless, whether this effect is an indication of the ability of 5FU to cross the blood-brain-barrier (BBB) or an alteration of the permeability of the same due to the angiogenesis process of the tumor is a matter of debate. In one study, though, evidence of 5FU partially crossing the BBB by diffusion was shown [81]. However, whether 5FU crosses the BBB partially or not, it has been shown in several clinical trials that the amount

of 5FU reaching the GBM tumor is insufficient to increase the median survival of the patients either alone or when combined with radiation or other drugs [82, 83]. In that sense, different groups have tried to develop new ways of delivering 5FU to GBM tumors. For instance, Menei et al showed that up to 8 times higher concentrations of 5FU could be obtained in brain tumors if biodegradable microspheres are used as carriers [84]. Another group has proposed the use of transferring-conjugated liposomal system with the same objective [85]. One of the most effective strategies is the direct intratumoral administration of 5-FU [86] but the short physiologic half-life of this drug has hampered achieving significant therapeutic gain even with the use of sustained-release polymer carrier systems [87, 88]. In that sense, a phase III trial using biodegradable carmustine-impregnated chips for sustained release in the post-resection tumor cavity, showed an improvement in median survival of only 2 months [89, 90]. Therefore, despite these efforts, to the best of our knowledge, there is no clinically available effectively way to deliver 5FU to GBM tumors. One possible solution to this problem could be the use of the prodrug 5- fluorocytosine (5-FC) together with an enzyme prodrug therapy approach. 5FC was initially synthesized in 1957 as an antitumor agent but it did not show any activity against tumors [91]. However, some years after its designed, 5-FC was found to be very effective against candidosis and cryptococcosis in mice and humans [92, 93]. Later on, 5-FC was also found to be a very effective antifungi agent (*Candida* spp. and *Cryptococcus neoformans) [94]*. 5FC enters the cell using the same transport system as cytosine, adenine and hypozanthine [95]. 5-FC itself does not show any biological activity but its action comes from its rapid conversion to 5-FU by the enzyme cytosine deaminase found in these fungi. In patients, 5-FC is administered orally and absorbed very rapidly [96, 97]. Between 76-89% is bioavailable

after administration attaining its concentration peak in serum 1-2 h after administration [96, 97]. Due to its solubility and size, it has a wide distribution in the body being able to cross the blood brain barrier with high efficacy [97-100]. Its half life on the body is typically between 3-4 hours but depends on the patient's renal function as it is mainly eliminated by the kidneys [96, 100, 101]. The regular dosage depends very much on the level of creatinine clearance of the patient but due to its relative low toxicity, concentrations that will result in bolus like concentrations after its conversion to 5FU could be administered [102]. Among the side effects, even though minors, we could mention nausea, vomiting and diarrhea, hepatotoxicity and bone marrow depression [94, 103]. However, it has been found that if the concentration of 5-FC is kept below 125 mg/L the more serious side effects like bone marrow depression are eliminated [102].

1.7 *Gene Directed Enzyme Prodrug Therapy and 5FU targeting*

A promising strategy based on enzyme prodrug therapy is suicide gene therapy [104-106], which commonly utilizes retroviruses to introduce genes, like cytosine deaminase (CD), into tumor cells. For instance, it is envisioned that after the insertion of the cytosine deaminase gene into the tumor, 5FC could be converted to 5FU intratumorally. However, this approach has had some problems. A Phase III clinical trial based on this rationale ultimately failed because a replication-defective retrovirus vector was employed to insert the CD gene into the genome of the cells leading to inadequate infection of and gene transfer to diffusely infiltrating glioma cells [107]. Therefore, the use of a virus that could actively replicate inside cells seems to be a key component for the success of this technique. However, in such situation, the virus should only be able to infect tumor cells if any therapeutic ratio improvement is expected. As it has been shown by Miller et al. Murine Leukemia Virus (MLV) could infect only actively diving

cells as its nucleocapsid complex contains no nuclear localization signal for active nuclear uptake [113] In that sense, a MLV that contains a transgene cassettes (CD) inserted at the env-3V UTR border has been shown to effectively transduce and propagate over multiple infection cycles [108-111]. This MLV-based retrovirus was actually able to achieve in situ amplification after the initial administration [112]. Based on this principle, Tai et al. have developed a murine leukemia virus (MLV)-based replication-competent retrovirus (RCR) vector, Toca 511 (AC3-YCD2), containing the CD gene. This characteristic allows for its highly efficient and tumor-selective replication and gene transfer [112]. Using a sensitive PCR assay that could detect as little as 35 copies of the virus sequence in 0.5 μg of DNA, Wang et al found no trace of the virus in peritumoral normal brain tissue or any other systemic organ while detecting a strong signal in the tumor [110]. The stability, tumor specificity and efficient infection of all rapidly dividing cells make AC3-yCD2 a highly promising method to treat GBM's. [108-110, 114]. As such, the results of the current Clinical phaseI/II trials using this approach are highly encouraging (www.clinicaltrials.gov NCT01156584). On the other hand, because radiation therapy is an important component in the conventional treatment of GBM, it is desirable to prove that radiotherapy could be effectively combined with this approach to obtain synergistic effect when both agents are applied together.

1.8 *Metabolic Directed Enzyme Prodrug Therapy*

Despite the promising results showed by the suicide gene therapy approach described above, this technique has some shortcomings and other methods of targeting drugs to tumors are also desirable [108-110, 114]. As we have discussed above, the ultimate goal in oncology is to exclusively target the tumor and completely spare the healthy tissue. To this end, researchers have searched for unique tumor markers and aimed to develop agents that have as high an

affinity and specificity as possible for the markers as new ways to target drugs to tumors. In this dissertation we suggest a novel shift in paradigm by demonstrating the feasibility of using a strategy that relies on low affinity for a marker founded in the very fundamental core of tumor biology. The Warburg Effect – describing the addiction of tumors to anaerobic respiration of glucose even in the presence of oxygen – is an exemplary difference between cancers and normal tissues. To date, however, aside from imaging, it has been difficult to exploit this universal distinction to target tumors with therapeutic purpose. A major immediate consequence of the Warburg Effect is excessive lactate production within the tumor which could be up to 8 fold higher [115, 116]. In fact, tumors with high concentrations of lactate (above 8.3 mM) are associated with a poor prognosis and low survival factors [116]. Similar results have been observed in head and neck adenocarcinomas where the mean lactate concentration is approximately 12.5 mM, with the most aggressive ones reaching over 20mM [116]. In contrast, low concentrations of lactate (below 8.3 mM) in tumors correlate with high survival in carcinomas of the uterine cervix [116]. Thus, if enzyme/switches could be specifically activated only by the generally high concentrations of lactate found in tumors but not by the lower but still absolutely high (millimolar) concentrations found in healthy tissues, then this universal metabolic difference could be exploited to achieve drug targeting. However, high-affinity switches that bind lactate in the micromolar range cannot be used to differentiate millimolar stoichiometric differences found in the physiological range of these metabolites. Under physiologic conditions, a switch with binding constant for lactate in the micromolar range would be saturated and no distinction in activity could be made between tumors and healthy tissues, Figure 2. On the contrary, if a low-affinity switch for lactate with a k_d on the order of millimolar were designed, then it would allow us to take full advantage of the difference in concentrations

between tumors and healthy tissues of these substrates (millimolar range). This concept is explained in Figure 2. with more detail.

1.9 *β-lactamase (BLA)*

In our approach, as the prodrug is going to be activated by the enzyme, the latter should not be normally present in human tissues. In this sense, β-lactamase (BLA) seems to be a good match. BLA is a monomeric enzyme that hydrolyzes the amide bond of the β-lactam ring and is not present in human cells [67]. Different prodrugs can be activated with BLA such as: LY 26607, cephalosporin doxorubicin prodrug C-DOX or the doxorubicin prodrug PRODOX [117-119]. In all cases, the drug showed higher activity than the prodrug, ranging from 5 fold for LY26607 to 20-fold for PRODOX. Other important prodrugs that have been used with BLA are the 7-(Phenylacetamido)-cephalosporin mustard (CM**)** and the potent antitumor drug 4'-carboxyphthalato (1,2-cyclohexanediamine) platinum [120, 121]. CM was 50-fold less cytotoxic toward human lung adenocarcinoma cells than its corresponding drug but this parameter was not reported for 4-carboxyphthalato (1,2-cyclohexanediamine) platinum [120]. On the other hand, BLA is also known for hydrolyzing cephalosporins and penicillins in E. coli bacteria, inactivating both antibiotics [67]. This mechanism has been used to create "switches" that respond to different biochemical signals and a 5-FU prodrug developed by our collaborators at John Hopkins University [67].

1.10 *Protein Switches*

BLA is a monomeric protein that on its own does not present switching behavior unless the molecule to be hydrolyzed would be competed out by adding a saturating amount of another molecule. However, such a switch would turn the enzyme from an active into an inactive state,

which would be opposite to our goal of activating the enzyme in the tumor. In nature, switches exist in the form of allosteric proteins, where the binding of a substrate at a site will affect the binding of another substrate at a different site in the same protein. In fact, allostery is the most direct, rapid, and efficient mechanism for the modulation and regulation of cellular function in response to changes in concentration of small molecules [122] . Allosteric regulation can be negative when the binding of one substrate prevents binding of another or positive when it works the other way around. As our mechanism requires activation of an enzyme, we will need an enzyme that is regulated by a positive allosteric effect.

Classes of proteins presenting either positive or negative allosteric effects were obtained by coupling BLA with a maltose binding protein (MBP) creating MBP-BLA hybrids [123-125]. In these articles, the authors describe the use of in vitro gene recombination and circular permutations to create a library of different protein switches where the activity of BLA was influenced by the activity of MBP [123-125]. MBP is a member of the periplasmic binding protein superfamily and is involved in chemotactic response and the transport of maltodextrins [123]. MBP, as all periplasmic binding proteins (PBP), consists of a single polypeptide chain that folds into two domains connected by a hinge region. In this place is where the single binding site for maltose is located [123]. In the absence of maltose, MBP exists in an open form. Maltose binding is concomitant with a 35^{o} bending motion around the hinge, resulting in the closed form of the protein [126]**.** It must be said that besides binding maltose, MBP also binds other maltooligosaccharides but the conformational change that is induced by them is ligand-dependent. When MBP binds maltotriose, the conformational change of MBP is identical to that when it binds maltose [127]. From the library mentioned above, a switch (RG13) was identified whose β-lactam hydrolysis activity was increased 25-fold in the presence of maltose [123, 127].

In such enzyme, the BLA domain of the RG13, when MBP is in its open state, exists in a compromised, less active conformation. In the ligand-bond state (maltose binding MBP), the BLA domain exists in a more normal, active conformation [123, 127]. In fact, it was shown that by using mutations of RG13 that are partially closed (closure angle equals to 9.5^{o} for I329W and 28.4^{o} for I329W/A96W) more sensitive switches were created; switches that respond to lower concentrations of maltose [123]. Another protein from the same library T164-165-H shows, in presence of maltose, kinetic parameters very similar to those, previously reported, for BLA. This result proves that in presence of maltose the enzyme was fully activated. This enzyme showed switching behaviors at concentrations of maltose that were less than 1 μM. Besides, the K_d for maltose-binding at 22 ^{o}C was 1.7(± 0.5) μM; indistinguishable from the K_d previously reported for maltose-binding to MBP [127]. These values prove that both enzymes when put together act toward their substrates as they were alone, but showing allosteric behaviors.

On the other hand, as the allosteric effect found by Ostermeier et al was on the same order of magnitude as those of many natural allosteric enzymes, the authors examined the biological effects of RG13 [123]. The switching activity of this protein was sufficient to result in an observable phenotype: maltose-dependent resistance to ampicillin of E. coli bacteria. The idea was that in its inactive form, RG13 would not be able to hydrolyze the antibiotic and therefore the bacteria cells would die with opposed results when maltose was present. Ostermeier et al. obtained that *E. coli* cells expressing RG13 had a minimum inhibitory concentration (MIC) for ampicillin that was 4-fold higher in the presence of 50 μM maltose (from 128 μg/ml to 512 μg/ml) [123]. In contrast, the addition to a plate of same concentrations of sugars that do not bind MBP, sucrose or glucose, did not affect the MIC [123]. These results is in complete agreement with Ostermeier et al who showed that the addition of MBP not binding sugars (galactose,

glucose, sucrose and lactose) at concentrations of 0.5 mM (10^3 higher than that at which maltose's effect can be observed) had no effect on MBP-BLA activity [127]. Only glucose at a concentration of 50 mM had an effect but this was proven later to be due to traces of maltose contamination in the glucose [127].

Finally, other sensors have been created by using different methods to combine BLA with other proteins [128, 129]. Dafydd et al combined BLA and the protein Cyt b, a small four helical bundle electron transfer protein, to create a sensor that responded to Haem binding, an important biological co-factor [128]. It is worth to note that Cyt b undergoes a major conformational change on haem binding, similar to MBP [128]. On the other hand, Doi N. and Yanagawa obtained a BLA inhibitor sensor by combining BLA with GFP, a green fluorescent protein [129].

1.11 *A low affinity switch as a proof of concept*

In the present dissertation, we will report that the allosteric protein switch (a fusion between maltose binding protein and TEM1 β-lactamase (MBP-BLA)) MBP317-347 [125] behaves as a high-affinity switch in the presence of maltose and maltose-like polysaccharides, being activated in the range of micromolar concentrations, and at the same time, it acts as a low-affinity switch for glucose, becoming active at physiological glucose concentrations. We propose that this allosteric switch could be used as a proof of concept for an alternative prodrug therapy strategy that we denominate Metabolically-directed Enzyme Prodrug Therapy (MDEPT).

2. Hypothesis

1. 5 FU, if administered as a high dose pulse, could radiosensitize cancer cells.
2. 5 FU, if administered as a high dose pulse, could be used together with radiofractionated regimes.

3. Tumors are radiosensitized if 5FU is obtained in the tumor area via gene therapy and 5FC administration.

4. Metabolic directed enzyme prodrug therapy might be a valid alternative to target 5FU to the tumor area.

3. Materials and Methods

3.1 Cell line

Human glioblastoma multiforme (GBM) cell lines U87MG-VIII and U87 were maintained in exponential growth as monolayer cultures by serial passage at 37 ^{o}C, 5% CO2, in DMEM 1X medium with 10% fetal calf serum and 100 IU/ml penicillin, 100 µg/mL Streptomycin and 0.25 µg/mL of Amphotericim B.

3.2 Clonogenic Assays

Cells were irradiated either as suspension cultures at 37 ^{o}C with complete culture medium in Eppendorf tubes, or after they were plated. When experiments involved irradiating cells in multiple fractions at least 24 hours apart, the first fraction was delivered while the cells were still on their T75-flask and they were only plated before the last fraction to avoid multiplicity problems. A RS320 Irradiation System (Gulmay Medical, Bethel, CT, USA) with the following technique was used: of 300kVp, HVL 3mmCu, 10mA, 1.743Gy/min at 34.7 cm FSD. Colonies were stained after 21 days using Try Pam Blue, and only colonies with more than 50 cells were counted.

3.3 5FU Exposure

Stock 5FU (Sigma-Aldrich, St. Louis, MO) stocks were stored at -70 ^{o}C. At the time of 5FU exposure, the cells were grown in T75-flasks and harvested by removal of the growth medium and addition of 0.25% trypsin. For High Dose Pulses (HDP), the cells were exposed to 5FU for one hour in 1.5mL microfuge tubes at 37C. The cells were subsequently washed and resuspended in fresh medium devoid of 5FU. For Low Dose Concentrations (LDC), like those usually used in CVI, two protocols were used. Either the cells were plated in medium containing 5FU during the entire period of colony formation, or were only exposed to 5FU for 48h. After exposure, the medium was removed and 10 ml of fresh medium was added.

3.4 Sublethal Damage Repair Experiment

Split-dose experiments were done immediately after cells were incubated in 1mM 5FU or medium for 1 hour. For pre-irradiation experiments, cells were incubated in 1mM 5FU or medium, immediately exposed to 0 or 2 Gy, and left for 24 hours before initiation of the split-dose exposure. For the split-dose experiments, cells were irradiated with two fractions of 2Gy separated by different time intervals (0, 1, 2, 4 hrs). After all the treatments, cells were allowed to grow colonies for 21 days. All survival fractions were normalized to that of the cells irradiated with a single fraction of 4Gy (0 hr time interval).

3.5 Effect of 5FU and radiation on the Cell Cycle

U87MG-VIII and colon cancer HCT-116 cells were plated and left to grow for two days. After which, the medium was change and new medium or medium containing 1mM or 0.5mM, for U87MG-VIII or HCT-116 respectively, was added for one hour. After washing the cells, they

were sham irradiated or irradiated with 2Gy and allowed to recover at 37^{o}C for 24 hours. Cells were then trypsinized, washed, and fixed/permeablized in 70% ethanol and stored at -20 ^{o}C for later analysis. Following two wash steps and treatment with 1µg/mL RNase in 0.5% bovine serum albumin (BSA) (Sigma-Aldrich) + Dulbecco's phosphate buffered saline (DPBS) (Mediatech) for 1 hour, the cells were stained with 20 µg/mL propidium iodine (Sigma-Aldrich). DNA content was analyzed using a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

3.6 Curve fitting and statistical analysis

The survival curves were fitted using Excel 2007 and the method of the least minimum squares. A p-value of 0.05 was considered significant. One-way repeated measures ANOVA or T-test analysis was used for the comparison of bioluminescence signals between groups. P value < 0.05 was considered significant. The statistical analyses were performed using Excel and Prism 5 (GraphPad Software). To calculate the dose modification factor (DMF), the alpha and betas from the linear quadratic model were calculated for each curve using Excel 2007. Then, the curves were extrapolated and the dose resulting on a 0.1 survival fraction estimated. Finally, the DMF was calculated as the control dose divided by the dose needed to cause the same effect (0.1 survival fraction) for each particular treatment.

3.7 Reagents

The design, isolation and characterization of the MBP-BLA switch MBP317-347 was done as previously described [125]. All reagents used in this dissertation were purchased from

Sigma-Aldrich, St. Louis, MO. The same were stored in a -70^{o}C freezer until the day of each experiment.

3.8 Activation Assay

All enzyme activation assays were carried out in 100 µL aliquots using 10 nM solution of MBP317-347 and different concentrations of the activators. All the samples were dissolved in DPBS buffer with 10 mM glycerol added to stabilize the protein. Activation of the enzymatic activity of the β-lactamase domain of MBP317-347 was quantified colorimetrically. The change in A_{486nm} of nitrocefin over time was measured with a spectrophometer. The slope of the A_{486nm} vs time (velocity of adsorbance change) was calculated using instrument software. All the experiments were carried out at room temperature.

3.9 Synthesis of Glucose from Cellobiose

Novozyme (Cellobiase), an enzyme that hydrolyzes cellobiose to obtain glucose, was purchased from Aldrich Sigma (activity = 253 Units/ml). One µL of Novyzym was added to 378 mM of cellobiose previously mixed with 1 mL of acetic acid buffer (pH 5). The sample was left at 37°C for 48 hours to allow full conversion of cellobiose to glucose.

3.10 Retroviral replicating and lentiviral vectors

The retroviral replicating vectors AC3-yCD2 (Toca 511, T511) and AC3-emd have been described previously [130, 131]. The backbone of both plasmids were generated based on murine leukemia virus (MLV); envelope sequence is changed to amphotropic envelope (4070A) and internal ribosome entity site (IRES)-yCD2 or IRES-GFP cassette is inserted into downstream sequence of envelope sequence. To product AC3-emd virus, 293T cells were transiently

transfected with pAC3-emd using Fugene-6 (Roche) as previously described [132]. Supernatants were harvested and filtered 48 hours later, and stored at -80 ^{o}C. Clinical grade of AC3-yCD2 was provided by Tocagen Inc. The self-inactivating (SIN) lentiviral vector containing firefly luciferase (pRRL-sin-cPPT-hCMV-Fluc2) was used in generating luciferase-expressing glioblastoma cells in this study. Titers of virus were determined by measuring viral p24 antigen concentration by ELISA as described previously [133].

3.11 Mice

Female Foxn1 *nu/nu* athymic mice (age, 6~8 weeks) were purchased from Harlan Laboratories Inc. and bred under specific pathogen-free conditions. All animal studies were conducted under protocols approved by the University of California at Los Angeles Animal Research Committee.

3.12 Bioluminescent assay

U87EMG-VIII-Fluc2 and U87MG-VIII-AC3-yCD2-Fluc2 cells, where Fluc2 and AC3-yCD2 indicate the insertion of the Firefly Luciferase and CD deaminase to U87MG-VIII cells were plated in 10 cm dishes and exposed to DMEM / 10% FBS containing 0.1 mM 5FC *in vitro*. After two hours exposure to 5FC, culturing medium was removed and replaced to fresh complete medium. A part of the each cell line was irradiated with 2 Gy or 4 Gy, and reminder was unirradiated. The cells (1 x 10^5 in 2 μl PBS) were inoculated into mouse right hemisphere in 2 minutes using Hamilton syringe and stereotaxic micro injector (Narishige, Tokyo, Japan), followed by holding needle for 5 minutes (n = 6 / group). Injection site coordinates were 1 mm anterior, 1.5 mm lateral and 3 mm deep from bregma and dura mater. Firefly luciferase signal expressions from brain tumors were examined by optical *in vivo* imaging system (Xenogen IVIS,

Alameda, CA) every 3 or 4 days until one of each group mice died for brain tumor development. Bioluminescence data was analyzed by Living Image™ Software (Xenogen).

4. Results

4.1 5FU as a radiosensitizer

4.1.1 LDC 5FU as a radiosensitizer

First, we wanted to confirm that U87MG-VIII cells are radiosensitized by 5FU under classical conditions of LDC exposure obtained by Byfield, *et al.* [35]. We plated cells with medium containing 0.001 mM of 5FU and immediately irradiated them with 1, 2, 4, or 6 Gy. The medium containing 5FU was left in the plates during the incubation time to replicate the conditions used by Byfield, *et al.* [35]. As shown in Figure 3, 5FU radiosensitizes U87MG-VIII cells with a DMF of 1.48 when administered under CVI-like conditions and corroborates one of the classical rules that radiosensitization is effected only if cells are exposed to low doses of 5FU for more than 24 hours *after* radiation *[134]*. An analogous experiment using 0.005 mM of 5FU was carried out, but the survival fraction of the cells was too low to be scored (data not shown).

On the other hand, to examine the corollary that radiosensitization disappears if the cells are exposed to low doses of 5FU *before* irradiation, the cells were exposed to 5FU (0.005 mM) for 48 hours, washed, and then irradiated. In agreement with what has been shown by Byfield, *et al. [35]* for HT29 cells, 5FU LDC does not radiosensitize U87MG-VIII cells if they are exposed to the drug before irradiation (Figure 3). In fact, a slightly radioprotective effect is evident with a

DMF of 0.84. This experiment was also performed using 0.001 mM of 5FU and as with 0.005 mM, radiosensitization was not seen (data not shown).

4.1.2 HDP 5FU exposure

To evaluate the effect of HDP 5FU as a model for bolus administration, we first exposed U87MG-VIII cells for 1 hour to 5FU concentrations ranging from 0.15 to 1mM. As it can be seen in Figure 4, U87MG-VIII cells are sensitive to HDP of 5FU in this range of concentration. Additionally, from Figure 4 it can be seen that at 2Gy the effect of 5FU and radiation is mainly additive which will be analyzed further below. For subsequent HDP timing experiments with respect to irradiation, based on the results shown in Figure 4, we selected 1-hour exposure to 1mM 5FU, which is the dose that resulted in survival fraction (SF) of 0.5. This dose-time combination resembles bolus administration used in the clinic although 1mM is near the maximum tolerable dose used *[135, 136]*. It should be noted, however, that in most cases, clinical levels are predetermined using cell lines that are less resistant than U87MG-VIII. Moreover, with recent advances in gene therapy, the delivery of doses in the range of 1mM or greater to brain tumors using suicide gene therapy and the prodrug 5-fluorocytosine (5FC) are very achievable *[132]*. Therefore, 1mM of 5FU for 1hr for U87MG-VIII is a reasonable choice to model the clinical setting.

4.1.3 HDP 5FU as a radiosensitizer

To re-evaluate the use of bolus 5FU as a radiosensitizer, and corroborate hypothesis 1, we performed clonogenic survival experiments of U87MG-VIII cells irradiated after exposure to 1 mM of 5FU for 1 hour. Figure 5a shows significant radiosensitization of U87MG-VIII cells

(DMF = 1.47). There was, however, no statistically significant difference in the survival fractions for doses smaller than 2Gy ($p > 0.05$). These results are in agreement with others, who showed that administration of 5FU does not change the shoulder of the survival curve *(Byfield et al. 1982; Smalley et al. 1992)*. Additionally, colon cancer HCT-116 cells were also exposed for 1 hour to HDP 5FU before irradiation and radiosensitization was also confirmed for this cell line (Figure 5b; DMF = 1.28). In this case a HDP of 0.5 mM of 5FU was used instead of 1mM as HCT-116 cells are more sensitive to 5FU than U87MG-VIII cells (data not shown).

Interestingly, Figure 6 shows that a HDP 1-hour exposure of U87MG-VIII cells to 1mM 5FU at time points 1 to 5 hours after irradiation resulted in survival fractions greater than that obtained if both agents had been administered separately. These experiments suggest that bolus 5FU would have to be administered before irradiation in the clinic.

4.1.4 HDP and fractionation of irradiation

Smalley *et al.* suggested that HDP 5FU would be ineffective for fractionated radiotherapy because the shoulder of the survival curve was not altered by 5FU, which we have also confirmed (Figure 5). The unexpected radioprotective effect of HDP 5FU if cells were exposed up to a few hours after irradiation (Figures 3 and 6) suggested to us that pre-irradiation might alter the inherent cellular response to subsequent stresses, whether it be to 5FU or to subsequent radiation fractions.

In order to test this hypothesis, number 2 in our dissertation, we designed a simple but novel variation to the classic clonogenic assay that allowed us to see if the shoulder of the survival curve for radiation response for subsequent radiation fractions was in fact changed by

the 5FU HDP plus the first fraction of radiation. The procedural timeline of this experiment is shown in Figure 7a. We exposed cells on Day 0 to HDP 5FU (or medium alone) followed immediately by irradiation with 2Gy – a pre-survival curve irradiation.

Twenty-four hours later, on Day 1, we carried out a conventional survival curve experiment with these cells – exposing them to 0, 1, 2, 4, or 6 Gy. It is important to keep in mind that the actual doses are 2 (2+0), 3 (2+1), 4 (2+2), 6 (2+4), or 8 (2+6) Gy. The goal in this experiment was to assess whether the shoulders of the survival curves of the first and second fractions are unchanged, as the paradigm declares, or different, as we hypothesized.

Figure 7b shows that, if U87MG-VIII cells had been exposed to HDP 5FU alone on Day 0, then no radiosensitization was observed 24 hours later (DMF = 1). Additionally, if the cells had been irradiated with 2Gy on Day 0, then 24 hours later on Day 1 the cells were more radioresistant than the cells mock irradiated on Day 0 (Figure 7c; DMF = 0.76). However, if cells had been exposed to HDP 5FU plus 2Gy on Day 0, then 24 hours later on Day 1 the cells were radiosensitized – markedly compared to the cells pre-treated on Day 0 with 2Gy alone (Figure 7c; DMF = 1.09 compared to non-pre-treated control; DMF = 1.44 compared to cells pre-treated with 2Gy alone). In fact, in contrast to Figure 5a, there is a statistically significant difference ($p < 0.05$) in SF at 2Gy (actually 2+2=4Gy) between the pre-irradiated cells that were exposed to HDP 5FU and those that were not. This effect is also reflected on the alpha/beta ratio that changes from 4.3 when cells have only been exposed to 2Gy 24 hours before to 3.30 when they have been exposed to both 5FU and 2Gy. Thus, the shape of the shoulder significantly changes following incubation in HDP 5FU if and only if U87MG-VIII cells were pre-irradiated immediately post-incubation. In an analogous series of experiments, HCT cells also showed

similar responses to pre-treatments except for a significant difference within the shoulder region (Figures 7d and 7e). Accordingly, Figure 7c suggests that the shoulder of the survival curve will not necessarily be reproduced after each radiation fraction but that the shoulder, which is unchanged after the first radiation fraction, is diminished for subsequent fractions.

4.1.5 HDP and Sublethal Damage Repair Experiments

To further examine the loss of the shoulder from the U87MG-VIII survival curves shown in Figure 7c, we carried out classical split-dose experiments. The schema is illustrated in Figure 8a. When the cells were exposed to HDP 5FU and then immediately to two 2-Gy fractions separated by 0, 1, 2, or 4 hours, SLDR was unaffected, in agreement with the current paradigm for 5FU radiosensitization (Figure 8b). However, when the cells were pre-exposed to HDP 5FU plus 2Gy 24 hours prior to the split-dose, SLDR was markedly reduced (Figure 8c). Interestingly, in the HDP 5FU plus radiation pre-exposure experiments, subsequent dose-splitting of two 2-Gy fractions by 1 hour is more detrimental to cell survival than a single 4 Gy dose as it could be seen in Figure 8c; this is a reproducible observation.

4.1.6 HDP and Cell cycle

5FU is known to cause G1 cell cycle arrest and early S phase leading to a relative depletion of cells in the G2 [137]. In order to evaluate any cell cycle effects on the results described in Figures 7 and 8, the percentages of cells in each of the phases were determined under the pre-treatment conditions described in Figures 7 and 8. These results are presented in Table I. For both, HCT-116 and U87MG-VIII cells, a slight increase of cells in G1 with a comcomitnat decrease in G2/M were observed 24 hours after they had been exposed to 5FU, radiation or both when compare to those that were sham-irradiated and sham-exposed to 5FU. Combination of radiation with 5FU did not significantly alter the cell cycle distribution compared to radiation alone.

4.2 Radiosensitization by gene therapy and 5FC

As we discussed on the introduction section, 5FU does not cross effectively the blood brain barrier. Therefore, for the radiosensitization effects of 5FU on GBM cells shown on section 4.1 to have a real impact in the clinic, different ways to deliver 5FU to brain tumors are needed. In this section, experiments related to the use of gene therapy and 5FC administration to intracellularly obtained 5FU in GBM patients will be layout.

4.2.1 HDP 5FU exposure

To evaluate the effect of HDP of 5FU (1 hour exposure) on U87MG-VIII cells that have been infected with the virus (U87MG-VIII+AC3-YCD2) cells, the same were exposed to different concentrations ranging from 100μM to 1mM. As it can be seen in Figure 10 a), the introduction of the gene AC3-yCD2 seems to make the cells U87MGVIII a little more

sensitive at low doses. However when the dose is increased to 500 and 1000 μM no difference in the sensitivity to 5FU could be observed. Based on the results of Figure 10 a), we chose 1mM 5FU exposure for 1 hr, for subsequent HDP timing experiments with respect to irradiation for U87GMVIII and U87GMVIII+AC3-yCD2 cells.

4.2.2 HDP 5FC exposure

To evaluate the effect of HDP of 5FC (3 hour exposure) in U87MG-VIII and U87MG-VIII+AC3-yCD2 cells, the same were exposed to different concentrations ranging from 100μM to1mM. As it can be seen in Figure 10 b), the introduction of the gene AC3-yCD2 that makes possible the conversion of 5FC to 5FU has a huge effect on the sensitivity of the cells to 5FC. For U87MGVIII+AC3-yCD2 cells, there is a higher killing effect per unit of 5FC at low doses. However, after 250 μM the slope becomes shallower. On the other hand, in the case of U87MGVIII, initially there is a mild decrease in the survival fraction at doses around 250 μM but this decrease disappears at higher doses. In any case, Figure 10 b) indicates that the introduction of the gene AC3-yCD2 highly sensitizes U87MGVIII cells respect to 5FC. Based on the results of Figure 10 b) we chose 1mM 5FC exposure for 3 hr, for subsequent HDP timing experiments with respect to irradiation.

4.2.3 HDP 5FU as a radiosensitizer

To evaluate the use of bolus 5FU as a radiosensitizer and to see if the insertion of the gene AC3-yCD2 changed the radiosensitivity of the cells, clonogenic survival curves of U87MG-VIII and U87MG-VIII+AC3-yCD2 cells irradiated after exposure to 1 mM of

5FU for 1 hour were performed. Figures 11 a) and b) show radiosensitization of both cell lines when they were exposed to 5FU (DMF equal to 1.22 in both cases). Additionally, as it can be seen on Figure 11 a) and b), the insertion of the gene AC3-yCD2 does not change the radiosensitivity of these cells with or without 5FU exposure. Moreover, it is also important to note that the radiosensitization seen in Figure 11 a) and b) is shown at high doses and not at the shoulder region in agreement with previous reports [43].

4.2.4 HDP 5FC as a radiosensitizer

To evaluate the use of bolus 5FC as a radiosensitizer and to see if the insertion of the gene AC3-yCD2 changed the radiosensitivity of the cells after exposure to 5FC, clonogenic survival curves of U87MG-VIII and U87MG-VIII+AC3-YCD2 cells irradiated after exposure to 1 mM of 5FC for 3 hours respectively were performed. These experiments, if successful, will corroborate our hypothesis number 3. As it can be seen in Figure 11 c), cells that contain the gene AC3-yCD2 are greatly radiosensitize after the exposure to 5FC (DMF =.182) while those that do not contain the AC3-yCD2 gene are mildly radiosensitized (DMF=1.1). Additionally, as it was shown above and it can be seen on Figure 11 c), the insertion of the gene AC3-yCD2 does not change the radiosensitivity of these cell lines if 5FC is not present. Moreover, the radiosensitization observed when U87MGVIII+AC3-yCD2 cells were exposed to 5FC was bigger than the one observed when the cells were exposed to 5FU. In fact, when these cells were exposed to 5FC, radiosensitization was obtained even at low doses contrary to what was observed for 5FU.

4.2.5 CD gene transduction in vivo. Implications for radiosensitivity

To evaluate the use of bolus 5FC as a radiosensitizer in vivo and to see if the insertion of the gene AC3-yCD2 changed the radiosensitivity of the cells after exposure to 5FC, luciferase-expressing glioblastoma cell lines were developed using a using pRRL-sin-cPPT-hCMV-Fluc2 lentivirus. These cells were transfected with the lentiviral vector at an MOI of 2.0 and expression of firefly luciferase was detected *in vitro* by optical imaging system. A direct correlation between the number of cells and luminescent signal was corroborated. After U87MG-VIII-Fluc2 had been obtained we also infected them with the virus AC3-yCD2 to obtain U87MG-VIII -AC3-yCD2-Fluc2 cells. Both cell lines were plated and exposed to 0.1 mM 5FC for 2 hours after which they were sham irradiated or exposed to 2Gy or 4Gy. These cells were later on inoculated into athymic mouse brain (1×10^5 cells / mouse) and the bioluminescence signal coming from Fluc2 was determined as a direct measurement of the brain tumor size at different times. These results could be seen in Figure 12. No significant difference between the size of the tumor after 10 days were seen for cells that were irradiated (2Gy or 4Gy) or sham irradiated which is a direct indication of how resistant U87MG-VIII cells are. All mice in these groups dye by day 14. On the other hand, if cells had been previously infected by AC3-yCD2 (U87MG-VIII -AC3-yCD2-Fluc2) and exposed to 5FC, a significant different ($*$: $p < 0.05$) in signal as early as in day 4 could be seen respect to the control group or the irradiated cells. Thes results also corroborate the efficacy of 5FC treatment for those cells that have been infected by AC3-yCD2. Finally, when both radiation and 5FC HDP are combined, a significant reduction of tumor size could be seen on day 17 compared to the other groups ($***$: $p < 0.001$).

4.3 Towards 5FU targeting via MDEPT

As we discussed in the introduction, despite the promising results showed by suicide gene therapy, new ways to target 5FU to turmors are needed [108-110, 114]. In the following experiments, we will present the results of a new approach that we have named Metabolic Directed Enzyme Prodrug Therapy. This approach, albeit in very early development, seems highly promising.

4.3.1 MBP317-347 is a high-affinity switch for maltose like polysaccharides

To corroborate that the BLA domain of the hybrid MBP317-347 was activated upon binding of maltose, colorimetric assays at different concentrations of the disaccharide were performed using the velocity of nitrocefin hydrolysis as an indicator of enzyme activation. As shown in Figure 13, maltose concentrations as low as 0.5 μM were sufficient to activate the switch; saturation was achieved at doses above 8 μM. These results are in agreement with a K_d for maltose-binding at 22 ^oC of 1.7(± 0.5) μM previously reported by Guntas et al [138]. MBP317-347 was also activated by micromolar concentrations of longer glucose polysaccharides with α(1,4) glycosidic bonds such as maltotriose, consistent with MBP's known ability to bind these compounds and undergo a similar conformational change upon binding. Figure 13 shows that the longer the size of the polysaccharide chain, the smaller the full activation of the enzyme when compared to maltose, presumably as a result of differences in the conformational change caused by these disaccharides. This trend was the same for all the polysaccharides except for maltopentaose and maltohexaose. From these experiments, we conclude that MBP317-347 is a high-affinity switch for α(1,4) polyglucoses responding at concentrations of few micromolar, and that it saturates above concentrations of approximately 8 μM.

4.3.2 MBP317-347 is a low-affinity switch for glucose

Maltose is a disaccharide of two glucose units joined by an α(1-4) bond. We hypothesized that the probability of finding two glucose molecules together in the same configuration as one molecule of maltose, is proportional to the square of the concentration of glucose; therefore, a millimolar glucose solution should also be able to activate our switch. To prove this, we performed an activation assay and exposed MBP317-347 to different concentrations of glucose. Figure 14 shows that MBP317-347 becomes active at 1 mM, behaving as a low-affinity switch for glucose, with a threshold-like sigmoidal response to increasing physiological concentrations of glucose (1-10 mM). As the concentration of glucose required to activate the switch is three orders of magnitude higher than that of maltose, it was necessary to rule out the possibility that the results shown in Figure 14 were not due to maltodextrin contamination of the glucose sample. We therefore measured the activation profile of cellobiose, an isomer of maltose that binds two molecules of glucose through a β(1,4) bond in contrast to an α(1,4) bond for maltose, before and after degradation to glucose.

As shown in Figure 15 a), cellobiose does not activate the switch even at concentrations of 100 mM. Glucose produced by cellobiase digestion of this same cellobiose sample was then used to determine activation of MBP317-347. As shown in Figure 15 b), the activation of MBP317-347 by glucose produced from cellobiose is indeed able to activate the switch at concentrations above 0.5 mM to a similar extent. This indicates that the previously described activation in Figure 14 is not due to contaminating traces of maltodextrins, but rather by a bona fide interaction between the switch and glucose itself. Consequently, we have shown that MBP317-347 is a low-affinity switch for glucose with a sigmoidal glucose-dose vs. activation response between 0 mM and 50 mM – a range that includes physiological concentrations [139].

5. Discussion

5.1 *Radiosensitization using 5FU bolus injection*

Current protocols for combinational 5FU chemoradiotherapy are based on Byfield *et al.*'s pioneering work [35], which dictates that the radiosensitizing effect of 5FU manifests only if present at least 24 hours after irradiation [35]. For more than 50 years, this study has been the basis for further investigations that support the idea that 5FU must be given as a low-dose CVI to maximize treatment outcomes when combined with radiation [23, 26, 27].

In order to evaluate if this assumption holds true, we carried out a comprehensive examination of the dose-time correlation of radiosensitization with 5FU. Opposing to previous believes, we showed that the highly aggressive and radiosensitive human GBM cell lines U87MG-VIII and HCT-116 can be radiosensitized by HDP of 5FU if the pulse is administered before irradiation. In fact, our results challenge the tenets of the current paradigm, which can be summarized as follows: 1) radiation-induced SLDR is not affected by the addition of 5FU, 2) radiosensitization is maximized only if cells are chronically exposed to drug for more than 24h following irradiation, 3) radiosensitization only occurred in cells that are sensitive to 5FU alone, and 4) CVI is deemed the best clinical option.

In that sense, we established the U87MG-VIII cells as a model system in accordance with current principles. We first showed that U87MG-VIII cells are indeed radiosensitized by low dose concentration (LDC) of 5FU for 48 hrs after irradiation Figure

3. In the same lieu, we did not observe radiosensitization of cells when incubated in LDC of 5FU for 48 hrs prior to irradiation (Figure 3) in agreement with Byfield et al.

Next, we examined the sensitivity of U87MG-VIII cells to HDP 5FU in order to establish the ideal BI concentrations for further experiments, and to verify that they were within a clinically relevant range. As it can be seen in Figure 4, U87MG-VIII is sensitive to 5FU administered for 1 hr in the range of 100-1000 uM. In the same lieu, HCT-116 cells were also found to be sensitive to 5FU in the same range (Data not shown). In the clinic, the tolerable dose for the method of delivery is limited by the toxicity. Although both CVI and HDP produce mucositis and diarrhea, HDP schedules cause greater leucopenia, while patients treated with CVI are affected by stomatitis and dermatitis [140-143]. The total tolerable 5FU dose for CVI ranges from 1,625 to 2,875 mg/m^2/wk, and from 500 to 750 mg/m^2/wk for HDP [144, 145]. Extensive experimental and clinical studies have examined the pharmacokinetic differences between HDP and CVI for 5FU as a single agent. Conventional HDP doses (400 to 600mg/m2) result in peak plasma concentrations in the near-millimolar range (100 to 1000 umol/L) followed by a rapid decline with a half-life of ≤20 minutes [23, 26, 27]. Consequently, it could be seen from above discussion that exposure of cells in vitro to HDP 5FU between 100 to 1000 umol/L for 1 hr would be clinically relevant. In that sense, we chose to expose U87GMVIII to 1mM for 1 hour for to asset these cell lines' radiosensitivity after HDP which are in both cases the LD50 respectively.

In that sense, as it can be seen in Figure 6, radiosensitization cannot be observed if the cells are irradiated 0-5hr before HDP 5FU exposure. In fact, the combination of both

agents result in less killing than the one would have been expected if they had acted separately. A possible explanation for this phenomenon could be the lack of 5FU uptake after irradiation due to cycle arrest cause by irradiation. It is important to note that these results might be a cell line-specific phenomenon, as it has been previously suggested that the rules for radiosensitization are not equally demonstrated for all cell lines [146].

On the other hand, contrary to the current paradigm, we did observe radiosensitization of U87GMVIII and HCT-116 cell lines when cells are irradiated after exposure to HDP 5FU Figure 5 a, b. Another noteworthy point from this perspective is the relationship between 5FU's effects and fractionated radiotherapy. The current paradigm states that radiation-induced SLDR is not affected by 5FU; i.e., there is little impact on the shoulder region of the survival curve. This observation spawned arguments that although radiosensitization could be exploited clinically at high radiation dose, it would be less effective with conventional fractionation schemes of radiotherapy. We have addressed this issue and demonstrated that in agreement with the current paradigm, the shoulder of the survival curve is unaffected with the addition of HDP 5FU as could be seen in Figure 5 a, b. However, we have also shown that a single short pulse of high-dose 5FU prior to the first fraction would affect the shoulder during subsequent fractions of radiation, Figure 7. As it is shown in Figure 7 c, e after the first 2Gy fraction cells become more resistant if they were not exposed to 5FU. This phenomenon could be explained if we take into account that the radiosensitivity of the cells follows a distribution. The first fraction would kill the more sensitive cells leaving the more resistant for subsequent fractions. However, this effect is dramatically reversed as it could be seen in Figure 7 c, e if cells are exposed to 5FU before the first fraction of 2Gy. If cells are only exposed to 5FU and not to 2Gy 24 hours before,

the radiosensitization effect disappears though, Figure 7 b,d. At this point we hypothesized that the radiosensitization produced in further fractions after the cells had been exposed to 5FU and 2Gy was due to a change in the SLDR system. This hypothesis was actually confirmed by our split-dose experiments shown in Figure 8 b,c, where changes on the SF under split doses were only observed in cells previously exposed to 5FU and radiation 24 hours before the experiment. The explanation for these results might rely on the mechanism of action of 5FU. As 5FU enters the cell, it is converted into active metabolites important in DNA and RNA biochemistry: FdU-triphosphate (FdUTP), fluorodeoxyuridine monophosphate (FdUMP), and fluorouridine triphosphate (FUTP) [6, 147]. FdUMP inhibits the enzyme thymidylate synthase (TS) by forming a reversible covalent bond [5, 148]. TS is active in the S cell cycle phase and catalyzes methylation of dUMP to dTMP, the sole de novo source of dT for DNA synthesis. On the other hand, FUTP is incorporated into RNA, which obstructs its normal processing and general functions. In general, short-lived proteins with mRNAs of rapid turnover are the most affected by FUTP incorporation into the RNA–like thymidine kinase (TK) [21], which incidentally, is responsible for the salvage conversion of dT to dTMP. As it was shown by Nord et al. the decrease of the activities of both TS and thymidine kinase (TK) last for up to 96 hours after HDP of 5FU [21]. In that sense, after cells are exposed to 5FU and irradiated, the existent pool of DTTP needed for the repair of DNA is still available and allows for efficient repair. However, after the cells are irradiated, the DTTP pool cannot be replaced in cells exposed to 5FU, and the DNA repair mechanism is compromised 24 hours after the first fraction of radiation and HDP of 5FU explaining the results observed in Figure. 8. Additionally, it is important to note that the decreased of sparing in cells when these ones are irradiated one hour apart

after having been exposed to 5FU and 2Gy 24 hours before the split doses experiment is intriguing. At this time we do not have a plausible explanation for this effect, as further study is required to reach any conclusions.

On the other hand, regarding translation to the clinic, our experiments suggest different possibilities. As we found, one single tolerable HDP of 5FU might radiosensitize the cells for at least 24 hours. These results indicate that the use of fractionated radiotherapy in combination with suicide gene therapy and the prodrug 5-fluorocytosine (5FC) currently undergoing clinical trial might be ideal [132]. 5FC is converted to 5FU by the enzyme cytosine deaminase [27]. By developing a retrovirus that contains a cytosine deaminase gene (AC3-YCD2) Hiraoka et al. were able to specifically target cancer cells and produce 5FU inside them [27]. Moreover due to 5FC low toxicity, they are able to administer it in the HDP range every day for a long period of time with minimal side effects. Administration of HDP of 5FC and its conversion to 5FU every day during a fractionated scheme would take advantage of the radiosensitization that occurs after each fraction. However, we must highlight that in order for this protocol to work, it is important to show that radiosensitization is produced after the 5FC is converted to 5FU in the cells. These propositions will be discussed further below.

Finally, we should also address the fact that as it now several clinical trials have failed to show radiosensitization after HDP of 5FU.First of all, these clinical trials were done using dose typical in fractionated schemes (1.8-2 Gy). However, as it was shown by us and suggested by other authors, the biggest radiosensitization is obtained for higher doses like those used in SBRT and SRS. In fact our results make 5FU HDP an ideal

candidate to be used together with these radiation protocols. Second, even for doses typical of fractionated regimes, we think that the scheduling between radiation and 5FU was suboptimal. As it was mentioned in the introduction, most of the irradiations in these clinical trials were performed without effect of 5FU. However, due to the fact that TS and TK activity will be decreased for up to 96 hours after HDP of 5FU and as result TTP will not be produced as shown by Nord et al. [21], we could hypothesize here that the radiosensitization seen in Figure 7 after the first fraction of radiation might last for up to 96 hours. As a result, the administration of one HDP of 5FU at the beginning of each week during the radiation therapy might render radiosensitization for the whole period and not only on the first and last week as the schemes used in previous clinical trials did. In that case, synergism between HDP 5FU and radiation should be expected.

5.2 *Radiosensitization via gene therapy and 5FC*

In this dissertation, we have shown using glioblastoma cell lines that 5FU, if administered as a BI, does indeed radiosensitize. Those results were further corroborated when we showed in Figure 11 that U87MG-VIII and U87MG-VIII-AC3yCD2 are radiosensitized after exposure to 5FU (LD50) for 1 hour. In order to exploit these findings to increase the therapeutic gain in the treatment of brain tumors, an efficacious method to deliver 5FU to brain tumors is critical. In this respect, the use of gene therapy seems to offer several advantages. As it has been previously discussed, 5FU could be effectively delivered to GBM tumors after treatment with an RCR vector encoding the yeast CD suicide gene [16], which results in conversion of the nontoxic, BBB-permeable prodrug 5FC to the highly toxic non-BBB-permeable 5FU. In fact, as mentioned earlier, this gene therapy approach is currently on clinical phase I/II trial (www.clinicaltrials.gov

NCT01156584) showing promising results. Therefore, in order to incorporate radiation into this current clinical trial, it behooved us to prove that 5FC does not radiosensitize cells that are not infected by AC3yCD2 and that it does radiosensitize those that are.

As illustrated in Figure 11 c), the radiosensitivity of U87MG-VIII and U87MG-VIII-AC3yCD2 is the same, indicating that infection with AC3yCD2 does not radiosensitize these cells. Further, the radiosensitivity effect by 5FU was not altered by the introduction of the AC3yCD2 gene. Therefore, infection by AC3yCD2 does not alter the cell's intrinsic sensitivity to 5FU, radiation, or 5FU-induced radiosensitivity. On the other hand, as shown in Figure 10 b), there is a large AC3yCD2-infection-dependence on sensitivity to 5FC of these cells. And accordingly, the U87MG-VIII-AC3yCD2 cells are greatly radiosensitized after HPD of 5FC.

On the other hand, Figure 11 c) demonstrates that 3 hours of exposure to 5FC considerably radiosensitizes the cells if they had been infected with AC3yCD2. The radiosensitization caused by the three-hour exposure to 5FC is larger than that caused by the 1-hour exposure to 5FU and is achievable even at low radiation doses, within the shoulder-region of the cell survival curve. These results were also corroborated by invivo clonogenic assays experiments as it could be seen in Figure 12. The data therefore suggest that HDP 5FC may affect sublethal repair of DNA to a greater extent than HDP 5FU. Additionally, these results make GBM treatment amenable to the combination of CD suicide gene therapy and either fractionated radiation therapy or SRS (stereotactic radiosurgery) treatment methods. Finally, these encouraging results could open the door for the incorporation of radiation into the protocols currently being carried out (NCT01156584). As Tai et al. and others have shown that 5FC to 5FU conversion using AC3yCD2 occurs only on the tumor cells, radiosensitization could be localized and the healthy

tissue spared [15, 20, 21], thus potentiating the synergistic therapeutic gain after the 5FC and radiation administration.

5.3 *Radiosensitization via metabolic directed enzyme prodrug therapy*

Currently, several methods for cancer drug targeting that rely on specific tumor markers are undergoing various levels of clinical trials. Among them, the suicide gene therapy approach that we have extensively discussed in this dissertation. However, problems such as marker mutation, differential marker expression among tumors, virus mutation and marker expression in normal cells still need to be resolved. We propose that a Metabolically Directed Enzyme Prodrug Therapy (MDEPT) based on exploiting the fundamental difference between tumors and normal tissues would circumvent many of these confounders. In the present dissertation, we show that MBP317-347 acts as a low-affinity switch that responds to glucose concentrations in the millimolar range. We have demonstrated that MBP317-347 could potentially be preferentially activated in tumor cells with high glucose content and that therefore, it could be used as a proof of concept for MDEPT. However, it is important to recognize that the use of MBP317-347 will be limited invivo, because it may become activated by high levels of serum glucose; although this should be controllable to a certain extent in animal studies. Moreover, unlike lactate, which is excreted and accumulates within tumors resulting in high local concentrations, especially in aggressive ones, glucose concentrations in the tumor are less predictable and sometimes contradictory [139, 149]. On the other hand, activation by maltose should not be a problem since all disaccharides and longer sugars are cleaved into monosaccharides before transported across the intestinal walls as glucose [150]. Additionally, the concentration of glucose in the bloodstream is similar to that found inside tumors and therefore, the switch will be also active in

the blood [151] . One way to circumvent these issues in vivo would be to use an amphiphilic prodrug that is converted to a polar drug by the switch. If the prodrug is converted outside the cells, in the blood, the drug will not be able to cross the cell membrane and its toxicity to healthy tissue will be diminished. On the other hand, in the tumor cells with high concentrations of glucose, once the prodrug cross the cell membrane it will be converted to a polar drug and it will be trapped inside the cell to exert is toxic effect.

Accordingly, testing whether cells exposed to high glucose concentration, such as the one found in cancer cells, are preferentially killed by conversion of a 5FU prodrug by MBP317-347 is currently ongoing. 5FU is one of the oldest chemotherapeutic drugs and it has successfully used in the fight against cancer for decades [1]. Additionally, It is important to note that the switching activity of this protein has already been shown to be sufficient to result in an observable phenotype: maltose-dependent resistance to ampicillin of *E. coli* bacteria [138] .

Finally, as glucose has certain limitations to be used as an activator in vivo due to its high concentration in blood and the limitation that this imposes in the type of prodrug used, alternative molecular switches could be developed. One excellent prospect for our proposed MDEPT approach is a low-affinity lactate switch. The difference in lactate concentrations between tumor and healthy tissues can be up to 8 fold compare with the 3-fold for glucose [115, 116]. Furthermore, because glycolysis is up-regulated in cancer cells in the absence of aerobic respiration, large quantities of lactate are produced, amplifying the difference in concentration of the molecule between tumors and healthy tissue. In fact, tumors with high concentrations of lactate (above 8.3 mM) are associated with a poor prognosis and low survival factors [116]. Similar results have been observed in head and neck adenocarcinomas where the mean lactate concentration is approximately 12.5 mM, with the most aggressive ones reaching over 20mM

[116]. In contrast, low concentrations of lactate (below 8.3 mM) in tumors correlate with high survival in carcinomas of the uterine cervix [116]. Additionally, as radiotherapy is less effective in treating the hypoxic areas of tumors, where lactate levels are especially high, MDEPT would be well suited as an adjunct to radiotherapy as well.

The development of physiologically relevant lactate switches is a real possibility. A low-affinity lactate periplasmic binding protein (LPBP) can be coupled to BLA. The coupling can be done by using the same method that Guntas et al. used when they designed the MBP-BLA switch [138]. In fact, Looger et al. designed three distinct LPBP's with Kd's equal to 0.95, 20 and 25 mM, respectively [152]. The coupling of those LPBP with high Kd's to BLA is expected to give lactate switches (LPBP-BLA) that will be activated upon physiological concentrations of lactate (1.5-20 mM) and would be preferentially activated in the tumor area.

6. Conclusions and future work

As it has been shown above, 5FU, after being administered as a LDC pulse, radiosensitizes U87MG-VIII and HCT-116 cell lines. The radiosensitization obtained was higher at high doses suggesting that 5FU might be an excellent candidate to be used together with SRS or SBRT protocols. On the other hand, we also showed that in the low

dose range, there was no significant difference between the survival fractions after irradiation of cells that had been expose to 5FU and those that had not. Similar results had prompted other authors to assert that 5FU would be a little value in fractionated regimes [43] . However, we saw that if cells had been exposed to 5FU and irradiated with 2Gy 24 hours before the survival curve experiment, then there is a significant difference in the survival fraction between cells that were exposed to 5FU and those that were not even in the low dose region. These results could be interpreted as obtaining radiosensitization for at least up to the second fraction of a fractionated radiation regime. However, in order for these results to have real impact on the fractionated regimes that are currently used in the clinic, radiosenstization should last for up to 96 hours. If we take into consideration that when 5FU HDP is administered, the activities of both TS and thymidine kinase (TK) decreased for 96 hours, [21]; then, it could be hypothesized that after the first 2 Gy the pool of thymidine will be used and the radiosensitization will not only last for 24 hours but for up to 96 hours. In that case, one could envision a regime where the patients are given high doses of 5FU at the beginning of each week during the fractionated radiation treatment to maximize outcomes. As 5FU acts preferentially in cancer cells, it should be expected that such regime will radiosensitize the cells thorough the duration of the radiation treatment and increase the therapeutic ratio. Additionally, another way to translate our results to the clinic as we showed above would be through the use of gene therapy and the conversion of 5FC to 5FU [132]. In this case, due the low toxicity of 5FC to mammalian cells, one could envision the same to be administered every day in the concentration range typical of bolus injection together with radiation to maximize the therapeutic ratio of both treatments. Our results in this area are highly encouraging and might support the incorporation of radiation

to the clinical trial given by the following ID number NCT01156584. Finally, a novel target mechanism that take advantage of the Warburg effect and the high concentrations of lactate present in tumors was discussed in this dissertation. Although much of the experimental work for Metabolic Directed Enzyme Prodrug Therapy is still ahead of us, we showed that at least theoretically, this mechanism could be successful. However, the following step, and definitely not a simple one, would be to design and obtain the lactate switch. We are confident that this task would be successfully completed in the near future and further work will be carried.

7. Figures and Tables

Figure 1.

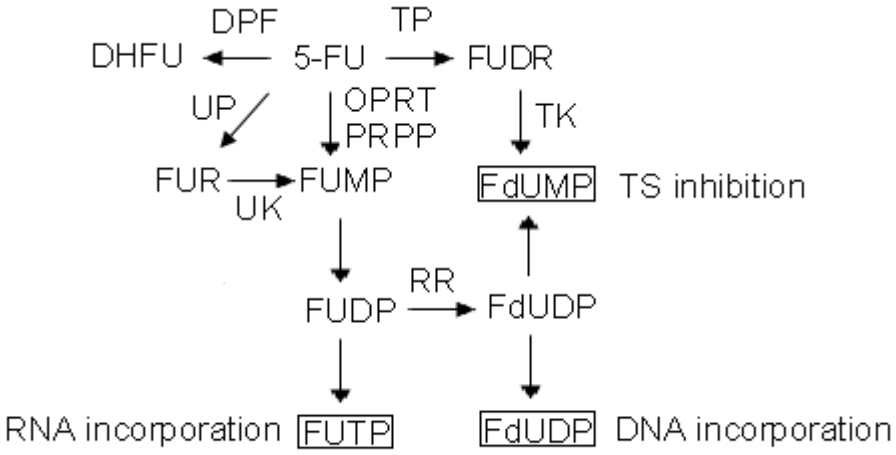


Figure 1. Catabolism and metabolism of 5-FU. Upon entrance to the cell, 5FU is converted to three different metabolites: fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate (FUTP). The mechanism and proteins involved in the conversion of 5FU to these metabolites is represented in this Figure. The name of all enzymes and cofactors involved are as follow: fluorouridine monophosphate (FUMP), orotate phosphoribosyltransferase (OPRT), phosphoribosyl pyrophosphate (PRPP), fluorouridine (FUR), uridine phosphorylase (UP), uridine kinase (UK), fluorouridine diphosphate (FUDP), fluorouridine triphosphate (FUTP), fluorodeoxyuridine diphosphate (FdUDP), ribonucleotide reductase (RR), fluorodeoxyuridine (FUDR), thymidine kinase (TK), Finally, Dihydropyrimidine dehydrogenase (DPD) directs the degradation on 5-FU to dihydrofluorouracil (DHFU) being the rate-limiting step of its catabolism.

Figure 2.

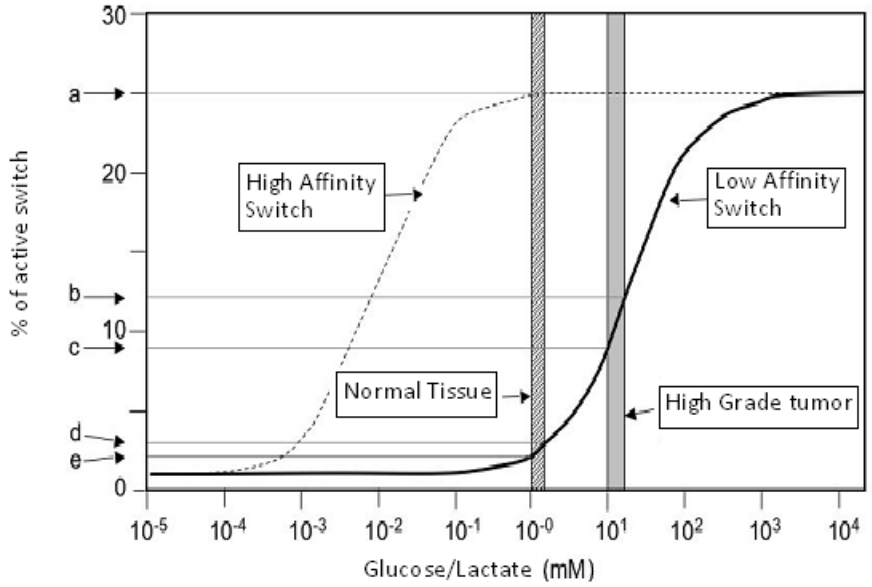


Figure 2. *Percent switch activity vs concentration of activator. The High-affinity Switch has been* plotted for an effector binding K_d of 10 μM (dashed line) and the low-affinity switch for K_d of 20 mM (solid line) for various hypothetical concentrations of glucose or lactate. The hatched region represents the typical range of concentration of these metabolites found in resting normal tissues and the gray region represents the range on high-grade head and neck cancers. Note that arrow 'a' illustrates that there would be no difference in the % of activation of the switch in normal tissues and tumors if the switch has high-affinity for glucose or lactate because even the low concentrations of these metabolites on tissues would bind. However, note that the % of activation is 2-5 times higher in the tumor than in the normal tissues (compare b-c to d-e) if a low-affinity switch is used.

Figure 3.

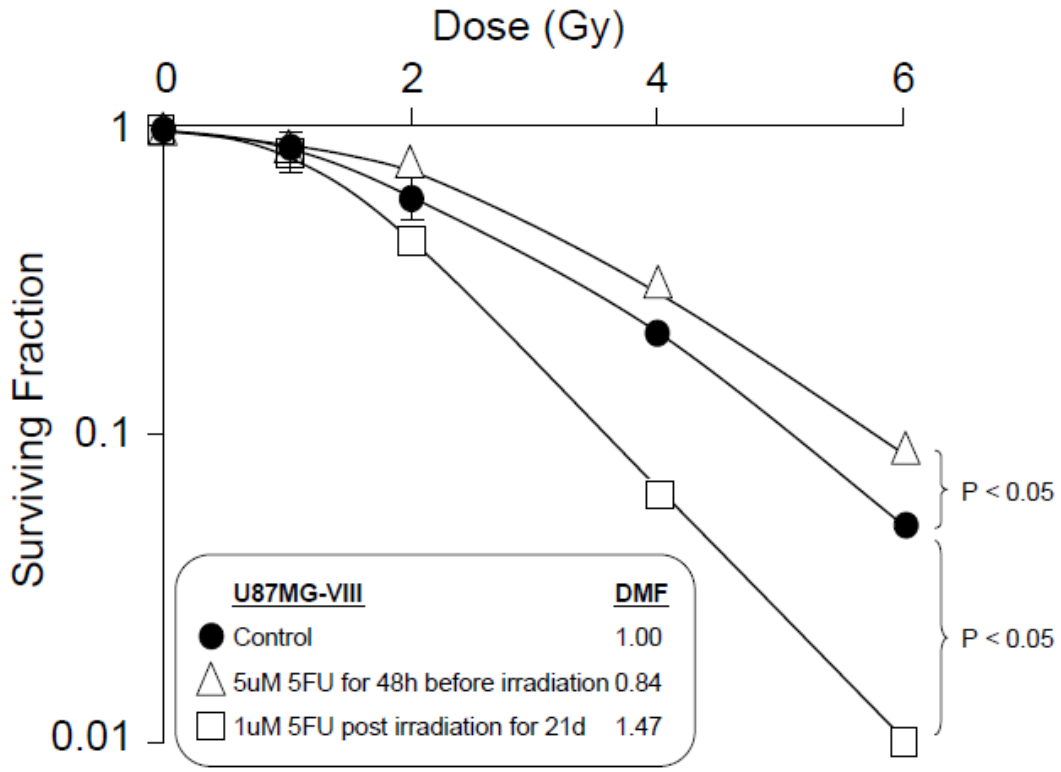


Figure 3. LDC 5FU after and before irradiation. *1μM post-irradiation for 21 days and control:* A known number of U87MG-VIII cells were irradiated on day 1 and cultured in medium alone or containing 1μM of 5FU. After 21 days, colonies were fixed, stained, and enumerated. 5*μM for 48h before irradiation:* 5FU87MG-VIII cells were exposed to 5 μM of 5FU for 48 hours after which the cells were washed, irradiated, and counted such that a known number was plated in medium alone. Each point represents the average of three experiments with 3 replicates each. In some cases, the error bars (standard errors) are obscured by experimental points. See Materials and Methods for DMF calculations.

Figure 4.

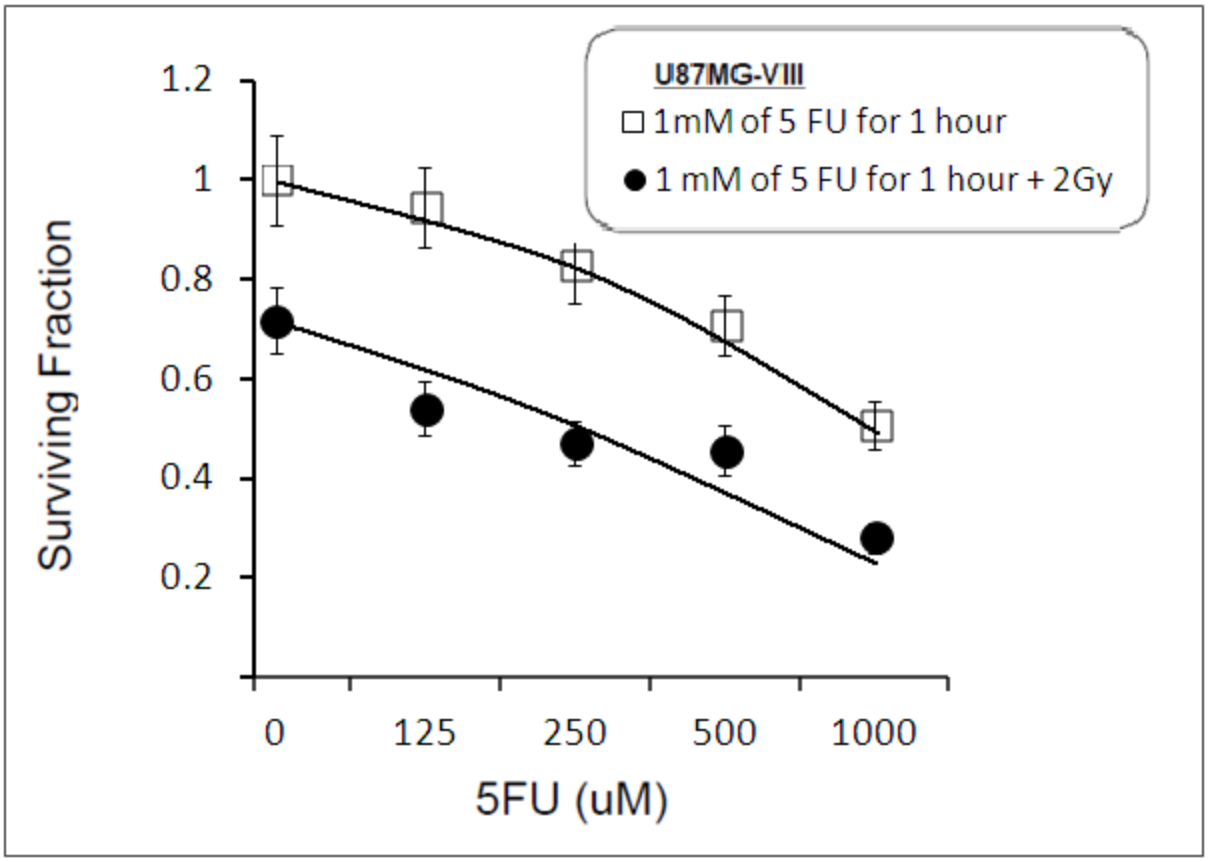
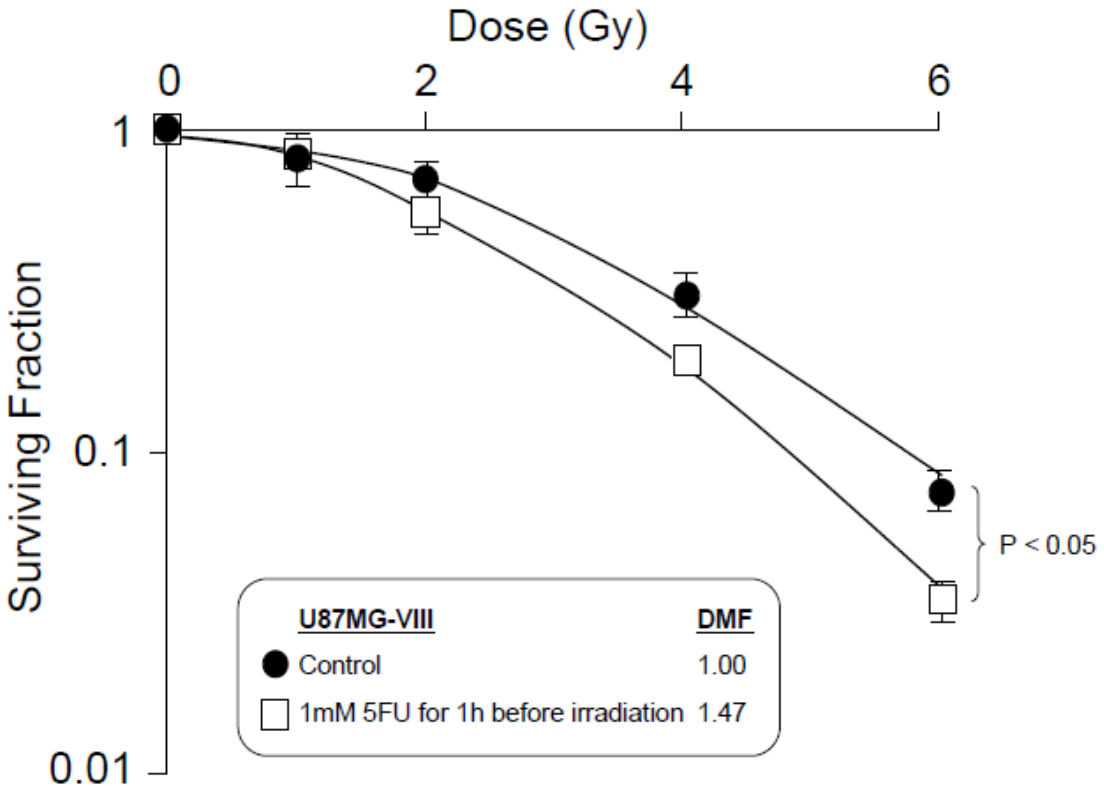


Figure 4. Effects of 5FU concentrations on U87MG-VIII cells. U87MG-VIII cells were incubated in 5FU for 1 hour (concentration range 100 μM – 1mM) mimicking the 10-15 minutes half-life of 5FU *in vivo*. The cells were washed, counted, and plated. After 21 days, colonies were fixed, stained, and enumerated. Each point represents the average of three experiments with 3 replicates each. Error bars represent standard errors.

Figure 5

a)


Dose (Gy)
0
2
4
6
1
0.1
0.01
Surviving Fraction
P < 0.05
U87MG-VIII
DMF
Control
1.00
1mM 5FU for 1h before irradiation
1.47

b)

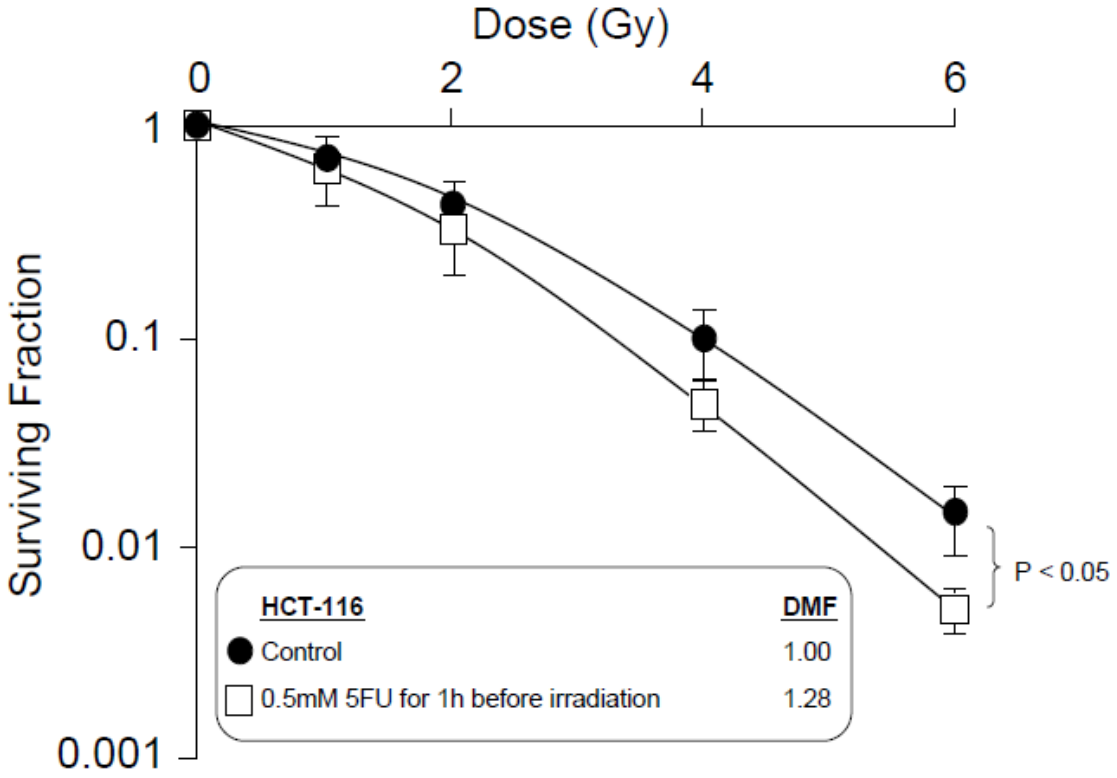
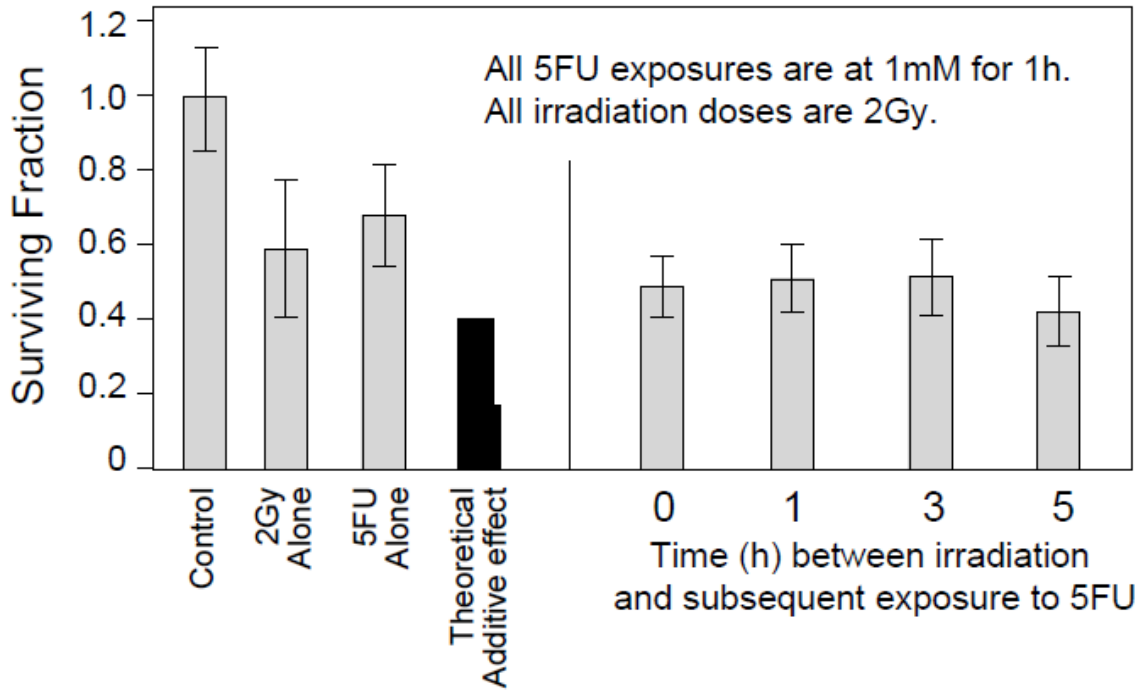


Figure 5. HDP 5FU followed 1hr later by irradiation. a) U87MG-VIII cells were exposed to 5FU (1mM) for 1 hour (HDP) or medium (control), washed and irradiated 1 hour later. Cells were counted and known numbers were plated in medium alone. After 21 days, colonies were fixed, stained, and enumerated. b) HCT-116 cells were exposed to 5FU (0.5 mM) for 1 hour (HDP) or medium (control), washed, and irradiated 1 hour later. Cells were counted and known numbers were plated in medium alone. After 21 days, colonies were fixed, stained, and enumerated. Each point represents the average of three experiments with 3 replicates each. In some cases, the error bars (standard errors) are obscured by experimental points. See Materials and Methods for DMF calculations.

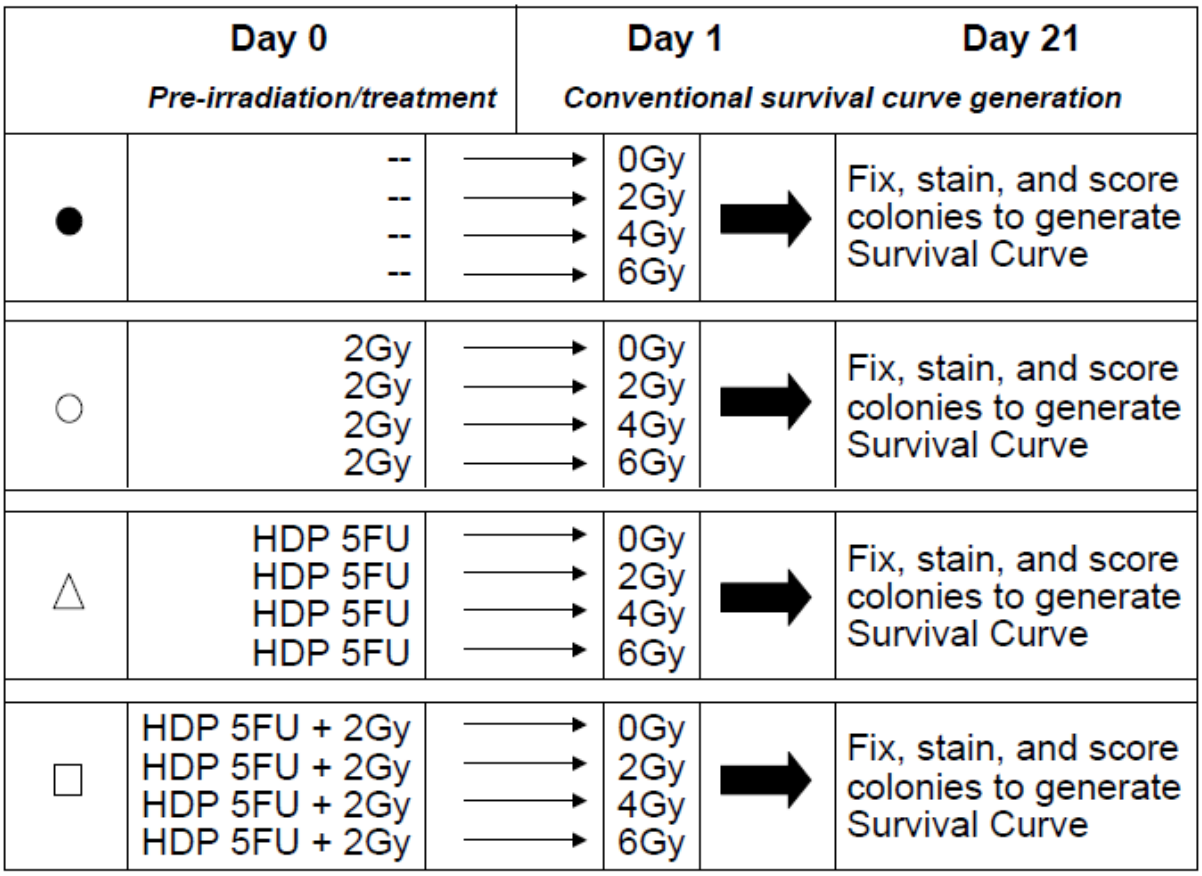
Figure 6.



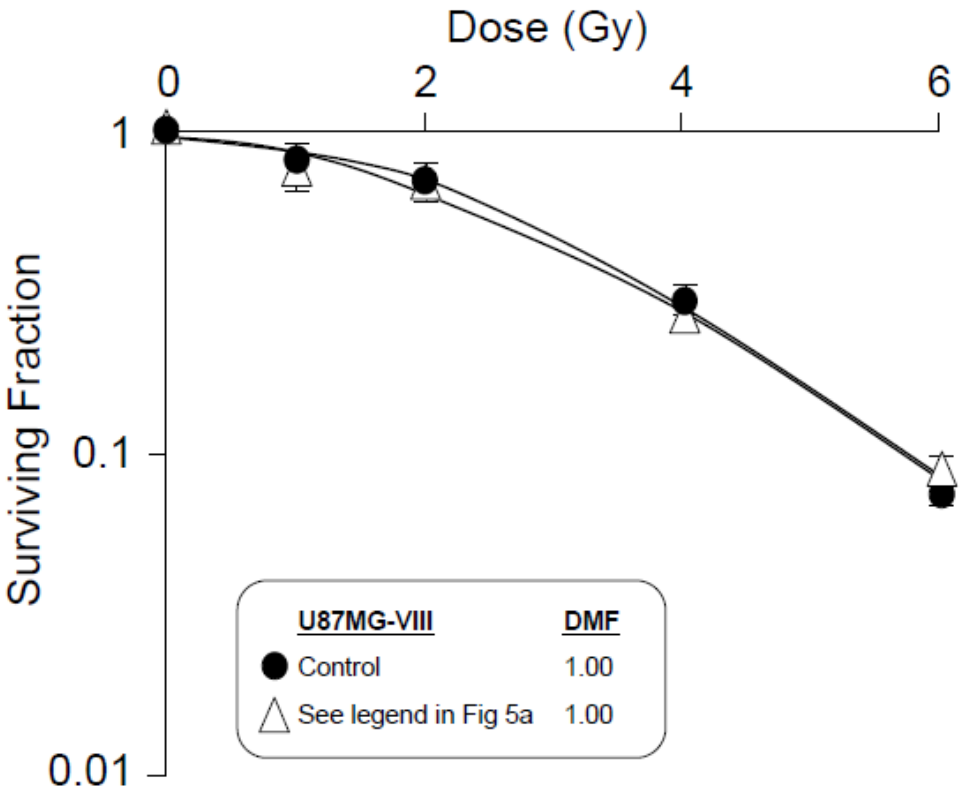
Effect of HDP 5FU when administered after irradiation. U87MG-VIII cells were incubated in 1mM of 5FU for 1 hr (HDP) at different times (0h-5h) after irradiation (2Gy). Cell were washed, counted, and a known number was plated in medium alone. Colonies were fixed, stained, and enumerated after 21 days. The survival fractions of cells only exposed to 5FU (5FU) or irradiation (2Gy) are shown together with the theoretical value of the survival fraction obtained if the effect of both agents was additive – that is, if there was no synergistic effect. A SF greater than the SF of the additive effect indicates that both agents together kill less than if they have been administered separately. Each point represents the average of three experiments with 3 replicates each. Error bars represent standard errors.

Figure 7.

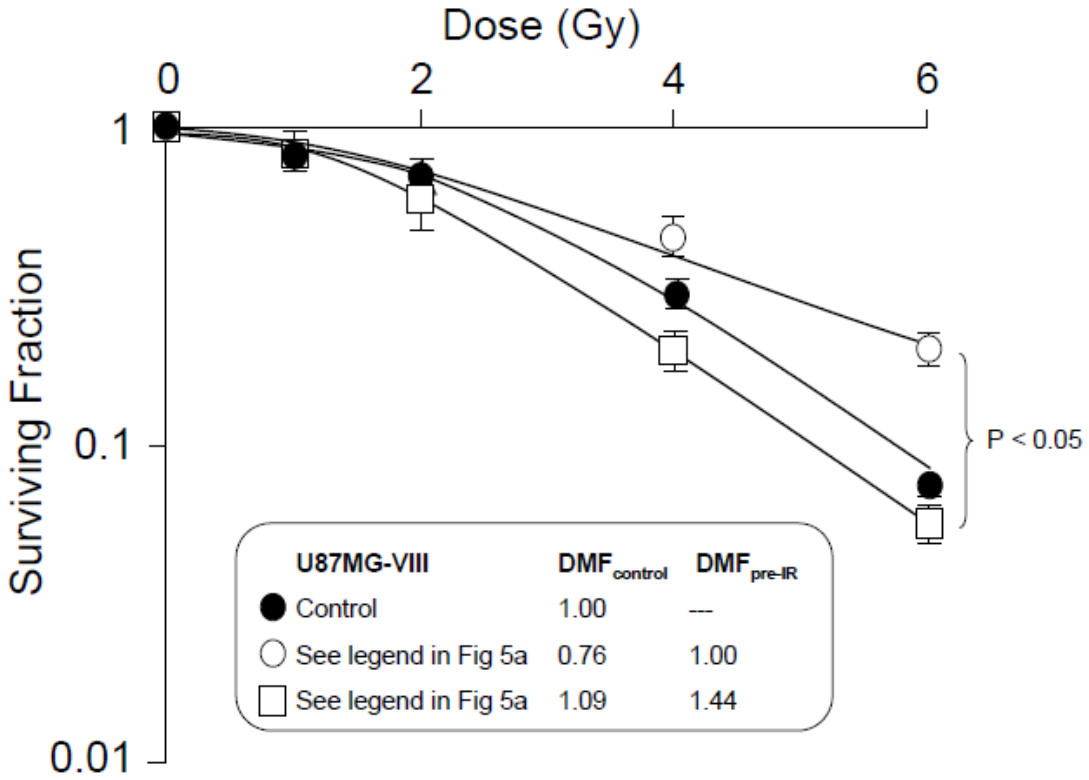
a)


Day 0
Pre-irradiation/treatment
Day 1
Conventional survival curve generation
Day 21
●
--
--
--
--
0Gy
2Gy
4Gy
6Gy
Fix, stain, and score colonies to generate Survival Curve
○
2Gy
2Gy
2Gy
2Gy
0Gy
2Gy
4Gy
6Gy
Fix, stain, and score colonies to generate Survival Curve
△
HDP 5FU
HDP 5FU
HDP 5FU
HDP 5FU
0Gy
2Gy
4Gy
6Gy
Fix, stain, and score colonies to generate Survival Curve
□
HDP 5FU + 2Gy
HDP 5FU + 2Gy
HDP 5FU + 2Gy
HDP 5FU + 2Gy
0Gy
2Gy
4Gy
6Gy
Fix, stain, and score colonies to generate Survival Curve

b)

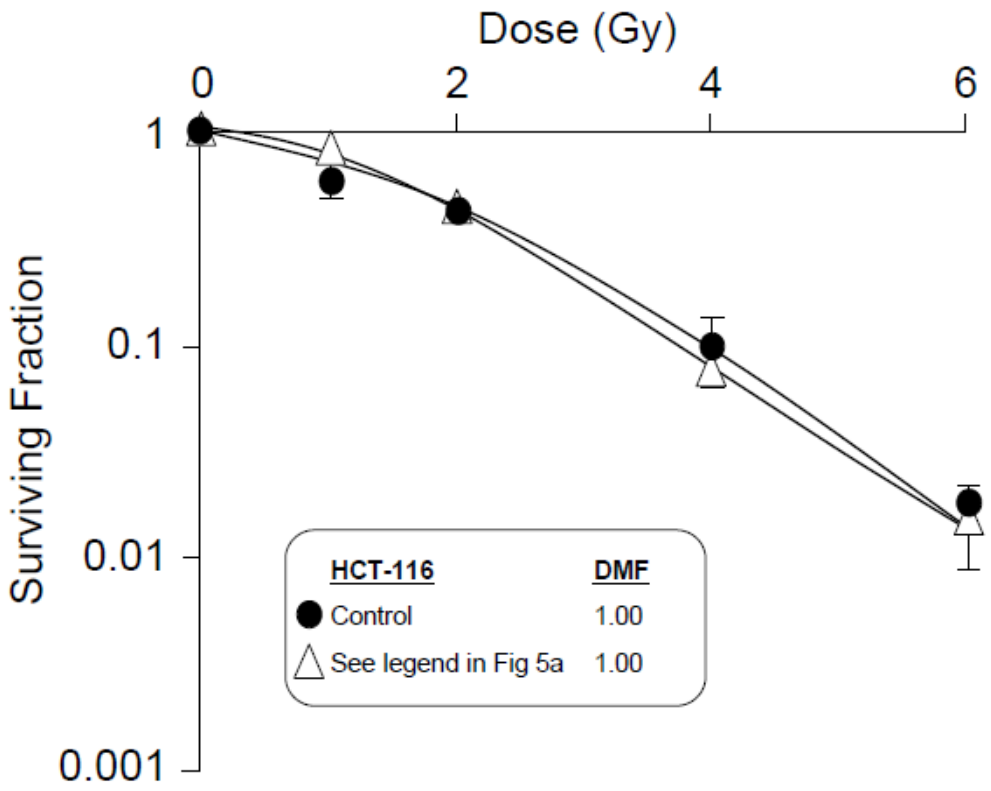

Dose (Gy)
0
2
4
6
1
0.1
0.01
Surviving Fraction
U87MG-VIII
DMF
Control
1.00
See legend in Fig 5a
1.00

c)


Dose (Gy)
0
2
4
6
1
0.1
0.01
Surviving Fraction
P < 0.05
U87MG-VIII
DMF control
DMF pre-IR
Control
1.00

See legend in Fig 5a
0.76
1.00
See legend in Fig 5a
1.09
1.44

d)


Dose (Gy)
0
2
4
6
1
0.1
0.01
0.001
Surviving Fraction
HCT-116
DMF
Control
1.00
See legend in Fig 5a
1.00

e)

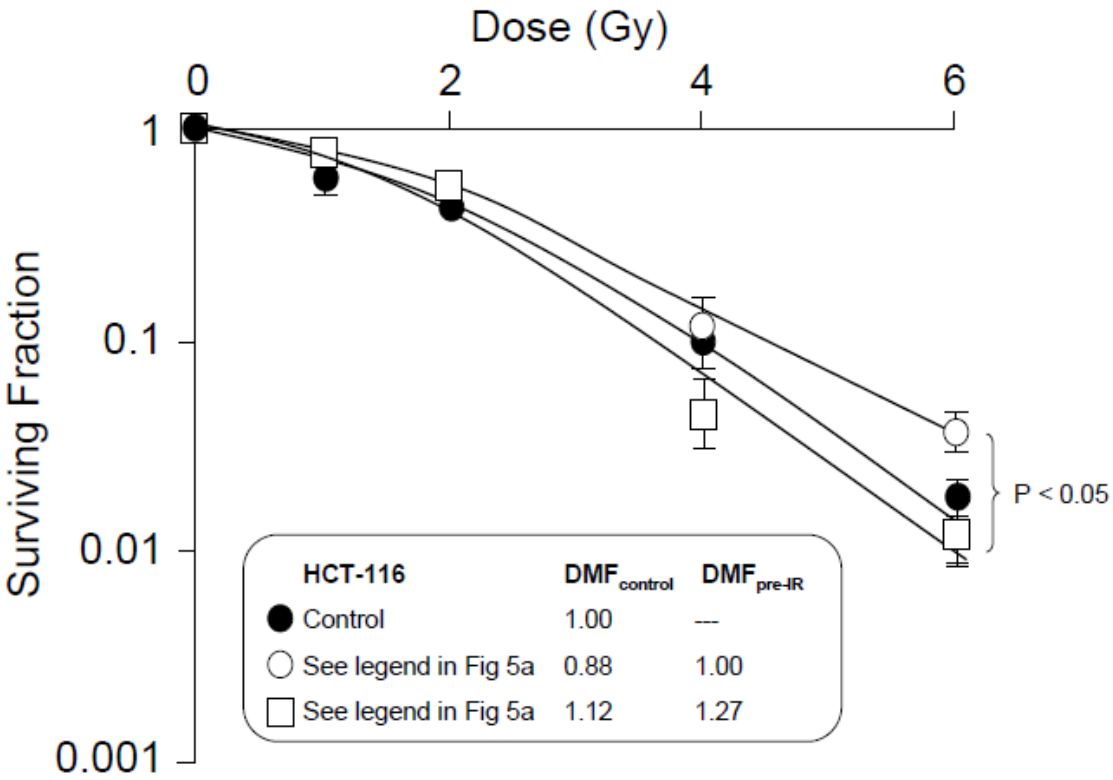
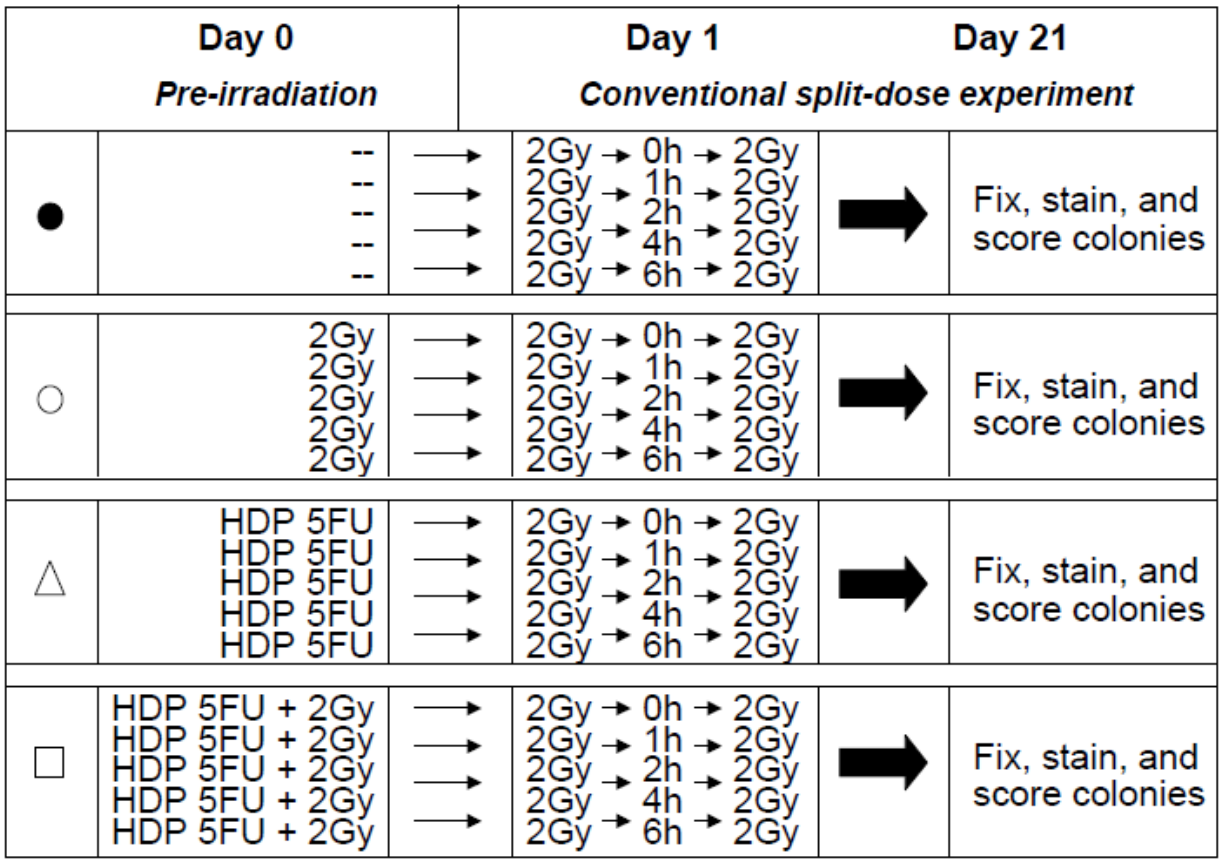


Figure 7. Pre-irradiation of cells with a single fraction of 2Gy affects HPD 5FU-induced radiosensitization. a) Legend and experimental schema for parts b, c, d, and e. b) U87MG-VIII cells were incubated in 1mM of 5FU (HDP) or medium (control) for 1 hour and mock irradiated. Twenty-four hours later known numbers of cells were plated and exposed to 0, 1, 2, 4, or 6 (additional) Gy. c) U87MG-VIII cells were incubated in 1mM of 5FU (HDP) or medium (control) for 1 hour and irradiated with 2Gy. Twenty-four hours later known numbers of cells were plated and exposed to 0, 1, 2, 4, or 6 (additional) Gy. Therefore, all the cells in fact received the dose indicated plus 2Gy (including the ones at 0 Gy). After 21 days, colonies were fixed, stained, and enumerated. d) HCT-116 cells treated as in part b except with 0.5mM

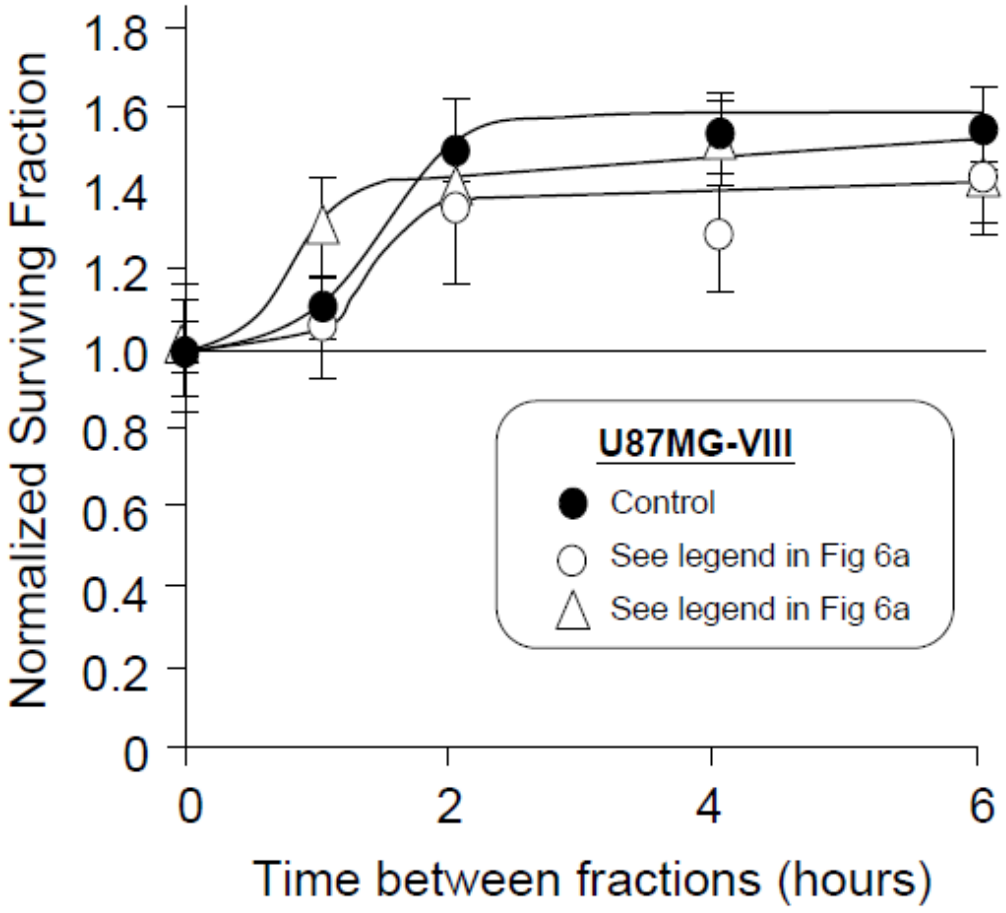
5FU. e) HCT-116 cells treated as in part c except with 0.5mM 5FU. Each point represents the average of three experiments with 3 replicates each. In some cases, the error bars (standard errors) are obscured by experimental points. See Materials and Methods for DMF calculations.

Figure 8.

a)



b)


Normalized Surviving Fraction
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
Time between fractions (hours)
U87MG-VIII
Control
See legend in Fig 6a
See legend in Fig 6a

c)

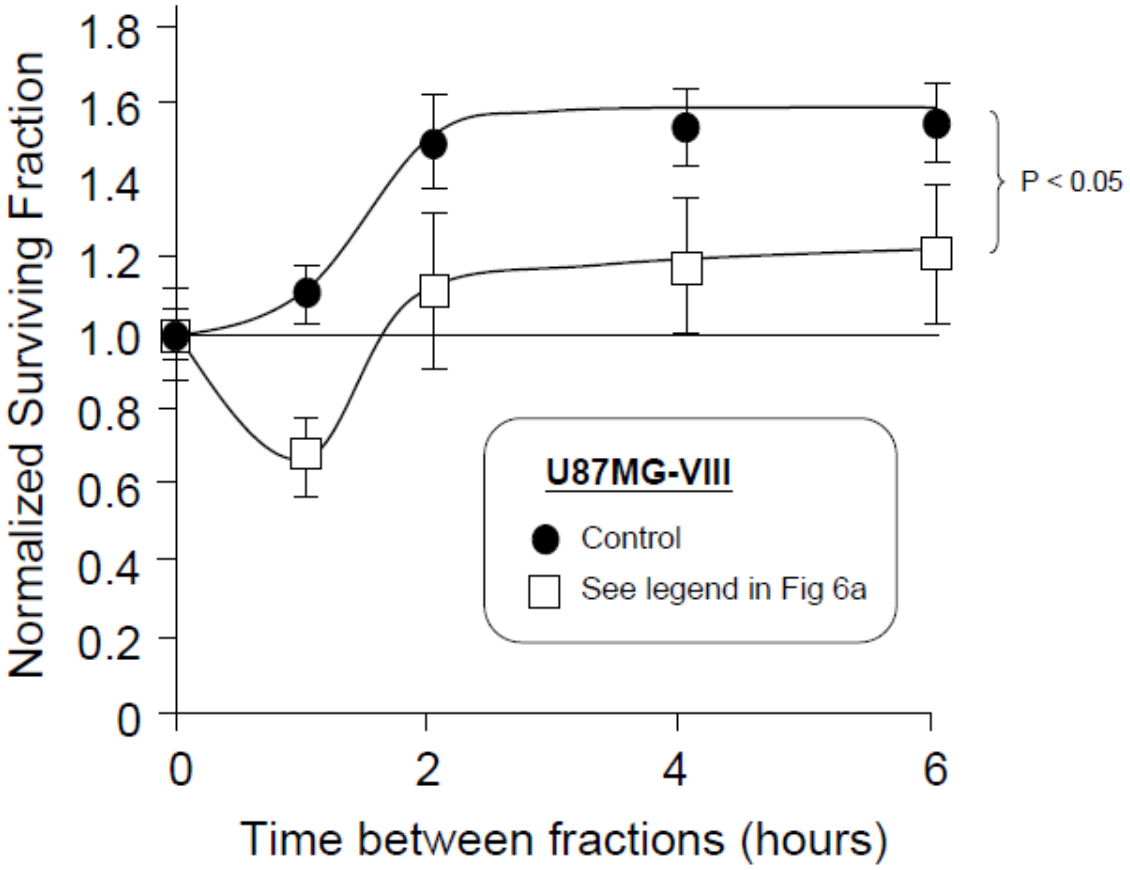


Figure 8. Pre-irradiation of U87MG-VIII cells with a single fraction of 2Gy and exposure to 1mM HDP 5FU affects the SLDR system 24 hrs later. a) Legend and experimental schema for parts b and c. b) U87MG-VIII cells were split into three groups. Cells in group #1 [control] were irradiated with two fractions of 2 Gy separated by 0, 1, 2, 4, or 6 hours on Day 1. Cells in group #2 [5FU HDP] were incubated in 1mM 5FU for 1 hour (HDP) and then irradiated as in the control group on Day 1. Cells in group #3 [2Gy 24 h before irradiation] were irradiated with 2Gy on Day 0, allowed to recover for 24 hours, and then treated as in group #1 with the split-dose protocol on Day 1. c) U87MG-VIII cells were split into two groups. Cells in group #1

[control] were irradiated with two fractions of 2 Gy separated by 0, 1, 2, 4, or 6 hours on Day 1. Cells in group #2 [(5FU HDP + 2Gy) 24h before irradiation] were incubated in 1mM 5FU for 1 hour and immediately irradiated with 2Gy on Day 0, allowed to recover for 24 hours, and then treated as in group #1 with the split-dose protocol on Day 1. All cells were counted, plated, and allowed to grow colonies for 21 days, after which they were fixed, stained and enumerated. In all cases, the SF was normalized to the SF corresponding to a single dose of 4Gy on Day 1. Error bars represent standard errors.

Figure 9.

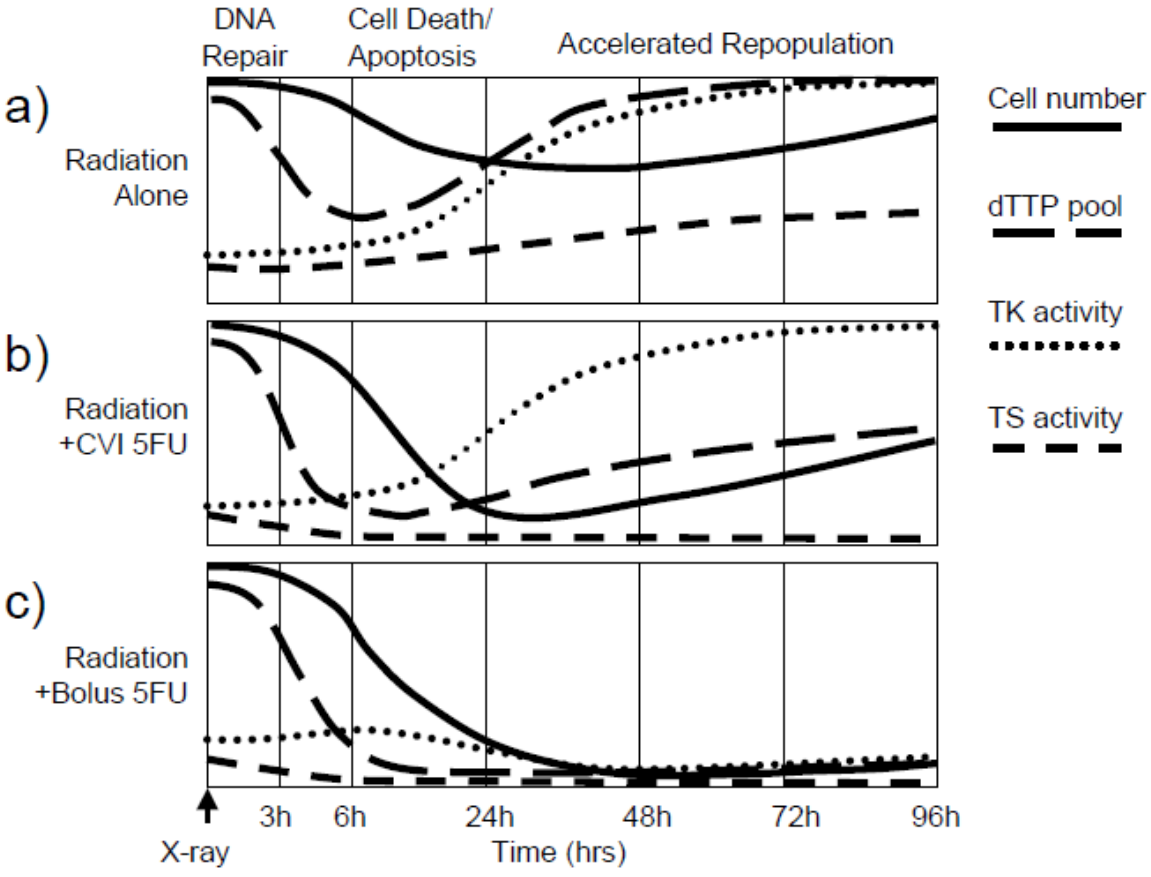
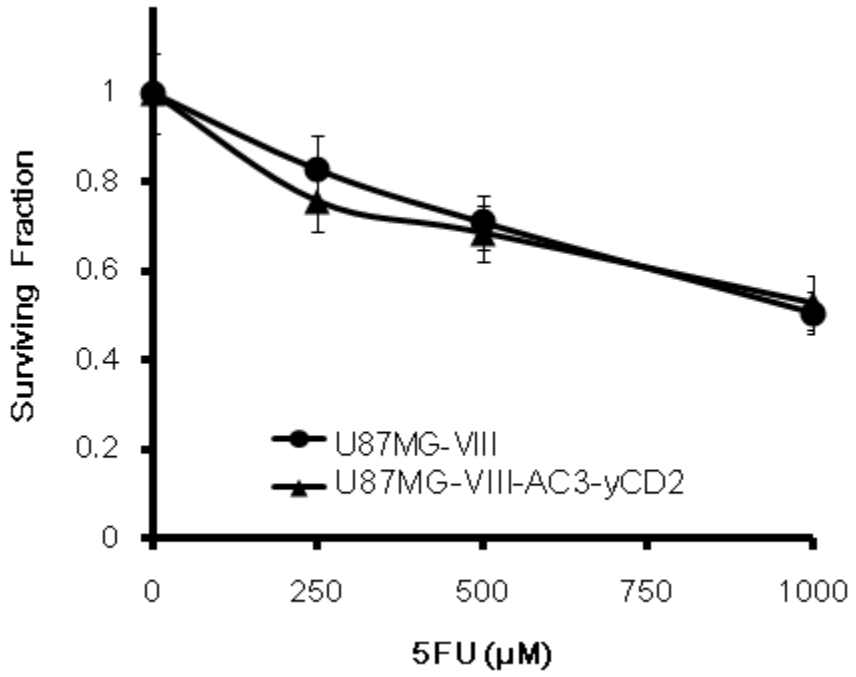


Figure 9. Effect of radiation and 5FU administered as CVI or bolus on cell response. Because only high-dose bolus can impair both TS and TK activity for days, affecting intracellular dTTP pools, it should be ideal in combination with fractionated radiotherapy. See text for full explanation.

Figure 10.

a)



b)

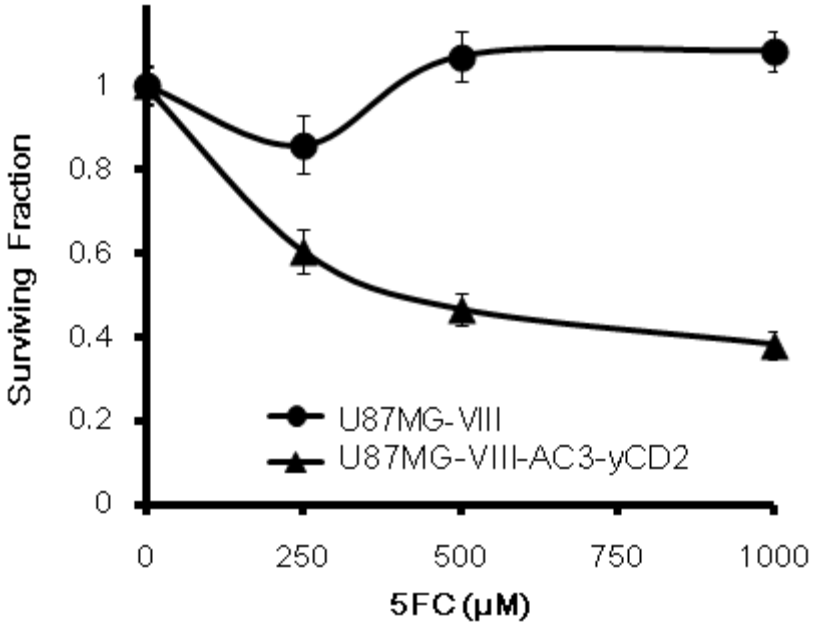
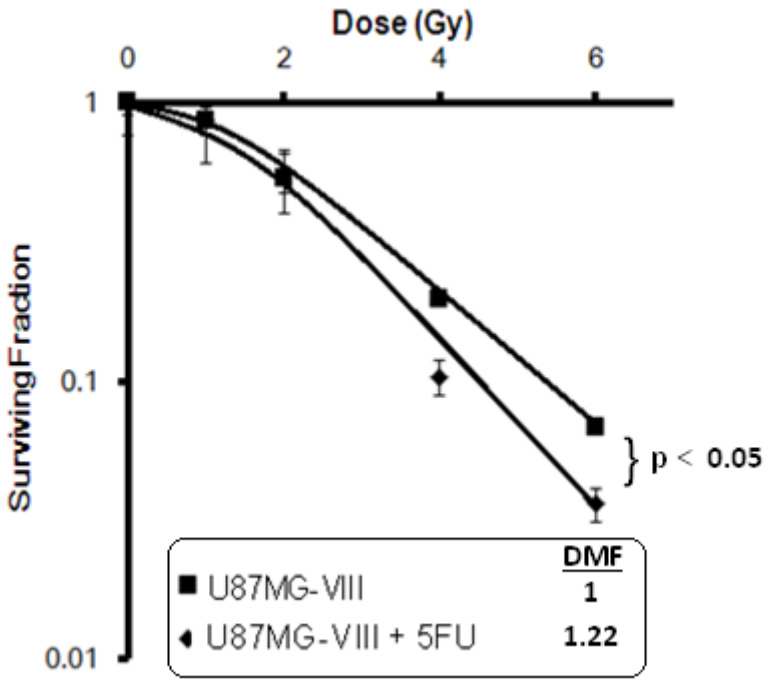


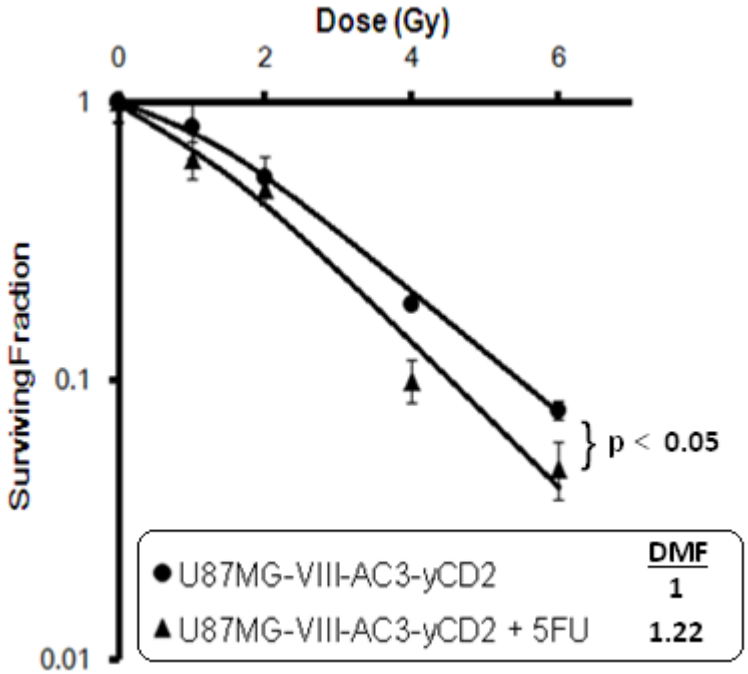
Figure 10. Effect of 5FU and 5FC on the survival fraction of U87MG-VIII and U87E U87MG-VIII -AC3-yCD2 cells. a) Effect of 5FU concentrations on the clonogenic survival fraction of U87MG-VIII and U87E U87MG-VIII -AC3-yCD2 cells. Cells were incubated in 5FU for 1 hour (concentration range 100 µM – 1mM). The cells were washed and plated, mimicking the 10-15 minutes half-life of 5FU in vivo. After 21 days, colonies were fixed, stained, and enumerated. Each point represents the average of three experiments with 3 replicates each. b) Effect of 5FC concentrations on the clonogenic survival fraction of U87EMG-VIII and U87 U87MG-VIII-AC3-yCD2 cells. Cells were incubated in 5FC for 3 hours (concentration range 100 µM – 1mM). The cells were washed and plated, mimicking the 1.4 hours half-life of 5FC in vivo. After 21 days, colonies were fixed, stained, and enumerated. Each point represents the average of three experiments with 3 replicates each.

Figure 11.

a)


Dose (Gy)
0
2
4
6
1
0.1
0.01
Surviving Fraction
p < 0.05
DMF
U87MG-VIII
1
U87MG-VIII + 5FU
1.22

b)


Dose (Gy)
0
2
4
6
1
0.1
0.01
Surviving Fraction
p < 0.05
DMF
U87MG-VIII-AC3-yCD2
1
U87MG-VIII-AC3-yCD2 + 5FU
1.22

c)

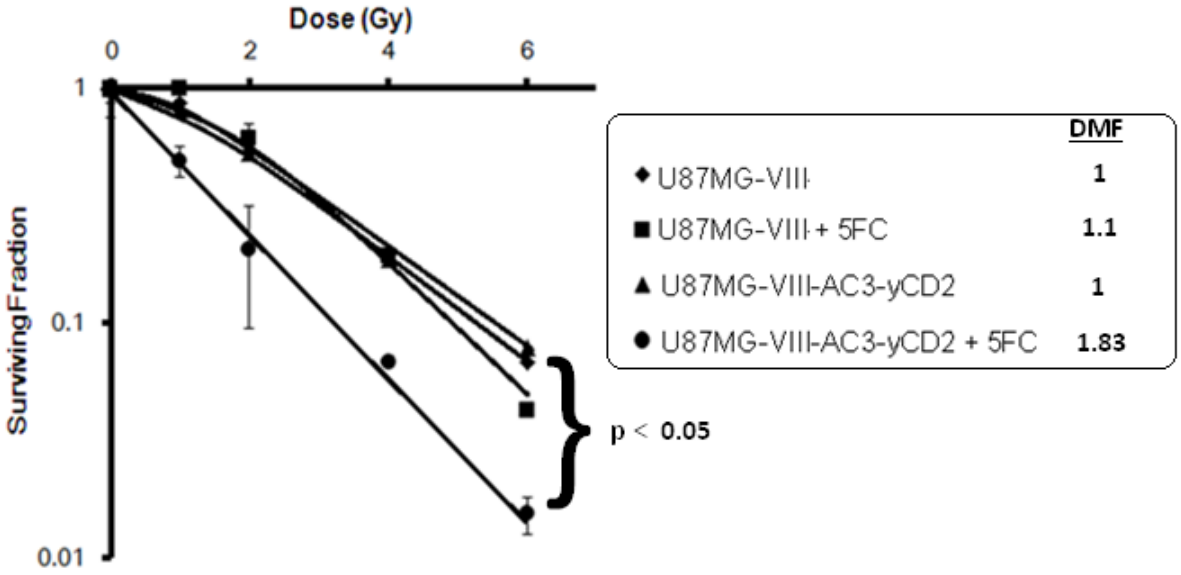


Figure 11. HDP 5FU followed 1hr later by irradiation. U87MG-VIII (a) and U87MG-VIII-AC3-yCD2 (b) cells were exposed to 5FU (1mM) or medium for 1 hour, washed, and irradiated 1 hour later. After 21 days, colonies were fixed, stained, and enumerated. Each point represents the average of three experiments with 3 replicates each. c) HDP 5FC followed by irradiation. U87MG-VIII and U87MG-VIII -AC3-yCD2 cells were exposed to 5FC (1 mM) or medium for 3 hours, washed, and irradiated 1 hour later. After 21 days, colonies were fixed, stained, and enumerated. Each point represents the average of three experiments with 3 replicates each.

Figure 12.

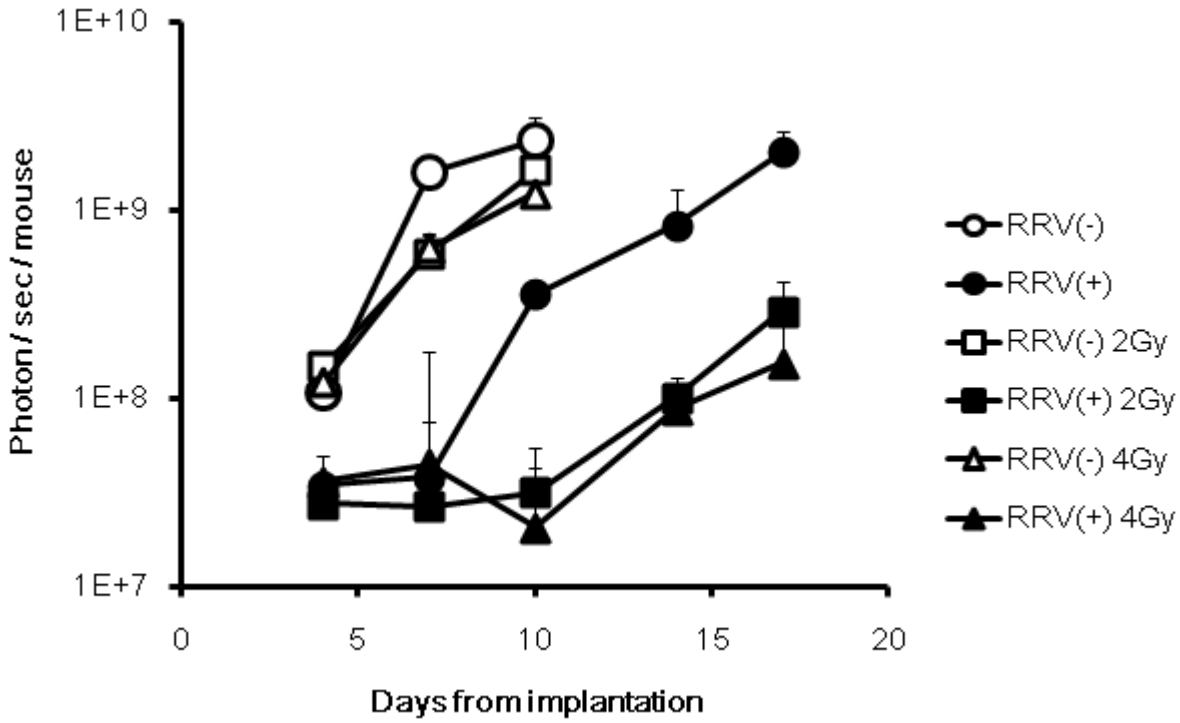


Figure 12. In vitro clonogenic assay. U87MG-VIII and U87 U87MG-VIII-AC3-yCD2 cells were exposed to 0.1 mM 5FC for two hours *in vitro* before the irradiation. After the exposure to 5FC, one third of each cell line was treated with 2 Gy or 4 Gy irradiation, and another one third was not irradiated. These cells were inoculated into athymic mouse brain on day 0 (1×10^5 cells / mouse). Bioluminescence of brain tumor was monitored every 3 or 4 days by optical *in vivo* imaging system (Xenogen). RRV(-),U87MG-VIII-Fluc2 cells without irradiation represented as white circle (○) ; RRV(+),U87MG-VIII-AC3-yCD2-Fluc2 cells without irradiation as black circle (●); RRV(-) 2Gy, U87MG-VIII-Fluc2 cells irradiated with 2 Gy as white rectangle (□); RRV(+) 2Gy, U87MG-VIII-AC3-yCD2-Fluc2 cells irradiated with 2 Gy as black rectangle (■); RRV(-) 4Gy, U87MG-VIII-Fluc2 cells irradiated with 4 Gy as white triangle (Δ); RRV(+) 4Gy, U87MG-VIII-AC3-yCD2-Fluc2 cells irradiated with 4 Gy as black triangle (▲), ∗: $p < 0.05$, ∗∗∗: $p < 0.001$.

Figure 13.

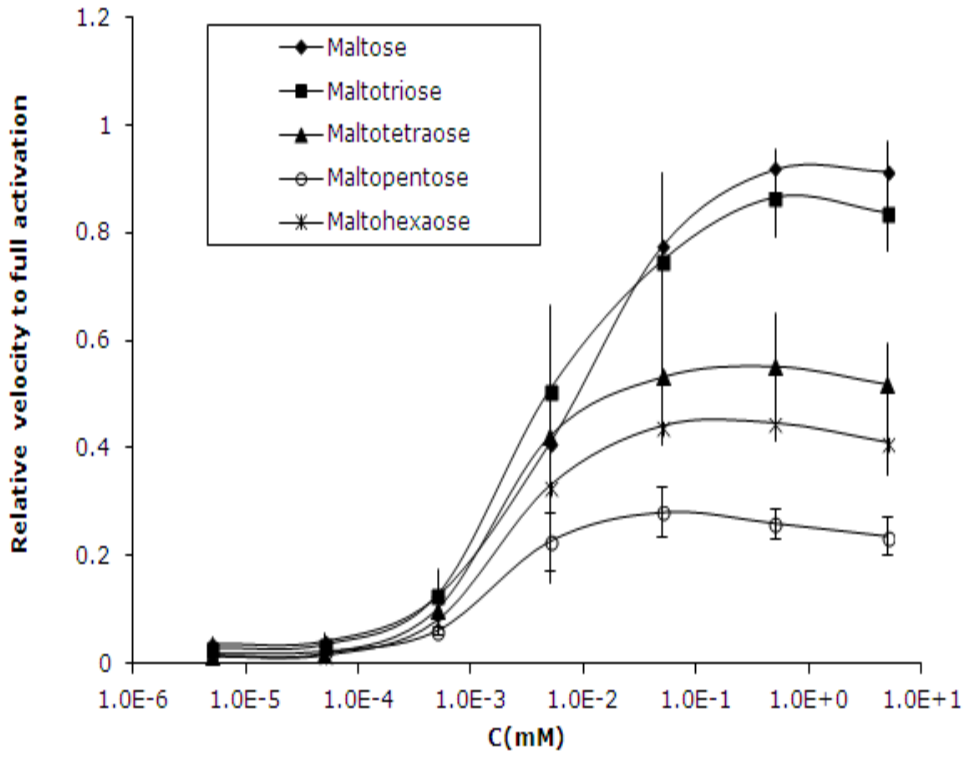


Figure 13. Relative velocity of hydrolysis of nitrocefin by MBP317-347 when activated by various oligosaccharides at different concentrations. The oligosaccharides are polymers of two (maltose), three (maltotriose), four (maltotetraose), five (maltopentaose), or six (maltohexaose) molecules of glucose linked via $\alpha(1,4)$ bonds.

Figure 14.

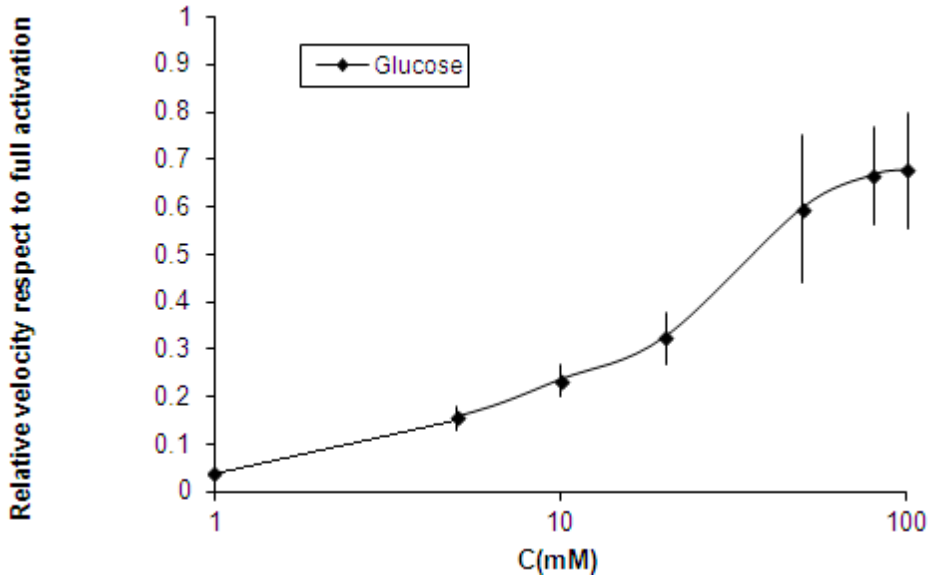
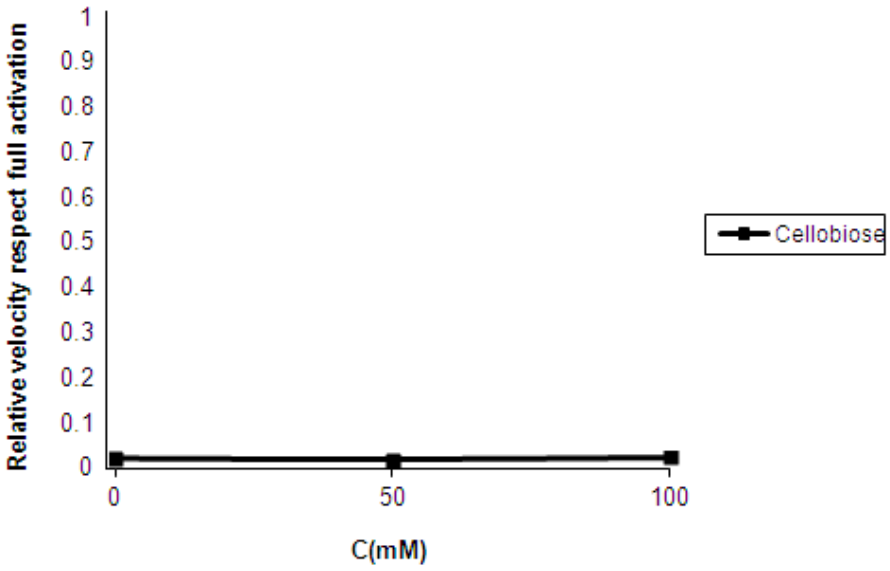


Figure 14. Relative velocity of hydrolysis of nitrocefin by MBP317-347 in the presence of glucose. Note that the range of glucose concentrations that activate the enzyme are typical concentrations found in healthy tissues and tumors.

Figure 15.

a)



b)

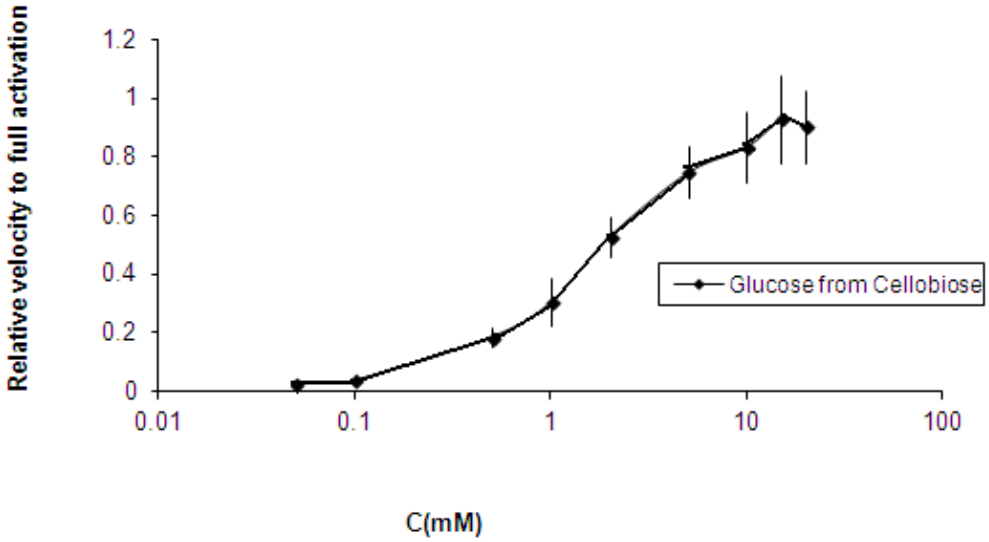


Figure 15. Relative velocity of hydrolysis of nitrocefin by MBP317-347 in the presence of cellobiose. a) Untreated cellobiose. b) Cellobiose treated with cellobiase for 48h at 37C.

Table I. Effect of 5FU and radiation on the cell cycle distribution of HCT-116 and U87MG-VIII cells.

Cell Line	Pre-experimental conditions (24h prior to cell cycle measurement)	Fraction of population in given phase (measured 24h after pre-experiment)		
		G1	S	G2/M
HCT-116	Exposed to medium 24h before experiment	0.67	0.24	0.10
	Exposed to HDP 5FU 24h before experiment	0.72	0.23	0.04
	Exposed to 2Gy 24h before experiment	0.68	0.27	0.04
	Exposed to HDP 5FU + 2Gy 24h before experiment	0.67	0.24	0.08
U87MG-VIII	Exposed to medium 24h before experiment	0.77	0.17	0.06
	Exposed to HDP 5FU 24h before experiment	0.85	0.11	0.04
	Exposed to 2Gy 24h before experiment	0.82	0.13	0.05
	Exposed to HDP 5FU + 2Gy 24h before experiment	0.82	0.11	0.07

Each value on the table represents the percent of cells in each phase of the cell cycle (G1, S and G2/M phases) for the given pre-experimental condition. In all cases, the cell distribution was measured 24 hours after the pre-experimental conditions indicated on the table. When cells were exposed to HDP 5FU, the medium containing 5FU was removed after 1 hour, the cells were washed, and new medium free of 5FU was added. HCT-116 cells were exposed to 0.5mM 5FU; U87MG-VIII cells were exposed to 1mM 5FU. Each number represents the mean of two independent experiments, n=2.

8. Bibliography

1. Heidelberger, C., et al., *Fluorinated pyrimidines, a new class of tumour-inhibitory compounds.* Nature, 1957. **179**(4561): p. 663-6.

2. McGinn CJ, L.T., *Recent advances in the use of radiosensitizing nucleosides.* Semin Radiat Oncol, 2001. **11**(4): p. 10.

3. Sobrero, A.F., C. Aschele, and J.R. Bertino, *Fluorouracil in colorectal cancer--a tale of two drugs: implications for biochemical modulation.* J Clin Oncol, 1997. **15**(1): p. 368-81.

4. Sommer, H. and D.V. Santi, *Purification and amino acid analysis of an active site peptide from thymidylate synthetase containing covalently bound 5-fluoro-2'-deoxyuridylate and methylenetetrahydrofolate.* Biochemical and Biophysical Research Communications, 1974. **57**(3): p. 689-95.

5. Santi, D.V., C.S. McHenry, and H. Sommer, *Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate.* Biochemistry, 1974. **13**(3): p. 471-81.

6. Longley, D.B., D.P. Harkin, and P.G. Johnston, *5-fluorouracil: mechanisms of action and clinical strategies.* Nat Rev Cancer, 2003. **3**(5): p. 330-8.

7. Houghton, J.A., D.M. Tillman, and F.G. Harwood, *Ratio of 2'-deoxyadenosine-5'-triphosphate/thymidine-5'-triphosphate influences the commitment of human colon carcinoma cells to thymineless death.* Clinical Cancer Research, 1995. **1**(7): p. 723-30.

8. Yoshioka, A., et al., *Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death.* Journal of Biological Chemistry, 1987. **262**(17): p. 8235-41.

9. Aherne, G.W., et al., *Immunoreactive dUMP and TTP pools as an index of thymidylate synthase inhibition; effect of tomudex (ZD1694) and a nonpolyglutamated quinazoline antifolate (CB30900) in L1210 mouse leukaemia cells.* Biochemical Pharmacology, 1996. **51**(10): p. 1293-301.

10. Mitrovski, B., et al., *Biochemical effects of folate-based inhibitors of thymidylate synthase in MGH-U1 cells.* Cancer Chemotherapy and Pharmacology, 1994. **35**(2): p. 109-14.

11. Lindahl, T., *An N-glycosidase from Escherichia coli that releases free uracil from DNA containing deaminated cytosine residues.* Proceedings of the National Academy of Sciences of the United States of America, 1974. **71**(9): p. 3649-53.

12. Grem, J.L. and P.H. Fischer, *Enhancement of 5-fluorouracil's anticancer activity by dipyridamole*. Pharmacology and Therapeutics, 1989. **40**(3): p. 349-71.

13. Glazer, R.I. and L.S. Lloyd, *Association of cell lethality with incorporation of 5-fluorouracil and 5-fluorouridine into nuclear RNA in human colon carcinoma cells in culture*. Molecular Pharmacology, 1982. **21**(2): p. 468-73.

14. Kanamaru, R., et al., *The inhibitory effects of 5-fluorouracil on the metabolism of preribosomal and ribosomal RNA in L-1210 cells in vitro*. Cancer Chemotherapy and Pharmacology, 1986. **17**(1): p. 43-6.

15. Ghoshal, K. and S.T. Jacob, *Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil.* Cancer Research, 1994. **54**(3): p. 632-6.

16. Santi, D.V. and L.W. Hardy, *Catalytic mechanism and inhibition of tRNA (uracil-5-)methyltransferase: evidence for covalent catalysis.* Biochemistry, 1987. **26**(26): p. 8599-606.

17. Randerath, K., et al., *Specific effects of 5-fluoropyrimidines and 5-azapyrimidines on modification of the 5 position of pyrimidines, in particular the synthesis of 5-methyluracil and 5-methylcytosine in nucleic acids.* Recent Results in Cancer Research, 1983. **84**: p. 283-97.

18. Patton, J.R., *Ribonucleoprotein particle assembly and modification of U2 small nuclear RNA containing 5-fluorouridine*. Biochemistry, 1993. **32**(34): p. 8939-44.

19. Doong, S.L. and B.J. Dolnick, *5-Fluorouracil substitution alters pre-mRNA splicing in vitro*. Journal of Biological Chemistry, 1988. **263**(9): p. 4467-73.

20. Samuelsson, T., *Interactions of transfer RNA pseudouridine synthases with RNAs substituted with fluorouracil.* Nucleic Acids Research, 1991. **19**(22): p. 6139-44.

21. Nord, L.D. and D.S. Martin, *Loss of murine tumor thymidine kinase activity in vivo following 5-fluorouracil (FUra) treatment by incorporation of FUra into RNA.* Biochem Pharmacol, 1991. **42**(12): p. 2369-75.

22. Ho, D.H., et al., *Comparison of 5-fluorouracil pharmacokinetics in patients receiving continuous 5-fluorouracil infusion and oral uracil plus N1-(2'-tetrahydrofuryl)-5-fluorouracil.* Clinical Cancer Research, 1998. **4**(9): p. 2085-8.

23. van Groeningen, C.J., et al., *Pharmacokinetics of 5-fluorouracil assessed with a sensitive mass spectrometric method in patients on a dose escalation schedule*. Cancer Res, 1988. **48**(23): p. 6956-61.

24. McDermott, B.J., H.W. van den Berg, and R.F. Murphy, *Nonlinear pharmacokinetics for the elimination of 5-fluorouracil after intravenous administration in cancer patients.* Cancer Chemotherapy and Pharmacology, 1982. **9**(3): p. 173-8.

25. van Groeningen, C.J., et al., *Pharmacokinetics of 5-fluorouracil assessed with a sensitive mass spectrometric method in patients on a dose escalation schedule.* Cancer Research, 1988. **48**(23): p. 6956-61.

26. Collins, J.M., et al., *Nonlinear pharmacokinetic models for 5-fluorouracil in man: intravenous and intraperitoneal routes.* Clin Pharmacol Ther, 1980. **28**(2): p. 235-46.

27. Fraile, R.J., et al., *Pharmacokinetics of 5-fluorouracil administered orally, by rapid intravenous and by slow infusion.* Cancer Res, 1980. **40**(7): p. 2223-8.

28. Chaudhuri, N.K., B.J. Montag, and C. Heidelberger, *Studies on fluorinated pyrimidines. III. The metabolism of 5-fluorouracil-2-C14 and 5-fluoroorotic-2-C14 acid in vivo.* Cancer Res, 1958. **18**(3): p. 318-28.

29. Cohen, J.L., et al., *Clinical pharmacology of oral and intravenous 5-fluorouracil (NSC-19893).* Cancer Chemotherapy Reports. Part 1, 1974. **58**(5 Pt 1): p. 723-31.

30. Finj, C. and W. Sadee, *Determination of 5-fluorouracil (NSC-19893) plasma levels in rats and man by isotope dilution-mass fragmentography.* Cancer Chemotherapy Reports. Part 1, 1975. **59**(2 Pt 1): p. 279-86.

31. Fleming, R.A., et al., *Correlation between dihydropyrimidine dehydrogenase activity in peripheral mononuclear cells and systemic clearance of fluorouracil in cancer patients.* Cancer Research, 1992. **52**(10): p. 2899-902.

32. Yngner, T., et al., *Anabolism versus catabolism of [5-3H]uridine and its relationship to ribonucleic acid labelling in mouse liver after partial hepatectomy.* Biochemical Journal, 1979. **178**(1): p. 1-8.

33. Pinedo, H.M. and G.F. Peters, *Fluorouracil: biochemistry and pharmacology.* Journal of Clinical Oncology, 1988. **6**(10): p. 1653-64.

34. Koenig, H. and A. Patel, *Biochemical basis for fluorouracil neurotoxicity. The role of Krebs cycle inhibition by fluoroacetate.* Archives of Neurology, 1970. **23**(2): p. 155-60.

35. Byfield, J.E., et al., *Pharmacologic requirements for obtaining sensitization of human tumor cells in vitro to combined 5-Fluorouracil or ftorafur and X rays.* Int J Radiat Oncol Biol Phys, 1982. **8**(11): p. 1923-33.

36. Clarkson, B., et al., *The Physiologic Disposition of 5-Fluorouracil and 5-Fluoro-2'-Deoxyuridine in Man.* Clin Pharmacol Ther, 1964. **5**: p. 581-610.

37. Aschele, C., et al., *Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules.* Cancer Res, 1992. **52**(7): p. 1855-64.

38. Sobrero, A.F., et al., *Synergism and lack of cross-resistance between short-term and continuous exposure to fluorouracil in human colon adenocarcinoma cells.* J Natl Cancer Inst, 1993. **85**(23): p. 1937-44.

39. Mori, A., et al., *Activity of continuous-infusion 5-fluorouracil in patients with advanced colorectal cancer clinically resistant to bolus 5-fluorouracil.* Cancer Chemother Pharmacol, 1993. **33**(2): p. 179-80.

40. Sobrero, A.F., et al., *Schedule-selective biochemical modulation of 5-fluorouracil: a phase II study in advanced colorectal cancer.* Clin Cancer Res, 1995. **1**(9): p. 955-60.

41. Guglielmi, A., et al., *In vitro synergism between 5-fluorouracil and natural beta interferon in human colon carcinoma cells.* Clin Cancer Res, 1995. **1**(11): p. 1337-44.

42. Evans, R.M., J.D. Laskin, and M.T. Hakala, *Assessment of growth-limiting events caused by 5-fluorouracil in mouse cells and in human cells.* Cancer Res, 1980. **40**(11): p. 4113-22.

43. Smalley, S.R., B.F. Kimler, and R.G. Evans, *5-Fluorouracil modulation of radiosensitivity in cultured human carcinoma cells.* Int J Radiat Oncol Biol Phys, 1991. **20**(2): p. 207-11.

44. Miller, R.C., et al., *Acute diarrhea during adjuvant therapy for rectal cancer: a detailed analysis from a randomized intergroup trial.* Int J Radiat Oncol Biol Phys, 2002. **54**(2): p. 409-13.

45. Denehy, T.R., et al., *Bolus mitomycin C and 5-FU with sequential radiation for poor-prognosis locally advanced cervical cancer.* Gynecol Oncol, 1996. **60**(1): p. 64-71.

46. Tepper, J.E., et al., *Adjuvant postoperative fluorouracil-modulated chemotherapy combined with pelvic radiation therapy for rectal cancer: initial results of intergroup 0114*. J Clin Oncol, 1997. **15**(5): p. 2030-9.

47. Gunderson, L.L.C., R.; Earle, J. D.; Wieand, H. S.; and C.K. Moertel, J.; Martenson, J.; Beart, R. W.; Kubista, *Adjuvant treatment of rectal cancer: randomized prospective*

study of irradiation + chemotherapy. Int. J. Radiat. Oncol. Biol. Phys., 1986. **12**.

48. Moertel, C.G.C., D. S.; Reitemeier, R. J., Colby, M. Y.; Holbrook, M. A., *Combined 5Fluorouracil and supervoltage radiation therapy of unresectable gastrointestinal cancer.* Lancet, 1969. **2**: p. 3

49. *Prolongation of the disease-free interval in surgically treated rectal carcinoma. Gastrointestinal Tumor Study Group.* N Engl J Med, 1985. **312**(23): p. 1465-72.

50. Moertel, C.G., et al., *Therapy of locally unresectable pancreatic carcinoma: a randomized comparison of high dose (6000 rads) radiation alone, moderate dose radiation (4000 rads + 5-fluorouracil), and high dose radiation + 5-fluorouracil: The Gastrointestinal Tumor Study Group.* Cancer, 1981. **48**(8): p. 1705-10.

51. Wen, P.Y. and S. Kesari, *Malignant gliomas in adults.* New England Journal of Medicine, 2008. **359**(5): p. 492-507.

52. Furnari, F.B., et al., *Malignant astrocytic glioma: genetics, biology, and paths to treatment.* Genes and Development, 2007. **21**(21): p. 2683-710.

53. Pelloski, C.E., et al., *Epidermal growth factor receptor variant III status defines clinically distinct subtypes of glioblastoma.* Journal of Clinical Oncology, 2007. **25**(16): p. 2288-94.

54. Stupp, R., et al., *Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.* New England Journal of Medicine, 2005. **352**(10): p. 987-96.

55. Ohka, F., A. Natsume, and T. Wakabayashi, *Current trends in targeted therapies for glioblastoma multiforme.* Neurol Res Int, 2012. **2012**: p. 878425.

56. Fruehauf, J.P., et al., *In vitro drug response and molecular markers associated with drug resistance in malignant gliomas.* Clinical Cancer Research, 2006. **12**(15): p. 4523-32.

57. Ma, J., et al., *Biochemical changes associated with a multidrug-resistant phenotype of a human glioma cell line with temozolomide-acquired resistance.* Biochemical Pharmacology, 2002. **63**(7): p. 1219-28.

58. Hermisson, M., et al., *O6-methylguanine DNA methyltransferase and p53 status predict temozolomide sensitivity in human malignant glioma cells.* Journal of Neurochemistry, 2006. **96**(3): p. 766-76.

59. Kokkinakis, D.M., et al., *Thresholds of O6-alkylguanine-DNA alkyltransferase which confer significant resistance of human glial tumor xenografts to treatment with 1,3-bis(2-chloroethyl)-1-nitrosourea or temozolomide.* Clinical Cancer Research, 2001. **7**(2): p. 421-8.

60. Srivenugopal, K.S., et al., *Ubiquitination-dependent proteolysis of O6-methylguanine-DNA methyltransferase in human and murine tumor cells following inactivation with O6-benzylguanine or 1,3-bis(2-chloroethyl)-1-nitrosourea.* Biochemistry, 1996. **35**(4): p. 1328-34.

61. Rich, J.N., et al., *Phase II trial of gefitinib in recurrent glioblastoma*. Journal of Clinical Oncology, 2004. **22**(1): p. 133-42.

62. Wen, P.Y., et al., *Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08*. Clinical Cancer Research, 2006. **12**(16): p. 4899-907.

63. Batchelor, T.T., et al., *AZD2171, a pan-VEGF receptor tyrosine kinase inhibitor, normalizes tumor vasculature and alleviates edema in glioblastoma patients.* Cancer Cell, 2007. **11**(1): p. 83-95.

64. Vredenburgh, J.J., et al., *Bevacizumab plus irinotecan in recurrent glioblastoma multiforme*. Journal of Clinical Oncology, 2007. **25**(30): p. 4722-9.

65. Friedman, H.S., et al., *Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma*. Journal of Clinical Oncology, 2009. **27**(28): p. 4733-40.

66. Chamberlain, M.C., *Emerging clinical principles on the use of bevacizumab for the treatment of malignant gliomas.* Cancer, 2010. **116**(17): p. 3988-99.

67. Xu, G. and H.L. McLeod, *Strategies for enzyme/prodrug cancer therapy.* Clin Cancer Res, 2001. **7**(11): p. 3314-24.

68. Niculescu-Duvaz, I., et al., *Recent developments in gene-directed enzyme prodrug therapy (GDEPT) for cancer.* Curr Opin Mol Ther, 1999. **1**(4): p. 480-6.

69. Nawa, A., et al., *Gene directed enzyme prodrug therapy for ovarian cancer: could GDEPT become a promising treatment against ovarian cancer?* Anticancer Agents Med Chem, 2008. **8**(2): p. 232-9.

70. Chung-Faye, G.A., et al., *In vivo gene therapy for colon cancer using adenovirus-mediated, transfer of the fusion gene cytosine deaminase and uracil phosphoribosyltransferase*. Gene Ther, 2001. **8**(20): p. 1547-54.

71. Syrigos, K.N. and A.A. Epenetos, *Antibody directed enzyme prodrug therapy (ADEPT): a review of the experimental and clinical considerations.* Anticancer Res, 1999. **19**(1A): p. 605-13.

72. Robinson, M.A., et al., *LEAPT: lectin-directed enzyme-activated prodrug therapy*. Proc Natl Acad Sci U S A, 2004. **101**(40): p. 14527-32.

73. Menei, P., et al., *Effect of stereotactic implantation of biodegradable 5-fluorouracil-loaded microspheres in healthy and C6 glioma-bearing rats.* Neurosurgery, 1996. **39**(1): p. 117-23; discussion 123-4.

74. Luppi, G., et al., *Multifocal leukoencephalopathy associated with 5-fluorouracil and levamisole adjuvant therapy for colon cancer. A report of two cases and review of the literature. The INTACC. Intergruppo Nazionale Terpia Adiuvante Colon Carcinoma*. Annals of Oncology, 1996. **7**(4): p. 412-5.

75. Moore, D.H., W.C. Fowler, Jr., and L.S. Crumpler, *5-Fluorouracil neurotoxicity*. Gynecologic Oncology, 1990. **36**(1): p. 152-4.

76. Kubo, O., et al., *[Treatment of malignant brain tumors with slowly releasing anticancer drug-polymer composites]*. No Shinkei Geka (Neurological Surgery), 1986. **14**(10): p. 1189-95.

77. Oda, Y., et al., *[Treatment of brain tumors with anticancer pellet--experimental and clinical study (author's transl)]*. No Shinkei Geka (Neurological Surgery), 1982. **10**(4): p. 375-81.

78. Ringkjob, R., *Treatment of intracranial gliomas and metastatic carcinomas by local application of cytostatic agents*. Acta Neurologica Scandinavica, 1968. **44**(3): p. 318-22.

79. Phuphanich, S., M. Jacobs, and A. Spiers, *Response of recurrent brain metastases in malignant melanoma to 5-fluorouracil and interferon-alpha therapy*. Journal of Neuroimaging, 1994. **4**(2): p. 114-6.

80. Wang, M.L., et al., *Capecitabine for 5-fluorouracil-resistant brain metastases from breast cancer*. American Journal of Clinical Oncology, 2001. **24**(4): p. 421-4.

81. Bourke, R.S., et al., *Kinetics of entry and distribution of 5-fluorouracil in cerebrospinal fluid and brain following intravenous injection in a primate*. Cancer Research, 1973. **33**(7): p. 1735-46.

82. Krishnasamy, S., et al., *Concomitant chemoradiotherapy, neutron boost, and adjuvant chemotherapy for anaplastic astrocytoma and glioblastoma multiforme*. Cancer Investigation, 1995. **13**(5): p. 453-9.

83. Edland, R.W., M. Javid, and F.J. Ansfield, *Glioblastoma multiforme. An analysis of the results of postoperative radiotherapy alone versus radiotherapy and concomitant 5-fluorouracil. (A prospective randomized study of 32 cases)*. American Journal of Roentgenology, Radium Therapy and Nuclear Medicine, 1971. **111**(2): p. 337-42.

84. Menei, P., et al., *Local and sustained delivery of 5-fluorouracil from biodegradable microspheres for the radiosensitization of glioblastoma: a pilot study*. Cancer, 1999. **86**(2): p. 325-30.

85. Soni, V., D.V. Kohli, and S.K. Jain, *Transferrin-conjugated liposomal system for improved delivery of 5-fluorouracil to brain*. Journal of Drug Targeting, 2008. **16**(1): p. 73-8.

86. Menei, P., et al., *Stereotaxic implantation of 5-fluorouracil-releasing microspheres in malignant glioma*. Cancer, 2004. **100**(2): p. 405-10.

87. Fournier, E., et al., *Therapeutic effectiveness of novel 5-fluorouracil-loaded poly(methylidene malonate 2.1.2)-based microspheres on F98 glioma-bearing rats.* Cancer, 2003. **97**(11): p. 2822-9.

88. Benoit, J.P., et al., *Development of microspheres for neurological disorders: from basics to clinical applications.* J Control Release, 2000. **65**(1-2): p. 285-96.

89. Westphal, M., et al., *A phase 3 trial of local chemotherapy with biodegradable carmustine (BCNU) wafers (Gliadel wafers) in patients with primary malignant glioma*. Neuro Oncol, 2003. **5**(2): p. 79-88.

90. Brem, H., et al., *Placebo-controlled trial of safety and efficacy of intraoperative controlled delivery by biodegradable polymers of chemotherapy for recurrent gliomas. The Polymer-brain Tumor Treatment Group*. Lancet, 1995. **345**(8956): p. 1008-12.

91. Duschinsky, R., Pleven, E. & Heidelberger, C, *The synthesis of 5-fluoropyrimidines.* Journal of the American Chemical Society 1957. **79**: p. 11.

92. Grunberg, E., E. Titsworth, and M. Bennett, *Chemotherapeutic Activity of 5-Fluorocytosine*. Antimicrob Agents Chemother (Bethesda), 1963. **161**: p. 566-8.

93. Tassel, D. and M.A. Madoff, *Treatment of Candida sepsis and Cryptococcus meningitis with 5-fluorocytosine. A new antifungal agent.* JAMA, 1968. **206**(4): p. 830-2.

94. Benson, J.M. and M.C. Nahata, *Clinical use of systemic antifungal agents*. Clinical Pharmacy, 1988. **7**(6): p. 424-38.

95. Polak, A. and M. Grenson, *Evidence for a common transport system for cytosine, adenine and hypoxanthine in Saccharomyces cerevisiae and Candida albicans.* European Journal of Biochemistry, 1973. **32**(2): p. 276-82.

96. Cutler, R.E., A.D. Blair, and M.R. Kelly, *Flucytosine kinetics in subjects with normal and impaired renal function*. Clinical Pharmacology and Therapeutics, 1978. **24**(3): p. 333-42.

97. Daneshmend, T.K. and D.W. Warnock, *Clinical pharmacokinetics of systemic antifungal drugs*. Clinical Pharmacokinetics, 1983. **8**(1): p. 17-42.

98. Bennet, J.E., *Flucytosine*. Annals of Internal Medicine, 1977. **86**(3): p. 319-21.

99. Block, E.R., et al., *Flucytosine and amphotericin B: hemodialysis effects on the plasma concentration and clearance. Studies in man*. Annals of Internal Medicine, 1974. **80**(5): p. 613-7.

100. Schonebeck, J., et al., *Pharmacokinetic studies on the oral antimycotic agent 5-fluorocytosine in individuals with normal and impaired kidney function.* Chemotherapy, 1973. **18**(6): p. 321-36.

101. Francis, P. and T.J. Walsh, *Evolving role of flucytosine in immunocompromised patients: new insights into safety, pharmacokinetics, and antifungal therapy.* Clinical Infectious Diseases, 1992. **15**(6): p. 1003-18.

102. Vermes, A., H.J. Guchelaar, and J. Dankert, *Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions.* J Antimicrob Chemother, 2000. **46**(2): p. 171-9.

103. Harder, E.J. and P.E. Hermans, *Treatment of fungal infections with flucytosine.* Archives of Internal Medicine, 1975. **135**(2): p. 231-7.

104. Miller, C.R., et al., *Intratumoral 5-fluorouracil produced by cytosine deaminase/5-fluorocytosine gene therapy is effective for experimental human glioblastomas.* Cancer Res, 2002. **62**(3): p. 773-80.

105. Lv, S.Q., et al., *Antitumor efficiency of the cytosine deaminase/5-fluorocytosine suicide gene therapy system on malignant gliomas: an in vivo study.* Med Sci Monit, 2009. **15**(1): p. BR13-20.

106. Menei, P., et al., *Local and sustained delivery of 5-fluorouracil from biodegradable microspheres for the radiosensitization of malignant glioma: a randomized phase II trial.* Neurosurgery, 2005. **56**(2): p. 242-8; discussion 242-8.

107. Rainov, N.G., *A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme.* Hum Gene Ther, 2000. **11**(17): p. 2389-401.

108. Logg, C.R., et al., *A uniquely stable replication-competent retrovirus vector achieves efficient gene delivery in vitro and in solid tumors.* Hum Gene Ther, 2001. **12**(8): p. 921-32.

109. Tai, C.K., et al., *Antibody-mediated targeting of replication-competent retroviral vectors.* Hum Gene Ther, 2003. **14**(8): p. 789-802.

110. Wang, W.J., et al., *Highly efficient and tumor-restricted gene transfer to malignant gliomas by replication-competent retroviral vectors.* Hum Gene Ther, 2003. **14**(2): p. 117-27.

111. Solly, S.K., et al., *Replicative retroviral vectors for cancer gene therapy.* Cancer Gene Therapy, 2003. **10**(1): p. 30-9.

112. Tai, C.K., et al., *Single-shot, multicycle suicide gene therapy by replication-competent retrovirus vectors achieves long-term survival benefit in experimental glioma.* Mol Ther, 2005. **12**(5): p. 842-51.

113. Miller, D.G., M.A. Adam, and A.D. Miller, *Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection.* Molecular and Cellular Biology, 1990. **10**(8): p. 4239-42.

114. Kievit, E., et al., *Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts.* Cancer Res, 1999. **59**(7): p. 1417-21.

115. Sahlin, K., et al., *Intracellular pH and bicarbonate concentration in human muscle during recovery from exercise.* J Appl Physiol, 1978. **45**(3): p. 474-80.

116. Walenta, S. and W.F. Mueller-Klieser, *Lactate: mirror and motor of tumor malignancy.* Semin Radiat Oncol, 2004. **14**(3): p. 267-74.

117. Rooseboom, M., J.N. Commandeur, and N.P. Vermeulen, *Enzyme-catalyzed activation of anticancer prodrugs.* Pharmacological Reviews, 2004. **56**(1): p. 53-102.

118. Meyer, D.L., et al., *Site-specific prodrug activation by antibody-beta-lactamase conjugates: regression and long-term growth inhibition of human colon carcinoma xenograft models.* Cancer Research, 1993. **53**(17): p. 3956-63.

119. Meyer, D.L., et al., *Site-specific prodrug activation by antibody-beta-lactamase conjugates: preclinical investigation of the efficacy and toxicity of doxorubicin delivered by antibody directed catalysis.* Bioconjugate Chemistry, 1995. **6**(4): p. 440-6.

120. Rodrigues, M.L., et al., *Development of a humanized disulfide-stabilized anti-p185HER2 Fv-beta-lactamase fusion protein for activation of a cephalosporin doxorubicin prodrug.* Cancer Research, 1995. **55**(1): p. 63-70.

121. Svensson, H.P., et al., *Monoclonal antibody-beta-lactamase conjugates for the activation of a cephalosporin mustard prodrug.* Bioconjugate Chemistry, 1992. **3**(2): p. 176-81.

122. Hanessian, S., et al., *Design, synthesis, and neuraminidase inhibitory activity of GS-4071 analogues that utilize a novel hydrophobic paradigm.* Bioorganic and Medicinal Chemistry Letters, 2002. **12**(23): p. 3425-9.

123. Liang, J., et al., *Ligand binding and allostery can emerge simultaneously.* Protein Science, 2007. **16**(5): p. 929-37.

124. Guntas, G. and M. Ostermeier, *Creation of an allosteric enzyme by domain insertion.* Journal of Molecular Biology, 2004. **336**(1): p. 263-73.

125. Guntas, G., et al., *Directed evolution of protein switches and their application to the creation of ligand-binding proteins.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(32): p. 11224-9.

126. Sharff, A.J., et al., *Crystallographic evidence of a large ligand-induced hinge-twist motion between the two domains of the maltodextrin binding protein involved in active transport and chemotaxis.* Biochemistry, 1992. **31**(44): p. 10657-63.

127. Guntas, G., S.F. Mitchell, and M. Ostermeier, *A molecular switch created by in vitro recombination of nonhomologous genes.* Chemistry and Biology, 2004. **11**(11): p. 1483-7.

128. Kim, J.R. and M. Ostermeier, *Modulation of effector affinity by hinge region mutations also modulates switching activity in an engineered allosteric TEM1 beta-lactamase switch.* Archives of Biochemistry and Biophysics, 2006. **446**(1): p. 44-51.

129. Edwards, W.R., et al., *Linking the functions of unrelated proteins using a novel directed evolution domain insertion method.* Nucleic Acids Research, 2008. **36**(13): p. e78.

130. Ostertag, D., et al., *Brain tumor eradication and prolonged survival from intratumoral conversion of 5-fluorocytosine to 5-fluorouracil using a nonlytic retroviral replicating vector.* Neuro Oncol, 2012. **14**(2): p. 145-59.

131. Perez, O.D., et al., *Design and selection of toca 511 for clinical use: modified retroviral replicating vector with improved stability and gene expression.* Molecular therapy : the journal of the American Society of Gene Therapy, 2012. **20**(9): p. 1689-98.

132. Hiraoka, K., et al., *Therapeutic efficacy of replication-competent retrovirus vector-mediated suicide gene therapy in a multifocal colorectal cancer metastasis model.* Cancer Res, 2007. **67**(11): p. 5345-53.

133. Stripecke, R., et al., *The use of lentiviral vectors in gene therapy of leukemia: combinatorial gene delivery of immunomodulators into leukemia cells by state-of-the-art vectors.* Blood Cells Mol Dis, 2003. **31**(1): p. 28-37.

134. Byfield, J.E., *5-Fluorouracil radiation sensitization--a brief review.* Invest New Drugs, 1989. **7**(1): p. 111-6.

135. Mosconi, S., et al., *The value of oxaliplatin in combination with continuous infusion +/- bolus 5-fluorouracil and levo-folinic acid in metastatic colorectal cancer progressing after 5FU-based chemotherapy: a GISCAD (Italian Group for the Study of Digestive Tract) cancer phase II trial.* Tumori, 2000. **86**(6): p. 465-9.

136. Carlomagno, C., et al., *Second-line chemotherapy with a hybrid-alternating regimen of bolus 5FU modulated by methotrexate and infusional 5FU modulated by folinic acid in*

patients with metastatic colorectal cancer pretreated with 5FU. A phase 2 study. Oncology, 2002. **63**(3): p. 219-25.

137. Lawrence, T.S., et al., *Fluorodeoxyuridine-mediated cytotoxicity and radiosensitization require S phase progression*. International Journal of Radiation Biology, 1996. **70**(3): p. 273-80.

138. Guntas, G., S.F. Mitchell, and M. Ostermeier, *A molecular switch created by in vitro recombination of nonhomologous genes*. Chem Biol, 2004. **11**(11): p. 1483-7.

139. Board, M., A. Colquhoun, and E.A. Newsholme, *High Km glucose-phosphorylating (glucokinase) activities in a range of tumor cell lines and inhibition of rates of tumor growth by the specific enzyme inhibitor mannoheptulose*. Cancer Research, 1995. **55**(15): p. 3278-85.

140. Herrmann, R., et al., *Sequential methotrexate and 5-fluorouracil (FU) vs. FU alone in metastatic colorectal cancer. Results of a randomized multicenter trial. The Association of Medical Oncology (AIO) of the German Cancer Society*. Ann Oncol, 1992. **3**(7): p. 539-43.

141. Woolley, P.V., et al., *A controlled trial of the effect of 4-hydroxypyrazolopyrimidine (allopurinol) on the toxicity of a single bolus dose of 5-fluorouracil*. J Clin Oncol, 1985. **3**(1): p. 103-9.

142. Seifert, P., et al., *Comparison of continuously infused 5-fluorouracil with bolus injection in treatment of patients with colorectal adenocarcinoma*. Cancer, 1975. **36**(1): p. 123-8.

143. Trump, D.L., et al., *Pharmacokinetic and pharmacodynamic analysis of fluorouracil during 72-hour continuous infusion with and without dipyridamole*. J Clin Oncol, 1991. **9**(11): p. 2027-35.

144. *Toxicity of fluorouracil in patients with advanced colorectal cancer: effect of administration schedule and prognostic factors. Meta-Analysis Group In Cancer*. J Clin Oncol, 1998. **16**(11): p. 3537-41.

145. *Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. Meta-analysis Group In Cancer*. J Clin Oncol, 1998. **16**(1): p. 301-8.

146. Khil, M.S., et al., *Radiosensitization by 5-fluorocytosine of human colorectal carcinoma cells in culture transduced with cytosine deaminase gene*. Clin Cancer Res, 1996. **2**(1): p. 53-7.

147. Wohlhueter, R.M., R.S. McIvor, and P.G. Plagemann, *Facilitated transport of uracil and 5-fluorouracil, and permeation of orotic acid into cultured mammalian cells*. J Cell Physiol, 1980. **104**(3): p. 309-19.

148. Klubes, P., et al., *Effects of 5-fluorouracil on 5-fluorodeoxyuridine 5'-monophosphate and 2-deoxyuridine 5'-monophosphate pools, and DNA synthesis in solid mouse L1210 and rat Walker 256 tumors.* Cancer Res, 1978. **38**(8): p. 2325-31.

149. Hirayama, A., et al., *Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry.* Cancer Research, 2009. **69**(11): p. 4918-25.

150. Lee, B.H., et al., *Modulation of starch digestion for slow glucose release through "toggling" of activities of mucosal alpha-glucosidases.* Journal of Biological Chemistry, 2012. **287**(38): p. 31929-38.

151. Sharma, H.S. and A. Sharma, *Nanowired drug delivery for neuroprotection in central nervous system injuries: modulation by environmental temperature, intoxication of nanoparticles, and comorbidity factors.* Wiley Interdiscip Rev Nanomed Nanobiotechnol, 2012. **4**(2): p. 184-203.

152. Looger, L.L., et al., *Computational design of receptor and sensor proteins with novel functions.* Nature, 2003. **423**(6936): p. 185-90.