Defining DNA Double Strand Breaks and the Role of Homologous Recombination in the Mechanism of Radiosensitization by Gemcitabine

by

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Chapter I

Introduction

Gemcitabine (Gemzar®; 2', 2'-difluoro-2'-deoxycytidine; dFdCyd) is a nucleoside analog that is similar in structure to the endogenous pyrimidine, deoxycytidine, differing only by the substitution of 2 hydrogens with fluorines at the 2' position (Fig. 1.1). Based on the activity of arabinosyl pyrimidines, such as 1-β-D arabinofuranosylcytosine (ara-C), against viruses and leukemias, the Eli Lilly Company designed new analogs containing 2'-difluoro substitutions with the intention of discovering a drug with improved resistance to inactivation and enhancing biological activity, and identified gemcitabine as a promising compound (Hertel 1990). Evaluation as an antiviral agent showed gemcitabine to have a narrow therapeutic window resulting in discontinuation for that purpose. However, investigation as a cancer chemotherapeutic showed that gemcitabine inhibited growth of leukemia cells *in vitro*, and demonstrated activity in a broad spectrum of solid tumors in murine models, providing a rationale for clinical experimentation (Hertel 1990). In 1996, gemcitabine was approved for use by the Food and Drug Administration as a first-line therapy against pancreatic cancer, and against non-small cell lung cancer in combination with cisplatin. Additional approvals came in 2004 for use in metastatic breast cancer in combination with paclitaxel, and in 2006 for relapsed ovarian cancer in combination with carboplatin.

Gemcitabine Transport

As a prodrug, gemcitabine requires phosphorylation within the tumor cell in order to elicit its anticancer activity. The first step in this process, entry of gemcitabine into the cell, is accomplished primarily through specific nucleoside transporters. There are at least 7 nucleoside transporters that are distinguished based on their substrate activity as well as their ability to transport nucleosides either to equalize the concentration of nucleoside within and outside the cell (equilibrative transporter), or against a concentration gradient (sodium-dependent concentrative transporter). The majority of gemcitabine transport occurs through two transporters, hCNT1 and hENT1, with hCNT1 displaying an approximately 20-fold greater affinity for gemcitabine than hENT1 (K_m = 18.3$\pm$7.2µM for hCNT1; K_m = 329$\pm$91uM for hENT1), but a similar efficiency of transport (K_m/V_{max} = 22.8$\pm$9.7 for hCNT1; K_m/V_{max} = 20.1+5.9 for hENT1) (Mackey 1998). Chemical inhibition of these transporters in different cell lines resulted in >39-fold increase in resistance to gemcitabine (Mackey 1998). The importance of transporters has also been demonstrated in patients with pancreatic cancer, in whom deficiency of hENT1 was associated with resistance to gemcitabine (Spratlin 2004), and hENT1 expression was correlated with response to gemcitabine (Giovannett 2006).

Gemcitabine Activation

As a prodrug, gemcitabine requires phosphorylation for activity, which is accomplished by endogenous cellular kinases. Subsequent to its entry into the tumor cell, gemcitabine is phosphorylated by deoxycytidine kinase (dCK) to the monophosphorylated form (dFdCMP) (Fig. 1.2). Gemcitabine is an excellent substrate for dCK, with a reported Km of 3.6 - 4.6µM versus 1.4 - 1.5µM for dCyd (Heinemann 1988, Bouffard 1993). Cells lacking dCK neither

accumulated phosphorylated forms of gemcitabine, nor did they exhibit cytotoxicity with gemcitabine incubation (Heinemann 1988), thus establishing dCK as the required kinase for the monophosphorylation. The importance of this initial phosphorylation *in vivo* was demonstrated by overexpression of dCK in HT-29 tumor xenografts in nude mice which resulted in increased accumulation and prolonged elimination of phosphorylated gemcitabine. In addition, tumor growth delay was enhanced compared to the non-overexpressing control (Blackstock 2001). In patients, this step was shown to be saturable at infusion rates of 350mg/m2 over 30min resulting in dFdCyd steady-state concentrations of 20μM (Grunewald 1991).

After formation of the 5'-monophosphate, gemcitabine is rapidly phosphorylated to the 5'-diphosphate, dFdCDP, by dCMP kinase, and to the 5'-triphosphate, dFdCTP, by nucleotide diphosphokinase (Fig. 1.2). dFdCTP accumulates to highest levels with respect to the mono- and diphosphate forms, accounting for >85% of the total phosphorylated gemcitabine (Heinemann 1988). Phosphorylation by dCK is the rate-limiting step in the activation of gemcitabine.

The active metabolite dFdCTP can accumulate within tumor cells to levels that far exceed the concentration of gemcitabine in the incubation medium. In HT29 cells, a 4h incubation with 10μM dFdCyd resulted in accumulation of dFdCTP to 450μM (Shewach 1994), and in CCRF-CEM cells, incubation with 10μM dFdCyd for 2h resulted in a cellular concentration of 525μM dFdCTP, which accounted for approximately 80% of metabolites (Heinemann 1992). In patients with leukemia, peak dFdCTP accumulation in leukemic cells occurred when plasma levels of dFdCyd reached approximately 20μM, which was achieved within 15min from the start of infusion of 350-1000mg/m2/30min (Abbruzzese 1991). Similarly, in patients with head and

neck tumors, dFdCTP attained levels that exceeded the concurrent amount of dFdCyd in the plasma (Eisbruch 2001).

The accumulation of dFdCTP to a higher concentration than that of the dFdCyd exposure is facilitated by the rapid phosphorylation of dFdCyd as well as the relatively slow half life of its triphosphate. Studies in solid tumor and leukemia cells have demonstrated that the half-life of dFdCTP is dependent on its initial concentration. Exposure of HT-29 cells to 0.1μM dFdCyd for 2h resulted in accumulation of dFdCTP to 30μM with a $t_{1/2}$ of 12h, whereas exposure to 3μM for 2h resulted in accumulation of 200μM dFdCTP with a $t_{1/2}$ of greater than 72h (Shewach 1994). This was also demonstrated in CCRF-CEM cells, where cellular dFdCTP concentrations below 50μM resulted in linear elimination with a half-life of approximately 2h, but biphasic elimination at concentrations above 100μM with a terminal half-life of 19h (Heinemann 1992).

Gemcitabine Deamination

The prodrug dFdCyd is deaminated by deoxycytidine deaminase to the inactive difluorodeoxyuridine. The prevalence of deoxycytidine deaminase in humans results in a short half-life of approximately 10 min for gemcitabine after IV infusion (Abbruzzese 1991). Although the deamination product difluorodeoxyuridine has a lengthy terminal half-life, no significant antitumor activity has been ascribed to this metabolite. The monophosphate, dFdCMP, can be deaminated by deoxycytidylate deaminase to difluorodeoxyuridylate, and this metabolite also has no significant activity. Interestingly, dFdCTP is a feedback inhibitor of deoxycytidylate deaminase (Fig. 1.2). This inhibition contributes to the lengthy half-life of dFdCTP, especially at high concentrations. Thus, despite a short half-life for dFdCyd *in vivo*,

the facile uptake through transport mechanisms and rapid phosphorylation combined with a long half-life all contribute to the once-weekly dosing schedule for dFdCyd in patients.

Active Metabolite: Gemcitabine Triphosphate (dFdCTP)

Studies have demonstrated that dFdCTP acts as a substrate for incorporation by DNA polymerases, with a K_m of 26.7μM for pol α and 45.8μM for pol ε. Additionally, the triphosphate functions as a competitive inhibitor of polymerases with respect to dCTP, with a K_i of 11.2μM for pol α and 14.4μM for pol ε (Huang 1991). The cumulative effects of dFdCDP and dFdCTP result in DNA synthesis inhibition and accumulation of cells into S-phase, however, cytotoxicity is not correlated directly with DNA synthesis inhibition, as ara-C, which results in greater inhibition than dFdCyd, produces less cytotoxicity (Ostruszka 2003). Instead, incorporation into DNA has been correlated with cytotoxicity (Huang 1991). Incorporation of dFdCMP into DNA is amplified by the difficulty in its removal, as demonstrated by an inability of pol ε to excise dFdCMP from DNA (Huang 1991), and a reduced excision activity of the Klenow fragment on incorporated gemcitabine (Gandhi 1996).

After its incorporation into DNA, dFdCMP slows chain elongation and, in a primer template system, can cause termination of DNA synthesis following addition of one nucleotide after dFdCMP (Huang 1991). In cells that survive incorporation of dFdCMP into the nascent strand of DNA and divide, the nucleotide analog then in the template produces further interference with replication. A study by Schy demonstrated that misinsertion of dTTP, dGTP, and dATP was 6, 150, and 200-fold more likely to occur opposite dFdCMP compared to dCMP in the template, respectively (Schy 1993). While a strong correlation between dFdCMP in DNA and cytotoxicity

has been established, the exact mechanism by which this fraudulent incorporation causes cytotoxicity is not known.

Active Metabolite: Gemcitabine Diphosphate (dFdCDP)

dFdCDP acts as an irreversible mechanism-based inhibitor (Silva 1998, van der Donk 1998) of ribonucleotide reductase (RR), an enzyme that provides the deoxynucleotides for DNA synthesis through conversion of rNDPs to their corresponding dNDPs, followed by phosphorylation to the dNTP substrates. RR is the rate-limiting enzyme in the biosynthesis of all four dNTPs. RR is composed of two subunits, a large 90kDa R1 subunit that is constitutively expressed and exhibits a half-life of >24h, and a small 45kDa R2 subunit that is expressed during S-phase and is rapidly degraded (t1/2=3h) (Eriksson 1984). The active enzyme is thought to exist as a heterotetramer, containing 2 subunits each of R1 and R2. Additionally, a p53 inducible subunit, p53R2, can substitute for the R2 subunit during damage repair (Tanaka 2000). The R2 subunit contains a dinuclear iron center which utilizes oxygen to generate a stable tyrosyl free radical on Y122 of R2 (Larsson 1986). This free radical is responsible for generating a transient thiyl radical on C439 of R1. The reduction process begins with removal of the 3' hydrogen of the ribose by the thiyl radical (Stubbe 1983), creating a substrate radical which facilitates removal of the 2' hydroxyl of the ribose, and ends following a series of radical transfers which generates the final dNDP product and restores the tyrosyl free radical. The R1 subunit contains two allosteric sites which regulate enzymatic activity (activity site) and substrate specificity (specificity site) (Eriksson 1997). Binding of ATP and dATP to the "activity site" results in increased and decreased activity, respectively. ATP and dATP also act as allosteric effectors at the "specificity site" where binding promotes reduction of CDP and UDP. In contrast, binding of dTTP to the

"specificity site" inhibits CDP and UDP reduction, but stimulates GDP reduction, whereas dGTP stimulates reduction of ADP and inhibits GDP reduction, illustrating a complex regulatory system that maintains a tightly balanced supply of dNTPs during repair and synthesis. The only nucleotide that does not act as an effector is dCTP. Additional regulation was suggested in a study of murine RR which proposed an additional allosteric site (hexamerization-site; h-site) that causes enhanced R_1 subunit interactions with increasing concentrations of ATP or dATP, resulting in formation of an active $R1_2$ dimer and $R1_6$ hexamer, as well as an intermediate inactive $R1_4$ tetramer (Kashlan 2002).

Interaction of dFdCDP with RR occurs through binding of the nucleotide analog to the substrate site of the enzyme. The enzyme attempts to abstract a fluorine at the 2'-position of dFdCDP, resulting in degradation of the nucleotide with inactivation of the essential tyrosine residue. RR inactivation by dFdCDP occurs with a 1:1 stoichiometry and results in release of 2 fluoride ions and 1 cytosine (Silva 1998, van der Donk 1998, Artin 2009), demonstrating cleavage of the glycosidic bond in the RR-mediated destruction of the nucleotide analog. In the presence or absence of reductant, covalent binding of the ribose of dFdCDP with the alpha subunit of RR, or loss of the essential tyrosyl free-radical through interaction with a product formed from the destruction of dFdCDP, respectively, results in inactivation of the enzyme (Artin 2009).

In tumor cells, dFdCyd is a potent inhibitor of RR with long lasting effects. In CCRF-CEM leukemia cells, the *in situ* RR activity was reduced to 50% by 0.3µM dFdCDP and the greatest effect on dNTP pools was depletion of dCTP (Heinemann 1990). Longer incubations reduced the levels of other dNTPs, with pyrimidine dNTPs decreasing prior to the purine dNTPs. In

contrast, in solid tumor cells the pattern of pool depletion is reversed, with dATP being the most sensitive to the effects of dFdCyd on RR, followed by dGTP and, at high concentrations, the pyrimidine dNTPs decrease (Shewach 1994, Lawrence 1996, Robinson 2001).

The excellent antitumor activity of gemcitabine is assisted by several mechanisms of self-potentiation. First, inhibition of RR by dFdCDP results in an imbalance in dNTP pools. As demonstrated in leukemia cells, the depletion of the dCTP pool is the most evident, resulting in reduced competition for incorporation of dFdCTP into DNA. Additionally, dFdCTP acts as an inhibitor of dCMP deaminase, a pathway for the elimination of gemcitabine, resulting in a lengthy half-life for dFdCTP. These mechanisms enhance the residence time of dFdCTP in the cell and decrease the barrier to incorporation into DNA, resulting in excellent DNA incorporation. These features contribute to gemcitabine's excellent antitumor activity in patients.

Radiosensitization

Antimetabolites, including several nucleoside analogs, have long been recognized for their ability to synergistically increase killing of tumor cells when combined with ionizing radiation (termed radiosensitization), and they are commonly employed in chemoradiotherapy regimens in patients (Shewach & Lawrence, JCO 2007). The nucleoside analogs, bromodeoxyuridine (BrdUrd) and iododeoxyuridine (IdUrd), are thought to produce radiosensitization through their incorporation into DNA. These drugs also produce an imbalance in dNTPs through the inhibition of thymidylate synthase (Heimburger 1991, Shewach 1992), though these effects were not thought to affect radiosensitization. However, fluorodeoxyuridine (FdUrd) is an excellent

inhibitor of thymidylate synthase with lesser effects on DNA incorporation, and it is a good radiosensitizer. Furthermore, the anticancer drug hydroxyurea is also a radiosensitizer, and its primary effect in cells is as a tyrosyl free-radical scavenger for RR, resulting in imbalances in dNTPs. This common link with inhibition of the biosynthesis of dNTPs between established radiosensitizers and gemcitabine provided the rationale for proposing that gemcitabine would be a good radiosensitizer.

Indeed, numerous studies have now demonstrated that dFdCyd is among the most potent of radiation sensitizers *in vitro* and *in vivo*. Importantly, the synergistic cell killing observed with dFdCyd and ionizing radiation is achievable using non-cytotoxic concentrations of dFdCyd, allowing for potent sensitization of cancer cells *in vivo*, thereby improving the therapeutic index. Radiosensitization with dFdCyd also occurs in patients, in which low doses of dFdCyd combined with standard radiotherapy can enhance tumor regression (Blackstock 1999, Eisbruch 2001). Despite the fact that dFdCyd is one of the most potent radiosensitizers known, the exact mechanism by which this occurs has not been fully elucidated.

Radiosensitization with dFdCyd is dependent on several factors. The sequence dependence was demonstrated in HT-29 cells in which irradiation following a 24h exposure to IC_{10} dFdCyd resulted in the best enhancement compared to irradiation prior to gemcitabine or 4h into drug exposure which resulted in biologically insignificant enhancement. This established that optimal radiosensitization occurs when gemcitabine precedes IR. Radiosensitization increased with greater duration of gemcitabine incubation prior to irradiation. Furthermore, incubation with increasing concentrations of gemcitabine for fixed periods of time, ranging from 4 hr to 24 hr,

also increased radiosensitization. Additionally, a shorter exposure (16h) to a higher concentration of dFdCyd (30nM) was able to produce a similar degree of radiosensitization as observed with a longer exposure (24h) to a lower concentration of dFdCyd (10nM) (Shewach 1994). A separate study in Panc-1 and BxPC-3 pancreatic cancer cell lines showed that radiosensitization depended on concentration and duration of exposure to gemcitabine (Lawrence 1996). These studies also highlight a strong correlate of RS with dFdCyd: dATP must be depleted by at least 80% for at least 4 hours prior to irradiation (Shewach 1994, Lawrence 1996, Robinson 2001). Finally, optimal RS by dFdCyd occurs in cells that accumulate into early S-phase (Shewach 1994, Robinson 2001, Latz 1998, Ostruszka 2000), as demonstrated in human glioblastoma cells where it was shown that U251 cells accumulating into S-phase were radiosensitized, but D54 cells exhibiting a G_1 block following dFdCyd exposure were not radiosensitized, despite the fact that S-phase cells are less radiosensitive than cells in other phases of the cell cycle.

It is well accepted that ionizing radiation kills tumor cells primarily through its ability to produce DNA double strand breaks (DSBs). Radiosensitization with other nucleoside analogs, such as BrdUrd, IdUrd, and FdUrd, is typically attributed to an increase in the induction, or a reduction in the rate of repair of the IR-induced DSBs (Bruso 1990, Heimburger 1991, Lawrence 1990, Ling 1990). However, several reports have demonstrated that this model does not account for radiosensitization with dFdCyd (Lawrence 1997, Rosier 2003). One drawback to these studies is that they used pulsed-field gel electrophoresis to measure DSBs, a relatively insensitive method that requires excessively high doses of IR. In addition, these studies evaluated the repair of IR-induced damage only during the first several hours after IR. Thus, while the mechanism by

which gemcitabine produces excellent radiosensitization has remained elusive, a more sensitive and more detailed exploration of the role of gemcitabine in IR-induced DNA damage is necessary.

Mismatch Repair and Gemcitabine-mediated Radiosensitization

Based on the findings that radiosensitization with dFdCyd did not occur through an increase in DSBs or a delay in their repair, other effects of dATP depletion that may lead to radiosensitization were sought. It is known that an imbalance in dNTPs can increase the frequency of mutations in DNA (Kunkel 1982), encouraging evaluation of a role for mismatches in DNA in the mechanism for radiosensitization with dFdCyd. In a recent study utilizing a shuttle vector reporter assay to measure mutation frequency in cells exposed to dFdCyd, mismatches in DNA were shown to occur only with radiosensitizing concentrations of dFdCyd (Flanagan 2007). In addition, cells that were defective in their ability to repair mismatches in DNA, i.e. mismatch repair (MMR) deficient cells that lacked a required MMR protein (MLH1), were better radiosensitized than cells proficient in MMR, consistent with an essential role for mismatches in DNA in RS with dFdCyd (Flanagan 2007, Robinson 2003). Specifically, HCT-116 + ch3 and HCT-116 1-2 cells (which are MMR proficient due to reintroduction of the chromosome harboring the MLH1 gene, or MLH1 cDNA, respectively) were not radiosensitized by IC_{10} or IC_{50} dFdCyd, while the isogenically matched MLH1- (MMR-) deficient cells were well radiosensitized at both concentrations. Only with highly cytotoxic concentrations of dFdCyd was radiosensitization observed in the MMR-proficient HCT116+ch3 cells (IC_{96}). Similarly, A549 lung cancer cells not normally radiosensitized by an IC_{10} of dFdCyd exhibited excellent radiosensitization following siRNA knockdown of MLH1 (~90% knockdown of

protein) (Flanagan 2007). Analysis of mutation frequency showed that the combination of dFdCyd and IR resulted in a higher frequency of mutations than dFdCyd or IR alone. The increased mutation frequency persisted for up to 96h only after the combination treatment, while that resulting from single treatments decreased to control levels within 24h (Flanagan 2007, Robinson 2003). Similar studies with FdUrd have now established that the FdUrd-mediated imbalance in dNTPs also produces mismatches in DNA that correlate with radiosensitization (Flanagan 2008). Taken together, these studies have established a causal association between mismatches in DNA and radiosensitization. However, still lacking is an understanding of why these mismatches produce radiosensitization.

Homologous Recombination and Radiosensitization by Gemcitabine

Cells have several pathways to address DNA damage caused by drugs or ionizing radiation. Because unrepaired DSBs are lethal to the cell, mechanisms to repair this damage are critically important for survival. As mentioned earlier, radiosensitization by gemcitabine is optimal in cells that show accumulation in early S-phase. Within S-phase, there are two major repair pathways involved with resolution of IR-induced damage: non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ is the major pathway involved in repair of DSBs and is active in all phases of the cell cycle. This pathway, which proceeds with limited to no end-processing, involves binding of Ku70/Ku80 proteins which serve to stabilize DNA ends, and to recruit the required repair factors to the sites of damage, including the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (Lieber 2010). Because NHEJ does not, or minimally utilizes regions of homology in repair, ligation of broken ends typically results in loss of genetic information making it an error-prone pathway. Evidence in Chinese hamster ovary

(CHO) cells deficient in essential components for end-joining (DNA-PKcs or Ku80) has shown dFdCyd-mediated RS is achievable regardless of NHEJ proficiency, suggesting that an intact NHEJ pathway is not necessary for radiosensitization to occur (van Putten 2001).

In contrast to NHEJ, HR is a pathway that utilizes regions of homology in directing repair, thus providing high-fidelity resolution of DNA damage. Here, DSB formation is followed by DNA resection resulting in 3' overhangs. Subsequent binding by Rad51 stimulates a search for homology with preference for a sister chromatid. As such, HR activity increases with increasing availability of a sister chromatid, as cells progress through S-phase and into G_2 (Hiom 2010). An important protein involved in HR is XRCC3, deficiency of which showed a 25-fold reduction in HR activity, as measured by a GFP reporter substrate (Pierce 1999). In a study utilizing the irs1SF CHO cells deficient in XRCC3, radiosensitization with gemcitabine was not achieved. However, excellent radiosensitization was observed in the parental HR-proficient AA8 cell line, suggesting a necessity for a functional HR pathway in radiosensitization by gemcitabine in CHO cells (Wachters 2003). These findings in rodent cells warrant investigation of whether HR is important in radiosensitization with gemcitabine in human cells and, if it is, an understanding of the type of damage induced by the combination of radiation and gemcitabine that requires HR is needed.

With roles identified for MMR and HR in radiosensitization with gemcitabine, it is still not clear whether these represent separate or interacting contributions. The major proteins involved in MMR, including MLH1, have been shown to be involved in homologous recombination (HR). Furthermore, MMR itself can influence the activity of repair of DSBs via HR. For instance, in

an analysis of HR activity involving repair substrates with increasing sequence divergence, wild-type cells showed a decrease in HR activity, whereas MMR-deficient cells did not, illustrating the ability of MMR to inhibit recombination between heterologous sequences (Elliot 2001).

Dissertation Rationale

The mechanism of radiosensitization by gemcitabine does not appear to fit the model of other radiosensitizers, such as BrdUrd, IdUrd, and FdUrd, whereby synergistic cell killing is a result of an increase in the amount of damage formed, or an inhibition of repair. However, assessment of DSBs was determined using a relatively insensitive technique requiring radiation that was well above clinically relevant amounts ($\geq$20Gy). With the more recent advance of γ-H2AX focus formation as a measure of DSBs, a more sensitive assay that allows evaluation of DSBs using clinically relevant concentrations of gemcitabine and radiation, Chapter 2 presents the effects of dFdCyd+IR on the appearance and resolution of DNA damage following IR/drug washout. While traditional studies of repair of IR-induced damage are limited to the first 4 hr after IR, Chapter 2 includes evaluation of extended time points (24-72h time points) in a much more comprehensive and sensitive analysis of DNA damage and repair under radiosensitizing conditions with gemcitabine.

A role for homologous recombination in radiosensitization by gemcitabine was established in CHO cells, in which dFdCyd combined with IR in HR-proficient AA8 cells (parental) exhibited synergy, but HR-deficient irs1SF cells (XRCC3 mutant) did not. The irs1SF cells were radiosensitized by hyperthermia, demonstrating that synergy was still achievable in an HR-

deficient background, and discounting the possibility that the increase in IR sensitivity observed in HR-deficient CHO cells was obscuring evidence of radiosensitization by dFdCyd. While a requirement for HR proficiency was demonstrated in radiosensitization by dFdCyd in these cells, the conditions of dATP pool depletion, which appear necessary for radiosensitization by dFdCyd, were not addressed. The focus of chapter 3 is on the effect of deoxynucleotide pool levels in response to different gemcitabine exposure conditions to determine whether radiosensitization is indeed dependent on functional HR, or whether the lack of radiosensitization in HR-deficient cells can be attributed to factors other than repair proficiency. Because results derived from rodent cells do not always correspond with findings in human cells, I wished to explore the role of HR in radiosensitization by gemcitabine using MCF-7 breast cancer cells as a model. Chapter 3 examines the effect of HR inhibition via shRNA knockdown of XRCC3 on the ability of gemcitabine to radiosensitize human tumor cells, along with evaluation of the type of DNA damage that ensues from HR activation, in an effort to further elucidate the role of HR in cell death with gemcitabine and IR.

The research presented in this dissertation demonstrates a requirement for functional homologous recombination in responding to late damage induced by the combination of gemcitabine and IR. A greater understanding of this process can help to optimize chemoradiotherapy with gemcitabine, as it suggests that combinations of gemcitabine and radiation would be most effective in HR-proficient tumors.

List of Abbreviations

dFdCMP, gemcitabine monophosphate; dFdCDP, gemcitabine diphosphate; dFdCTP, gemcitabine triphosphate; dCyd, deoxycytidine; ara-C, 1-β-D arabinofuranosylcytosine; hENT, human equilibrative nucleoside transporter; hCNT, human concentrative nucleoside transporter; dCK, deoxycytidine kinase; dFdCyd, 2', 2'-difluoro-2'-deoxycytidine, gemcitabine; CDP, cytidine diphosphate; UDP, uridine diphosphate; GDP, guanosine diphosphate; dGTP, deoxyguanosine triphosphate; dTTP, deoxythymidine triphosphate; dCTP, deoxycytidine triphosphate; rNDP, ribonucleotide diphosphate; dNDP, deoxynucleotide diphosphate; dNTP, deoxynucleotide triphosphate; RR, ribonucleotide reductase; RS, radiosensitization; ATP, adenosine triphosphate; dATP, deoxyadenosine triphosphate; BrdUrd, bromodeoxyuridine; IdUrd, iododeoxyuridine; FdUrd, fluorodeoxyuridine; MMR, mismatch repair; DSB, double strand break; HR, homologous recombination; NHEJ, nonhomologous end joining; DNA-PK_{cs}, DNA protein kinase catalytic subunit; CHO, Chinese hamster ovary

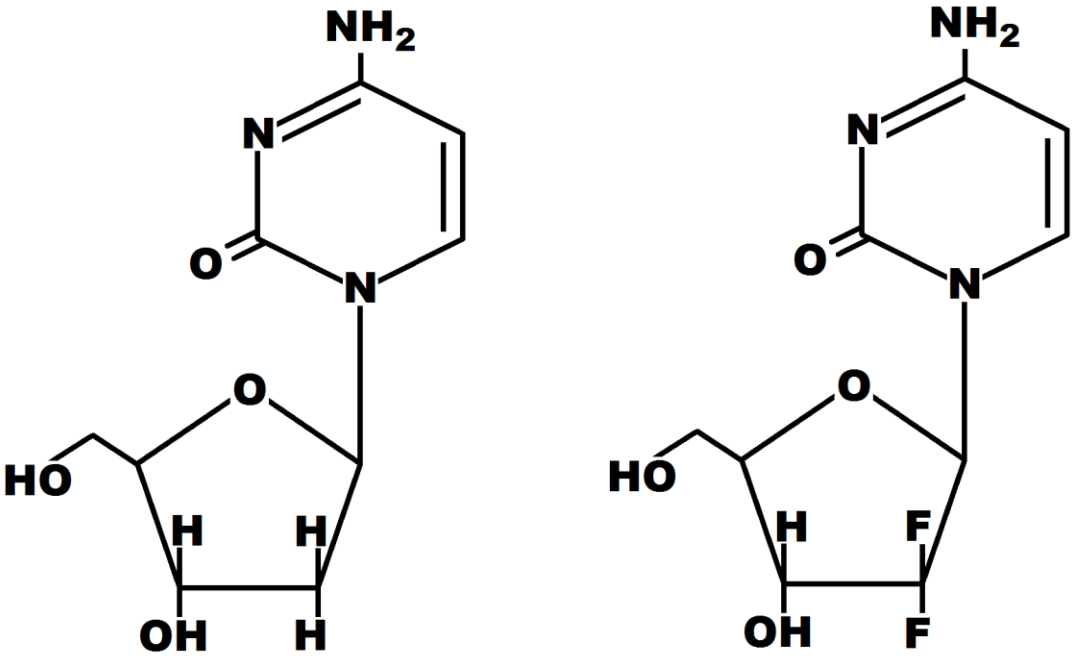

Figure 1.1. Chemical structures of deoxycytidine and gemcitabine.

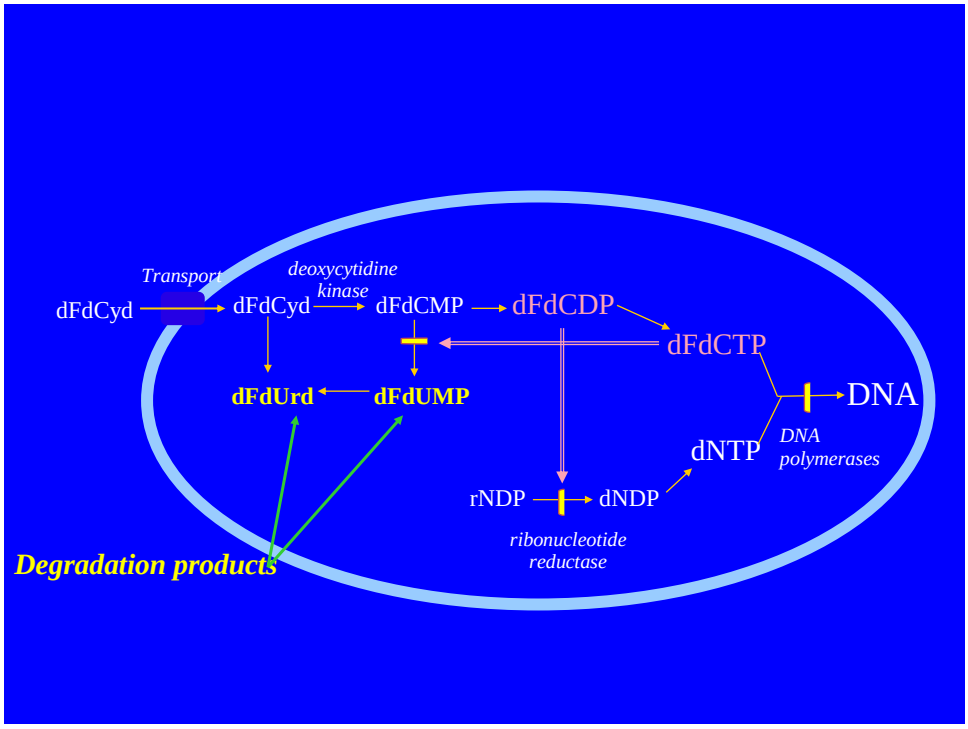

Figure 1.2. Pathway of gemcitabine metabolism

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Chapter II

Late DNA Damage Correlates with Radiosensitization by Gemcitabine

Abstract

The antitumor drug gemcitabine (2',2'-difluorodeoxycytidine; dFdCyd) is well recognized for its potent radiosensitization of tumor cells *in vitro* and *in vivo*. However, the exact mechanism by which dFdCyd enhances cell death when administered prior to ionizing radiation (IR) has remained elusive. Here we have used γ-H2AX foci to sensitively detect DNA damage during an extended time period following dFdCyd and/or IR treatment. MCF-7 breast cancer cells displayed excellent radiosensitization with dFdCyd at its IC_{10} (10nM) and IC_{90} (300nM) (radiation enhancement ratios 1.7 – 2.7). Measurement of DNA damage demonstrated a dose-response relationship between dFdCyd or IR and γ-H2AX foci formation, providing an appropriate basis for quantitative measurement of the effects of drug combined with IR under radiosensitizing conditions. After a 24 hr incubation with dFdCyd followed by 2 Gy IR, there was no consistent difference in either the appearance of γ-H2AX foci or their resolution within the first 4 hr after treatment, relative to either drug or IR alone, consistent with previous reports using pulsed field gel electrophoresis. However, at 24 – 48 hr after drug washout/IR, there was an 18 to 19-fold (IC_{10}+2Gy) and 15 to 30-fold (IC_{90}+2Gy) increase in γ-H2AX foci, exceeding the maximal increase in foci within the first four hours by approximately 4-fold. This increase was significantly higher than the 5 to 10-fold (IC_{10}) or 6 to 16-fold (IC_{90}) increase observed with

dFdCyd only, and distinct from IR alone, which did not show a late increase in γ-H2AX foci. These data demonstrate for the first time a difference in DNA damage under radiosensitizing conditions compared to that with either dFdCyd or IR alone. Furthermore, this late DNA damage is characteristic of dFdCyd but not IR, indicating that IR sensitizes cells to the mechanism by which dFdCyd produces cell death. While previous studies of IR combined with other antimetabolites have ascribed radiosensitization to either increased DNA damage or decreased repair shortly after drug and IR exposure, the data here demonstrate a novel mechanism for dFdCyd in which late DNA damage plays a role not previously recognized.

Introduction

Gemcitabine (2', 2'-difluoro-2'-deoxycytidine; dFdCyd) is a nucleoside analog with broad clinical activity in patients with solid tumors (Anderson 1994, Kaye 1994, Rothenburg 1996, Fossella 1997). In addition to its activity as a chemotherapeutic agent, dFdCyd is well recognized for its ability to synergistically increase ionizing radiation (IR)-induced cell killing (Shewach 2007). This radiosensitizing activity has been demonstrated in a wide variety of human tumor cell lines in vitro (Shewach 1994, Lawrence 1996, Ostruszka 2000, Robinson 2001) and in mouse tumor models (Milas 1999, Joschko 1997, Fields 2000). Importantly, the combination of dFdCyd with concurrent radiotherapy has enhanced antitumor activity in patients with solid tumors, including head and neck (Eisbruch 1997), pancreatic (McGinn 2001) and non-small cell lung cancer (Trodella 2002). However, despite numerous studies of dFdCyd and IR *in vitro* and *in vivo*, the exact mechanism for radiosensitization has not been completely elucidated. While it is expected that synergistic cytotoxicity produced by combining two modalities, both of which produce cell death through DNA damage, would result in synergistic DNA damage, prior

studies have not shown any difference in DNA damage or its repair with dFdCyd and IR under radiosensitizing conditions (Lawrence 1997, Rosier 2003).

Similar to other antimetabolites, dFdCyd requires phosphorylation within the tumor cell in order to exert either its chemotherapeutic or radiosensitizing effects. Its antitumor activity has been attributed to the triphosphorylated metabolite, dFdCTP, which causes apoptosis through its incorporation into DNA (Huang 1997, Ewald 2008). The diphosphorylated metabolite, dFdCDP, is a mechanism-based inhibitor of ribonucleotide reductase (Heinemann 1990, Baker 1991), resulting in depletion of dATP in solid tumor cells (Shewach 1994, Robinson 2001), which contributes to cytotoxicity. Inhibition of ribonucleotide reductase has been identified as requisite for radiosensitization, whereas no correlation exists between DNA incorporation of dFdCTP and radiosensitization (Shewach 1994, Flanagan 2007). The dFdCDP-mediated depletion of dATP causes misincorporation of endogenous dNTPs into DNA which, if not repaired prior to irradiation, results in radiosensitization (Flanagan 2007, Robinson 2003).

Despite the elucidation of mismatches in DNA as the lesions leading to radiosensitization, the exact effects on induction or repair of DNA damage have not been identified. Indeed, prior studies by us and others have demonstrated that neither increased DNA damage nor inhibition of its repair immediately after IR can explain the radiosensitizing ability of dFdCyd (Lawrence 1997, Gregoire 1998). Others have suggested that dFdCyd affects S-phase DNA structures or stability, or residual DNA damage as mechanisms for the radiosensitization. These studies all relied on pulsed-field gel electrophoresis (PFGE) to identify DNA double strand breaks (DSBs). One limitation of PFGE, however, is the requirement for extremely high single doses of radiation ($\geq$10 Gy) in order to observe DSBs. Such exposures to

high doses of radiation would be expected to induce excessive DNA damage that could preclude determination of increased damage when combined with dFdCyd.

Because PFGE is not sensitive enough to measure DSBs with clinically relevant concentrations of dFdCyd and IR at which radiosensitization has been demonstrated *in vitro* and *in vivo*, we utilized a more sensitive assay for measuring DNA damage. It has been reported that phosphorylation of the variant histone H2AX occurs in response to DNA damage, and the phosphorylated histone (γ-H2AX) then accumulates in punctate foci at sites of DSBs (Rogakou 1999). Furthermore, the number of γ-H2AX foci that accumulate is proportional to the number of DSBs (Sedelnikova 2002). Thus we have used immunostaining for γ-H2AX foci as a more sensitive and quantitative measure of DSBs following dFdCyd and IR alone or in combination. The results demonstrate that, while DNA damage and its repair did not differ between dFdCyd or IR alone or in combination within the first four hr after treatment, a dramatic increase in DSBs was observed at 24 – 48 hr after treatment with dFdCyd but not IR alone. Furthermore, the late accumulation of DSBs increased synergistically with dFdCyd + IR compared to either treatment alone. Thus, our studies demonstrate for the first time a rationale for radiosensitization based on augmentation of late-occurring DNA DSBs with dFdCyd.

Materials and Methods

Cytotoxicity and Radiosensitization

MCF-7 breast cancer cells were maintained in RPMI 1640 media (Invitrogen) supplemented with 10% calf serum (Invitrogen), and 2mM L-glutamine (Fisher Scientific). Cells were incubated in T75 culture flasks at 37^0C, 5% CO_2. Clonogenic survival was used to determine cytotoxicity to dFdCyd alone and radiosensitization in response to dFdCyd and IR.

Cytotoxicity was determined by incubating cells with graded doses of gemcitabine for 24 hours. Cells were trypsinized and seeded onto 6 well culture plates at a density of 100 and 200 cells per well (each density was plated in triplicate). Colony formation was allowed to proceed for 12-14 days at 37^0C, 5% CO_2, after which plates were fixed with 3:1 methanol:acetic acid and stained with 0.4% crystal violet. Radiosensitization was determined similarly, except cells were irradiated with 0-10 Gy following 24h dFdCyd exposure, and seeded onto 6 well culture plates at varying densities depending on the extent of cell kill for each condition.

γ-H2AX staining

MCF-7 cells were seeded onto 8 well chambered slides at a density of 3000 cells per well and allowed to grow for 3 days. Individual wells were then treated with either gemcitabine for 24 hours, 2 Gy radiation, or a combination of the two. Following drug washout or radiation treatment, cells were processed for confocal imaging. This included a 10 min. permeabilization/fixation step with a 50:50 solution of acetone:methanol, a 1 hr block with 10% goat serum in phosphate buffered saline (PBS), a 1 hr incubation with an antibody directed against γ-H2AX (Trevigen), and a 1 hr incubation with an AlexaFluor 488 conjugated secondary antibody (Molecular Probes). Washes using PBS or 10% goat serum were performed between each step. Following the processing steps, the slides were mounted with ProLong antifade reagent (Molecular Probes) and were allowed to dry for at least 2 days before viewing on an Olympus confocal microscope. The gain and contrast were set to allow for visualization of foci across all drug and IR conditions, and were kept constant for all slides within each experiment. Images were obtained using Fluoview software, and individual cells were scored for γ-H2AX foci.

Results

Cytotoxicity and Radiosensitization. MCF-7 breast cancer cells were sensitive to low concentrations of dFdCyd alone during a 24 hr incubation, with IC_{10} and IC_{90} values of 10 and 300 nM, respectively (Table 2.1). When MCF-7 cells were incubated with these concentrations of dFdCyd for 24 hr followed by irradiation, they were well radiosensitized with radiation enhancement ratios (RERs) ranging from 1.9 to 2.7 (Table 2.1). These data demonstrate synergy with dFdCyd and IR, as radiosensitization was apparent after correction for survival with drug alone, and furthermore occurred even at a non-cytotoxic (IC_{10}) concentration of dFdCyd. With a D-bar value of 2.6, this cell line is moderately sensitive to IR and thus the excellent RER values cannot be attributed simply to a highly radiosensitive cell line.

Drug Concentration and Time Dependence of DNA DSBs. We wished to determine whether γ-H2AX foci, a surrogate for DSBs, would correlate with dFdCyd concentrations and doses of IR that produced radiosensitization. As illustrated in figure 2.1, γ-H2AX staining increased with dFdCyd concentration and time during a 24 hr incubation, relative to controls. IR administered at 2 Gy either alone or in combination with IC_{10} dFdCyd also produced discrete γ-H2AX foci within 1 hr after irradiation that were similar in appearance to those produced by dFdCyd alone (Fig. 2.2). Quantitation of the foci demonstrated a dose-response relationship between a wide range of dFdCyd concentrations and γ-H2AX foci formation (Fig. 2.3A). Furthermore, γ-H2AX foci increased over the range of 0.3 to 2.1 Gy (Fig. 2.3B), and continued to increase up 10 Gy, however, coalescing of foci occurred with $\geq$ 5 Gy (data not shown), which prevented

quantitation. For the following studies, we chose 2 Gy IR because higher doses of IR produced γ-H2AX foci that were too dense to count.

Early repair of DNA damage following dFdCyd or IR alone or in combination. With measurable differences in γ-H2AX foci over the range of dFdCyd concentrations used for radiosensitization, we then evaluated the appearance and repair of DNA damage under radiosensitizing conditions. As in previous studies, we initially evaluated DNA damage and its repair over a 4 hr period following drug and/or IR. We hypothesized that, if increased DNA damage or decreased DNA repair were the mechanism of radiosensitization for dFdCyd, then there would be a significant and consistent change in γ-H2AX foci between controls and drug plus IR treated cells. As illustrated in table 2.1, radiosensitization was apparent with all concentrations of dFdCyd evaluated.

Following dFdCyd exposure alone, foci formation was highest between 0 and 2 hr after drug washout (Fig. 2.4). By 4 hr, γ-H2AX foci formation in dFdCyd-only treated cells was not significantly different from the untreated controls. Irradiation with 2 Gy alone resulted in peak levels of foci between half and 1 hr post-irradiation, and the number of foci returned to control levels within 4 hr.

When cells were exposed to dFdCyd for 24 hr followed by 2 Gy IR, the subsequent pattern of γ-H2AX foci formation was similar to that of radiation alone. For all dFdCyd concentrations in combination with IR, the highest increase in foci occurred within 1 hr after irradiation followed by a decline in numbers of foci to near control levels within 4 hr. Using the IC_{50} and the IC_{90} for dFdCyd plus IR, there was a greater increase in foci with the drug and radiation combination within 1 hr after irradiation than with either treatment alone. However,

the increase in γ-H2AX foci was not additive (Table 2.2). In contrast, with IC_{10} dFdCyd, radiation or drug alone produced a higher increase in foci than the combination. Thus, despite the similarity in RER values for IC_{10} and IC_{50} dFdCyd, the patterns of γ-H2AX foci formation were different at these early time points.

The repair of DNA damage was visualized by the decrease in γ-H2AX foci after its peak at 1 hr. At all dFdCyd concentrations, by 4 hr after drug washout/IR the number of γ-H2AX foci decreased to a similar level as that of dFdCyd or IR alone. Thus, there was no apparent slowing of DNA repair after dFdCyd plus IR. These studies demonstrate that DNA damage or its repair in the first four hr after treatment does not account for radiosensitization with dFdCyd.

DNA damage at late timepoints following dFdCyd or IR alone or in combination. We extended the evaluation of γ-H2AX foci to include later time points up to 72 h post IR/drug treatment. These late time points are justified based on our previous report that radiosensitization with dFdCyd is associated with the appearance of mutations in DNA, which persisted for up to 96h with the combination of dFdCyd and IR. Following drug washout and irradiation, a 30 min time point was included as an internal reference for comparison to the previous 4 hr study. As there was no substantial difference in the extent of radiosensitization with IC_{10} or IC_{50} dFdCyd, only the non-cytotoxic IC_{10} and the cytotoxic IC_{90} for dFdCyd are included here. MCF7 cells exposed to dFdCyd or 2 Gy IR alone or in combination exhibited changes in γ-H2AX foci at 30 min post-treatment (Fig. 2.5) similar to those observed in Fig. 2.4. At the extended time points, dFdCyd alone produced γ-H2AX foci that were significantly higher at 48 hr after drug washout compared to those at 30 min or 24 hr. Ionizing radiation alone produced an increase in γ-H2AX foci within the first 30 min, however there was no further

increase in DNA damage until 72 hr, at which time cell death was apparent. Interestingly, with the combination of dFdCyd and IR there was a highly synergistic increase in γ-H2AX foci at 24 – 48 hr post-treatment compared to dFdCyd or IR alone. The combination of IC_{10} dFdCyd with IR produced an increase in γ-H2AX foci to 20 ± 1.2-fold over control at 24 hr and 18.3 ± 3.0-fold at 48 hr ($p<0.001$), compared to the 30 min time point (5.2±0.5-fold over untreated cells). With the combination of IC_{90} dFdCyd and IR, γ-H2AX foci increased to 15.7 ± 1.3 and 30.8 ± 2.8-fold over control at 24 and 48h-post treatment, respectively. By 72 hr, there was a decrease in γ-H2AX foci in cells treated with dFdCyd and IR but not with either modality alone, consistent with the decreased numbers of cells under radiosensitizing conditions observed in the slides, due to cell death. No substantial changes in γ-H2AX foci were observed in untreated control cells during the 72 hr time period (data not shown).

Discussion

The potent radiosensitizing ability of dFdCyd has been well documented in preclinical models *in vitro* and *in vivo* as well as in patients (Shewach 1994, Lawrence 1996, Fields 2000, Blackstock 1999, Eisbruch 2001). However, the mechanism responsible for the radiosensitizing effect of dFdCyd has not been fully elucidated. Radiation sensitizers typically enhance cytotoxicity either through increasing radiation-induced DSBs, or decreasing the rate of their repair (Shewach 2007). Using PFGE to measure DSBs, we previously reported that altering the induction or repair of DSBs does not account for the radiosensitization observed with dFdCyd (*Lawrence 1997*). One limitation to this approach is that PFGE is relatively insensitive and requires high doses of IR to measure DNA damage (Lawrence 1997, Rosier 2003). Here we have used a more sensitive technique, measuring γ-H2AX foci formation, to evaluate the role of

DNA damage in radiosensitization with dFdCyd (Paull 2000). The results demonstrate that radiosensitization with dFdCyd cannot be explained by an increase in DSBs or a decrease in the rate of their repair, similar to the results with PFGE. Additionally, while IR or dFdCyd alone showed dose-response relationships with γ-H2AX, no such relationship existed for the combination at these time points highlighting that this was not the time at which a difference in DNA damage could be correlated with RS. In contrast, a synergistic increase in γ-H2AX foci was observed by 24 – 48 hr post drug washout/IR, which strongly suggests that late-occurring DNA damage is responsible for radiosensitization with dFdCyd.

Analysis of γ-H2AX foci is commonly used as a measure of DSBs (Bonner 2008). However, most studies use a single or limited number of time points to evaluate this damage. Here we have performed a kinetic study of DNA damage, using γ-H2AX foci, with dFdCyd and IR alone or in combination. The results demonstrate that dFdCyd alone produces increasing DNA damage during drug incubation. This is consistent with a recent study that detected dFdCyd-induced γ-H2AX foci formation in acute myelogenous leukemia cells following exposure to 10 and 100 nM dFdCyd for 2 hr, concentrations that inhibited DNA synthesis by over 90% within 2 hr (Ewald 2008).

Because cell death with dFdCyd and IR individually results from DNA damage, it was expected that the synergistic cell killing observed with the combination of dFdCyd and IR would produce a significant increase in DNA damage. Consistent with this hypothesis, both dFdCyd and IR produced a dose-dependent increase in γ-H2AX foci. However, the combination of the two modalities resulted in either a decrease (IC_{10}), or a less than additive increase (IC_{50}, IC_{90}) in DNA damage during the first 4 hr after drug washout. Thus, there was no consistent increase in DNA damage during this early time period with dFdCyd concentrations that produced equivalent

radiosensitization (IC_{10}, IC_{50}). Furthermore, the rate of resolution of foci within 4 hr after drug washout/IR was not altered at any of the concentrations of dFdCyd compared to drug or radiation alone. Thus, these results, using a more sensitive assay, support the prior findings with PFGE and demonstrate that radiosensitization with dFdCyd is not due to an increase in DNA damage or a decrease in its repair during the first 4 hr after dFdCyd and IR.

Evaluation of later time points, up to 72 hr after dFdCyd washout and IR, revealed a late increase in DNA damage with dFdCyd alone or in combination with IR, that did not occur with IR alone. Importantly, the combination of dFdCyd with IR resulted in a synergistic increase in γ-H2AX foci up to 3.8-fold greater than either treatment alone, at both 24 and 48 hr. The findings that this synergistic increase occurred earlier with the combination than with dFdCyd alone, was higher with greater radiosensitization, and that it occurred at two consecutive time points strongly suggests that this late-occurring DNA damage is crucial for radiosensitization. A previous study in MCF-7 cells showed that the fraction of cells undergoing apoptosis in response to IC_{50} dFdCyd + 5Gy IR was below 8% at time points up to 72h post washout/IR, similar to untreated control cells. Thus, the synergistic increase in γ-H2AX foci in response to IC_{10} dFdCyd + 2Gy IR is not likely accounted for by DNA fragmentation as a consequence of programmed cell death. Furthermore, these data suggest that IR greatly amplifies the mechanism responsible for dFdCyd-mediated cell death, i.e. IR magnifies the effects of dFdCyd.

A previous study, as assessed by constant field electrophoresis, suggested that dFdCyd increased residual DNA damage from IR, apparent at 24 hr after a 2 hr incubation with dFdCyd followed by IR in BxPC3 pancreatic cancer cells (Weiss 2003). These studies utilized high concentrations of dFdCyd (0.5 – 1000 μM) which likely could have produced DSBs in the absence of IR. In our hands, this cell line was highly sensitive to dFdCyd (IC_{10} = 10 nM for 24

hr incubation), therefore without controls for DNA damage induced by the drug alone, it is difficult to interpret these results. A recent study identified increased S-phase specific DNA damage immediately after IR in WIDR cells treated with a moderately toxic concentration of dFdCyd, although this damage was repaired within 1 hr (Jensen 2008). In our current study, we also identified an increase in DSBs at early time points at one concentration of dFdCyd, however, when we evaluated two other concentrations this relationship wasn't consistent. Nevertheless, it may be important to identify the cell cycle phase and exact type of damage more specifically in order to refine our understanding of radiosensitization with dFdCyd.

Taken together, these data allow us to propose a mechanism that accounts for the late increase in DNA damage. Following dFdCyd incubation, cells accumulated in early S-phase due to both dFdCTP incorporation into DNA and inhibition of dNTP synthesis (Robinson 2003). The imbalance in dNTPs allows mutations in DNA to accumulate during limited DNA synthesis (Flanagan 2007). After dFdCyd washout, declining dFdCTP and normalizing dNTPs provides conditions amenable for re-establishment of DNA synthesis. The dramatic increase in γ-H2AX foci may reflect either an attempt to restart stalled replication forks, or alternatively it may correspond to attempted repair of fraudulent nucleotide incorporation or mismatches in DNA. The finding that the increase in γ-H2AX foci continues for at least 24 hr suggests that this late DNA damage is unrepairable, resulting in radiosensitization. Intriguingly, others have suggested that radiosensitization with dFdCyd requires homologous recombination (HR) repair (Wachters 2003), a pathway whose role in DNA damage repair increases as cells progress through S-phase. Because HR is inhibited by divergent DNA sequences (Elliot 2001), DNA mismatches will prevent the attempted repair by HR. If mismatch repair is unable to correct all of the mismatches produced by dFdCyd-mediated dNTP imbalance, as suggested by the persistence and increasing

mismatches with time after dFdCyd washout and irradiation (Flanagan 2007), then HR would fail eventually resulting in cell death.

These results suggest a novel mechanism for radiosensitization with dFdCyd. Compared to other antimetabolite radiosensitizers, dFdCyd does not alter the induction or repair of radiation-induced DNA damage. Rather, the addition of radiation prevents the repair of late DNA damage resulting from dFdCyd exposure, resulting in increased cell death. It is not clear why radiation prevents repair of this damage. One possibility is that the necessary repair of lesions produced from the combination of radiation with dFdCyd overwhelms mismatch repair, such that the DNA mismatches persist and prevent HR repair when it is needed in late S-phase. We have recently demonstrated that persistence of mismatches is also important in radiosensitization with fluorodeoxyuridine (Flanagan 2008), and it would be of interest to determine whether repair of late DNA damage contributes to radiosensitization with this antimetabolite as well. This novel mechanism has clinical implications, as mechanisms to increase DNA mismatches in tumors treated with dFdCyd would be expected to contribute to improved radiosensitization.

List of Abbreviations

dFdCDP, gemcitabine diphosphate; dFdCTP, gemcitabine triphosphate; dCyd, deoxycytidine; dATP, deoxyadenosine triphosphate; dNTP, deoxynucleotide triphosphate; IR, ionizing radiation; PFGE, pulsed-field gel electrophoresis; DSB, double strand break; IC, inhibitory concentration; RER, radiation enhancement ratio; AUC, area under the curve

[dFdCyd] (nM)	Survival at 0 Gy	Radiosensitization Enhancement Ratio (RER)	D-bar (no drug)
0	100	---	2.6 $\pm$ 0.2
10	90%	1.9 $\pm$ 0.1	
80	50%	1.7 $\pm$ 0.3	
300	10%	2.7 $\pm$ 0.1	

Table 2.1. Cytotoxicity and radiosensitization of MCF-7 breast cancer cells with dFdCyd. Survival of MCF-7 cells were determined in response to 24h exposure to the indicated concentrations of dFdCyd. Radiation enhancement ratios were determined by dividing the area under the survival curve (AUC) for IR alone by the AUC for the indicated concentrations of dFdCyd in combination with IR, which has been corrected for sensitivity to dFdCyd alone.

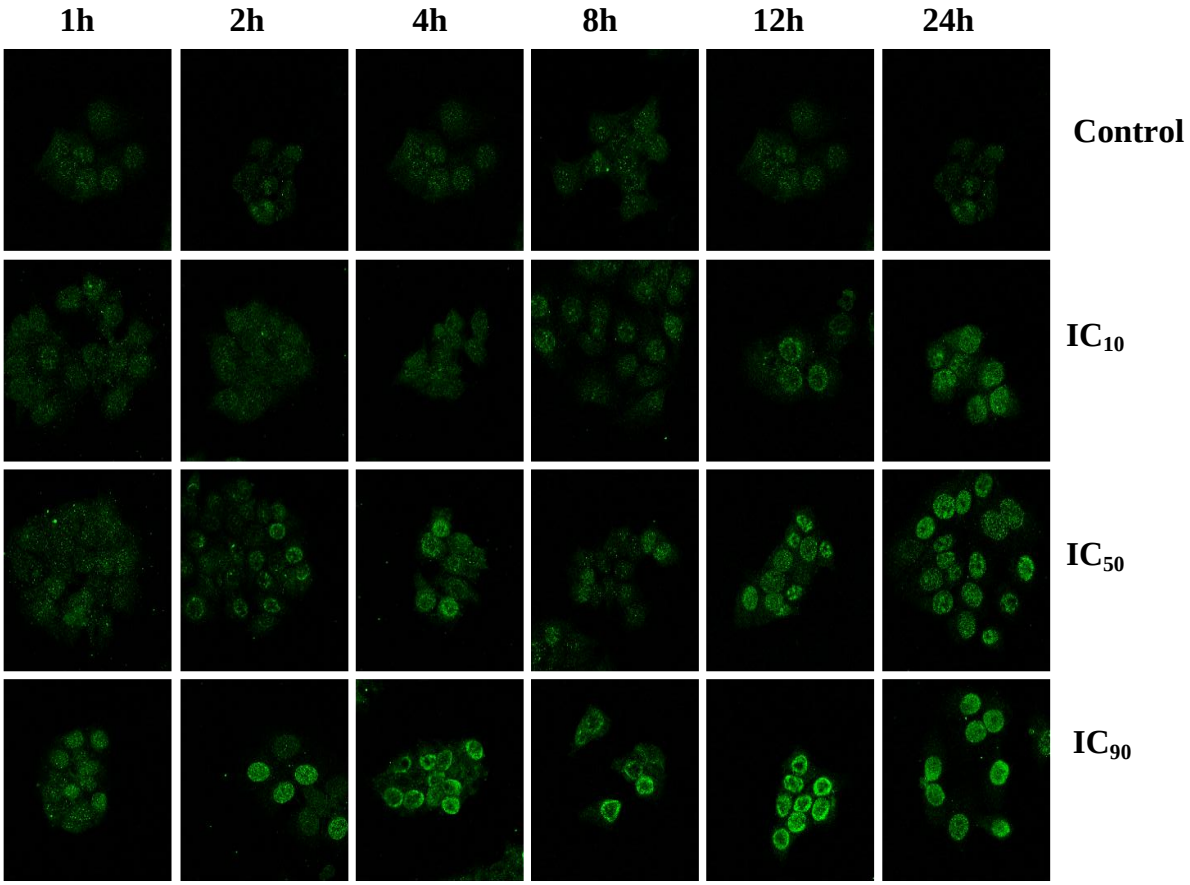


Figure 2.1. Time and concentration dependent γ-H2AX foci formation in MCF-7 cells. MCF-7 cells were exposed to the indicated concentrations of dFdCyd for up to 24h and visualized by confocal microscopy.

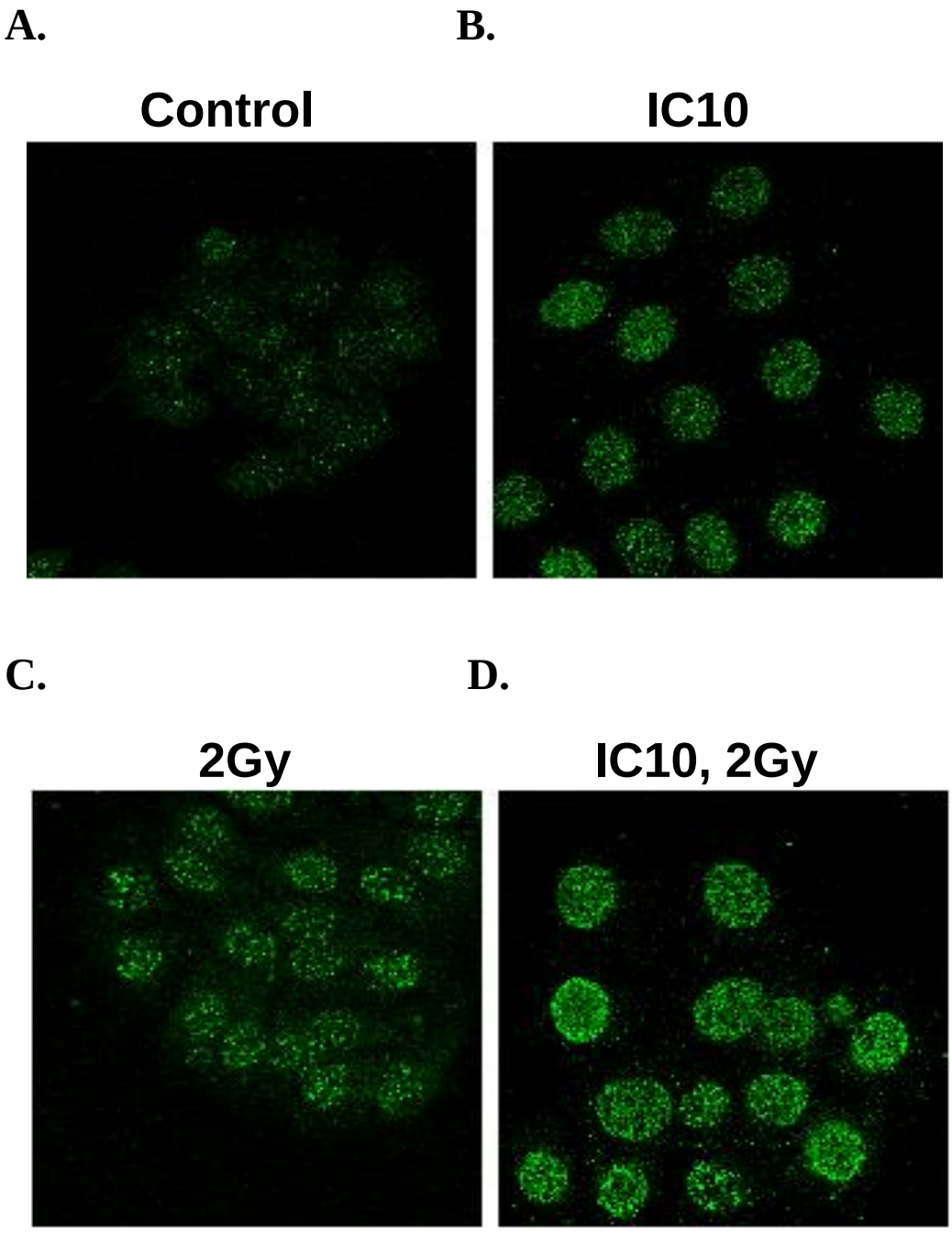
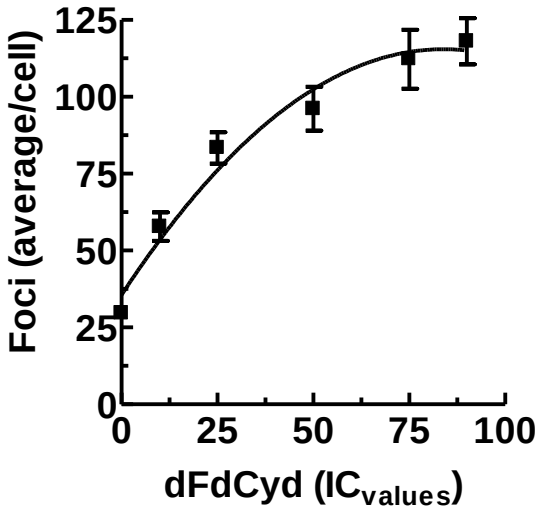


Figure 2.2. Representative images of MCF-7 cells. A. Untreated, B. cells exposed to dFdCyd for 24 hr, C. cells visualized 30 minutes following exposure to 2 Gy IR, D. cells exposed to dFdCyd for 24 hr followed by 2 Gy IR and visualized 30 minutes post-IR.

A.



B.

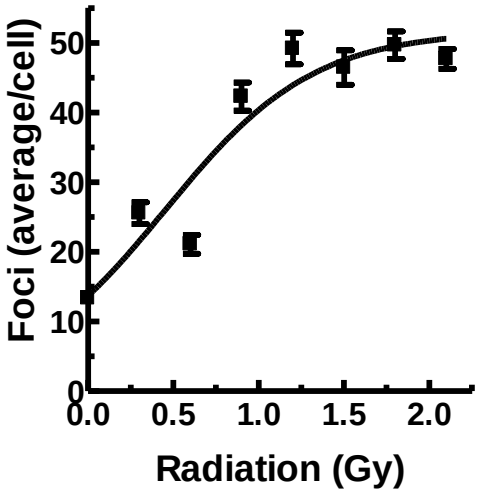


Figure 2.3. Dose response relationship between dFdCyd concentration (A) or radiation dose (B) and y-H2AX foci formation.

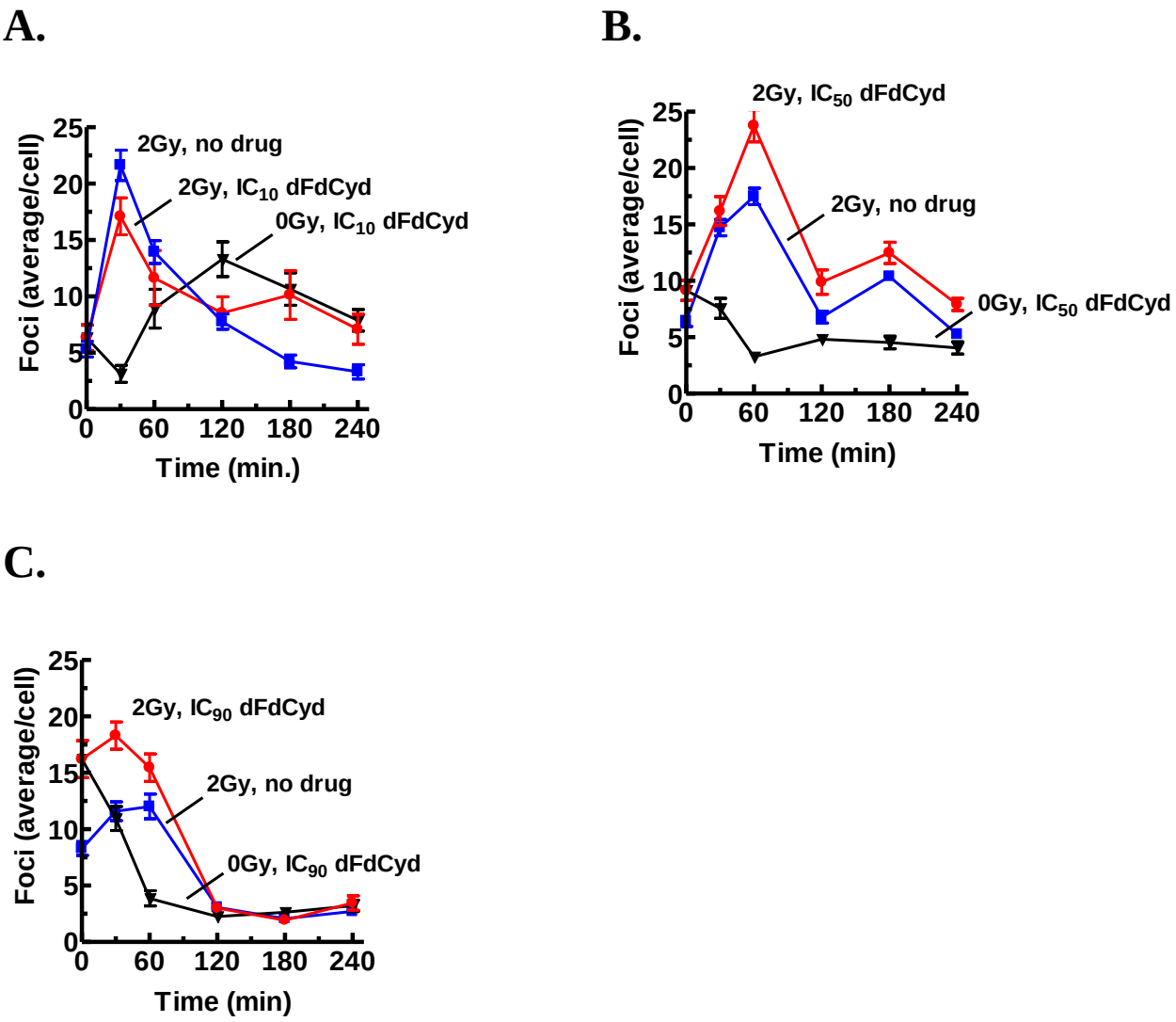


Figure 2.4. Effect of combined exposure to dFdCyd and IR on γ-H2AX induction. IC_{10} (A), IC_{50} (B), or IC_{90} (C) of dFdCyd. MCF-7 cells were exposed to drug alone (▼), 2 Gy IR (■), or the combination (●). Time represents time post-washout/post IR.

	Foci (fold increase)	RER
2Gy	4.06	
IC10	1.19	
2Gy, IC10	3.21	1.9 ± 0.1
2Gy	2.72	
IC50	1.42	
2Gy, IC50	3.69	1.7 ± 0.3
2Gy	1.4	
IC90	1.96	
2Gy, IC90	2.21	2.7 ± 0.1

Table 2.2. Comparison of γ-H2AX foci in MCF-7 breast cancer cells with dFdCyd. Peak foci formation within the first 4h post drug washout/IR was compared between each condition over control (2Gy, IC_{10}, combination). Peak induction values represented as fold increase (relative to untreated controls) are included with enhancement ratios

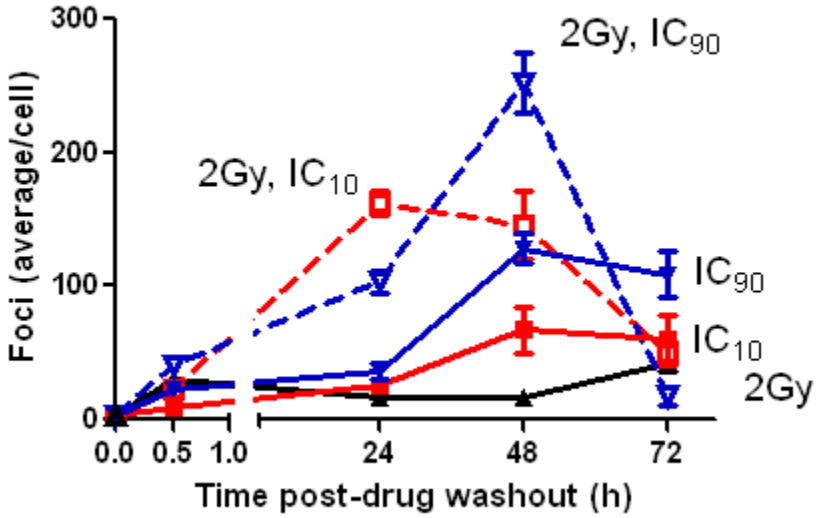


Figure 2.5. Effect of combined exposure to dFdCyd and IR on late γ-H2AX induction. The appearance of γ-H2AX foci in MCF-7 cells was quantified for up to 72h post drug washout/IR, in response to IC_{10} (■),IC_{10} + 2Gy (□), IC_{90} (▼), IC_{90} + 2Gy (▽), or 2Gy (▲)

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Chapter III

Homologous Recombination has a Necessary Role in the Appearance of Late DNA Double Strand Breaks and Radiosensitization by Gemcitabine

Abstract

Gemcitabine (2',2'-difluorodeoxycytidine; dFdCyd) is an antimetabolite that causes a synergistic enhancement in cell killing (radiosensitization; RS) when combined with radiation. Correlations have been established with RS by dFdCyd and depletion of dATP by >80% for at least 4h, accumulation of cells into S-phase, and persistence of mismatches in DNA. A dependence for active homologous recombination (HR) has also been demonstrated, however, a precise mechanism for RS by dFdCyd has not been determined. Previously, we showed that RS was correlated with a synergistic increase in γ-H2AX foci at late time points (≥24h post drug washout/IR) that greatly exceeded initial levels, and we hypothesized that this marked a causal event in RS by dFdCyd. Here we verify a role for HR in RS of CHO cells, and expand the investigation to include the MCF-7 human breast cancer cell line. We show that lentiviral-mediated shRNA knockdown of XRCC3, an essential HR protein, results in radiation enhancement ratios (RERs) of 0.87±0.22 and 1.17±0.19, values that do not represent RS, compared to cells transduced with a non-specific (NS) shRNA, or nontransduced control cells which exhibit excellent RS with RERs of 1.43±0.19 and 1.67±0.17, respectively. This indicates that suppression of HR inhibits RS. Analysis of late γ-H2AX foci in response to the combination of dFdCyd and IR revealed significant 43-48% and 34-58% decreases in foci levels at 24 and

48h post drug washout/IR in cells transduced with each of two XRCC3 shRNA constructs compared to non-transduced control cells. On the other hand, the level of foci in cells transduced with the NS shRNA was not significantly different from the non-transduced control MCF-7 cells. Similarly, XRCC3 transduced cells showed a reduction in levels of phospho-ATM at 24 hr compared to control cells exposed to dFdCyd + IR. These data demonstrate a dependence for functional HR in RS by dFdCyd in human tumor cells, and further show that the late increase in γ-H2AX foci, which marks a crucial event in RS, is reduced in cells where HR is disrupted through shRNA knockdown of XRCC3. Furthermore, these data suggest that the late damage represented by γ-H2AX foci, is comprised of DNA double strand breaks, whose formation is influenced by HR.

Introduction

Gemcitabine (2',2'-difluoro-2'-deoxycytidine; dFdCyd) is a nucleoside analog that produces a synergistic enhancement in cell killing (radiosensitization; RS) when combined with ionizing radiation (IR). The DNA double strand break (DSB) is thought to be the most detrimental lesion formed in response to ionizing radiation, and other radiosensitizing nucleoside analogs, such as bromodeoxyuridine (BrdUrd), iododeoxyuridine (IdUrd) and fluorodeoxyuridine (FdUrd), are thought to elicit synergy through an increase in the formation, or a decrease in the rate or extent of repair of these DSBs (Bruso 1990, Heimburger 1991, Lawrence 1990, Ling 1990). There are two major pathways involved in the repair of DSBs in mammalian cells, the error-prone nonhomologous end joining (NHEJ) pathway, which involves ligation of blunt ends or ends containing small regions of homology resulting in DSB resolution with loss of information, and homologous recombination (HR) which utilizes a homologous template, with preference for a

sister chromatid, resulting in virtually error-free DSB repair (Chapman 2012). Studies in Chinese hamster ovary (CHO) cells containing mutations of essential NHEJ proteins (Ku80 or DNA-PKcs) that rendered them NHEJ inactive showed that RS by gemcitabine was still achieved, suggesting NHEJ to be dispensable for RS by dFdCyd (van Putten 2001). On the other hand, evaluation of gemcitabine-mediated synergy in CHO cells containing mutations in key HR proteins (XRCC2 and XRCC3) showed that RS was not achieved in an HR-deficient background, suggesting that HR is essential for RS by dFdCyd in CHO cells (Wachters 2003). While this study in CHO cells implicates HR in RS with gemcitabine, it is not clear whether this applies to the mechanism of synergy with gemcitabine and IR in human tumor cells. For example, RS with gemcitabine is concentration-dependent in several different types of human tumor cells (Shewach 1994, Lawrence 1996, Robinson 2001), whereas the study by Wachters (2003) showed similar RS with 0.5 and 5 μM gemcitabine in CHO cells. In addition, RS by gemcitabine in many different human tumor cell lines has been correlated strongly with a depletion of dATP pools by $\geq$80%, due to gemcitabine-mediated inhibition of dNTP synthesis via ribonucleotide reductase (Shewach 1994, Lawrence 1996, Robinson 2001). In addition, RS with gemcitabine is dependent on schedule and duration of exposure in human tumor cells, but not on the accumulation of gemcitabine triphosphate or its incorporation into DNA (Lawrence 1996, Robinson 2003). Thus we wished to explore further the mechanism of RS with gemcitabine, and to determine whether the effects of mutations in HR on RS in these cells could be explained by alterations in the effects of gemcitabine on dNTP pools. Furthermore, we wished to evaluate the role of HR in RS with gemcitabine in human tumor cells, in which the required effects of gemcitabine on dNTPs and DNA replication is known. In particular, we wished to determine the role of HR in our recent finding that RS with gemcitabine is correlated

with a synergistic increase in DNA damage at late time points. Thus we have undertaken studies of gemcitabine metabolism and RS in the HR-proficient Chinese hamster AA8 cells and HR-deficient (XRCC3-deficient) irs1SF cells, along with studies of HR deficiency on RS in MCF-7 human breast cancer cells.

Materials and methods

Cell Lines

The Chinese hamster cell lines, AA8 (HR-proficient) and irs1SF (XRCC3-deficient, HR-deficient), were grown in MEMα medium (Invitrogen) supplemented with 10% FBS (Invitrogen), L-glutamine (Fisher Scientific), and penicillin/streptomycin at 37^0C, 5% CO_2, and maintained at exponential growth. MCF-7 breast cancer cells were grown in RPMI supplemented with 2mM L-glutamine and 20% bovine serum. 293T cells were maintained in DMEM supplemented with 2mM L-glutamine and 10% FBS. CHO cell doubling time was measured over a period of 72h. Doubling times were calculated during exponential growth using the following equation:

Doubling time = $((\ln(\text{final cell \#}) - \ln(\text{initial cell \#}))/\text{time})^{-1} \times 0.693$

For evaluation of cell cycle progression, MCF-7 cells were exposed to IC_{10} dFdCyd for 24h and harvested at time points up to 72h post drug washout. Cells were labeled with BrdUrd for 30 min prior to harvest, then fixed and stained with PI. Cells were processed and analyzed by flow cytometry as previously described (Robinson 2001).

Cytotoxicity and Radiosensitization

Cells were seeded into T25 flasks at a density of 2.5 to 3 x 10^5 cells per flask. Cells were exposed to a range of dFdCyd concentrations for specified times, harvested by trypsinization, diluted and approximately 100 viable cells/well were plated in triplicate in six well plates. The plates were incubated at 37^0C, 5% CO_2 until surviving cells grew into colonies of at least 50 cells. Plates with AA8 cells were incubated for 7 days, and irs1SF for 12-14 days cells to accommodate the difference in doubling times between the cells. MCF-7 plates were incubated for 12 to 14 days. Following the incubation period, cells were fixed in 75% methanol/25% acetic acid then stained with 0.4% crystal violet. Colonies with at least 50 cells were counted. Each experiment was performed at least three times. For determination of RS, cells were incubated with dFdCyd for specified periods of time prior to irradiation. Cells were irradiated with 2-10 Gy IR, and plated as above. Results were fit to a linear-quadratic equation, and the areas under the resulting survival curves (AUC) were used to calculate the radiation enhancement ratio (RER), which is defined as the AUC for IR alone divided by the AUC for dFdCyd + IR (Fertil 1984).

dNTP Pools

Cells were grown in T75 flasks and exposed to specific concentrations of dFdCyd. Following incubation, cells were harvested by trypsinization and enumerated using a Coulter counter. At least 10^7 cells were collected for each condition. Collected cells were pelleted, washed with ice-cold PBS, and repelleted prior to acid extraction of nucleotides with perchloric acid. Samples were neutralized and frozen prior to HPLC separation on a strong anion exchange column eluted with a gradient of 0.15-0.60M NH_4H_2PO4 (JT Baker). For dNTP analysis, neutralized samples were applied to an Affi-gel (BioRad) boronate column to separate dNTPs from rNTPs. Prior to HPLC, samples were acidified with phosphoric acid to lower the pH to match that of the running

buffers. The dNTPs were detected by UV absorbance at 254 or 281 nm, and quantitated by comparison of peak areas to that of known amounts of standards.

shRNA suppression of XRCC3

Lentiviral components (generously supplied by the Dr. Mikhail Nikiforov, Roswell Park Cancer Institute) and shRNA (XRCC3#1 and XRCC3#4; Sigma) plasmids were isolated and purified from bacterial stocks, and transfected into 293T cells to propagate virus. Following incubation, media containing virus was appropriately diluted, supplemented with polybrene, and filter sterilized before being used to transduce AA8 or MCF-7 cells. Positively transduced cells were selected with puromycin. Cells were assessed for RS by dFdCyd following knockdown of XRCC3, and knockdown was confirmed by western blot at time points preceding and following plating of the experiments.

Analysis of γ-H2AX foci formation by confocal microscopy

Cells (untreated or shRNA treated) were grown on chambered slides. After incubation with drug, the cells were washed with PBS and then fixed and permeabilized with acetone/methanol (50:50 v/v) for 10 min. The fixed cells were then washed with PBS, blocked with 10% goat serum for 1 h, incubated with γ-H2AX primary antibody (1:400 dilution; Millipore, Billerica, MA) for 1 h, washed, incubated with AlexaFluor 488 conjugated goat anti-mouse secondary antibody (1:200 dilution; Molecular Probes, Eugene, OR) for 1 h, washed and mounted with ProLong antifade kit (Molecular Probes, Eugene, OR). Slides were imaged with a Zeiss LSM510 confocal microscope using a 60x objective lens. Images of representative cell populations were captured, and γ-H2AX foci were counted visually. At least 5 - 16 cells per well were counted with triplicate wells per condition.

Histone extraction

Histones were extracted according to the protocol by Ward and Chen (Ward 2001). Briefly, non-transduced and shRNA-transduced MCF-7 cells were grown in T75 flasks and lysed in NETN buffer (150mM NaCl, 1mM EDTA, 20mM Tris (pH 8), 0.5% Nonidet P-40) at appropriate time points following exposure to dFdCyd, IR, or dFdCyd + IR. Lysates were centrifuged to isolate the insoluble pellets, which were exposed to 0.1N HCl overnight at 4^0C to extract the histones. Histone extracted samples were then analyzed via western blot.

Western blot

Trypsinized and washed cells were incubated in lysis buffer for 10 min. on ice, and supernatants were collected following centrifugation. Protein concentrations were determined using a protein assay from BioRad. For total H2AX and γ-H2AX, proteins were separated on a 15% polyacrylamide gel. XRCC3 and actin were separated on a 10% gel, and total ATM and pATM were separated on a 7% gel. All separated proteins were transferred to an Immobilon-P membrane (Millipore) for 2h at 200mA, except ATM and pATM, which were transferred at 350mA overnight. Blocked membranes were incubated with appropriate primary antibody overnight at 4^0C followed by 1h with secondary antibody. Membranes were thoroughly washed with Tris-buffered saline + 0.1% Tween 20 between each step. Protein bands were visualized using an enhanced chemiluminescence kit (Pierce) and quantified using Image-J software (NIH). Antibody for XRCC3 was kindly provided by Dr. Patrick Sung (Yale University). Antibodies were obtained commerically for total H2AX (Cell Signaling), γ-H2AX (Millipore), actin (Calbiochem), pS1981 ATM and total ATM (Epitomics).

Results

CHO cells

Cytotoxicity and Radiosensitization with dFdCyd in CHO Cells

We established sensitivity to dFdCyd for the HR proficient AA8 and HR-deficient irs1SF CHO cells during a 4h exposure over a wide range of drug concentrations. As shown in Fig. 3.1, both the AA8 and irs1SF cells exhibited comparable sensitivity across a range of dFdCyd concentrations during a 4h incubation. The pattern of cell kill was also similar between cell lines, with the most drastic decreases in survival occurring at concentrations up to 0.2μM dFdCyd, with little additional decrease in survival at concentrations >0.2μM dFdCyd. Additionally, despite the 10-fold difference in concentration, cytotoxicity at 0.5μM was similar to 5μM dFdCyd (~ 75% decrease in survival). A previous publication that evaluated exposure of these cell lines to 0.5μM and 5μM dFdCyd only reported lower sensitivity in irs1SF cells (~30% and 50% decreased survival, respectively) and similar sensitivity in AA8 cells (~50% and 75% decreased survival, respectively) (Wachters 2003)

When AA8 and irs1SF cells were incubated with 0.5μM and 5μM dFdCyd for 4h prior to exposure to 2Gy IR, only the HR-proficient AA8 cells were radiosensitized (Fig. 3.2), consistent with the previous report (Wachters 2003). The radiation enhancement ratios (RER) were 0.9 ± 0.1 and 0.8 ± 0.1, in irs1SF cells exposed to 4h 0.5μM and 5μM dFdCyd, respectively, values indicating a lack of RS. Compared to the irs1SF cells, AA8 cells showed RERs of 1.6 ± 0.3 and 2.4 ± 0.5 in AA8 cells exposed to 4h 0.5μM and 5μM dFdCyd, respectively, values that demonstrate excellent RS. Furthermore, RERs in AA8 cells were significantly greater than those in irs1SF cells ($p<0.01$ for both concentrations of dFdCyd).

It was noted that the AA8 cells grew at twice the rate of the irs1SF cells, with doubling times of 10h and 20h, respectively. To equalize drug exposure according to the percentage of cell doubling time, the effect of 2h and 4h exposure to 0.5μM and 5μM dFdCyd in AA8 cells on RS

was compared to 4h and 8h exposures in irs1SF cells. As shown in Table 3.1, RERs achieved for the 2h incubation were similar to those achieved with the 4h incubation in AA8 cells at both concentrations. On the other hand, RS was not observed in irs1SF cells.

Because RS using highly cytotoxic concentrations of dFdCyd (IC_{75}) can be difficult to interpret, lower drug concentrations were evaluated for one cell doubling time in the AA8 and irs1SF cells, similar to the conditions that have produced RS with dFdCyd in numerous human tumor cell lines. Under these conditions, irs1SF cells were shown to be more sensitive to dFdCyd than AA8 cells (Fig. 3.3). When cells were incubated for equal periods of time, the AA8 cells showed increased sensitivity over irs1SF cells with both a 10h and 20h exposure (Fig. 3.4). This is likely due to the 2-fold increase in doubling rate of the AA8 cells over irs1SF cells. When cells were incubated with dFdCyd at the corresponding IC_{10} and IC_{50} for 10 or 20h prior to irradiation, no RS was observed in either the AA8 or irs1SF cells (Fig. 3.5).

Effect of dFdCyd on dNTPs in CHO Cells

In human tumor cells, we have demonstrated that depletion of dATP by $\geq$80% for at least 4 hr is necessary for RS with dFdCyd (Shewach 1994, Lawrence 1996). In both AA8 and irs1SF cells, high concentrations of dFdCyd (0.5 or 5 μM) decreased dATP to nearly undetectable levels (>90% in AA8 and >85% in irs1SF cells) for incubation times $\geq$2h (Fig. 3.6). In contrast, long incubations (10h or 20h) with noncytotoxic to moderately cytotoxic concentrations (IC_{10} and IC_{50}) of dFdCyd showed less than 80% decrease in dATP in AA8 cells at the conclusion of the incubation (Fig. 3.7), likely explaining the lack of RS under those conditions in that cell line. However, irs1SF cells showed >80% dATP pool depletion at IC_{10}, and undetectable levels at IC_{50}, yet RS did not occur. Thus, lack of RS in the irs1SF cells cannot be attributed to insufficient dATP depletion.

Examination of dFdCTP accumulation showed similar levels in both AA8 and irs1SF cells, in response to short incubations with high concentrations of dFdCyd (Fig. 3.8). This is consistent with the similarity in sensitivity to dFdCyd in these two cell lines during a 4h incubation (Fig. 3.1), as dFdCTP is thought to be the primary metabolite responsible for cytotoxicity. Taken together, the data support a role for HR in RS with dFdCyd in CHO cells.

Effect of XRCC3 knockdown in AA8 cells

The irs1SF cell line is deficient in XRCC3, a protein required for HR, and exhibits a 25-fold decrease in recombination compared with the HR-proficient AA8 cell line as a consequence (Pierce 1999). These cells were derived through exposure to a mutagen (ICR-191) followed by selection of mutagenized colonies that exhibited increased x-ray sensitivity after exposure to 1Gy (Fuller and Painter 1998). In order to determine whether the lack of RS with dFdCyd observed in irs1SF cells was due to XRCC3-mediated HR deficiency rather than nonspecific effects that might have arisen through mutagenesis, AA8 cells were treated with an shRNA directed against XRCC3 and RS evaluated. AA8 cells transduced with shRNA exhibited 20% reduction in XRCC3 protein levels by western blot analysis (data not shown). Subsequent incubation with 5μM dFdCyd followed by IR resulted in a decrease in RS (RER=1.58), compared to non-transduced (RER=2.26) and non-specific (NS) shRNA-transduced (RER=1.97) AA8 cells (Fig. 3.9). These data further support an important role for HR in RS with dFdCyd in CHO cells.

MCF-7 cells

Cytotoxicity and Radiosensitization with dFdCyd in MCF-7 cells

While the results in the CHO cells strongly implicate HR in RS with dFdCyd, it was important to determine whether a similar role for HR was required for RS in human tumor cells. Indeed, the inability of the HR-proficient AA8 cells to be radiosensitized at non-cytotoxic to moderately

cytotoxic concentrations of dFdCyd during one cell cycle doubling time highlighted important differences between the CHO cells and many human tumor cell lines that readily radiosensitize with such treatment. Transduction of MCF-7 cells with two different XRCC3 shRNA constructs resulted in strong growth inhibition initially, with recovery in cell growth kinetics observed after 1 to 2 weeks. Thus, cells were typically used for experiments between 17 and 21 days following transduction, when their growth rate returned to normal. Cells were collected at the starting and ending points of each experiment and typically showed 40% - 60% knockdown of XRCC3 protein levels by Western blot analysis (Fig. 3.10). No substantial reduction in XRCC3 was observed in cells transduced with the non-specific shRNA.

Assessment of sensitivity to dFdCyd showed equal survival between non-transduced MCF-7, NS shRNA-, and XRCC3 shRNA-transduced cells (Fig. 3.11), demonstrating that inhibition of HR did not alter sensitivity to gemcitabine alone. When these cells were exposed to IC_{10} (10nM) dFdCyd for 24h, RS was observed in the MCF-7 cells (RER=1.67$\pm$0.17) and NS shRNA-transduced cells (RER=1.43$\pm$0.19). However, cells in which XRCC3 was suppressed by treatment with either of two XRCC3 shRNA constructs did not exhibit RS (RER=0.87$\pm$0.22 for XRCC3 #1; RER=1.17$\pm$0.08 for XRCC3#4). Inhibition of dFdCyd-mediated RS by decreasing HR appears to be due to an increased sensitivity of XRCC3 shRNA-transduced cells to IR alone, and a decrease in sensitivity to the combination of IC_{10} dFdCyd and IR (Fig. 3.12).

Effect of dFdCyd on cell cycle and dATP depletion

To determine whether the XRCC3 knockdown may have altered the required metabolic effects that correlate with RS, effects of the shRNA treatment on cell cycle position and dATP depletion were evaluated. Cell cycle analysis showed S-phase accumulation following a 24h incubation with IC_{10} dFdCyd in control and shRNA-treated cells (data not shown). Analysis of dNTPs

showed >85% depletion of dATP in all cells by 8h following addition of IC_{10} dFdCyd, and >95% depletion by 16h (Fig. 3.13). By 24h, dATP pools began to recover, increasing to 75% of untreated control in MCF-7 cells, and to 45%, 31%, and 34% in NS, XRCC3 #1, and XRCC3 #4 shRNA-transduced cells, respectively, as we have observed previously under radiosensitizing conditions in MCF-7 cells (Robinson 2001). Thus, suppression of XRCC3 did not alter the effects of dFdCyd on dATP and cell cycle redistribution as required for RS. Taken together, these results implicate the decrease in XRCC3 as the mechanism responsible for the decrease in RS.

Role of HR in dFdCyd-mediated increase in late DNA damage

Previous studies evaluating DNA damage during the first four hours after dFdCyd and IR have demonstrated no significant difference in DSBs for the combination treatment compared to each modality alone (Lawrence 1997, Rosier 1999). Recently, we observed a late (at 24 and 48h post drug washout/IR) synergistic increase in DSBs, as evidenced by γ-H2AX foci, that corresponded to RS by dFdCyd, which we suggest marks a crucial event in RS. Considering that decreasing HR by knockdown of XRCC3 diminished the degree of RS, we wished to determine whether the pattern of γ-H2AX foci showed a corresponding decrease in DNA damage to identify the point at which HR promotes RS. The effect of XRCC3 knockdown on DNA damage was determined by comparing the number of γ-H2AX foci in shRNA-transduced cells to the number observed in MCF-7 control cells at late time points after dFdCyd $\pm$ IR. Following incubation with IC_{10} dFdCyd alone, a similar level of γ-H2AX foci in NS shRNA-transduced cells was observed compared with non-transduced MCF-7 cells at 24 and 48h after drug washout (Fig. 3.14). In contrast, there was a >20% decrease in γ-H2AX foci in XRCC3 shRNA-transduced cells vs. control MCF-7 cells at 24 and 48h after dFdCyd washout, however, this was

not statistically significant ($p>0.05$). When dFdCyd was combined with IR, no significant difference in γ-H2AX foci was observed in NS shRNA-transduced cells compared to control MCF-7 cells. ($p>0.05$ at 24h and 48h). A much more marked decrease was observed in cells transduced with the XRCC3 shRNAs, in which the XRCC3 #1 construct showed a 43% ($p<0.001$) and 48% ($p<0.01$) decrease in γ-H2AX foci and the XRCC3 #4 construct showed a 34% ($p<0.01$) and 58% ($p<0.01$) decrease, compared to MCF-7 cells at the 24 and 48h time points, respectively. In response to IR alone, γ-H2AX foci had returned to near baseline by 24 and 48h in all cells (data not shown). These results implicate HR in the late increase in DNA damage resulting from exposure to dFdCyd and IR.

Characterization of late DNA damage caused by dFdCyd

To further characterize the damage represented by late γ-H2AX foci, we evaluated phosphorylated ATM (pATM) by western blot as another measure of DSBs following with dFdCyd $\pm$ IR and in the context of XRCC3 knockdown (Fig. 3.15). Untreated cells, regardless of XRCC3 knockdown, showed no detectable pATM, however, exposure to 2Gy IR, IC_{10} dFdCyd, or the combination each resulted in measurable levels of pATM by the 0.5h time point under all treatment conditions (Fig. 3.16). In cells treated with IC_{10} dFdCyd alone, pATM was highest at 0.5h and declined by 4h in all cells. Relative to dFdCyd alone, pATM was elevated 2-fold at 4h with the combination of dFdCyd + IR in non-transduced and NS shRNA-treated cells, and 5- to 12-fold in XRCC3 shRNA treated cells. At the 24h time point, the non-transduced or NS shRNA treated cells treated with dFdCyd + IR continued to show a 3- to 5-fold increase in pATM compared to dFdCyd only. However, in cells transduced with either of the two XRCC3 shRNA constructs, this difference was completely eliminated, with pATM reduced to the low level observed with dFdCyd alone (Fig. 3.17). These results suggest that HR is responsible for

the elevation of pATM at 24h post-drug washout in cells treated with dFdCyd and IR. By 48h, pATM under all conditions showed a pattern of decline to near baseline. Exposure to 2Gy IR alone resulted in increasing pATM through 4 or 24h post-IR, at which times it exceeded the pATM in nearly all other treatment groups. While the reason for the large increase in pATM at 24h with IR alone is not clear, it appears independent of HR as XRCC3 knockdown diminished this effect by only 20.4% to 30.5%. Taken together, these results support a role for HR in producing increased DSBs at 24 hr after dFdCyd and IR exposure, resulting in RS in the MCF-7 cells.

Discussion

Gemcitabine is one of the most potent antimetabolite radiation sensitizers known, with excellent radiosensitization occurring both *in vitro* and *in vivo*. In human tumor cells, RS with dFdCyd has been correlated with dATP depletion, due to dFdCDP-mediated inhibition of ribonucleotide reductase, and accumulation of cells in early S phase, whereas dFdCTP and its incorporation into DNA are associated with cytotoxicity but not RS (Shewach 1994, Lawrence 1996, Latz 1998, Ostruszka 2000, Robinson 2003). The imbalance in dNTPs produced by dFdCyd results in mismatched nucleotides in DNA which, if not repaired prior to irradiation, result in RS (Flanagan 2007). It should be noted that these mismatches do not refer to incorporation of dFdCMP into DNA. Although mismatches in DNA are thought to be necessary for RS, the mechanism by which these lesions enhance cell death with IR and the type of DNA damage they elicit have not been fully elucidated. Recently we described a correlation between a late increase in DSBs and RS with gemcitabine. A prior report implicated HR in radiosensitization with gemcitabine (Wachters 2003), although the mechanism by which HR resulted in RS was not known. Here we have evaluated a role for HR in the late DSBs observed under radiosensitizing

conditions with dFdCyd. The results demonstrated that HR did not play a role in the early damage produced by dFdCyd and IR, but HR was necessary for the late DNA damage to fully develop. Appearance of γ-H2AX foci and phosphorylated ATM at late time points verified that this damage was DSBs. Moreover, decreasing HR via shRNA suppression of XRCC3, a protein required for HR, prevented radiosensitization. Thus, these data suggest that HR is activated in an attempt to repair late DNA damage induced by dFdCyd, and IR cripples the ability of the cell to repair this damage resulting in RS.

A prior study implicated HR in RS with dFdCyd using CHO cell lines either proficient or deficient in HR (Wachters 2003). However, dNTPs were not evaluated in this study, and therefore it is not known whether RS in HR proficient CHO cells correlates with dATP depletion, as has been demonstrated in many human tumor cell lines. The results presented here suggest that dATP depletion is important for RS with dFdCyd in HR proficient AA8 cells, as RS occurred only when dATP was decreased by >80%. However, the lack of RS in HR-deficient irs1SF cells could not be attributed to insufficient dATP depletion or low phosphorylation of dFdCyd. Furthermore, partial knockdown of XRCC3 in the HR-proficient AA8 cells decreased RS by dFdCyd, eliminating the possibility that lack of RS in irs1SF cells was due to a non-specific effect caused by mutagenesis in creating the cell line, and supporting the importance of an intact HR pathway for RS. These studies confirm the prior report that HR is necessary for RS in CHO cells, and justify investigation of HR in radiosensitization by dFdCyd in human tumor cells.

To render human tumor cells defective in HR, we decreased the amount of XRCC3 by using lentivirus-delivered shRNA to suppress expression of XRCC3. Human MCF-7 breast cancer cells were used for these studies because they have been well characterized with respect to

dFdCyd effects on dNTPs, cell cycle progression and DNA damage under radiosensitizing conditions. XRCC3 knockdown was only partial, possibly due to the deleterious effects on cell survival associated with complete lack of HR in cells. Nevertheless, this resulted in excellent inhibition of dFdCyd-mediated RS that could not be explained by altered effects on dNTPs. This partial knockdown increased sensitivity to radiation alone somewhat, though not nearly to the extent observed with irs1SF likely due to the complete deficiency of wild type XRCC3 in the CHO cell line. In addition, XRCC3 knockdown in MCF-7 cells decreased cytotoxicity with the combination of dFdCyd and IR relative to NS shRNA or untreated control cells, similar to the results with the partial XRCC3 knockdown in the AA8 cells. These results demonstrate an important role for HR in RS with dFdCyd in MCF-7 human tumor cells.

In prior studies (chapter 2), late DNA damage was observed with dFdCyd alone and in combination with IR, but not with IR alone, demonstrating that IR is sensitizing MCF-7 cells to the DNA damage produced by dFdCyd. This late damage was decreased with XRCC3 knockdown, as assessed by γ-H2AX foci formation. Furthermore, XRCC3 knockdown decreased pATM at a late time point (24h post drug washout $\pm$ IR) in cells exposed to dFdCyd and IR relative to cells treated with dFdCyd alone, with no clear effect at earlier time points. Taken together, these data demonstrate that HR is important in mediating the DNA damage at late but not early time points after dFdCyd exposure alone or with IR. This is consistent with the cell cycle results, in which cells are in early S phase by the conclusion of dFdCyd exposure and slowly progress through mid and late S after drug washout, where it is known that HR repair of DNA plays a greater role because of the increased availability of sister chromatids. In contrast, the cell depends upon NHEJ primarily in DSB repair immediately after dFdCyd $\pm$ IR when cells are in early S phase.

The results demonstrate that XRCC3 knockdown decreased DNA damage with dFdCyd combined with IR. However, the pATM and the γ-H2AX foci results do not clearly demonstrate an effect of XRCC3 knockdown on DNA damage with dFdCyd alone. Thus, it is not clear whether IR is simply augmenting the ability of HR to produce damage with dFdCyd alone, or whether the addition of IR allows for a new, HR-mediated mechanism of producing DNA damage. Further studies, including evaluation of pATM and γ-H2AX foci simultaneously in fixed cells, may allow greater understanding of the mechanism of DNA damage with dFdCyd alone. In either case, the synergistic increase in DNA damage with the combination of dFdCyd and IR at late time points well explains the excellent RS.

It is not clear why the pATM results show increasing DSBs with IR alone through 24h post irradiation, whereas γ-H2AX foci demonstrated decreasing DNA damage after 30 – 60 min post-irradiation. While pATM has been observed 24h after irradiation in other cell types, typically it is highest within the first hour after irradiation. Nevertheless, a strong role for HR in repair of IR-induced damage was not demonstrated with the partial XRCC3 knockdown, consistent with the idea that NHEJ is primarily responsible for repair of IR-induced DSBs in an asynchronous population of cells.

Thus, these data suggest that HR is activated in an attempt to repair damage induced by dFdCyd as replication is restarted, and IR cripples the ability of the cell to repair this damage resulting in RS. Mechanistically, we propose that this occurs as a result of dFdCDP-mediated dATP depletion, producing mismatched nucleotides in DNA and slowing of progression through S-phase. Cells can usually repair many mismatches and recover from slowed progression. However, the added damage due to IR slows progression to a greater degree, resulting in a greater need for HR as cells attempt to restart replication in the first S-phase. As cells attempt to

utilize HR, mismatches are encountered which are known to produce a barrier to successful completion of HR (Elliot 2001), resulting in excess cell death and thus RS.

We have demonstrated previously that mismatches in DNA, which are the necessary lesions for RS with dFdCyd, also play an important role in RS with other antimetabolites that decrease dNTPs, such as FdUrd (Flanagan 2008). In view of the results presented here, it is intriguing to speculate that these antimetabolites may also produce late DNA damage via HR that will be augmented by IR and contribute to radiosensitization. Further studies are warranted.

The results presented here suggest that the combination of dFdCyd and IR would be best suited for use in cancers that are HR-proficient. It would be of interest to determine whether the level of HR influences radiosensitization by dFdCyd, since many types of cancer exhibit an increase in this repair activity. For instance, the overexpression of Rad51 in non-small cell lung cancer was shown to correlate with significantly reduced survival (Qiao 2005), and a screen of several breast cancer cell lines revealed a significant elevation of HR efficiency in cancer cells vs. normal breast cells (Mao 2009). If the level of HR does determine the degree of radiosensitization by dFdCyd, this could be a promising therapeutic approach for patients with tumors exhibiting overactive HR.

List of Abbreviations

dFdCyd, 2',2'-difluoro-2'-deoxycytidine, gemcitabine; dFdCDP, gemcitabine diphosphate; dFdCTP, gemcitabine triphosphate; dCyd, deoxycytidine; dATP, deoxyadenosine triphosphate; dNTP, deoxynucleotide triphosphate; IR, ionizing radiation; RS, radiosensitization; HR, homologous recombination; CHO, Chinese hamster ovary; shRNA, short hairpin RNA; BrdUrd, bromodeoxyuridine; IdUrd, iododeoxyuridine; FdUrd, fluorodeoxyuridine; DSB, double strand break; NHEJ, nonhomologous end joining; AUC, area under the curve; RER, radiation enhancement ratio; HPLC, high-performance liquid chromatography; NS, non-specific; ATM, Ataxia Telangiectasia Mutated; pATM, phospho-S1981 ATM.

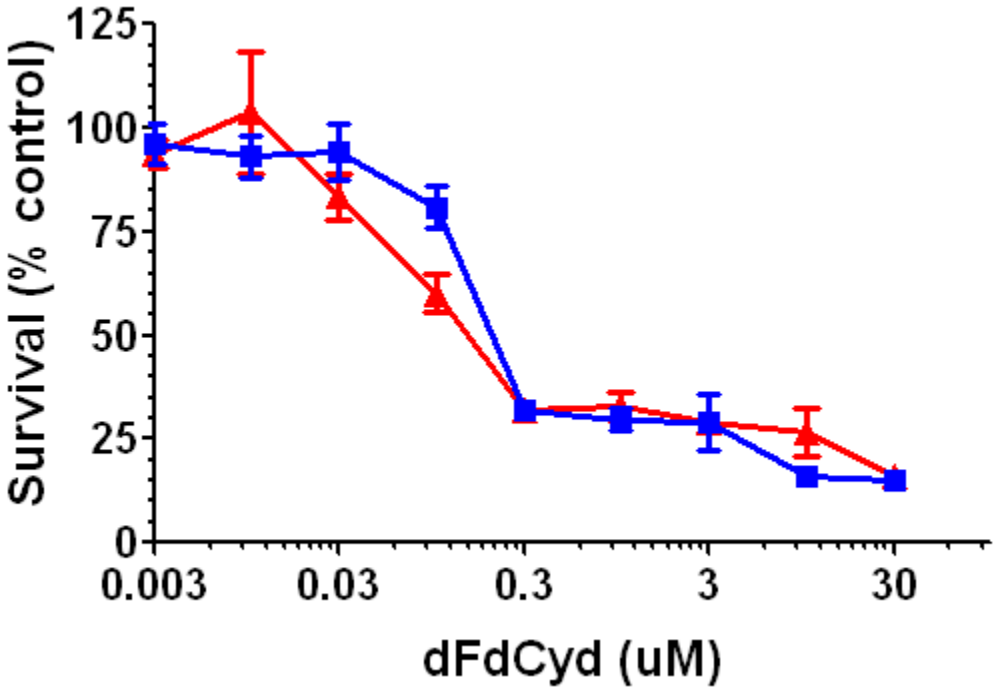
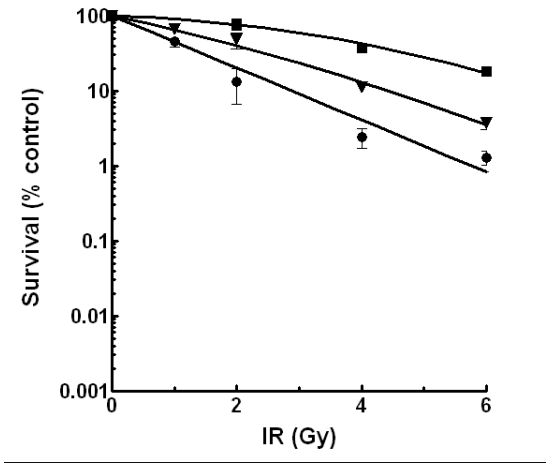


Fig. 3.1. Sensitivity of AA8 and irs1SF CHO cells to 4h dFdCyd. Cytotoxicity was determined by clonogenic survival in AA8 (■) and irs1SF (▲) exposed to the indicated concentrations of dFdCyd for 4h.

A.



B.

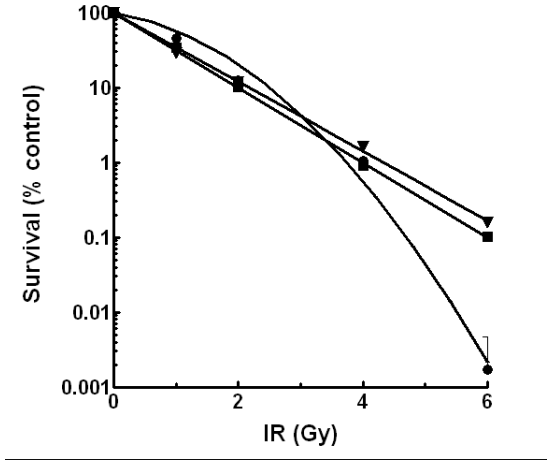


Fig. 3.2. Radiosensitization by dFdCyd in CHO cells. AA8 (A) and irs1SF (B) cells incubated with 5uM (●), 0.5uM (▼), or left untreated (■) were exposed to the indicated amounts of IR. Radiosensitization was determined as described in "Materials and Methods".

Cell Line	Time (h)	[dFdCyd] (μM)	RER
AA8	**2**	**0.5**	**1.5±0.1**
		5	**2.1±0.2**
AA8	**4**	**0.5**	**1.6±0.3**
		5	**2.4±0.5**
irs1SF	**2**	**0.5**	**1.0+0.1**
		5	**0.7+0.1**
irs1SF	**4**	**0.5**	**0.9±0.1**
		5	**0.8±0.1**

Table 3.1. Comparison of radiation enhancement ratios (RER) in CHO cells. RERs were determined in AA8 cells and irs1SF cells were incubated with either 0.5uM or 5uM dFdCyd for 2 or 4h.

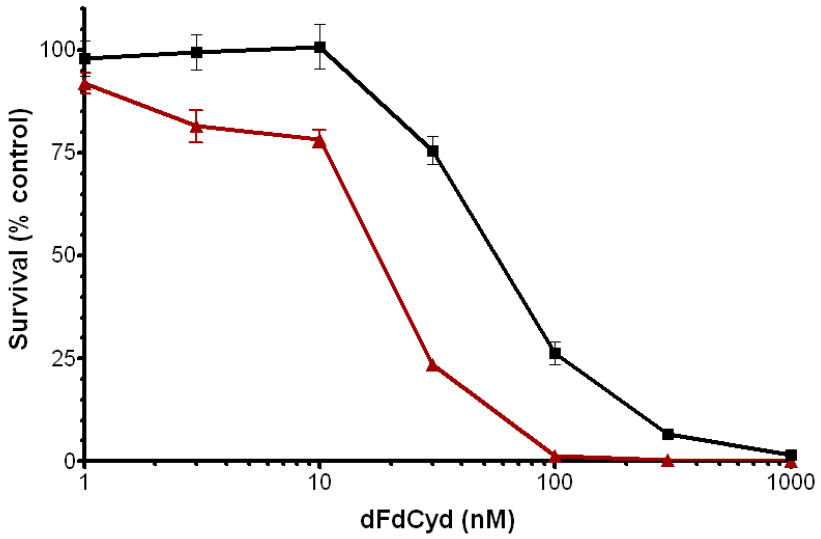
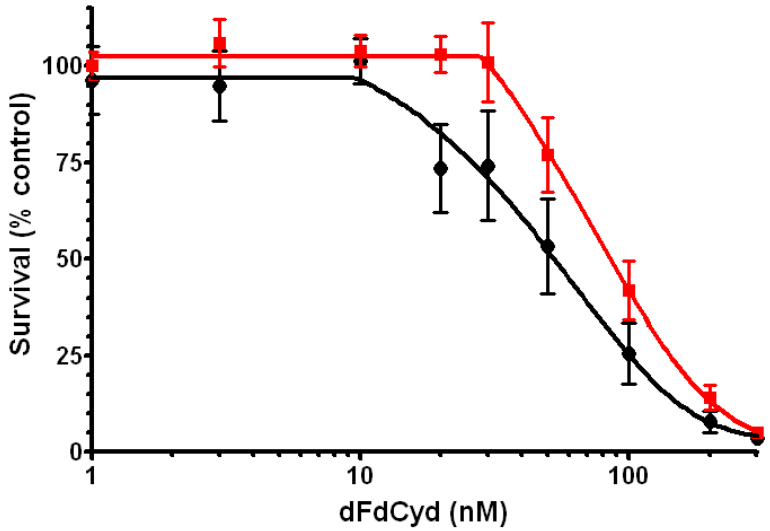


Fig 3.3. Sensitivity to dFdCyd in CHO cells based on doubling time. Cytotoxicity was compared between AA8 (■) and irs1SF (▲) cells exposed to the indicated concentrations of dFdCyd for 10h and 20h, respectively, exposures that take into account doubling time variation.

A.



B.

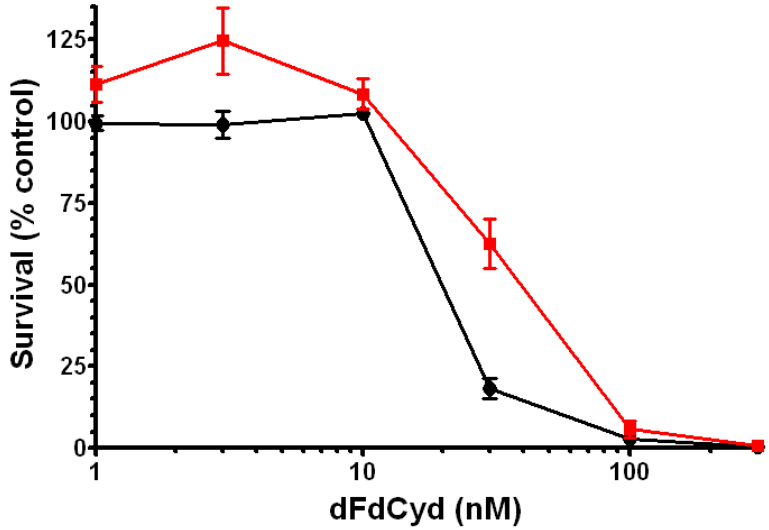
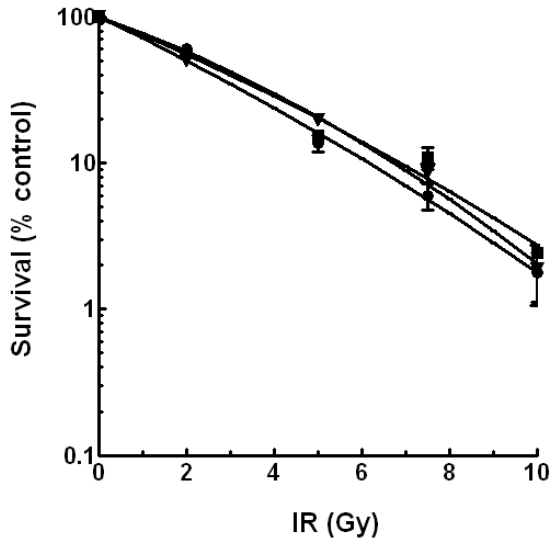


Fig. 3.4 Effect of equal dFdCyd exposure on CHO cell survival. Sensitivity to dFdCyd was determined in AA8 (●) and irs1SF (■)CHO cells exposed to the indicated concentrations of dFdCyd for equal durations of 10h (A), or 20h (B).

A.



B.

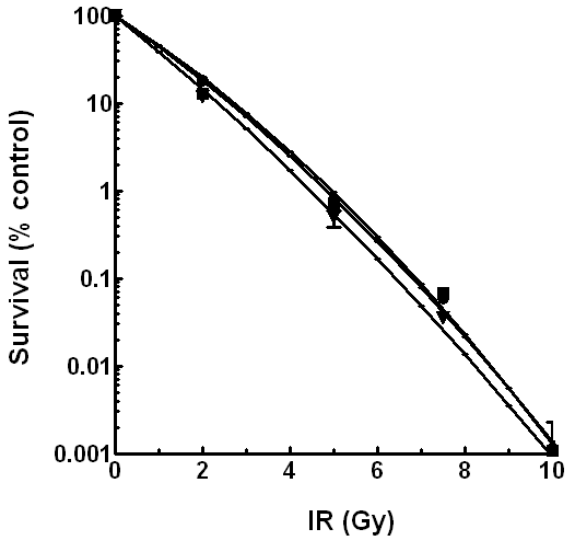
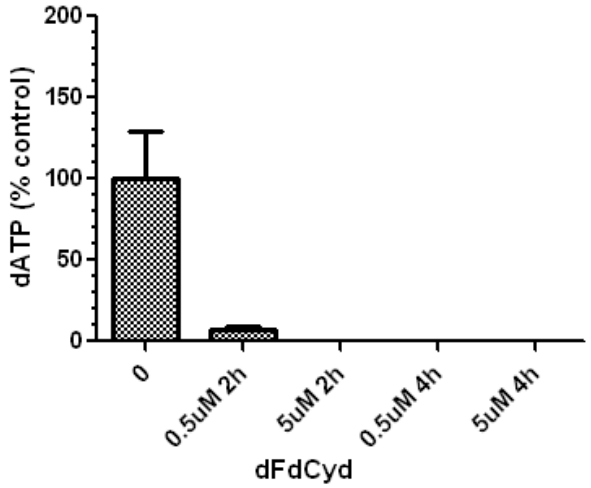


Fig. 3.5 Determination of radiosensitization in response to low and moderately toxic concentrations of dFdCyd. AA8 (A) and irs1SF (B) cells, either untreated (■) or exposed to IC_{10} (▼) or IC_{50} (●) dFdCyd, were examined for radiosensitivity.

A.



B.

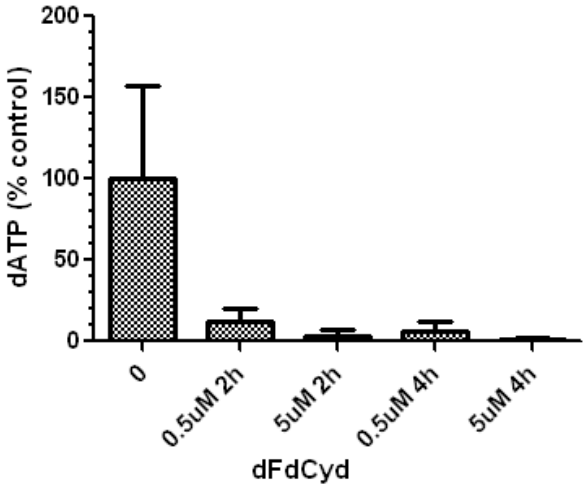
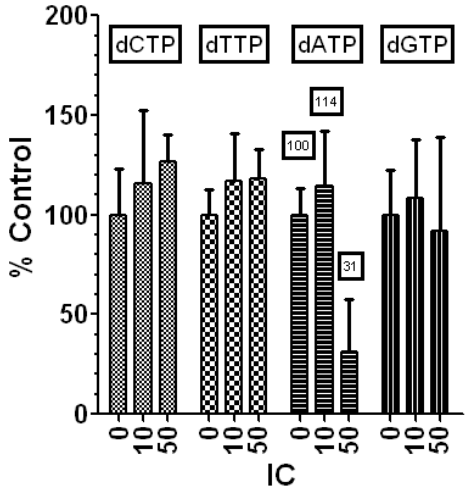


Fig. 3.6. Effect of high concentration dFdCyd on depletion of dATP in CHO cells. AA8 (A) and irs1SF (B) cells were exposed to 0.5 or 5μM dFdCyd for 2 or 4h.

A.



B.

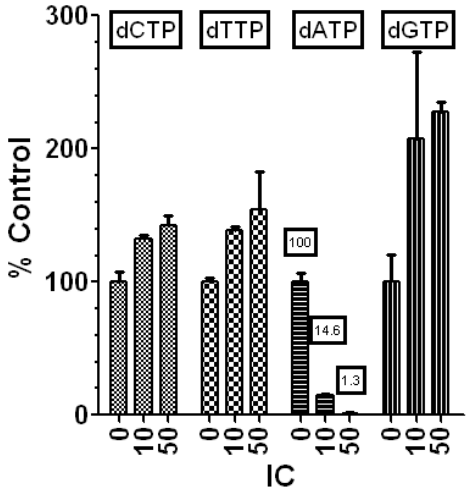


Fig. 3.7. Effect of low concentration dFdCyd, based on doubling time, in depletion of dATP in CHO cells. AA8 (A) and irs1SF (B) cells were untreated or exposed to IC_{10} or IC_{50} dFdCyd. IC values were calculated with respect to doubling times of 20h and 10h for irs1SF and AA8 cells, respectively, with drug incubation times matching doubling times. Indicated are the levels of dATP in response to dFdCyd, with respect to untreated control cells.

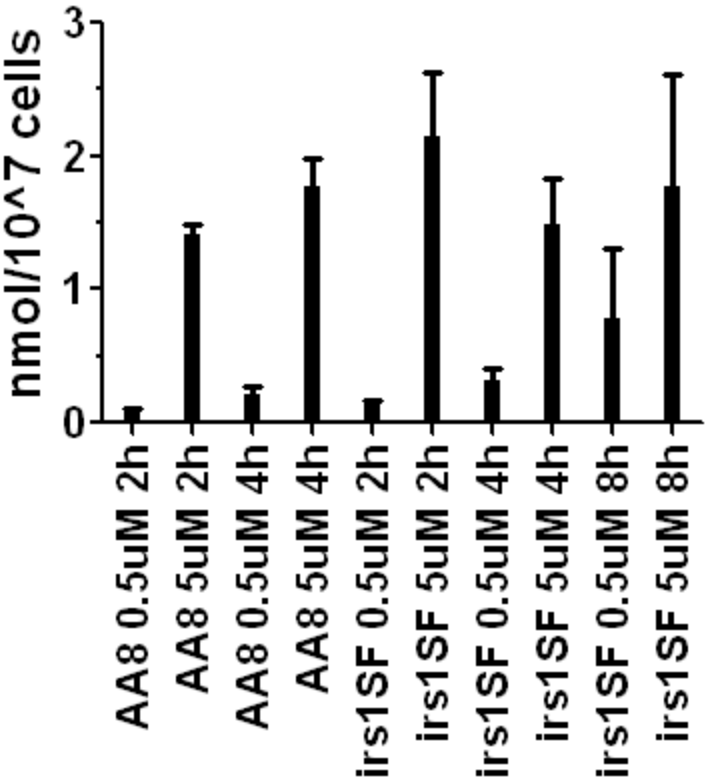


Fig. 3.8. Effect of high concentration dFdCyd on dFdCTP accumulation in CHO cells. dFdCTP levels were analyzed by HPLC in AA8 and irs1SF cells exposed to 0.5 and 5μM dFdCyd for 2 or 4h. The 8h incubations with dFdCyd in irs1SF cells were included to account for doubling time differences.

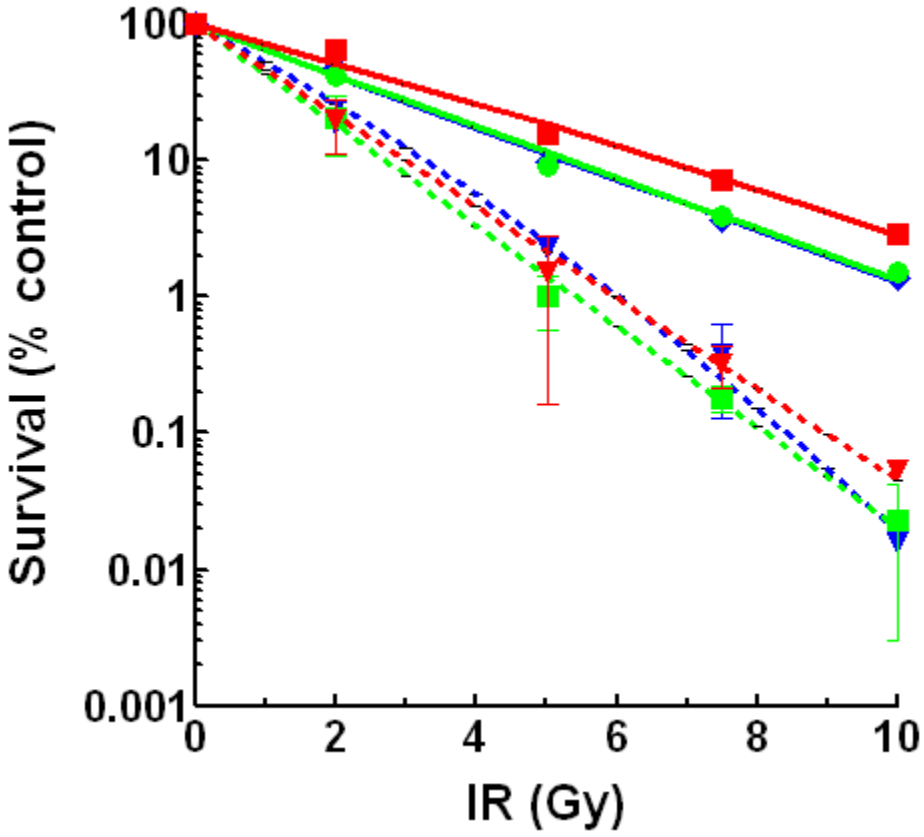


Fig. 3.9. Effect of XRCC3 knockdown on radiosensitization by dFdCyd in AA8 cells. An shRNA against XRCC3 was used to transduce AA8 cells. Radiosensitization in response to 5uM dFdCyd was determined as described in "Materials and Methods."

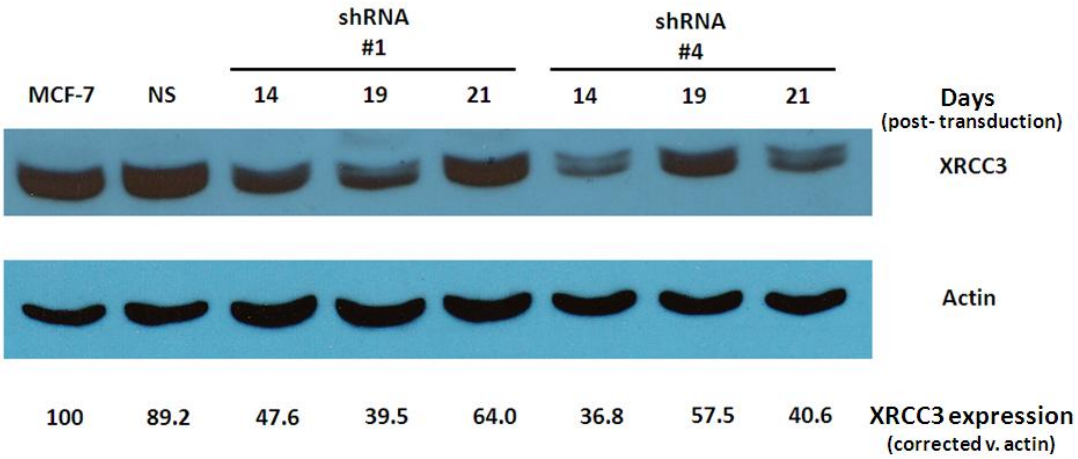


Fig. 3.10. Western blot analysis of XRCC3 knockdown in MCF-7 cells. The levels of XRCC3 were determined in cells transduced with each of two XRCC3 shRNAs at the indicated days post-transduction. Non-transduced MCF-7 cells, and cells transduced with NS shRNA were included as controls. The XRCC3 levels indicated are % MCF-7 control (untreated) cells, corrected for loading with actin.

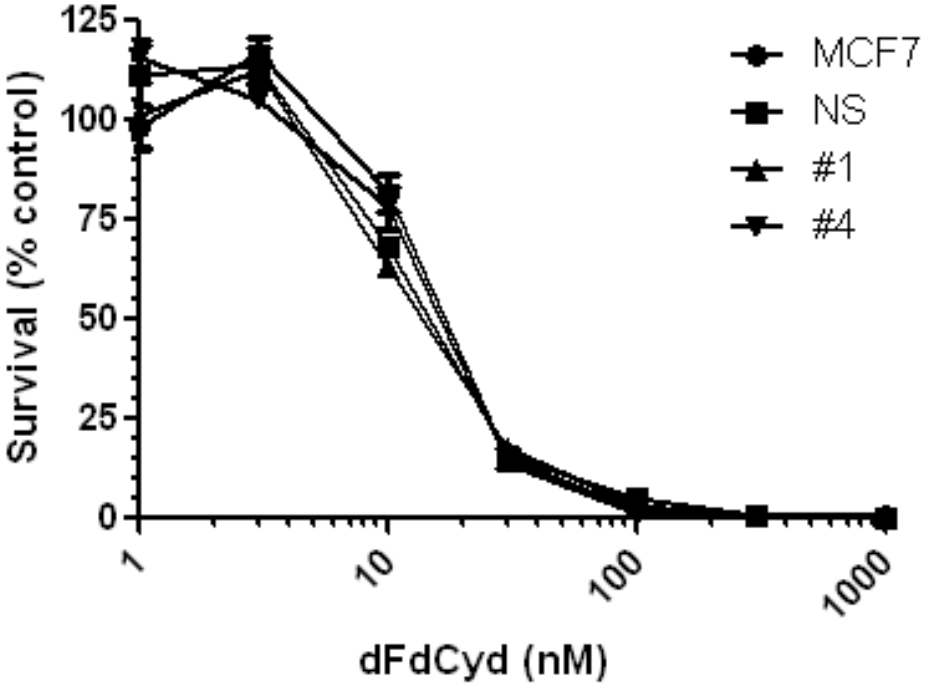


Fig. 3.11. Sensitivity of shRNA-transduced MCF-7 cells to dFdCyd. Cells transduced with NS shRNA, or each of two XRCC3 shRNAs, as well as non-transduced MCF-7 cells were exposed to the indicated concentrations of dFdCyd for 24h, and cytotoxicity was determined by clonogenic survival.

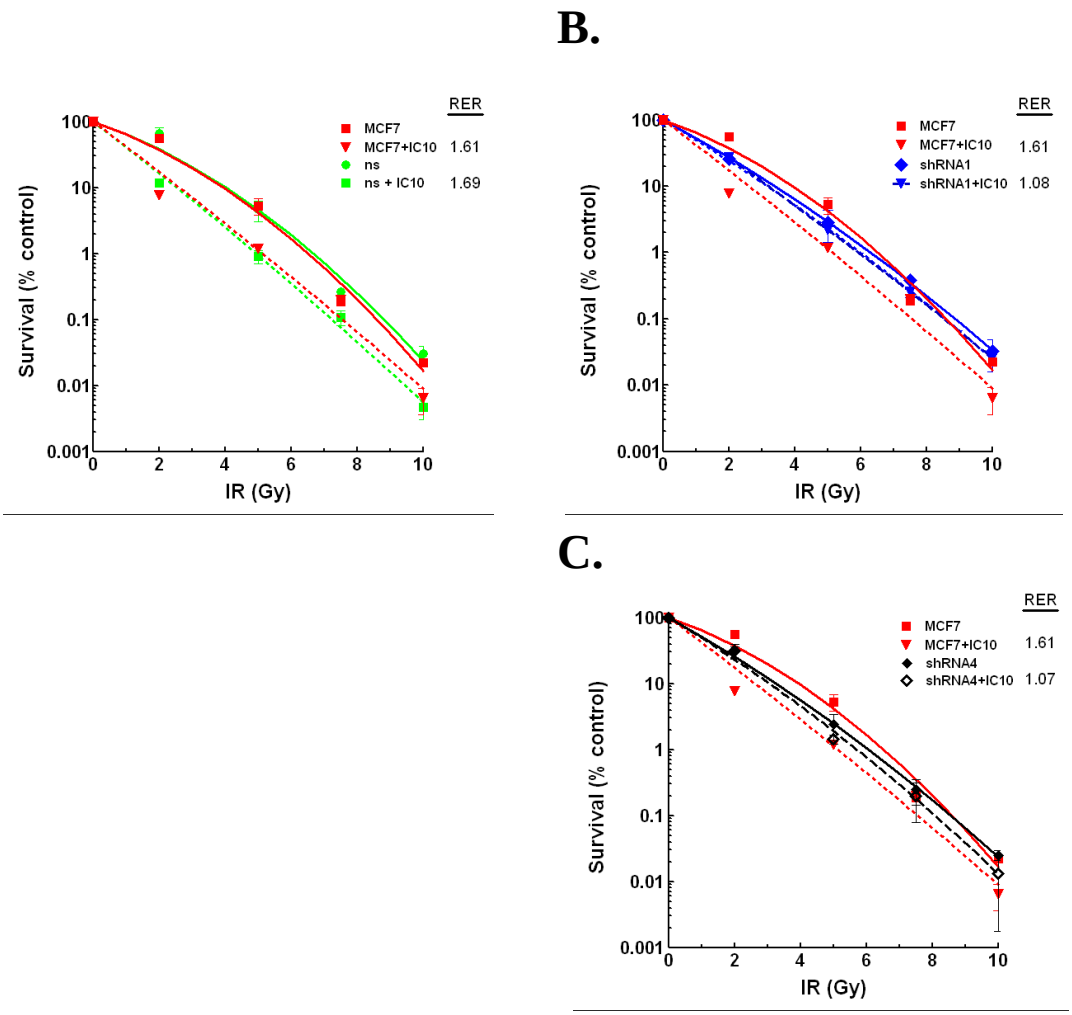


Fig. 3.12. Effect of XRCC3 knockdown on radiosensitization by dFdCyd. The ability of IC_{10} dFdCyd to radiosensitize MCF-7 cells was compared to NS (A), XRCC3#1 (B), and XRCC3#4 (C) shRNA-transduced cells. RERs for each comparison are included. This is a representative experiment of studies performed 2 (shRNA1) and 4 (shRNA4) times.

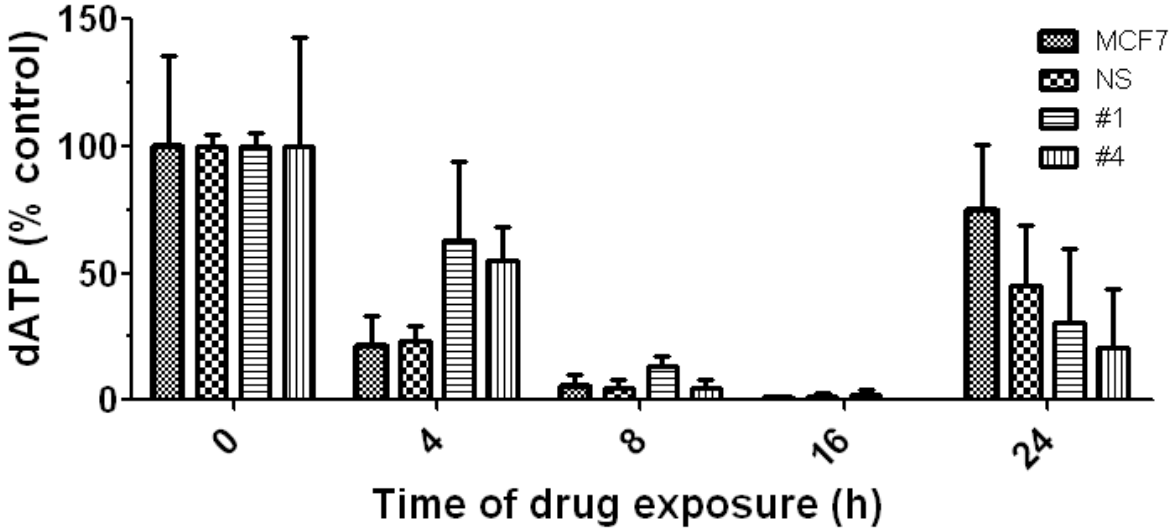
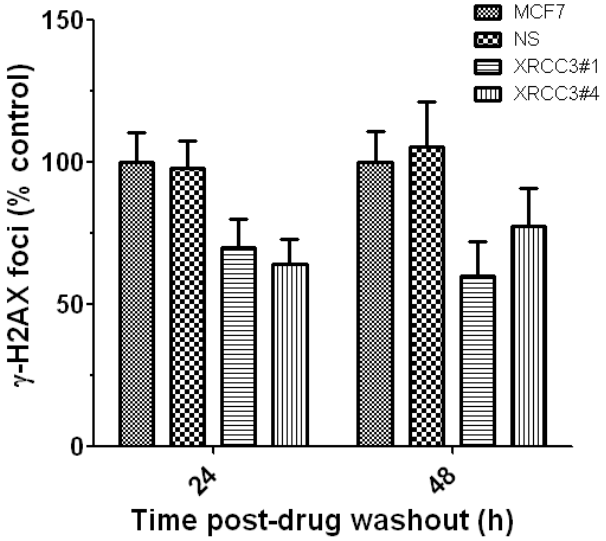


Fig. 3.13. Kinetics of dATP pool depletion in non-transduced and shRNA-transduced MCF-7 cells. The depletion of dATP was monitored over a 24h incubation with IC_{10} dFdCyd in MCF-7 (small squares), NS (large squares), XRCC3#1 (horizontal stripes), and XRCC3#4 (vertical stripe) shRNA-transduced cells.

A.



B.

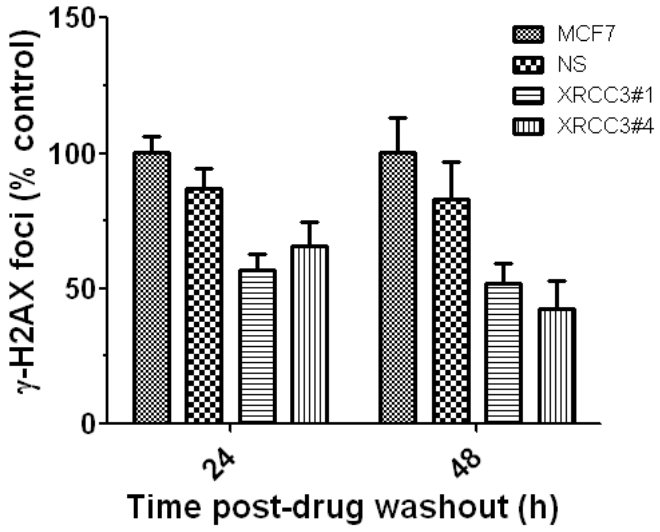


Fig. 3.14. Reduced γ-H2AX foci formation with XRCC3 knockdown.
γ-H2AX foci were quantified from confocal images, and the levels in shRNA-transduced cells were compared to non-transduced MCF-7 cells at 24h and 48h, in response to IC_{10} dFdCyd alone (A) and IC_{10} dFdCyd + 2Gy (B).

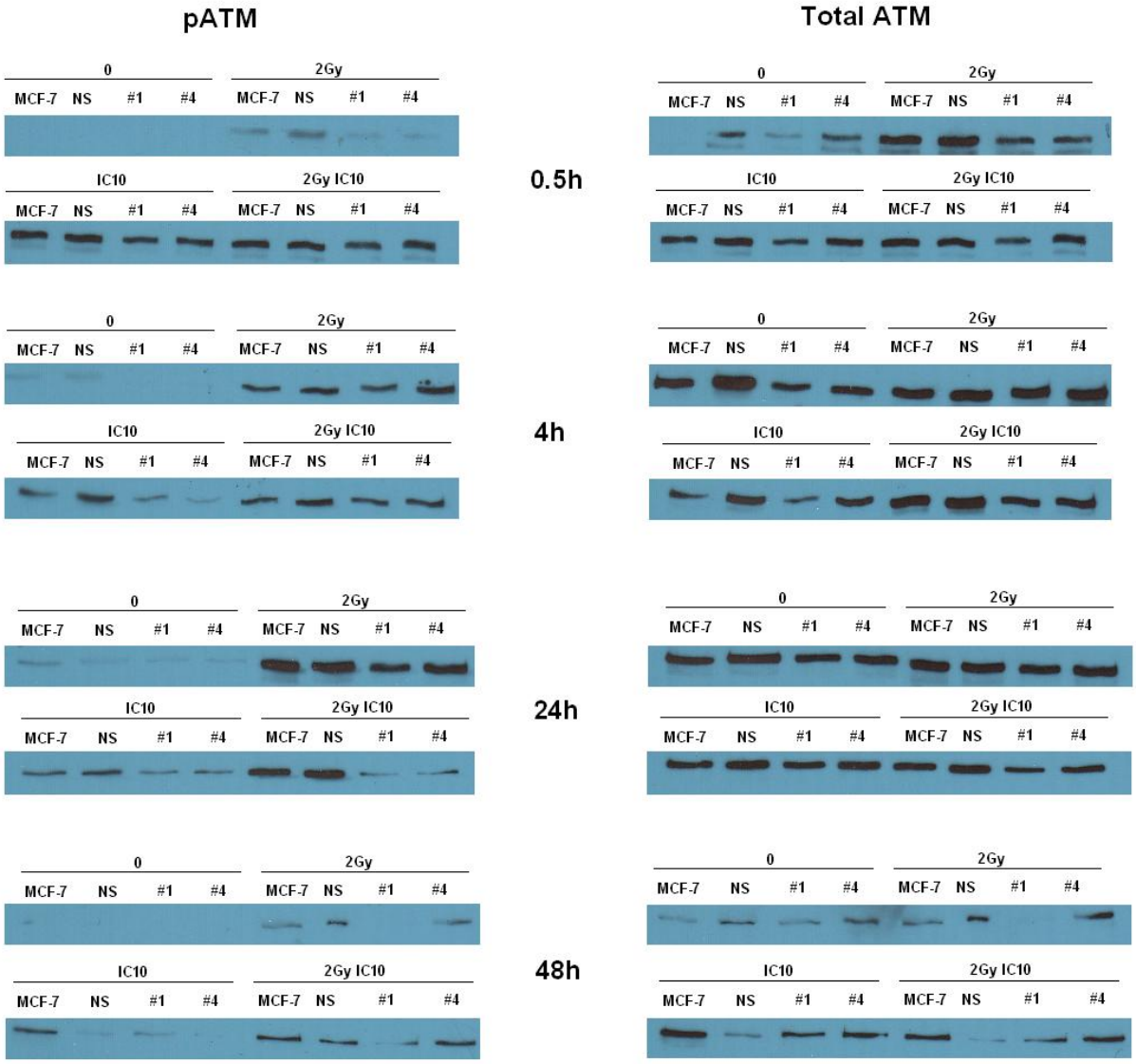


Fig. 3.15. Western blot analysis of pATM in response to IR alone, dFdCyd alone, or the combination. Non-transduced MCF-7 cells and those transduced with either NS, XRCC3#1, or XRCC3#4 shRNAs were exposed to 2Gy, IC_{10} dFdCyd, or the combination, and levels of pATM were measured for up to 48h. Total ATM was used as a loading control.

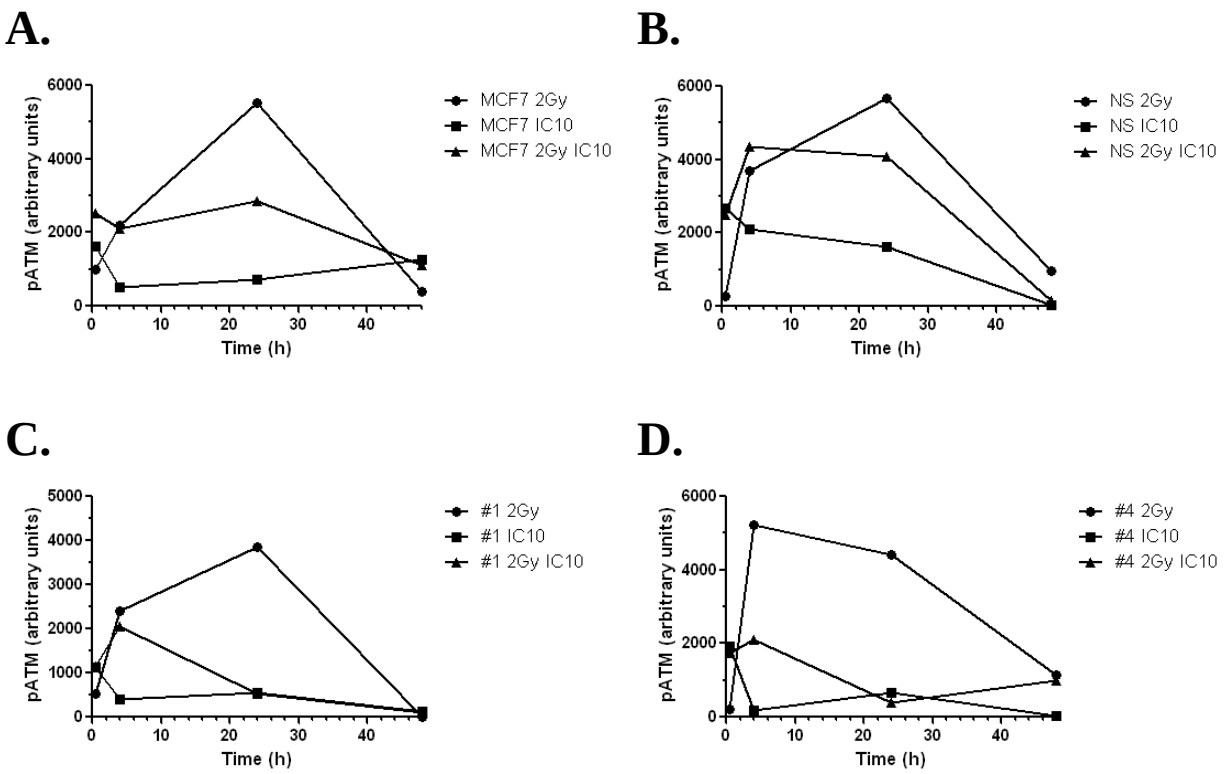


Fig. 3.16. Pattern of ATM phosphorylation in response to IR alone, dFdCyd alone, or the combination. Levels of pATM in control (A) MCF-7 cells, or (B) NS, (C) XRCC3#1, and (D) XRCC3#4 shRNA-transduced cells were determined by western blot and quantified using Image-J software. Time 0h represents the time of drug washout/IR.

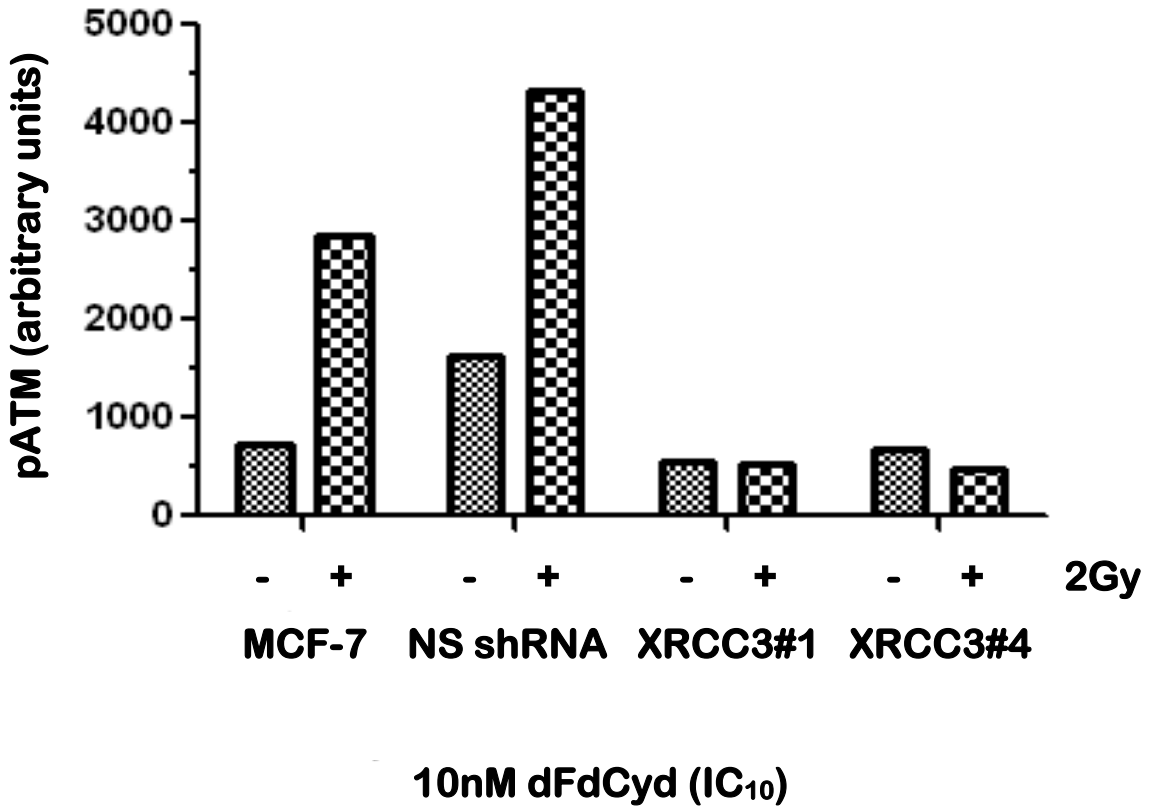


Fig. 3.17. Pattern of ATM phosphorylation at 24h. Levels of pATM were determined by western blot and quantitated using Image-J software for comparison of cells exposed to 24h IC_{10} dFdCyd, with (+) or without (-) 2Gy IR.

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Chapter IV

Conclusions

Gemcitabine is a nucleoside analog that produces a synergistic enhancement in cell killing when combined with ionizing radiation. This can be achieved using non-toxic concentrations of dFdCyd allowing for an improved therapeutic index. Radiosensitization by dFdCyd has been demonstrated in a wide variety of human solid tumor cell lines and in patients (Shewach 2007, Blackstock 1999, Eisbruch 2001), however, despite being one of the most potent radiosensitizers available, the exact mechanism of RS by dFdCyd has yet to be elucidated. Understanding this mechanism could improve future treatments, because optimizing synergy could allow for increased efficacy in tumor response while minimizing toxicity, further improving the therapeutic index. This dissertation explores this mechanism and identifies late DNA damage as a causal event in RS by dFdCyd, and these studies further identify HR in development of these late DNA double strand breaks (DSBs).

Following transport into the cell, activation of dFdCyd occurs through the rate-limiting phosphorylation of the prodrug by dCK. Subsequent phosphorylation steps yield dFdCDP and dFdCTP. The triphosphate form accumulates to the highest levels in the cell, with respect to the dFdCyd metabolites (Heinemann 1988), and its incorporation into DNA is correlated with cytotoxicity (Huang 1991, Ewald 2008), but not radiosensitization (Shewach 1994, Lawrence,

1996, Flanagan 2007, Robinson 2003). On the other hand, dFdCDP, an irreversible mechanism-based inhibitor of RR (Silva 1998, van der Donk 1998), causes dNTP pool imbalances, and this action is correlated with RS. In fact, radiosensitization by dFdCyd is dependent on ≥80% depletion of dATP (Shewach 1994, Lawrence 1996, Robinson 2001) and the highest synergy is observed in cells that accumulate into S-phase of the cell cycle (Shewach 1994, Robinson 2001, Latz 1998, Ostruszka 2000). Studies have demonstrated that the decrease in dATP in cells undergoing DNA replication causes mismatches in DNA which, if not corrected prior to irradiation, results in RS. Furthermore, a causal relationship between mismatches in DNA and RS by dFdCyd was established, identifying mismatched nucleotides in DNA as necessary lesions for RS with dFdCyd (Flanagan 2007).

Exposure to ionizing radiation (IR) results in the formation of several types of damage to the DNA, and of this damage, double strand breaks (DSBs) are considered to be the most cytotoxic. The mechanism whereby other radiosensitizing nucleoside analogs elicit synergy, such as BrdUrd, IdUrd, and FdUrd, involves an increase in the formation, or a decrease in the rate or extent of repair of these DSBs within the first few hours following IR (Bruso 1990, Heimburger 1991, Lawrence 1990, Ling 1990). However, radiosensitization by dFdCyd does not fit this model, as determined by pulsed-field gel electrophoresis (Lawrence 1997, Rosier 2003), and as shown in Chapter 2, with analysis of γ-H2AX foci formation. While nonhomologous end-joining (NHEJ) was shown to be dispensable for RS by dFdCyd (van Putten 2001), a study by Wachters *et al*. (2003) demonstrated a requirement for homologous recombination (HR) in RS by dFdCyd using isogenically-matched CHO cells differing in proficiency of XRCC3, a required HR protein. This was perplexing, as dFdCyd causes accumulation of cells into early S-phase, a

stage expected to primarily utilize nonhomologous end-joining (NHEJ) for repair of DSBs. Furthermore, studies from different laboratories demonstrated that RS with dFdCyd did not involve increased DNA damage nor delayed repair in the first few hours after IR (Lawrence 1997, Rosier 2003) thus the mechanism by which HR might influence RS was not clear.

Because RS by dFdCyd did not correspond with an increase in DNA damage or a decrease in repair within the first 4h following IR, we hypothesized that an increase in DNA damage, which could be attributed to RS by dFdCyd, must be occurring at a later stage. Studies in Chapter 2 focusing on early as well as late time points revealed a second increase in γ-H2AX foci in response to dFdCyd alone and dFdCyd combined with IR, but not with IR alone, which was hypothesized to mark a crucial event in radiosensitization. This increase was synergistic in cells treated with dFdCyd and IR compared to individual treatments at corresponding time points, and it greatly exceeded the level of foci formation immediately following IR, thus indicating greater DNA damage. This correlation between DNA damage formation at late time points with radiosensitization, with greater damage formation observed with greater radiosensitization, suggested that the synergistic increase in γ-H2AX foci marked a causative event for RS by dFdCyd. Furthermore, the late increase coincided with times where HR activity would be expected to increase, due to progression of cells through S-phase and hence restarting of replication, resulting in greater availability of a sister chromatid for HR repair. These findings, together with the dependence for active HR in radiosensitization by dFdCyd in CHO cells (Wachters 2003), suggested a possible involvement of HR in the late damage observed with RS conditions.

The role of HR in radiosensitization by dFdCyd was evaluated in CHO cells prior to investigation in human tumor cells in order to address several issues. First, the link between functional HR and RS by dFdCyd was demonstrated in a single report which required confirmation. Second, the concentrations of dFdCyd used were moderately to highly cytotoxic. Finally, the mechanism of RS by dFdCyd was not evaluated with respect to dNTP levels. With this in mind, I hypothesized that the lack of radiosensitization by dFdCyd in HR-deficient cells was due to insufficient depletion of dATP, potentially due to metabolic differences caused by the exposure to mutagens to obtain a cell line deficient in XRCC3. Experiments presented in Chapter 3 in CHO cells were performed which confirmed the requirement for HR in radiosensitization by dFdCyd, and provided additional support for dATP depletion in RS. However, whereas human cells show better radiosensitization following exposure to a noncytoxic concentration of dFdCyd for one doubling time than to short exposure at high concentrations (Shewach 1994), the AA8 CHO cell line was only radiosensitized by the latter condition, suggesting the existence of significant differences between CHO and human cells in their response to dFdCyd. The inability of AA8 cells to radiosensitize in response to the non-cytotoxic concentration was attributable to insufficient depletion of dATP. The reason for this inadequate depletion is unknown, however, this might be accounted for by the finding that dFdCTP, which accumulates to the highest levels with respect to the gemcitabine metabolites, was undetectable following exposure to low concentration dFdCyd for one doubling time (data not shown). With undetectable dFdCTP, dFdCDP, which is the metabolite responsible for RR inhibition, would be approximately 10 times lower (Heinemann 1988). The low amount of dFdCTP could be due to increased deamination of dFdCyd in CHO cells, inefficient activation at low concentrations, or a shorter half life for dFdCTP at low concentrations as this parameter has

been shown to be dependent on the concentration of dFdCTP (Heinemann 1992, Shewach 1994). This would explain the lack of dATP depletion as due to lesser inhibition of RR.

Analysis of sensitivity to dFdCyd in AA8 cells revealed increasing cytotoxicity at concentrations up to 0.2µM, and little to no increase in sensitivity at concentrations above 0.2µM. As a result, the cytotoxicity observed at 0.5µM was similar to that observed at 5µM dFdCyd. In CCRF-CEM cells, accumulation of dFdCTP and its incorporation into DNA showed a concentration-dependent relationship, however, even when dFdCTP accumulation reached a plateau with higher concentrations of dFdCyd, incorporation, which was directly correlated with cytotoxicity, continued to increase (Huang 1991). This is in contrast to CHO cells, which showed continued accumulation of dFdCTP at concentrations that did not produce an increase in cytotoxicity. Despite this difference, assuming the same relationship between incorporation of the drug into DNA and cytotoxicity exists in CHO cells, the absence of increased sensitivity suggests that incorporation is saturated at concentrations above 0.2µM. However, despite equal cytotoxicity at 0.5µM and 5µM dFdCyd, there was a trend towards increasing RER at the higher concentration, suggesting that RS by dFdCyd is independent of incorporation.

This would correspond with RS in human cells, which has been shown to be independent of dFdCTP accumulation or incorporation into DNA (Lawrence 1996, Robinson 2003). Future work measuring incorporation of dFdCTP into DNA will be necessary to determine the exact relationship between this parameter and incorporation into DNA, and the subsequent relationship to cytotoxicity.

In human tumor cells, RS by dFdCyd depends on $\geq$ 80% dATP depletion for at least 4h (Shewach 1994, Lawrence 1996, Robinson 2001). This situation was different in AA8 cells, which were able to be radiosensitized by exposure to high concentration dFdCyd for 2h. This might be explained by doubling time differences that revealed an AA8 doubling time half that of irs1SF cells and many human tumor cells, thus allowing for a shorter dATP depletion period in RS. These differences could not account for the lack of RS in irs1SF cells, consistent with a role for dATP depletion and functional HR in radiosensitization by dFdCyd, however further analysis of cell cycle is needed to determine whether the lack of RS in irs1SF cells is due to insufficient S-phase accumulation. Species specific differences in the role that each repair pathway plays in coping with the damage caused by dFdCyd and IR could also be an issue. For instance, the relationship between the extent of dATP depletion and production of mismatches in DNA in CHO cells may differ compared to that in human tumor cells. Similarly, while HR has been shown to play a role in RS with dFdCyd in CHO cells, it is not guaranteed that this repair pathway is equally important in human cells.

Although rodent systems are frequently used to model human pathways, the metabolic differences demonstrated in this dissertation bring into question whether the mechanism of RS in CHO cells is applicable to human cells. A focus of Chapter 3 was to determine whether the necessity for HR in radiosensitization by dFdCyd could be translated to human cells. Indeed, shRNA knockdown of XRCC3 in MCF-7 breast cancer cells inhibited RS by dFdCyd, which could not be attributed to inadequate depletion of dATP, corresponding to the findings in CHO cells and highlighting the importance of HR in radiosensitization by dFdCyd in human cells.

I then hypothesized that mechanism by which HR facilitates RS with dFdCyd was through the production of late DNA damage. Evaluation of γ-H2AX foci in the context of XRCC3 knockdown revealed an HR-dependence in the appearance of late occurring damage. In this study, XRCC3 knockdown resulted in decreased γ-H2AX foci, compared to non-transduced and cells transduced with a nonspecific shRNA, at corresponding time points, suggesting that HR is required for the synergistic increase in late foci. Additionally, this requirement was corroborated using a second XRCC3 shRNA, reducing the possibility that these findings were due to non-specific effects. Nevertheless, it would be helpful to further identify a role for HR in RS with dFdCyd through investigation of other HR proteins. As such, the characterization of Rad51, which is required for strand invasion during HR, in RS and in the late formation of DNA damage with dFdCyd would be an important focus for future work.

The mechanism of action for some nucleoside analog radiosensitizers (BrdUrd, IdUrd, and FdUrd) has been attributed primarily to an increase in the formation, or a decrease in the repair of DSBs (Bruso 1990, Heimburger 1991, Lawrence 1990, Ling 1990), suggesting that, in these cases, drug is sensitizing cells to the effects of IR. However, the observation that late DNA damage occurred with dFdCyd alone and in combination with IR, but not with IR alone, suggests the opposite, that IR sensitizes MCF-7 cells to the DNA damage produced by dFdCyd, thus distinguishing dFdCyd from other nucleoside radiosensitizers. Considering that the other nucleoside radiosensitizers also decrease dNTPs, it would be important to determine whether the late DNA damage observed with dFdCyd occurs with BrdUrd, IdUrd and FdUrd, and whether this effect contributes in part to radiosensitization.

While the low cytotoxic concentration of dFdCyd (IC_{10}) increased γ-H2AX foci at late time points, the response to dFdCyd alone does not represent damage that is cytotoxic because 90% of cells survive when exposed to this concentration. However, the combination of dFdCyd and IR results in a synergistic increase in damage at late time points, and this additional damage leads to an inability to complete HR, subsequently resulting in RS. These events correspond to the time that cells resume replication and progress through S-phase, when HR activity increases with increased availability of a sister chromatid. Additional analysis of other markers of DSBS could clarify whether this is the case.

The studies here demonstrated that exposure to dFdCyd alone, or with IR, activates homologous recombination 24 – 48h after drug washout, because suppression of the HR required protein, XRCC3, decreases the activation of HR and radiosensitization by dFdCyd. Additionally, knockdown of XRCC3 corresponds with a decrease in the formation of late γ-H2AX foci in response to dFdCyd alone or dFdCyd combined with IR, but not with IR alone. Taken together, these data suggest that with dFdCyd alone, as dNTP pools are restored and as replication is restarted, HR is activated in an attempt to repair damage induced by dFdCyd. Addition of IR overwhelms the ability of the cell to repair this damage, as demonstrated by the persistence of mismatches in DNA only under RS conditions (Flanagan 2007), where peak mutation frequency was observed beyond 24h following drug washout/IR and increased to approximately 5-fold above untreated control levels (0.09%), as determined by a shuttle vector assay containing an 85 base pair reporter sequence. Extrapolation of this data suggests that one nucleotide is misincorporated into DNA for approximately every 19,000 correctly incorporated nucleotides (0.45% mutation frequency within 85 base pairs). Assuming that these mutations are randomly

distributed throughout the genome, this density of mismatches would not be expected to inhibit HR. However, the stalling of replication forks in early-S might result in an increased density of mismatched nucleotides in DNA that are clustered within close proximity of the stalled forks, increasing the chance that these mismatches would prevent repair by HR due to rejection of heterologous sequences. This would result in prolonged stalling of replication forks, and collapse of these forks would create DSBs, leading to increased cell death and RS.

Proposed mechanism of radiosensitization by dFdCyd

S-phase is regarded as the most radioresistant phase of the cell cycle, due to the presence of sister chromatids which allow HR to accurately repair DSBs (Chapman 2012). However, the situation might be different when dNTP pools are imbalanced, and when cells are aggregated in early S-phase, where NHEJ is thought to be the predominant pathway for repair because DNA replication has just begun, and availability of a sister chromatid is scarce. Radiosensitization with dFdCyd begins with depletion of dATP, which results in mismatches in DNA, and slowing of progression through S-phase. While dFdCyd can increase mismatches in DNA (Flanagan 2007), cells are capable of repairing most of these mismatches and recovering from the slowed progression when exposed to low concentrations of dFdCyd alone. The circumstances change with the additional damage due to IR, which slows progression further as cells negotiate the formation of potentially lethal DSBs. I hypothesize that there is a greater need for HR in order to restart replication when dFdCyd and IR are combined. However, when mismatches in DNA are encountered, which have been shown to persist for up to 96h in response to radiosensitization by dFdCyd (Flanagan 2007), HR is unable to complete repair, due to the inhibition of recombination

caused by the areas of increased sequence divergence (Elliot 2001). I further hypothesize that this causes failed repair resulting in unrepaired DSBs, leading to cell death, as demonstrated by the persistence of γ-H2AX foci from 24-48h, and a decrease in cell number at 72h post drug washout/IR (Fig. 4.1). In HR-deficient cells, repair is managed by the inaccurate NHEJ pathway, resulting in increased sensitivity to IR alone, but a lack of synergy when combined with dFdCyd because a futile attempted repair cycle is not entered.

Clinical Implications

The results of this dissertation suggest that patients with HR-proficient cancers will benefit most from the combined treatment of dFdCyd and IR. Furthermore, the requirement for HR for RS with dFdCyd suggests that cancers with high HR activity might be better radiosensitized by dFdCyd than those with low activity. Further elucidation of this possibility could provide a strategy for identifying patients with tumors exhibiting high HR activity who would benefit most from the combination of dFdCyd and IR. Relevant to this point, a recent screen identified an increase in HR activity in several breast cancer cell lines compared to normal breast cells (Mao 2009). Additionally, overexpression of Rad51 has been identified in non-small cell lung cancer and is associated with significantly reduced survival time (Qiao 2005), thus, a treatment that is known to exploit HR could be extremely beneficial.

Analysis of MCF-7 breast and SKOV-3 ovarian cancer cell lines resistant to cis-diamminedichloroplatinum (II) (CDDP), a DNA cross-linking agent, showed overexpression of Brca1, an HR protein, compared to wild-type cells, which was directly attributable to resistance

(Husain 1998). If increasing HR activity improves radiosensitization by dFdCyd, then these CDDP-resistant cells might be more sensitive to RS with dFdCyd. Likewise, RS by dFdCyd could be beneficial for the treatment of any cancer that has become refractory to initial treatment due to increased HR activity.

Interest in poly ADP ribose polymerase (PARP) inhibitors for use in cancer has increased, however, these inhibitors are most effective in HR-deficient cells due to an inability to repair PARP inhibitor induced collapsed replication forks (Bryant 2005, Farmer 2005). Those patients who are resistant to PARP inhibitors likely have cancers that are proficient for HR, thus a treatment that exploits HR, such as dFdCyd + IR, may be useful in patients resistant to PARP inhibitor treatment.

Future Directions

Radiosensitization by dFdCyd is dependent on active homologous recombination, therefore identification of HR specific biomarkers, such as Rad51 and Brca1, would aid in identifying cancers where treatment with dFdCyd + IR would be effective. Additionally, if cancers with higher HR activity are better radiosensitized by dFdCyd, determining the levels of these proteins would identify those HR-proficient cancers that are most susceptible to RS by dFdCyd. This could be especially relevant for cancers that exhibit overexpression of HR proteins, such as those mentioned in the previous section.

HR inhibition was achieved through knockdown of XRCC3 using two different shRNA constructs, decreasing the likelihood that the reduced DNA damage and inhibition of RS caused

by knockdown were due to non-specific effects, however, to rule out cell line specific effects, knockdown of XRCC3 in additional cell lines could be performed. The XRCC3 protein is relatively uncharacterized, and although it has been shown to be essential for HR, its exact role has not been definitively elucidated. Therefore, it is possible that the effects on radiosensitization and DNA double strand break formation are due to a role for XRCC3 outside of HR. Future work focusing on other crucial HR components, such as those mentioned above, would address this issue, as would the use of HR-deficient cancer cells, such as CAPAN-1 cells (Brca2 mutant).

Gemcitabine combined with IR resulted in a synergistic increase in DNA double strand breaks at late time points which correlated with RS. It would also be interesting to determine whether other radiosensitizers that are known to inhibit ribonucleotide reductase or produce imbalances in dNTP pools, which result in persistent mismatches in DNA leading to radiosensitization, also cause a synergistic increase in damage at late time points as a mechanism of radiosensitization. If so, it would suggest that use of these radiosensitizers, like dFdCyd, would be most effective against HR-proficient cancers.

RS by dFdCyd has been shown to be dependent on factors such as schedule dependence, depletion of dATP, S-phase accumulation, and persistence of mismatches in DNA, however, the mechanism whereby these requirement come together to cause synergy has remained elusive. This dissertation demonstrates an essential role for HR in RS by dFdCyd, and for the first time, shows a synergistic increase in DSBs at late time points which correlates with RS. Knowledge of this mechanism may lead to improved therapy with dFdCyd and radiation by utilizing it only

in patients with tumors that are HR-proficient, while avoiding toxicity without efficacy in patients with HR-deficient tumors.

List of Abbreviations

dFdCyd, 2',2'-difluoro-2'-deoxycytidine, gemcitabine; dFdCDP, gemcitabine diphosphate; dFdCTP, gemcitabine triphosphate; dCyd, deoxycytidine; dCK, deoxycytidine kinase; dATP, deoxyadenosine triphosphate; dNTP, deoxynucleotide triphosphate; IR, ionizing radiation; RS, radiosensitization; HR, homologous recombination; CHO, Chinese hamster ovary; shRNA, short hairpin RNA; BrdUrd, bromodeoxyuridine; IdUrd, iododeoxyuridine; FdUrd, fluorodeoxyuridine; DSB, double strand break; NHEJ, nonhomologous end joining; RER, radiation enhancement ratio; ATM, Ataxia Telangiectasia Mutated; pATM, phospho-S1981 ATM.

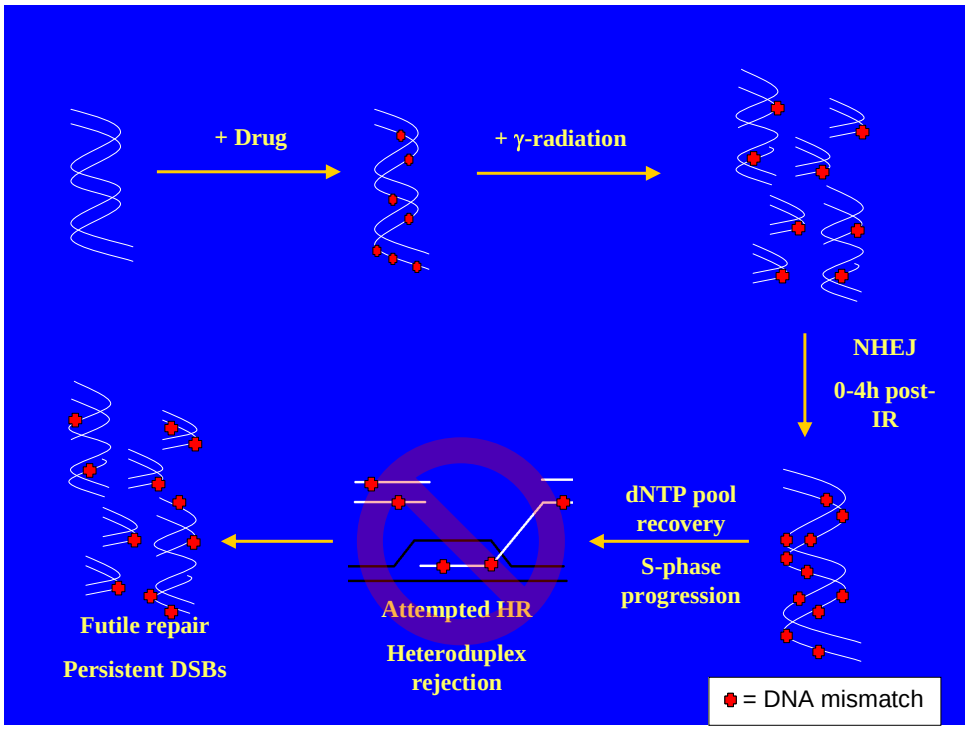

Figure 4.1. Proposed mechanism of radiosensitization by gemcitabine. Incubation of cells with dFdCyd results in misincorporated nucleotides. Exposure to IR causes DNA double strand breaks which are repaired, but results in persistent mismatches in DNA. Following dNTP pool recovery, which coincides with reduction of cellular dFdCTP, cells begin to progress through S-phase, which requires homologous recombination to restart stalled replication forks. However, due to the persistence in mismatches creating heterologous sequences, HR is unable to complete, resulting in failed repair, persistent double strand breaks, and radiosensitization.

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