THE EFFECTS OF p53 AND p53R2 EXPRESSION ON GEMCITABINE-MEDIATED CYTOTOXICITY AND RADIOSENSITIZATION

by

Joe Barber

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Pharmacology)
in The University of Michigan
2009

Doctoral Committee:

Professor Donna S. Shewach, Chair
Professor Thomas E. Carey
Professor Mats E. D. Ljungman
Assistant Professor Christine E. Canman

Acknowledgements

To my parents and my family, none of this would have been possible without your love and support, each one of you shares in this achievement.

I would also like to acknowledge Donna Shewach and former and current members of the Shewach laboratory (Brian, Leo, Blaine, Paul, Jessica, Mike, Sheryl, Maryanne) for all of their help during my dissertation studies, as well as making the lab an interesting and entertaining place to be.

To the Association of Multicultural Scientists, Namandje', L'Aurelle, Tigwa, Anwar, Ezra, Jane, Janice (the nice twin), and so many others, words can not describe how much all of you helped me on this journey.

To my thesis committee (Donna, Mats, Tom, Chris), thank you for all of your suggestions, critiques, comments, and even criticisms, I have grown by leaps and bounds as a scientist thanks to all of you.

I would like to sincerely thank Mary Davis and Susan in Radiation Oncology for all their help in irradiating samples.

I would also like to thank the Biomedical Research Flow Core for all of its help with my flow cytometry work.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

In the late 1980's, Eli Lilly and Company published a report detailing the efforts to synthesize new fluorinated nucleoside analogs to discover agents with unique biological activity [1]. Several fluorinated compounds were generated using 2-deoxy-2,2-difluoro-D-ribose as a backbone. By replacing a hydrogen atom with a fluorine atom in a metabolite, a compound with new biological activity and with similar substrate activity may be created since the van der Waals radius of fluorine (1.35 Å) is similar to that of hydrogen (1.20 Å) [2], while the electronegativity of fluorine (χ = 4.1) is much greater than that of hydrogen (χ = 2.2) [3]. Nucleoside analogs with substitutions of fluorine atoms at the C2' position have been demonstrated to increase the stability of the glycosidic bond and prevent its enzymatic cleavage [4,5]. 2-deoxy-2,2,-difluoro-D-ribose was used as the main building block for a series of nucleoside analogs in a program focused on identifying new antitumor agents. Several compounds were created during this study, with the most promising being 2',2'-difluoro-2'-deoxycytidine (gemcitabine, dFdCyd[1], Gemzar®), which will be the subject of this dissertation (Fig. 1.1)   After synthesis, dFdCyd was shown to have better activity than cytosine arabinoside (1- -D-arabinofuranosylcytosine, AraC), an anticancer agent, in a number of leukemia cell lines as well as solid tumors in mice [1,6].
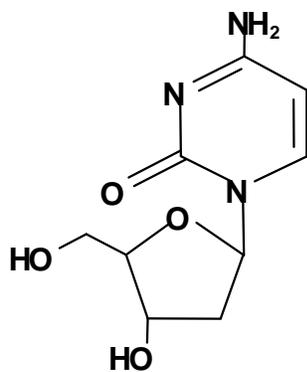
2'-deoxycytidine
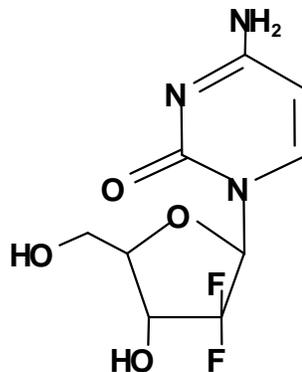
**2', 2' -difluoro -2'-deoxycytidine
(Gemcitabine)**

Figure 1.1. Structures of deoxycytidine and gemcitabine.

dFdCyd has been examined in numerous cell lines and cancer models by a number of research groups since its synthesis in 1988. It was approved by the Food and Drug Administration in 1996 for the treatment of advanced pancreatic cancer, unique in that it was based mostly on quality of life improvement [7], although dFdCyd did provide a small but significant increase in survival compared to the standard treatment with 5-fluorouracil [8]. In addition, dFdCyd is also approved for treating locally advanced or metastatic non-small cell lung cancer, breast cancer, and ovarian cancer [9].

In addition to its use in a chemotherapeutic regimen, dFdCyd can also enhance cell death induced by ionizing radiation, a process known as radiosensitization [10,11]. Radiosensitization is defined as synergistic cell killing achieved when a chemotherapeutic and ionizing radiation combine to produce a greater than additive cytotoxic effect. This could be a powerful treatment modality, since low, non-cytotoxic concentrations of dFdCyd can produce excellent radiosensitization. Clinical trials with dFdCyd and concurrent radiation have produced promising results. In a University of Michigan study, weekly dFdCyd at $\leq$ one-fourth of a standard chemotherapeutic dose produced excellent enhancement of standard radiotherapy-induced cell killing, with nearly 80 % of patients achieving a complete pathologic regression of their tumor compared to the expected 30-40 % with radiotherapy alone [12]. However, issues did arise with toxicity to normal tissue. The combination of dFdCyd and radiotherapy has also been active in patients with pancreatic cancer given either at low dose [13,14] or full dose with titration of radiotherapy with acceptable toxicity [15].

To maximize the antitumor activity of dFdCyd and radiation while controlling normal tissue toxicity, a greater understanding of the factors that contribute to radiosensitization is necessary. This dissertation will evaluate the roles of p53, a cell cycle protein induced by DNA damaging agents such as dFdCyd and radiation; ribonucleotide reductase, an important cellular target of dFdCyd; and p53R2, a novel ribonucleotide reductase subunit, in the radiosensitizing mechanism of dFdCyd. The introductory chapter of this dissertation examines dFdCyd in great detail by describing its metabolism, cytotoxicity, and radiosensitization. The introduction will also describe ribonucleotide reductase and p53R2. Later chapters examine the roles of p53 and p53R2 expression on the cytotoxic and radiosensitizing properties of dFdCyd.

Mechanism of Action

To exert its biologic effects, dFdCyd must be transported into the cell, which can be accomplished through either equilibrative (human Equilibrative Nucleoside Transporters, hENT, sodium independent) or concentrative (human Concentrative Nucleoside Transporters, hCNT, sodium dependent) nucleoside transporters: hENT1 (equilibrative sensitive), hENT2 (equilibrative insensitive), hCNT1 (concentrative insensitive). Nucleoside transporters are classified as equilibrative or concentrative sensitive based on the effectiveness of nitrobenzylthioinosine (NMBPR) inhibition [16]. Nucleoside transporters vary greatly in their expression levels, as well as in their ability and capacity to transport dFdCyd. The hENT1 and hCNT1 transporters have been shown to be the most efficient in dFdCyd transport [17].
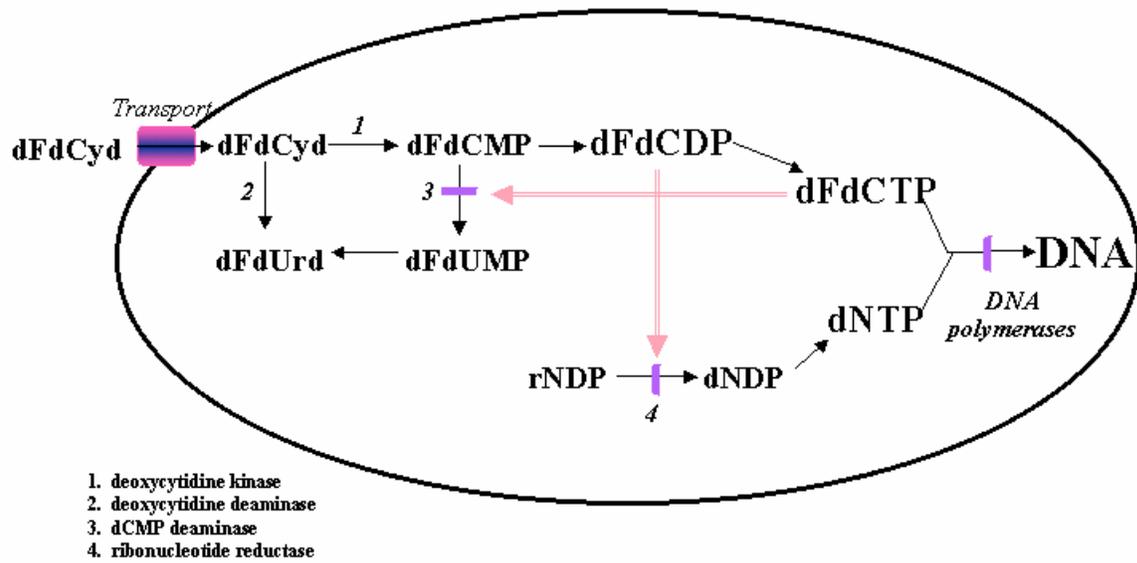
Figure 1.2. Metabolism of dFdCyd.

Another study by Mackey et al demonstrated that the hCNT1 transporter had a much higher affinity for dFdCyd, with an apparent K_m of 24 M, compared to hENT1 (K_m = 160 M) and hENT2 (K_m = 740 M) [18]. However, the role of hENT1 in dFdCyd transport was solidified by research employing the thymidylate synthase inhibitors 5-fluorouracil and raltitrexed [19]. Exposure to either of these agents increased the expression of hENT1 in pancreatic cancer cell lines and a resulting increase in dFdCyd cytotoxicity, representing a potential therapeutic benefit.

After transport into the cell, dFdCyd must be activated via phosphorylation (Fig 1.2). dFdCyd is initially phosphorylated by dCK (deoxycytidine kinase) to dFdCMP, which is the rate-limiting step of dFdCyd metabolism. The affinity of dCK for dFdCyd (K_m = 1.4 M) is similar to that of the endogenous substrate dCyd (K_m = 3.6 M). Further sequential phosphorylation by dCMP kinase and nucleoside diphosphokinase results in the production of the active metabolites, 2', 2'-difluorodeoxycytidine diphosphate (dFdCDP) and 2', 2'-difluorodeoxycitydine triphosphates (dFdCTP). dFdCyd is inactivated via deamination in either its unphosphorylated or monophosphorylated forms by deoxycytidine deaminase and dCMP deaminase, respectively, resulting in the production of dFdUrd and dFdUMP [20,21].

dFdCDP is a potent and irreversible inhibitor of ribonucleotide reductase (RR) [22,23], the enzyme responsible for the conversion of ribonucleoside diphosphates to their corresponding deoxyribonucleoside diphosphates, which, after further phosphorylation to the corresponding triphosphates, are essential precursors for

DNA synthesis. Inhibition of RR by dFdCDP is considered most important for dFdCyd-mediated radiosensitization [24]. dFdCDP was first discovered to inhibit ribonucleotide reductase activity in leukemia cells [23]. Further research uncovered that dFdCDP was a potent mechanism-based inactivator of ribonucleotide reductase with a stoichiometric relationship of 1:1 [22,25]. This inhibition resulted in depletion of deoxynucleotide triphosphate (dNTP) pools and stalled DNA synthesis [23,26]. However, the dNTP most depleted as a result of dFdCyd exposure differs in leukemia cells and solid tumor cells. In leukemic cells, dCTP is the most depleted dNTP, whereas dATP exhibits the largest decrease in solid tumor cells incubated with dFdCyd [11,23].

dFdCTP can inhibit DNA synthesis either through competition with dCTP for DNA polymerases or via enhancing the incorporation of dFdCMP into the elongating DNA strand [26,27]. The apparent K_i values of dFdCTP for DNA polymerases alpha and epsilon were 11.2 M and 14.4 M, respectively [26]. The ratios of the apparent K_m values for incorporation of dFdCTP and dCTP into DNA were 21.8 and 22.9 for DNA polymerases alpha and epsilon, respectively [26]. dFdCTP is the metabolite responsible for cytotoxicity, and a study by Huang *et al* confirmed that incorporation of dFdCMP into DNA was the lethal event [26]. It has been suggested that DNA chain termination contributes to dFdCyd-induced cell killing [28]. Although dFdCyd has been reported to be a masked chain terminator in *in vitro* DNA primer extension assays in which a single nucleotide is added after dFdCMP [26], this does not appear to be true chain termination, as [^{3}H]-dFdCyd has been detected in full length DNA in intact cells [29].

7

dFdCyd and Radiation

Nucleoside analogs have long been considered attractive agents to combine with ionizing radiation for a number of reasons. As DNA synthesis inhibitors, nucleoside analogs potentially could inhibit the repair of DNA damage induced by ionizing radiation or potentiate radiation-induced damage such as DNA double strand breaks (DSBs). Since nucleoside analogs are preferentially cytotoxic to proliferative cells, they could decrease the number of tumor cells and slow tumor repopulation during fractionated radiotherapy. Tumor shrinkage in turn could increase tumor oxygenation and deter the deleterious effects of hypoxia on radiation response. However, unlike other antimetabolite radiosensitizers such as 5-fluoro-2'-deoxyuridine (FdUrd) and 5-bromo-2'-deoxyuridine (BrdUrd), dFdCyd does not either potentiate radiation-induced DSBs or inhibit their repair [30,31].

Hydroxyurea is a chemotherapeutic agent that has been shown to produce radiosensitization as a result of inhibition of ribonucleotide reductase and subsequent dNTP pool depletion [32]. As dFdCyd was shown to deplete dATP pools in cancer cell lines via ribonucleotide reductase inhibition, it was also proposed to be a radiosensitizer [10,11]. After initial studies demonstrating potent radiosensitization by dFdCyd in HT-29 colon carcinoma cells [11,33], dFdCyd was subsequently determined to be a radiosensitizer in head and neck, pancreatic, and breast cancer cell lines [10,31,34,35].

Radiosensitization can be calculated in a number of ways both experimentally and clinically, one of which being the radiation enhancement ratio, which is

calculated by dividing the area under the curve for control cells by that of drug-treated cells [36]. Since these values are normalized for untreated survival, overlapping curves would result in an enhancement ratio of 1.0, which represents additivity. Enhancement ratios > 1.0 represent synergy or radiosensitization, whereas values less than 1.0 represent antagonism or radioprotection. While enhancement ratios > 1.0 represent radiosensitization, only values of 1.4 or greater are considered clinically significant. Radiosensitization with dFdCyd varies with cell line, drug concentration, and schedule of administration, yet radiation enhancement ratios (RER) as high as 3.0 have been reported [37]. A major advantage of dFdCyd as a radiosensitizer compared to agents such as hydroxyurea is that dFdCyd can induce radiosensitization at concentrations that are 1000 fold lower than typical plasma concentrations achieved clinically [38]. Conversely, hydroxyurea requires concentrations of nearly its maximal tolerated dose to produce radiosensitization.

Radiosensitization of solid tumor cells has been demonstrated to occur optimally when dFdCyd exposure precedes radiation. Excellent radiosensitization has been observed when cells are exposed to dFdCyd for 24 hr, and immediately irradiated [11]. Other studies have analyzed radiosensitization when irradiation does not immediately follow drug exposure, and reported lowered radiosensitization [39]. Studies have demonstrated that dFdCTP has a long half-life and as a result has extended biological activity [11,30]. Depending on the concentration of dFdCyd during incubation, radiosensitization can occur up to 2 days after drug washout in HT-29 cells [30].

Despite much intense effort, the exact mechanism of dFdCyd-mediated radiosensitization has yet to be elucidated. However, two correlative factors have been identified for radiosensitization in solid tumors, dATP depletion and S-phase accumulation prior to irradiation. Studies have demonstrated that depletion of dATP by at least 80% of control values for at least 4 hr is required for radiosensitization [11,24,34,35]. Accumulation of cells in S-phase during dFdCyd exposure appears to be required for radiosensitization, as Latz et al demonstrated that this is the cell cycle phase most sensitive to radiosensitization by dFdCyd [39]. Radiosensitization was reduced in synchronized cells incubated with dFdCyd during either G1 or G2/M. A correlation between S-phase accumulation and radiosensitization by dFdCyd has been noted in several studies [24,35,40].

dFdCyd Resistance

The inherent or induced resistance of tumors to dFdCyd is a major clinical issue. dFdCyd has multiple targets, and up- or down-regulation of these targets can confer resistance. Like other nucleoside analogs, dFdCyd is hydrophilic and cannot traverse cell membranes by passive diffusion [16]. Alterations in activity of nucleoside transporters have been shown to confer resistance to ara-C in leukemia cells [41,42]. BIBW22BS, a highly efficient inhibitor of equilibrate nucleoside transport, was found to reduce dFdCyd cytotoxicity by as much as 100-fold in a variety of cancer cell lines [43]. Mackey *et al* subsequently determined that cells with a nucleoside transport deficiency were highly resistant

to dFdCyd [17]. However, other reports have not found a similar relationship between nucleoside transport and dFdCyd resistance [19,44,45].

Phosphorylation of dFdCyd to dFdCMP by dCK is the rate limiting step in dFdCyd activation, consequently giving dCK a pivotal role in dFdCyd resistance. In fact, the most frequently described form of acquired dFdCyd resistance *in vitro* is dCK deficiency. Ruiz van Harparen *et al* issued the first report describing dFdCyd resistance conferred through an alteration in dCK [46]. In this study, stepwise exposure to dFdCyd resulted in a 30,000-fold dFdCyd-resistant cell line with dCK being undetected by western blotting. This group and others have subsequently described a correlation between intrinsic resistance to dFdCyd and dCK deficiency in a panel of solid tumor cell lines [46-49]. The correlation between dCK and dFdCyd resistance was strengthened by several studies which demonstrated a reversal of dFdCyd resistance by transfection of dCK into dCK-deficient cell lines *in vitro* [49] and *in vivo* [50].

In addition to the expression of dCK, regulation of dCK through phosphate donors and feedback inhibition can also alter dFdCyd sensitivity. Shewach *et al* proved that UTP was the most efficient phosphate donor for dCK [51], and other groups later found decreased UTP pools in dFdCyd-resistant murine leukemia and human ovarian cancer cells [46,52]. dCTP is a feedback inhibitor of dCK [53], and increased dCTP pools were found in KB human epidermoid carcinoma cells resistant to dFdCyd [48].

Another important cellular target of dFdCyd is ribonucleotide reductase, and several studies have implicated it as a cause of dFdCyd resistance. In human

K562 cells, a 3-fold increase in RR activity resulted in a 45-fold dFdCyd resistance [47]. In KB cells, a 2-fold increase in RR activity resulted in a 10-fold increase in dFdCyd resistance [48]. Overexpression of the R2 subunit has been implicated as a cause of dFdCyd resistance [54]. Conversely, inhibition of RR expression and activity, either by chemotherapeutics [55] or by siRNA silencing [56], increased dFdCyd sensitivity in a number of solid tumor cell lines. Increased activity of RR results in increased dNTP pools and this can potentially decrease dFdCyd sensitivity through three mechanisms. Firstly, an increase in dNTPs, particularly, dCTP, inhibits dCK activity through feedback inhibition, and this could potentially result in decreased dFdCyd phosphorylation and incorporation into DNA [6,21,48]. Secondly, dNTPs compete with dFdCTP for incorporation into DNA, and increased dNTP pools should decrease dFdCTP incorporation and cytotoxicity. Finally, Plunkett *et al* have shown that increased dCTP pools increased deoxycytidine deaminase activity, and this could serve to reduce dFdCyd sensitivity [21].

dFdCyd is degraded through a number of enzymes, and alterations in their expression and activity can affect dFdCyd sensitivity. 5'-Nucleotidase (5'NT) opposes the activity of nucleoside kinases by converting nucleotides back to nucleosides. In dFdCyd-resistant K562 variants, resistance was associated with increased 5'NT activity [47]. Also, HEK293 cells overexpressing 5'NT via transfection displayed a 22-fold reduction in dFdCyd sensitivity [57]. dFdCyd is also degraded via deamination by deoxycytidine deaminase (dCDA) and dCMP-deaminase. Most of the evidence suggests that dCDA does not play a major role

in intrinsic resistance to dFdCyd, although dCDA up-regulation may be a pathway of acquired resistance [58]. Although there have been many studies on resistance in dFdCyd in experimental systems, there are not enough data to draw conclusions on the mechanism of clinical dFdCyd resistance.

p53

The most frequently mutated gene in cancer cells is the tumor suppressor gene *TP53*, resulting in malfunction of its product, p53 [59]. Several lines of evidence indicate that p53 has an important role in tumor suppression. The effect of p53 status on the sensitivity of cancer cells to chemotherapeutic agents or radiation depends on the dual role of p53 as a guardian of genomic integrity and a mediator of cell death [60]. In unstressed cells, p53 is sequestered by MDM2 through its N-terminal domain, and this targets p53 for degradation by ubiquitination [61,62]. p53 is markedly upregulated after DNA damage and serves to block progression through the cell cycle or lead to cell death [63]. The decision to save or eliminate cells depends on several factors, including insult, degree of damage, and oncogenic status of the cell [64,65].

The p53 gene has been mapped to chromosome 17. p21^{WAF}, whose gene product is induced by p53, is a well known inhibitor of G1 cyclin-dependent kinases [66]. Following exposure to ionizing radiation, p53 is believed to initiate cell cycle arrest at the G1/S border through its interaction with p21^{WAF}. p53 also protects cells through initiation of repair processes such as nucleotide excision repair [67] and induce senescence [68]. Conversely, p53 can induce programmed cell death following irreparable DNA damage. p53 is believed to induce

apoptosis through induction of genes such as *bax* [69] and *fas* [70]. However, transactivation-deficient p53 cells can also induce apoptosis, indicating that p53 can induce apoptosis through transactivation-independent mechanisms as well [71].

dFdCyd, either alone or in combination with other agents, can induce p53 and cause cell cycle arrest as a result of DNA damage. As a result, much work has been dedicated to examining the role of p53 in dFdCyd sensitivity, either alone or when combined with other modalities. Following disruption of p53 in RKO cells, Chen *et al* observed a decrease in both dFdCyd sensitivity and apoptosis compared to the parental cells [72]. However, radiosensitization was not altered in this study. Galmarini *et al* described increased sensitivity to dFdCyd in wt p53 breast cancer cells compared to p53 deficient counterparts [73]. In human bladder cell lines, Kielb *et al* did not observe a difference in dFdCyd sensitivity [74]. In radiosensitization studies, Ostruszka *et al* observed radiosensitization in a mt p53 but not a wt p53 glioblastoma cell line [24]. In this study, wt p53 D54 cells accumulated in G1 in response to dFdCyd, whereas mt p53 U251 cells accumulated in S-phase following dFdCyd addition. However, other studies did not observe a difference in S-phase accumulation and radiosensitization between wt p53 and p53-deficient cells [35,75,76].

Ribonucleotide Reductase

As previously mentioned, ribonucleotide reductase is an important cellular target of dFdCyd, and is particularly essential in the radiosensitizing mechanism of dFdCyd. Ribonucleotide reductase is the enzyme responsible for the

conversion of ribonucleotide diphosphates to their corresponding

deoxyribonucleotide diphosphates, providing DNA precursors for DNA replication

and repair [77-80]. This is the rate-limiting step in DNA synthesis, and is the target

of several anticancer agents such as dFdCyd. There have been three main

classes of RR described based on different metal cofactors for the catalytic

activity [80,81]. Class I enzymes are founds in all eukaryotic organisms, as well as

some prokaryotes and viruses. Class I enzymes have been further divided into

three subcategories (Ia, Ib, Ic) based on polypeptide sequence homology and

allosteric regulation, with human RR belonging to Class Ia [82,83]. Synthesis of

deoxyribonucleotides is tightly regulated, as a failure in the control of the

amounts of dNTPs can lead to genetic abnormalities or cell death.

   Much of the structure and function studies on class I RRs have been

performed in E. coli and murine systems. RR is composed of two subunits, R1

and R2. R1 is an 85 kDa protein, and it harbors the active site for substrate

binding, allosteric regulatory sites, and redox active disulfides which are essential

for substrate reduction [84-86]. R2 is a 45 kDa protein which houses an oxygen-

linked diferric iron center and the tyrosyl radical that initiates substrate reduction

[77,87]. As a result of these different properties, R1 has been termed the regulatory

subunit, and R2 has been termed the catalytic subunit [86-88]. Although crystal

structures for both R1 and R2 have been determined separately, no crystal

structure of the RR holoenzyme has been elucidated. It has been proposed that

Class I RRs exist in a tetrameric holoenzyme with a $_2$$_2$ structure [87,89]. An $_6$$_6$

hexamer has also been proposed, and its formation is suggested to occur

through binding of ATP to the hexamerization site (H-site) [90,91]. However, this finding remains largely controversial.

Ribonucleotide reductase activity is controlled through its allosteric and regulatory sites [77]. The overall enzyme activity is regulated by binding of ATP (stimulation) or dATP (inhibition) to the activity site on R1. A balanced supply of dNTPs is maintained through binding of allosteric effectors (dATP or ATP, TTP, dGTP) to the specificity site, also on R1. Binding of dATP or ATP to the specificity site stimulates reduction of CDP and UDP, binding of TTP stimulates reduction of GDP, and binding of dGTP stimulates reduction of ADP.

RR activity is also controlled through the cell cycle by transcriptional mechanisms. The genes encoding R1 and R2 are located on different chromosomes in both human and murine cells [92,93]. R1 levels are constant and in excess through the cell cycle in proliferating cells, and it has a half-life of 18-24 h [94]. Conversely, R2 shows an S-phase specific expression with a half-life of 3-4 h. Expression of R2 is detected first in early S-phase, with the protein accumulating until it is rapidly degraded in mitosis [95]. R2 degradation has been linked to a KEN box in its N-terminal region that is recognized by the Cdh1-anaphase promoting complex that is activated in mitotic cells [96]. This complex targets R2 for degradation via ubiquitination.

p53R2

In 2000, Tanaka *et al* described the discovery of new p53 target genes through the use of differential display methods [97]. In this study, a 5.5 kb transcript was detected after exposure of SW480-LOWTP53 cells, a p53-

S phase Replication R2 ⟶ S phase dependent induction ⟶
dNTP supply for S phase replication

DNA damage repair p53R2 ⟶ p53 dependent induction by
DNA damage ⟶ dNTP supply for DNA repair

Fig 1.3. Proposed Mechanisms of R2 and p53R2 activity. In unstressed cells, R2 is activated via S-phase dependent genes, and when paired with R1, provides dNTPs for DNA replication. Following DNA damage, p53R2 is activated via p53, and when paired with R1, provides dNTPs for DNA repair. (Adapted from Nature 404 (6773) 42-49 (2000))

expressing cell line that had been established from a p53 mutant parental cell line, to 5 mM isopropylthiogalactoside (IPTG). This transcript was not detected following exposure to IPTG in a matching cell line which had mutant p53. Using a 132-kb differential display fragment as a probe to screen a cDNA library, an almost complete cDNA sequence consisting of 4,995 nucleotides was obtained. This cDNA sequence incorporated an open reading frame encoding a 351-amino acid sequence with 80% sequence homology to the human ribonucleotide reductase small subunit, R2, as well as significant homology to the yeast small subunit counterparts, RNR2, and RNR4. As a result of this protein being induced in a p53-dependent fashion, this protein was termed p53R2 (p53-inducible ribonucleotide reductase small subunit 2 homologue). p53R2 or similar variants have been subsequently discovered in mice and *A. albopictus* mosquito cells [98,99]. Homology exists throughout the peptide sequences except in the N-terminal region. The KEN box that is recognized by Cdh1-anaphase promoting complex in R2 is not found in p53R2, and as a result p53R2 can be induced in any phase of the cell cycle [96]. p53R2 is activated in a p53-dependent fashion in response to DNA damage, and has been suggested to play a role in DNA repair through supplying dNTPs (Fig 1.3). Conversely, R2 expression is linked to E2F and is induced only in S-phase cells to provide dNTPs for DNA replication.

Since its discovery, the role of p53R2 in ribonucleotide reduction has been extensively characterized. p53R2 was initially found to be induced in response to exposure to ionizing radiation or adriamycin in a p53-dependent manner [97]. Guillet *et al* later determined that p53R2 formed an active RR complex with R1 *in*

vitro, and that ribonucleotide reductase activity increased in response to UV radiation in G0/G1 synchronized cells [100]. Yamaguchi *et al* examined ribonucleotide reductase activity in response to ionizing radiation and found a correlation between p53R2 expression and RR activity [101]. In this study, p53R2 was also shown to accumulate in the nucleus in response to DNA damage. This finding was further strengthened by later work by Xue *et al* in which p53R2 dissociated from p53 and translocated from the cytoplasm to the nucleus in response to DNA damage, and colocalized with R1 [102]. This translocation was not observed in p53 mutant cell lines. Synthetic heptapeptide competition assays confirmed that p53R2 and R2 share the same binding site on R1, although p53R2 had lowered affinity for R1 and lowered RR activity compared to R2 *in vitro* [103]. When paired with R1, these two subunits have also demonstrated differing sensitivities to hydroxyurea and DFO in *in vitro* systems.

In addition to its role in providing dNTPs for DNA repair, p53R2 has other roles in protecting cells from damaging insults. Two different studies in mice have confirmed that p53R2-null mice die by 14 weeks of age due to kidney failure [99,104]. Since these animals developed normally until they were weaned, it was postulated that p53R2 may have a role in combating oxidative stress. Subsequent findings have confirmed that p53R2 has redox activity and is important in the cellular response to oxidative stress [105]. Interestingly, this redox activity is tied to its ribonucleotide reductase activity, as mutation of tyrosyl residues essential for ribonucleotide reductase results in ablation of redox

activity. In contrast, R2 was shown to have the opposite effect of promoting oxidative stress.

Recent findings have described a role for p53R2 in preventing metastasis. Immunohistological studies on primary and metastatic colon carcinoma samples suggested a negative correlation between p53R2 expression and metastasis [106,107]. Suppression of p53R2 expression was found to increase both invasion and migration. Conversely, overexpression of p53R2 by gene transfection reduced cell invasion potential by as much as 54%. Increased expression of R2 was associated positively with metastasis, although the results were not statistically significant.

Dissertation Rationale

The purpose of this dissertation was to elucidate the mechanisms by which dFdCyd potentiates radiation-induced cell killing. Much work in this laboratory has been dedicated to this endeavor, and we have previously identified dATP depletion and S-phase accumulation prior to radiation exposure as correlative factors. Studies undertaken were based on earlier findings in this laboratory and others and ideas formulated over the past few years.

While there is a considerable literature on the role of p53 in cytotoxicity with dFdCyd, there has been less attention directed at understanding the role of p53 in dFdCyd-mediated radiosensitization [24,35,75]. In particular, work in a pair of glioblastoma cell lines, D54 (wt p53) and U251 (mt p53), appeared to indicate a role for p53 in radiosensitization [24]. In response to dFdCyd, U251 cells accumulated in S-phase and were subsequently radiosensitized, whereas D54

20

cells accumulated in G1 and were not radiosensitized. Both cell lines were depleted of dATP similarly. It was hypothesized that p53 could prevent or reduce radiosensitization clinically by inducing a G1 block and preventing S-phase accumulation.

Therefore, in the studies presented in Chapter II, we have evaluated the role of p53 on the ability of dFdCyd to produce radiosensitization by utilizing a high, clinically relevant concentration of dFdCyd in MCF-7 breast carcinoma cells. It was important to determine whether this high concentration of dFdCyd would result in a high and rapid induction of p53, and whether this would in turn cause cell cycle arrest in G1, thus preventing radiosensitization as cells would not accumulate in S-phase. Although we were able to induce p53 rapidly in response to IC_{90} dFdCyd, we still observed radiosensitization of MCF-7 cells.

During the completion of this project, a study describing a p53-inducible ribonucleotide reductase subunit was published [97]. Because of the essential role of ribonucleotide reductase inhibition in dFdCyd-mediated radiosensitization, it was a natural progression to study the potential roles of p53R2 in our radiosensitizing model. Since p53R2 can provide dNTPs in response to DNA damage, the potential exists that this activity could work to alter radiosensitization through maintenance of dNTP pools.

Although studies have evaluated the response of p53R2 to radiation, very little had been published examining the induction of p53R2 in response to chemotherapeutics. In addition, most studies that evaluated p53 in response to dFdCyd were published before the discovery of p53R2, so it was unclear if

dFdCyd exposure resulted in p53R2 expression in wt p53 cell lines. Therefore, in Chapter III we initiated the first study to our knowledge that closely examined the kinetics of induction of p53R2 and R2 in response to a chemotherapeutic, either used alone or in combination with ionizing radiation. We hypothesized that dFdCyd exposure would result in an upregulation of p53R2 as a result of DNA damage, and an upregulation of R2 due to S-phase accumulation. Conversely, we predicted that exposure to ionizing radiation would result in an upregulation of p53R2 due to DNA damage and a downregulation of R2 as a result of cells progressing out of S-phase and accumulating in G1 and G2/M. p53R2 expression was induced within 4 hr of dFdCyd addition in two wt p53 cell lines, even at low, non-cytotoxic concentrations. Expression of p53R2 was also rapidly induced in response to ionizing radiation. p53R2 expression appeared unaffected by cell cycle distribution.

Chapter III further examines p53R2 by evaluating whether induction of p53R2 alters radiosensitization. We utilized two sets of cell lines matched for p53 status, MCF-7 (wt p53) and MCF/Adr (mt p53) breast carcinoma cells, and HCT116 (wt p53) and HCT116$^{p53-/-}$ colon carcinoma cells. We hypothesized that radiosensitization would be greater in cell lines with mutant p53, thus theoretically lacking p53R2, than in those with functional p53 and p53R2. This would be due to the activity of p53R2 in perturbing dNTP depletion in response to dFdCyd exposure. However, the two sets of matched cell lines demonstrated little difference in radiosensitization. In addition, no difference in dATP depletion

22

in response to dFdCyd exposure was noted at equitoxic concentrations in the matched cell lines.

Although the cell lines previously used were matched for p53 status, they demonstrated differences in sensitivity to dFdCyd and ionizing radiation as single agents. In addition, these cell lines have been grown separately over several years and may have acquired additional differences that may have altered drug and/or radiation sensitivity. As a result Chapter IV further examines the role of p53R2 in dFdCyd-mediated radiosensitization by examining the effects of suppression of p53R2 on radiosensitization. This will allow for the determination of the effects of p53R2 induction on radiosensitization in a single cell line. Initially, MCF-7 breast cancer cells were utilized and the effects of p53R2 silencing by siRNA were determined. We predicted that p53R2 silencing would increase dFdCyd-mediated radiosensitization as a result of increased dNTP depletion. Although we were able to inhibit dFdCyd-mediated increases in p53R2 expression, this suppression did not alter radiosensitization. We then chose a second cell line, the A549 lung carcinoma cell line, and attempted to increase radiosensitization by suppression of p53R2. This cell line was previously demonstrated to not be radiosensitized by IC_{10} dFdCyd, and dATP was depleted by 65% of control levels. Radiosensitization was observed at the IC50, and dATP was depleted to >80% of control levels. This suggested that p53R2 may have prevented radiosensitization at low dFdCyd concentrations by maintaining dNTP pools. In this cell line, silencing of p53R2 did result in increased radiosensitization. Nucleotide pool studies demonstrated that

silencing of p53R2 resulted in increased dNTP depletion, which may have

accounted for increased radiosensitization.

[1]The footnotes used are: dFdCyd, 2',2'-difluoro-2'-deoxycytidine; dCyd,

deoxycytidine; dFdCMP, 5'-monophosphate of dFdCyd; dFdCDP, 5'-diphosphate

of dFdCyd; dFdCTP, 5'-triphosphate of dFdCyd; dNTP, deoxynucleotide

triphosphate; dFdUrd, 2',2'-difluoro-2'-deoxyuridine; dFdUMP, 5'-monophosphate

of dFdUrd, dATP, deoxyadenosine triphosphate, dCTP, deoxycytidine

triphosphate, TTP, thymidine triphosphate, dGTP, deoxyguanosine triphosphate;

RR, ribonucleotide reductase; dCK, deoxycytidine kinase.

References

1. Hertel, L. W., Boder, G. B., Kroin, J. S., Rinzel, S. M., Poore, G. A., Todd, G. C., and Grindey, G. B. Evaluation of the antitumor activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine). Cancer Res., *50*: 4417-4422, 1990.

2. Heidelberger, C., Danenberg, P. V., and Moran, R. G. Fluorinated pyrimidines and their nucleosides. Adv.Enzymol.Relat Areas Mol.Biol., *54*: 58-119, 1983.

3. Ojima I, McCarthy J.R, and Welch J.T. Biomedical Frontiers of Fluorine Chemistry. American Chemican Society . 1996.
   Ref Type: Abstract

4. Gudmundsson, K. S., Freeman, G. A., Drach, J. C., and Townsend, L. B. Synthesis of fluorosugar analogues of 2,5,6-trichloro-1-(beta-D-ribofuranosyl)benzimidazole as antivirals with potentially increased glycosidic bond stability. J.Med.Chem., *43*: 2473-2478, 2000.

5. Stoeckler, J. D., Bell, C. A., Parks, R. E., Jr., Chu, C. K., Fox, J. J., and Ikehara, M. C(2')-substituted purine nucleoside analogs. Interactions with adenosine deaminase and purine nucleoside phosphorylase and formation of analog nucleotides. Biochem.Pharmacol., *31*: 1723-1728, 1982.

6. Heinemann, V., Hertel, L. W., Grindey, G. B., and Plunkett, W. Comparison of the cellular pharmacokinetics and toxicity of 2',2'-difluorodeoxycytidine and 1-beta-D-arabinofuranosylcytosine. Cancer Res., *48*: 4024-4031, 1988.

7. Hui, Y. F. and Reitz, J. Gemcitabine: a cytidine analogue active against solid tumors. Am.J.Health Syst.Pharm., *54*: 162-170, 1997.

8. Burris, H. A., III, Moore, M. J., Andersen, J., Green, M. R., Rothenberg, M. L., Modiano, M. R., Cripps, M. C., Portenoy, R. K., Storniolo, A. M., Tarassoff, P., Nelson, R., Dorr, F. A., Stephens, C. D., and Von Hoff, D. D. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J.Clin.Oncol., *15*: 2403-2413, 1997.

9. PDR Staff. Physicians Desk Reference 2003. Thomson, 2003.

10. Rockwell, S. and Grindey, G. B. Effect of 2',2'-difluorodeoxycytidine on the viability and radiosensitivity of EMT6 cells in vitro. Oncol.Res., *4*: 151-155, 1992.

11. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W., and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells. Cancer Res., *54*: 3218-3223, 1994.

12. Eisbruch, A., Shewach, D. S., Bradford, C. R., Littles, J. F., Teknos, T. N., Chepeha, D. B., Marentette, L. J., Terrell, J. E., Hogikyan, N. D., Dawson, L. A., Urba, S., Wolf, G. T., and Lawrence, T. S. Radiation concurrent with gemcitabine for locally advanced head and neck cancer: a phase I trial and intracellular drug incorporation study. J.Clin.Oncol., *19*: 792-799, 2001.

13. Blackstock, A. W., Bernard, S. A., Richards, F., Eagle, K. S., Case, L. D., Poole, M. E., Savage, P. D., and Tepper, J. E. Phase I trial of twice-weekly gemcitabine and concurrent radiation in patients with advanced pancreatic cancer. J.Clin.Oncol., *17*: 2208-2212, 1999.

14. Pipas, J. M., Mitchell, S. E., Barth, R. J., Jr., Vera-Gimon, R., Rathmann, J., Meyer, L. P., Wagman, R. S., Lewis, L. D., McDonnell, C., Colacchio, T. A., and Perez, R. P. Phase I study of twice-weekly gemcitabine and concomitant external-beam radiotherapy in patients with adenocarcinoma of the pancreas. Int.J.Radiat.Oncol.Biol.Phys., *50*: 1317-1322, 2001.

15. McGinn, C. J., Zalupski, M. M., Shureiqi, I., Robertson, J. M., Eckhauser, F. E., Smith, D. C., Brown, D., Hejna, G., Strawderman, M., Normolle, D., and Lawrence, T. S. Phase I trial of radiation dose escalation with concurrent weekly full-dose gemcitabine in patients with advanced pancreatic cancer. J.Clin.Oncol., *19*: 4202-4208, 2001.

16. Cass, C. E. Nucleoside Transport. *In* N. H. Georgopapadakou (ed.), Nucleoside Transport in Antimicrobial and Anticancer Chemotherapy, pp. 1-403. New York: Marcel Dekker, 1995.

17. Mackey, J. R., Mani, R. S., Selner, M., Mowles, D., Young, J. D., Belt, J. A., Crawford, C. R., and Cass, C. E. Functional nucleoside transporters are required for gemcitabine influx and manifestation of toxicity in cancer cell lines. Cancer Res., *58*: 4349-4357, 1998.

18. Mackey, J. R., Yao, S. Y., Smith, K. M., Karpinski, E., Baldwin, S. A., Cass, C. E., and Young, J. D. Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J.Natl.Cancer Inst., *91*: 1876-1881, 1999.

19. Rauchwerger, D. R., Firby, P. S., Hedley, D. W., and Moore, M. J. Equilibrative-sensitive nucleoside transporter and its role in gemcitabine sensitivity. Cancer Res., *60*: 6075-6079, 2000.

20. Bouffard, D. Y., Laliberte, J., and Momparler, R. L. Kinetic studies on 2',2'-difluorodeoxycytidine (Gemcitabine) with purified human deoxycytidine

kinase and cytidine deaminase. Biochem.Pharmacol., *45*: 1857-1861, 1993.

21. Plunkett, W., Huang, P., Searcy, C. E., and Gandhi, V. Gemcitabine: preclinical pharmacology and mechanisms of action. Semin.Oncol., *23*: 3-15, 1996.

22. Baker, C. H., Banzon, J., Bollinger, J. M., Stubbe, J., Samano, V., Robins, M. J., Lippert, B., Jarvi, E., and Resvick, R. 2'-Deoxy-2'-methylenecytidine and 2'-deoxy-2',2'-difluorocytidine 5'-diphosphates: potent mechanism-based inhibitors of ribonucleotide reductase. J.Med.Chem., *34*: 1879-1884, 1991.

23. Heinemann, V., Xu, Y. Z., Chubb, S., Sen, A., Hertel, L. W., Grindey, G. B., and Plunkett, W. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. Mol.Pharmacol., *38*: 567-572, 1990.

24. Ostruszka, L. J. and Shewach, D. S. The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine. Cancer Res., *60*: 6080-6088, 2000.

25. van der Donk, W. A., Yu, G., Perez, L., Sanchez, R. J., Stubbe, J., Samano, V., and Robins, M. J. Detection of a new substrate-derived radical during inactivation of ribonucleotide reductase from Escherichia coli by gemcitabine 5'-diphosphate. Biochemistry, *37*: 6419-6426, 1998.

26. Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B., and Plunkett, W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. Cancer Res., *51*: 6110-6117, 1991.

27. Ruiz, v. H., V, Veerman, G., Vermorken, J. B., and Peters, G. J. 2',2'-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem.Pharmacol., *46*: 762-766, 1993.

28. Storniolo, A. M., Allerheiligen, S. R., and Pearce, H. L. Preclinical, pharmacologic, and phase I studies of gemcitabine. Semin.Oncol., *24*: S7, 1997.

29. Ross, D. D. and Cuddy, D. P. Molecular effects of 2',2'-difluorodeoxycytidine (Gemcitabine) on DNA replication in intact HL-60 cells. Biochem.Pharmacol., *48*: 1619-1630, 1994.

30. Lawrence, T. S., Chang, E. Y., Hahn, T. M., and Shewach, D. S. Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine). Clin.Cancer Res., *3*: 777-782, 1997.

31. Rosier, J. F., Beauduin, M., Bruniaux, M., De Bast, M., De Coster, B., Octave-Prignot, M., Scalliet, P., and Gregoire, V. The effect of 2'-2' difluorodeoxycytidine (dFdC, gemcitabine) on radiation-induced cell lethality in two human head and neck squamous carcinoma cell lines differing in intrinsic radiosensitivity. Int.J.Radiat.Biol., *75*: 245-251, 1999.

32. McGinn, C. J., Shewach, D. S., and Lawrence, T. S. Radiosensitizing nucleosides. J.Natl.Cancer Inst., *88*: 1193-1203, 1996.

33. Shewach, D. S. and Lawrence, T. S. Radiosensitization of human tumor cells by gemcitabine in vitro. Semin.Oncol., *22*: 68-71, 1995.

34. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W., and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine. Int.J.Radiat.Oncol.Biol.Phys., *34*: 867-872, 1996.

35. Robinson, B. W. and Shewach, D. S. Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines. Clin.Cancer Res., *7*: 2581-2589, 2001.

36. Fertil, B., Dertinger, H., Courdi, A., and Malaise, E. P. Mean inactivation dose: a useful concept for intercomparison of human cell survival curves. Radiat.Res., *99*: 73-84, 1984.

37. Pauwels, B., Korst, A. E., de Pooter, C. M., Lambrechts, H. A., Pattyn, G. G., Lardon, F., and Vermorken, J. B. The radiosensitising effect of gemcitabine and the influence of the rescue agent amifostine in vitro. Eur.J.Cancer, *39*: 838-846, 2003.

38. Shewach, D. S. and Lawrence, T. S. Gemcitabine and radiosensitization in human tumor cells. Invest New Drugs, *14*: 257-263, 1996.

39. Latz, D., Fleckenstein, K., Eble, M., Blatter, J., Wannenmacher, M., and Weber, K. J. Radiosensitizing potential of gemcitabine (2',2'-difluoro-2'-deoxycytidine) within the cell cycle in vitro. Int.J.Radiat.Oncol.Biol.Phys., *41*: 875-882, 1998.

40. Pauwels, B., Korst, A. E., de Pooter, C. M., Pattyn, G. G., Lambrechts, H. A., Baay, M. F., Lardon, F., and Vermorken, J. B. Comparison of the sulforhodamine B assay and the clonogenic assay for in vitro chemoradiation studies. Cancer Chemother.Pharmacol., *51*: 221-226, 2003.

41. Gati, W. P., Paterson, A. R., Larratt, L. M., Turner, A. R., and Belch, A. R. Sensitivity of acute leukemia cells to cytarabine is a correlate of cellular es nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. Blood, *90*: 346-353, 1997.

42. Wiley, J. S., Jones, S. P., Sawyer, W. H., and Paterson, A. R. Cytosine arabinoside influx and nucleoside transport sites in acute leukemia. J.Clin.Invest, *69*: 479-489, 1982.

43. Jansen, W. J., Pinedo, H. M., van der Wilt, C. L., Feller, N., Bamberger, U., and Boven, E. The influence of BIBW22BS, a dipyridamole derivative, on the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine in vitro and in human tumour xenografts. Eur.J.Cancer, *31A*: 2313-2319, 1995.

44. Dumontet, C., Bauchu, E. C., Fabianowska, K., Lepoivre, M., Wyczechowska, D., Bodin, F., and Rolland, M. O. Common resistance mechanisms to nucleoside analogues in variants of the human erythroleukemic line K562. Adv.Exp.Med.Biol., *457*: 571-577, 1999.

45. Hammond, J. R., Lee, S., and Ferguson, P. J. [3H]gemcitabine uptake by nucleoside transporters in a human head and neck squamous carcinoma cell line. J.Pharmacol.Exp.Ther., *288*: 1185-1191, 1999.

46. Ruiz, v. H., V, Veerman, G., Eriksson, S., Boven, E., Stegmann, A. P., Hermsen, M., Vermorken, J. B., Pinedo, H. M., and Peters, G. J. Development and molecular characterization of a 2',2'-difluorodeoxycytidine-resistant variant of the human ovarian carcinoma cell line A2780. Cancer Res., *54*: 4138-4143, 1994.

47. Dumontet, C., Fabianowska-Majewska, K., Mantincic, D., Callet, B. E., Tigaud, I., Gandhi, V., Lepoivre, M., Peters, G. J., Rolland, M. O., Wyczechowska, D., Fang, X., Gazzo, S., Voorn, D. A., Vanier-Viornery, A., and Mackey, J. Common resistance mechanisms to deoxynucleoside analogues in variants of the human erythroleukaemic line K562. Br.J.Haematol., *106*: 78-85, 1999.

48. Goan, Y. G., Zhou, B., Hu, E., Mi, S., and Yen, Y. Overexpression of ribonucleotide reductase as a mechanism of resistance to 2,2-difluorodeoxycytidine in the human KB cancer cell line. Cancer Res., *59*: 4204-4207, 1999.

49. van der Wilt, C. L., Kroep, J. R., Bergman, A. M., Loves, W. J., Alvarez, E., Talianidis, I., Eriksson, S., van Groeningen, C. J., Pinedo, H. M., and Peters, G. J. The role of deoxycytidine kinase in gemcitabine cytotoxicity. Adv.Exp.Med.Biol., *486*: 287-290, 2000.

50. Blackstock, A. W., Lightfoot, H., Case, L. D., Tepper, J. E., Mukherji, S. K., Mitchell, B. S., Swarts, S. G., and Hess, S. M. Tumor uptake and elimination of 2',2'-difluoro-2'-deoxycytidine (gemcitabine) after deoxycytidine kinase gene transfer: correlation with in vivo tumor response. Clin.Cancer Res., *7*: 3263-3268, 2001.

51. Shewach, D. S., Reynolds, K. K., and Hertel, L. Nucleotide specificity of human deoxycytidine kinase. Mol.Pharmacol., *42*: 518-524, 1992.

52. Bergman, A. M., Pinedo, H. M., Jongsma, A. P., Brouwer, M., Ruiz, v. H., V, Veerman, G., Leyva, A., Eriksson, S., and Peters, G. J. Decreased resistance to gemcitabine (2',2'-difluorodeoxycitidine) of cytosine arabinoside-resistant myeloblastic murine and rat leukemia cell lines: role of altered activity and substrate specificity of deoxycytidine kinase. Biochem.Pharmacol., *57*: 397-406, 1999.

53. Datta, N. S., Shewach, D. S., Mitchell, B. S., and Fox, I. H. Kinetic properties and inhibition of human T lymphoblast deoxycytidine kinase. J.Biol.Chem., *264*: 9359-9364, 1989.

54. Zhou, B. S., Hsu, N. Y., Pan, B. C., Doroshow, J. H., and Yen, Y. Overexpression of ribonucleotide reductase in transfected human KB cells increases their resistance to hydroxyurea: M2 but not M1 is sufficient to increase resistance to hydroxyurea in transfected cells. Cancer Res., *55*: 1328-1333, 1995.

55. Jung, C. P., Motwani, M. V., and Schwartz, G. K. Flavopiridol increases sensitization to gemcitabine in human gastrointestinal cancer cell lines and correlates with down-regulation of ribonucleotide reductase M2 subunit. Clin.Cancer Res., *7*: 2527-2536, 2001.

56. Duxbury, M. S., Ito, H., Zinner, M. J., Ashley, S. W., and Whang, E. E. RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine. Oncogene, *23*: 1539-1548, 2004.

57. Hunsucker, S. A., Spychala, J., and Mitchell, B. S. Human cytosolic 5'-nucleotidase I: characterization and role in nucleoside analog resistance. J.Biol.Chem., *276*: 10498-10504, 2001.

58. Bergman, A. M., Pinedo, H. M., and Peters, G. J. Determinants of resistance to 2',2'-difluorodeoxycytidine (gemcitabine). Drug Resist.Updat., *5*: 19-33, 2002.

59. Vousden, K. H. and Lu, X. Live or let die: the cell's response to p53. Nat.Rev.Cancer, *2*: 594-604, 2002.

60. Lane, D. P. Cancer. p53, guardian of the genome. Nature, *358*: 15-16, 1992.

61. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. Mdm2 promotes the rapid degradation of p53. Nature, *387*: 296-299, 1997.

62. Kubbutat, M. H., Ludwig, R. L., Ashcroft, M., and Vousden, K. H. Regulation of Mdm2-directed degradation by the C terminus of p53. Mol.Cell Biol., *18*: 5690-5698, 1998.

63. Kastan, M. B., Canman, C. E., and Leonard, C. J. P53, cell cycle control and apoptosis: implications for cancer. Cancer Metastasis Rev., *14*: 3-15, 1995.

64. Ljungman, M. Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. Neoplasia., *2*: 208-225, 2000.

65. Sionov, R. V. and Haupt, Y. The cellular response to p53: the decision between life and death. Oncogene, *18*: 6145-6157, 1999.

66. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell, *75*: 805-816, 1993.

67. McKay, B. C., Francis, M. A., and Rainbow, A. J. Wildtype p53 is required for heat shock and ultraviolet light enhanced repair of a UV-damaged reporter gene. Carcinogenesis, *18*: 245-249, 1997.

68. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell, *88*: 593-602, 1997.

69. Miyashita, T. and Reed, J. C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell, *80*: 293-299, 1995.

70. Owen-Schaub, L. B., Zhang, W., Cusack, J. C., Angelo, L. S., Santee, S. M., Fujiwara, T., Roth, J. A., Deisseroth, A. B., Zhang, W. W., Kruzel, E., and . Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol.Cell Biol., *15*: 3032-3040, 1995.

71. Haupt, Y., Rowan, S., Shaulian, E., Vousden, K. H., and Oren, M. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. Genes Dev., *9*: 2170-2183, 1995.

72. Chen, M., Hough, A. M., and Lawrence, T. S. The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. Cancer Chemother.Pharmacol., *45*: 369-374, 2000.

73. Galmarini, C. M., Clarke, M. L., Falette, N., Puisieux, A., Mackey, J. R., and Dumontet, C. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. Int.J.Cancer, *97*: 439-445, 2002.

74. Kielb, S. J., Shah, N. L., Rubin, M. A., and Sanda, M. G. Functional p53 mutation as a molecular determinant of paclitaxel and gemcitabine susceptibility in human bladder cancer. J.Urol., *166*: 482-487, 2001.

75. Pauwels, B., Korst, A. E., Andriessen, V., Baay, M. F., Pattyn, G. G., Lambrechts, H. A., Pooter, C. M., Lardon, F., and Vermorken, J. B. Unraveling the mechanism of radiosensitization by gemcitabine: the role of TP53. Radiat.Res., *164*: 642-650, 2005.

76. Robinson, B. W., Im, M. M., Ljungman, M., Praz, F., and Shewach, D. S. Enhanced radiosensitization with gemcitabine in mismatch repair-deficient HCT116 cells. Cancer Res., *63*: 6935-6941, 2003.

77. Jordan, A. and Reichard, P. Ribonucleotide reductases. Annu.Rev.Biochem., *67*: 71-98, 1998.

78. Kolberg, M., Strand, K. R., Graff, P., and Andersson, K. K. Structure, function, and mechanism of ribonucleotide reductases. Biochim.Biophys.Acta, *1699*: 1-34, 2004.

79. Reichard, P. and Ehrenberg, A. Ribonucleotide reductase--a radical enzyme. Science, *221*: 514-519, 1983.

80. Reichard, P. From RNA to DNA, why so many ribonucleotide reductases? Science, *260*: 1773-1777, 1993.

81. Stubbe, J. and Der Donk, W. A. Protein Radicals in Enzyme Catalysis. [Chem. Rev. 1998, 98, 705minus sign762. Chem.Rev., *98*: 2661-2662, 1998.

82. Hogbom, M., Stenmark, P., Voevodskaya, N., McClarty, G., Graslund, A., and Nordlund, P. The radical site in chlamydial ribonucleotide reductase defines a new R2 subclass. Science, *305*: 245-248, 2004.

83. Jordan, A., Pontis, E., Atta, M., Krook, M., Gibert, I., Barbe, J., and Reichard, P. A second class I ribonucleotide reductase in Enterobacteriaceae: characterization of the Salmonella typhimurium enzyme. Proc.Natl.Acad.Sci.U.S.A, *91*: 12892-12896, 1994.

84. Eriksson, M., Uhlin, U., Ramaswamy, S., Ekberg, M., Regnstrom, K., Sjoberg, B. M., and Eklund, H. Binding of allosteric effectors to ribonucleotide reductase protein R1: reduction of active-site cysteines promotes substrate binding. Structure., *5*: 1077-1092, 1997.

85. Sintchak, M. D., Arjara, G., Kellogg, B. A., Stubbe, J., and Drennan, C. L. The crystal structure of class II ribonucleotide reductase reveals how an allosterically regulated monomer mimics a dimer. Nat.Struct.Biol., *9*: 293-300, 2002.

33

86. Uhlin, U. and Eklund, H. Structure of ribonucleotide reductase protein R1. Nature, *370*: 533-539, 1994.

87. Nordlund, P. and Eklund, H. Structure and function of the Escherichia coli ribonucleotide reductase protein R2. J.Mol.Biol., *232*: 123-164, 1993.

88. Nordlund, P., Sjoberg, B. M., and Eklund, H. Three-dimensional structure of the free radical protein of ribonucleotide reductase. Nature, *345*: 593-598, 1990.

89. Stubbe, J., Nocera, D. G., Yee, C. S., and Chang, M. C. Radical initiation in the class I ribonucleotide reductase: long-range proton-coupled electron transfer? Chem.Rev., *103*: 2167-2201, 2003.

90. Cooperman, B. S. and Kashlan, O. B. A comprehensive model for the allosteric regulation of Class Ia ribonucleotide reductases. Adv.Enzyme Regul., *43*: 167-182, 2003.

91. Kashlan, O. B. and Cooperman, B. S. Comprehensive model for allosteric regulation of mammalian ribonucleotide reductase: refinements and consequences. Biochemistry, *42*: 1696-1706, 2003.

92. Brissenden, J. E., Caras, I., Thelander, L., and Francke, U. The structural gene for the M1 subunit of ribonucleotide reductase maps to chromosome 11, band p15, in human and to chromosome 7 in mouse. Exp.Cell Res., *174*: 302-308, 1988.

93. Yang-Feng, T. L., Barton, D. E., Thelander, L., Lewis, W. H., Srinivasan, P. R., and Francke, U. Ribonucleotide reductase M2 subunit sequences mapped to four different chromosomal sites in humans and mice: functional locus identified by its amplification in hydroxyurea-resistant cell lines. Genomics, *1*: 77-86, 1987.

94. Bjorklund, S., Skog, S., Tribukait, B., and Thelander, L. S-phase-specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. Biochemistry, *29*: 5452-5458, 1990.

95. Thelander, M., Graslund, A., and Thelander, L. Subunit M2 of mammalian ribonucleotide reductase. Characterization of a homogeneous protein isolated from M2-overproducing mouse cells. J.Biol.Chem., *260*: 2737-2741, 1985.

96. Chabes, A. L., Pfleger, C. M., Kirschner, M. W., and Thelander, L. Mouse ribonucleotide reductase R2 protein: a new target for anaphase-promoting complex-Cdh1-mediated proteolysis. Proc.Natl.Acad.Sci.U.S.A, *100*: 3925-3929, 2003.

97. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature, *404*: 42-49, 2000.

98. Jayachandran, G. and Fallon, A. M. The mosquito ribonucleotide reductase R2 gene: ultraviolet light induces expression of a novel R2 variant with an internal amino acid deletion. Insect Mol.Biol., *13*: 231-239, 2004.

99. Kimura, T., Takeda, S., Sagiya, Y., Gotoh, M., Nakamura, Y., and Arakawa, H. Impaired function of p53R2 in Rrm2b-null mice causes severe renal failure through attenuation of dNTP pools. Nat.Genet., *34*: 440-445, 2003.

100. Guittet, O., Hakansson, P., Voevodskaya, N., Fridd, S., Graslund, A., Arakawa, H., Nakamura, Y., and Thelander, L. Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. J.Biol.Chem., *276*: 40647-40651, 2001.

101. Yamaguchi, T., Matsuda, K., Sagiya, Y., Iwadate, M., Fujino, M. A., Nakamura, Y., and Arakawa, H. p53R2-dependent pathway for DNA synthesis in a p53-regulated cell cycle checkpoint. Cancer Res., *61*: 8256-8262, 2001.

102. Xue, L., Zhou, B., Liu, X., Qiu, W., Jin, Z., and Yen, Y. Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p53R2 as well as hRRM2 subunits. Cancer Res., *63*: 980-986, 2003.

103. Shao, J., Zhou, B., Zhu, L., Qiu, W., Yuan, Y. C., Xi, B., and Yen, Y. In vitro characterization of enzymatic properties and inhibition of the p53R2 subunit of human ribonucleotide reductase. Cancer Res., *64*: 1-6, 2004.

104. Powell, D. R., Desai, U., Sparks, M. J., Hansen, G., Gay, J., Schrick, J., Shi, Z. Z., Hicks, J., and Vogel, P. Rapid development of glomerular injury and renal failure in mice lacking p53R2. Pediatr.Nephrol., *20*: 432-440, 2005.

105. Xue, L., Zhou, B., Liu, X., Wang, T., Shih, J., Qi, C., Heung, Y., and Yen, Y. Structurally dependent redox property of ribonucleotide reductase subunit p53R2. Cancer Res., *66*: 1900-1905, 2006.

106. Liu, X., Zhou, B., Xue, L., Shih, J., Tye, K., Lin, W., Qi, C., Chu, P., Un, F., Wen, W., and Yen, Y. Metastasis-suppressing potential of ribonucleotide reductase small subunit p53R2 in human cancer cells. Clin.Cancer Res., *12*: 6337-6344, 2006.

107. Liu, X., Zhou, B., Xue, L., Yen, F., Chu, P., Un, F., and Yen, Y. Ribonucleotide reductase subunits M2 and p53R2 are potential biomarkers for metastasis of colon cancer. Clin.Colorectal Cancer, *6*: 374-381, 2007.

Chapter II

THE EFFECTS OF HIGH [dFdCyd] ON p53 EXPRESSION, CELL CYCLE DISTRIBUTION, AND RADIOSENSITIZATION IN MCF-7 BREAST CANCER CELLS

Summary

Gemcitabine (2', 2'-difluorodeoxycytidine, dFdCyd) is a nucleoside analog with potent radiosensitizing properties in solid tumor cells *in vitro* and *in vivo*. Radiosensitization with dFdCyd has correlated with dATP depletion, mediated by inhibition of ribonucleotide reductase by dFdCyd diphosphate, and accumulation of cells in S-phase. Although dFdCyd can induce p53, a conclusive role for this cell cycle regulatory protein in radiosensitization by dFdCyd has not been demonstrated. Previous studies were performed using low concentrations of dFdCyd that induced only low levels of p53. Using wild-type p53 expressing MCF-7 cells, we evaluated the effect of a higher and more clinically relevant concentration of dFdCyd to determine whether this would result in higher induction of p53, which may prevent S-phase accumulation and radiosensitization. Following a 24 hr exposure to dFdCyd at its IC_{90}, high expression of p53 was induced rapidly, and greater than 80% of the cells accumulated in S-phase. While 5 Gy also induced high expression of p53, cells accumulated in G1 and G2/M with <10% of cells remaining in S-phase after 24 hr. Evaluation of p53 expression in each phase of the cell cycle demonstrated

that this cell cycle regulatory protein was induced in S-phase and G2/M cells after dFdCyd treatment, whereas radiation induced p53 expression in G1 and G2/M, consistent with the observed cell cycle blocks. The combination of IC_{90} dFdCyd followed by ionizing radiation produced an enhancement ratio of 2.7, significantly higher than that produced by the IC_{10} or IC_{50} of dFdCyd (<1.9). Taken together, these results demonstrate that exposure to a high, more clinically relevant dose of dFdCyd can result in high and rapid induction of p53 primarily in S-phase and G2/M cells. This does not preclude accumulation of cells in S-phase and therefore does not prevent dFdCyd-mediated radiosensitization.

Introduction

Gemcitabine (dFdCyd, Gemzar®) is a nucleoside analogue that has shown excellent clinical activity in a variety of solid tumors, including pancreatic [1-4] and non-small cell lung cancers [5-10]. In addition to its activity as a chemotherapeutic agent, studies have demonstrated that dFdCyd can sensitize cancer cells to the cytotoxic effects of ionizing radiation [11-14]. This radiosensitizing effect has been demonstrated in cultured cells, and the combination has been shown to be at least additive in animal models [15-20]. Preliminary clinical studies indicate that dFdCyd can act as a radiosensitizer in patients as well [21-24].

In tumor cells, dFdCyd is phosphorylated to two biologically important metabolites, dFdCDP and dFdCTP [25]. dFdCDP has been shown to be a potent inhibitor of ribonucleotide reductase, and this inhibition results in depletion of deoxynucleotides essential for DNA synthesis [25,26]. In solid tumor cells, inhibition

38

of ribonucleotide reductase results in dATP depletion primarily and a slowing of DNA synthesis [14,27]. dFdCTP can inhibit DNA synthesis either through competition with dCTP for DNA polymerases or via the incorporation of dFdCMP into the elongating DNA strand [28,29]. The mechanism for radiosensitization by dFdCyd has not yet been fully elucidated. Many radiosensitizers work through increasing radiation-mediated damage or interfering with the rate of DNA repair. However, dFdCyd does not increase radiation-induced double strand breaks or inhibit their repair [11,30]. Correlative studies have shown that the dFdCDP-mediated decrease in dATP is important for radiosensitization [11,14,27,31]. Cell cycle studies have suggested that S-phase accumulation prior to irradiation is important for radiosensitization as well [14,32,33].

Previous work from this and other laboratories has evaluated the role of p53 expression on cytotoxicity and radiosensitization with dFdCyd [34-41]. These studies have demonstrated that expression of wild-type p53 can enhance cytotoxicity of dFdCyd in some, but not all, cell lines. A clear role for p53 has not been demonstrated in radiosensitization with dFdCyd. In D54 glioblastoma cells, which express wild-type p53, addition of dFdCyd at the IC_{50} did not allow accumulation of cells in S-phase, and these cells were not radiosensitized by dFdCyd. Evaluation of isogenic MCF-7 cell lines expressing either wild-type or mutant p53 demonstrated that both cell lines were equally well radiosensitized by dFdCyd at non-cytotoxic or moderately cytotoxic (IC_{50}) concentrations [40]. Chen *et al* used matched RKO colorectal carcinoma cell lines that were p53 wild-type or null and reported that neither cell line could be radiosensitized with dFdCyd [34].

The latter two studies demonstrated that, under conditions in which dFdCyd treatment induced p53 expression, it did not alter S-phase accumulation prior to irradiation or subsequent radiosensitization. However, these studies were carried out using concentrations of dFdCyd ranging from 10 to 80 nM, far lower than the typical clinically useful doses resulting in plasma levels in the µM range. We noted in the MCF-7 cells that 80 nM dFdCyd induced p53 expression slowly, requiring approximately 8 hours for maximal expression, and cells were able to progress into S-phase during drug incubation. It was then important to ask whether higher concentrations of dFdCyd, more similar to the typical plasma levels achieved in patients, would produce a more rapid induction of p53 expression that may induce a G1 block as observed in D54 cells. If that occurred, we would predict that dFdCyd would not act as a radiosensitizer. The studies presented here have evaluated a high concentration of dFdCyd (IC_{90}) which promotes a more rapid and higher induction of p53 compared to dFdCyd at its IC_{10} and IC_{50}, and then assessed subsequent effects of high dose dFdCyd on cell cycle progression and radiosensitization. A preliminary account of these findings has been reported previously in abstract form [42].

Materials and Methods

Cell Culture and Drug Preparation. The MCF-7 breast carcinoma cells were cultured in RPMI medium (GIBCO Laboratories, Grand Island, NY), supplemented with 10% calf serum (GIBCO), and 2 mM L-glutamine (Fisher Scientific, Fair Lawn, NJ). Cells were maintained in logarithmic growth as a monolayer in T75 culture flasks at 37°C in a humidified atmosphere containing

40

5% CO_2. Gemcitabine was a generous gift from Eli Lilly and Co. (Indianapolis, IN), which was dissolved in PBS to obtain a stock solution of 10 mM and sterilized before diluting further with PBS to achieve final concentrations of 1, 10, and 100 μM.

Cell Survival Assay. Cells were assayed for clonogenic survival as described previously [14]. Briefly, cells were plated in T25 culture flasks at a concentration of 200,000 cells per flask and allowed to grow for approximately two days. After treatment with dFdCyd and/or ionizing irradiation, cells were reseeded at known densities and allowed to grow for 10-14 days and colonies were stained with crystal violet. Cell survival was expressed as a percentage of the control survival, after correcting for plating efficiency. Radiation survival data from dFdCyd-treated cells were corrected for plating efficiency using an unirradiated plate treated with dFdCyd under the same conditions. Cell survival curves were fit using a linear-quadratic equation [43]. Radiation sensitivity is expressed in terms of the mean inactivation dose, which represents the area under the cell survival curve.   Radiosensitization is expressed as an enhancement ratio, which is defined as the mean inactivation dose (control)/mean inactivation dose (dFdCyd).

Irradiation of Cells. Monolayer cultures of cells were irradiated using Co^{60} (AECL Theratron 80) at 1-2 Gy/min. Dosimetry was carried out using an ionizing chamber connected to an electrometer system that was directly traceable to a National Institute of Standards and Technology standard. All cells were irradiated at room temperature.

Western Blot Analysis. After harvesting, cell pellets were incubated with 50 μl

lysis buffer, vortexed, and centrifuged. The supernatant was used for analysis

after determination of protein concentration with a Bio-Rad (Bio-Rad

Laboratories, Hercules, CA) protein assay. For each sample, 50 µg protein was

loaded onto a 10% polyacrylamide gel. After electrophoresis for 2 hours at 150

V, the protein was transferred onto Immobilon-P transfer membrane (Millipore

Corp., Bedford, MA) for 2 hours at 125 mA. The membranes were incubated

overnight with a 5% milk solution at 4°C to block n on-specific binding sites. The

membrane was then incubated with a 1:500 dilution of p53 (Ab-6) monoclonal

mouse IgG antibody (Calbiochem) for 2 hours followed by incubation with a

1:20000 dilution of secondary anti-mouse IgG horseradish peroxidase linked

antibody for 1 hour at room temperature. After the primary and secondary

antibody incubations, the membranes were washed with Tris-buffered saline with

0.1% Tween-20 three times for 15 minutes each. Proteins that bound the

antibodies were visualized using an enhanced chemiluminescence detection

system (Pierce, Rockford, IL).

Flow Cytometry Analysis. Control or drug-treated cells were incubated in the

dark with 30 µM bromodeoxyuridine (BrdUrd) for 15 minutes prior to the

conclusion of the dFdCyd incubation period or for 15 minutes prior to irradiation,

and processed as described previously [44]. Briefly, following BrdUrd exposure,

cells were harvested, fixed in ethanol and stored at 4°C. On the day of analysis,

cells were incubated with PBS containing 0.5 mg/ml RNase A (Boerhinger-

Mannheim, Germany) and centrifuged, followed by 0.1 N HCl containing 0.7%

Triton-X 100 (Sigma Chemical Co.). Following centrifugation, cells were

suspended in HPLC water and centrifuged. The supernatant was removed and cell pellets were incubated with a mouse anti-BrdUrd (PharMingen, San Diego, CA) or anti-p53 (Calbiochem) antibody, followed by a FITC-conjugated goat anti-mouse antibody (Sigma). After centrifugation, cells were resuspended in 0.5 ml propidium iodide (18 μg propidium iodide (Sigma), 40 mg RNAse A/ml) in PBS then analyzed with the Coulter (Beckman Coulter, Fullerton, CA) EPICS Elite flow cytometer by the University of Michigan Biomedical Research Core Facilities Flow Laboratory.

Results

Effect of dFdCyd on p53 Expression

Previously, we demonstrated that dFdCyd at its IC_{10} poorly induced p53 expression in MCF-7 cells, however higher p53 expression was induced by IC_{50} dFdCyd and 5 Gy irradiation [40]. In the current study, MCF-7 cells were incubated for 24 hr with dFdCyd at its IC_{10} (10 nM), IC_{50} (80 nM), or IC_{90} (300 nM) and p53 expression was evaluated periodically. Using Western blot analysis, an increase in p53 expression was evident within 2 h of exposure to the IC_{50} for dFdCyd, and p53 expression remained elevated for the remained of the drug exposure period (Fig 2.1). Conversely, p53 expression did not increase until 8 hr after drug addition at the IC_{10} of dFdCyd. Incubation with the IC_{90} for dFdCyd resulted in a larger and more rapid increase in p53 expression compared to IC_{50} treatment. Treatment with 5 Gy also resulted in a rapid and substantial increase in p53 expression within 2 h after irradiation, and remained elevated throughout the 24 hr timecourse. The IC_{90} for dFdCyd was used in the subsequent studies to
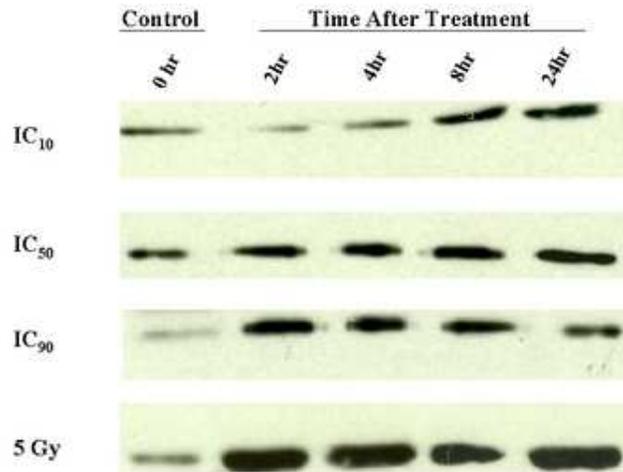
Figure 2.1. Effect of dFdCyd or radiation on p53 expression. Western blots were performed in MCF-7 cells treated with either dFdCyd at its A) IC_{10}, B) IC_{50}, C)IC_{90}, or D) 5 Gy irradiation. Cells were harvested at times indicated and analyzed via Western blotting as described in "Materials and Methods."

determine the effect of high and rapid induction of p53 on cell cycle progression and radiosensitization with dFdCyd.

Cell Cycle Analysis

Dual parameter (PI/BrdUrd) flow cytometry was used to evaluate the effect of the IC_{90} of dFdCyd on cell cycle progression. Following addition of 300 nM dFdCyd, there was a steady accumulation of cells into S phase throughout the incubation period, while the percentage of cells in G1 and G2/M phases decreased (Fig 2.2A, Table 2.1). By 24 h after dFdCyd addition, greater than 80% of the MCF-7 cells had accumulated in S-phase. As expected, this highly cytotoxic concentration of dFdCyd resulted in a profound decrease in DNA synthesis as evidenced by a decrease in BrdUrd incorporation to less than 20% of control levels by 4 hr after dFdCyd addition (Fig. 2.2A). Cell number remained constant over this time period, consistent with the inhibition of DNA synthesis. Following dFdCyd washout, cells began to progress through S-phase and then into G_2/M and G1, as evidenced by the flow cytometry results and 70% increase in cell number 24 hr post-washout.

In contrast to the results with dFdCyd, treatment with 5 Gy resulted in a decrease in the S-phase population, with accumulation of MCF-7 cells in G2/M and G1. The S-phase population began to decrease within 4 hr after irradiation, declining to <5% by 24 hr post irradiation (Fig. 2.2A, Table 2.1). Both G1 and G2/M populations increased after irradiation, with approximately 47 % of cells in G1 and 30% of cells in G2/M by 24 hr post-irradiation. Cells appeared to progress through the cell cycle by 48 h after irradiation, with a decrease in the

45

Fig 2A
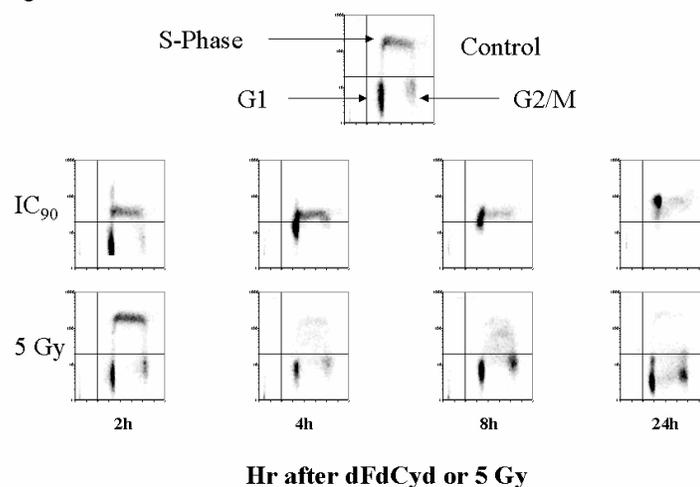
Fig 2B
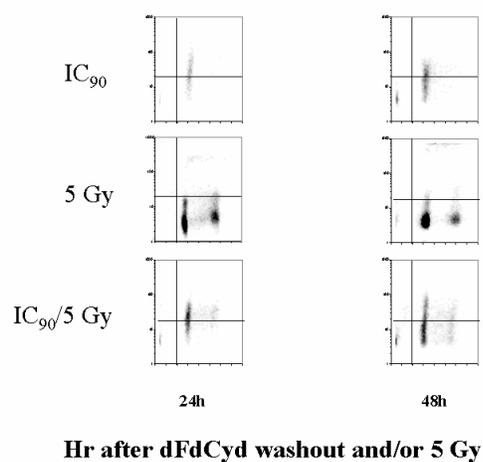
Figure 2.2. Effect of IC$_{90}$ dFdCyd and/or 5 Gy Ionizing Irradiation on Cell Cycle Distribution. MCF-7 cells were either incubated for up to 24 hr with 300 nM dFdCyd, exposed to 5 Gy ionizing radiation, or dFdCyd for 24 hr followed by irradiation, then assayed for cell cycle distribution using dual parameter flow cytometry. Histograms shown are from times A) during dFdCyd incubation or B) following washout. Times represent hours after exposure to dFdCyd or irradiation. The X-axis represents increasing PI incorporation, and the Y-axis represents increasing BrdUrd incorporation.

46

IC$_{90}$ dFdCyd

Time (hr)	G1	S	G2/M	A	S$_{NI}$
0	37.5	35.0	8.3	10.2	9.0
2	54.2	38.5	5.5	0.8	0.9
4	44.6	50.3	2.2	1.9	1.0
8	22.7	71.7	0.4	4.4	0.4
24	7.0	84.3	0.2	6.9	1.4

5 Gy

Time (hr)	G1	S	G2/M	A	S$_{NI}$
0	35.0	49.6	10.6	3.4	4.1
2	32.0	48.5	13.9	3.5	2.1
4	41.1	22.3	22.3	5.0	9.4
8	43.5	19.2	25.5	2.7	9.2
24	47.5	4.5	29.8	2.2	5.9

Table 2.1. Cell Cycle Distribution of MCF-7 Cells During Exposure to dFdCyd or Ionizing Radiation. MCF-7 cells were either incubated for up to 24 hr with 300 nM dFdCyd or exposed to 5 Gy ionizing radiation, then assayed for cell cycle distribution using dual parameter flow cytometry. Time 0 hr represents the time of addition of dFdCyd or exposure to radiation.

IC$_{90}$

Time (hr)	G1	S	G2/M	A	S$_{NI}$
24	37.6	43.1	3.9	10.7	4.6
48	42.4	30.3	3.2	14.1	10.1

5 Gy

Time (hr)	G1	S	G2/M	A	S$_{NI}$
24	64.8	8.5	10.7	7.8	5.0
48	56.7	15.3	15.5	5.3	4.9

IC$_{90}$/5Gy

Time (hr)	G1	S	G2/M	A	S$_{NI}$
24	11.0	77.7	1.8	6.4	3.1
48	22.6	53.4	6.4	14.0	3.6

Table 2.2. Cell Cycle Distribution of MCF-7 Cells Following a 24 hr Exposure to dFdCyd and/or Ionizing Radiation. MCF-7 cells were incubated with 300 nM dFdCyd for 24 h and/or irradiated with 5 Gy ionizing radiation, then assayed for cell cycle distribution using dual parameter flow cytometry. Times indicate hours following drug removal.

percentage of cells in G2/M while cells in G1 and S-phase increased (Fig 2.2B, Table 2.2). Cell number also increased by 60% at 48 h post-irradiation, indicating that cells were actively dividing at this timepoint. However, cells did not return to a normal cell cycle distribution pattern for at least 72 hr after irradiation (Fig. 2.2B, data not shown). After cells were incubated with dFdCyd at its IC_{90} for 24 h followed by irradiation with 5 Gy, the cell cycle distribution pattern was similar to that observed with dFdCyd alone (Fig. 2B, Table 3). Greater than 75% of the cells remained in S-phase for at least 24 h after drug washout. Although the G1 and G2/M populations began to increase at 48 hr post-washout, their percentages remained below those observed in untreated cells.

After exposure to dFdCyd or 5 Gy alone or in combination, there was no significant increase in the apoptotic population throughout the time course studied. However, the S_{NI} population (cells with S-phase DNA content but not incorporating BrdUrd) appeared to increase somewhat after 24 hr in cells that were irradiated compared to controls.

Radiosensitization

After observing that the IC_{90} of dFdCyd elevated p53 expression but did not prevent cells from accumulating in S-phase, the ability of this concentration of dFdCyd to radiosensitize MCF-7 cells was determined. In previous studies, MCF-7 cells were radiosensitized at IC_{10} and IC_{50} concentrations, with radiation enhancement ratios (RER) of 1.9 $\pm$ 0.1 and 1.7 $\pm$ 0.3, respectively [40]. Radiation sensitivity was greatly enhanced with IC_{90} dFdCyd, with an RER value of 2.7 $\pm$ 0.1, a marked increase over that observed at either the IC_{10} or IC_{50}.

Cell Cycle Dependent p53 Expression

Since treatment of MCF-7 cells with either the IC_{90} for dFdCyd or 5 Gy radiation induced a similar pattern of p53 expression but resulted in drastically different cell cycle distribution patterns, we wished to determine whether there was a difference in the phase of the cell cycle in which p53 was induced with these treatments. For this study, a two-parameter flow cytometric procedure was used in which MCF-7 cells were stained with PI to determine cell cycle position, and p53 expression was measured by antibody fluorescence. Using this technique, a low level of p53 expression was observed in most cells in the absence of radiation or drug treatment (data not shown). Therefore, we expressed the results as the -fold increase in p53 expression in cells in each cell cycle phase after dFdCyd addition or irradiation compared to control untreated cells. Incubation with dFdCyd at its IC_{90} resulted in increases in p53 expression in all three cell cycle phases (Table 3.3). Increased p53 expression was detected as early as 4 h after dFdCyd addition, and it remained elevated for the remainder of the incubation. In contrast, treatment with ionizing irradiation resulted in increases in p53 expression primarily in G1 and G2/M. Induction of p53 in G1 cells was evident within 2 hr after irradiation, and an elevated level of p53 was sustained for at least 24 hr. Increases in p53 expression in G2/M were less dramatic and appeared to decrease after 2 hr, while only minimal changes were observed in S-phase cells.

	300 nM		
Time (hr)	G1	S	G2/M
0	1	1	1
2	1.1	1.1	1.2
4	1.6	1.4	1.6
8	1.5	1.5	1.5
24	2.2	1.9	1.6

	5 Gy		
Time (hr)	G1	S	G2/M
0	1	1	1
2	2.0	1.1	1.6
4	1.9	1.1	1.2
8	1.6	0.9	0.9
24	1.7	0.9	1.4

Table 2.3. Fold Increase in p53 Expression Following Exposure to 300 nM dFdCyd or 5 Gy Ionizing Irradiation. MCF-7 cells were treated with either 300 nM dFdCyd for up to 24 hr or exposed to 5 Gy irradiation. At the timepoints indicated, cells were harvested and assayed as described in Materials and Methods. Results are shown as the fold increase in p53 expression compared to control untreated cells.

Discussion

The tumor suppressor protein, p53, is induced following exposure to a variety of DNA damaging agents, including chemotherapy drugs and ionizing radiation. Expression of p53 following DNA damage results in a series of events that may lead to a G1 or G2 cell cycle arrest, or apoptosis [45]. While previous studies have not demonstrated a conclusive role for p53 in radiosensitization with dFdCyd, those studies used moderate doses of dFdCyd that induced low or a slow increase in expression of p53 [34-41]. Here we wished to determine whether a higher, more clinically relevant concentration of dFdCyd would result in greater induction of p53 and, consequently, prevent the S-phase accumulation that appears to be required for radiosensitization. The results demonstrated that a highly cytotoxic dose of dFdCyd induced p53 in S-phase cells in a time-dependent manner. However, the rapid induction of p53 following dFdCyd addition did not prevent S-phase accumulation, and therefore treatment with ionizing radiation after dFdCyd produced excellent radiosensitization.

The results demonstrated that the IC_{90} for dFdCyd induced higher levels of p53 compared to the IC_{10} or IC_{50} for dFdCyd. In the presence of the rapid and high induction of p53 and strong inhibition of DNA synthesis, cells readily progressed into S-phase at the IC_{90}. These results are similar to those observed at lower concentrations of dFdCyd in MCF-7 and other cell lines [34,40] but different from the wild-type p53 expressing D54 glioblastoma cells which exhibited a G1 block after dFdCyd exposure [33]. The mounting evidence indicates that p53 expression does not produce G1 accumulation following dFdCyd exposure, and

the results observed in the D54 cell line appear to be unique to that particular cell line.

The increase in p53 expression in S-phase cells following dFdCyd exposure likely reflects the two major cellular effects of dFdCyd on DNA replication. dFdCTP can inhibit replicative DNA polymerases, and its incorporation into DNA has been correlated with cytotoxicity [28]; both are events which may induce p53. In addition, inhibition of ribonucleotide reductase results in dNTP pool depletion and cessation of DNA synthesis in S-phase cells. Since imbalances in dNTP pools can lead to misincorporation events in DNA and eventually cell death [46], this may also lead to induction of p53. Thus, there are several mechanisms by which dFdCyd can induce p53 in S-phase cells. The induction of p53 by dFdCyd in G1 and G2/M cells is not as clear. It is possible that some cells were able to progress out of S-phase with unrepaired DNA lesions (e.g. mismatched nucleotides, dFdCMP in DNA). If these lesions were detected during G2, this may have resulted in induction of p53 in an effort to repair the DNA damage. This population may also represent cells in which p53 was induced during S-phase, and the cells simply progressed into G2/M with elevated p53 levels. Although the percentage of cells in G2/M decreased during drug exposure, the total number of cells in this cell cycle phase was low and therefore an influx of a small number of S-phase cells could have resulted in the increased p53 expression in G2/M cells. The slow increase in the percentage of G2/M cells is consistent with either of these explanations. The increase in p53 expression in G1 cells is more difficult to reconcile, as it is unlikely that cells progressed from

S-phase through G2/M to G1 with elevated p53. It is most likely that a portion of cells detected as G1 cells by this technique are actually early S-phase cells, since PI staining cannot distinguish between cells in early S-phase and G1 as they have similar DNA content. Consistent with this idea, the PI/BrdUrd method, which determines cell cycle distribution more accurately (Table 1), demonstrated a nearly 50% increase in the number of cells in early S-phase by 4 hr after dFdCyd addition, the time at which an increase in p53 expression in G1 cells was observed. Thus, we believe it is most likely that the increase in p53 expression in G1 cells actually represents p53 induction in early S-phase cells.

Whereas dFdCyd induced expression of p53 primarily in S- and G2/M cell cycle phases, following ionizing radiation p53 induction was apparent only in G1 and G2/M. As S-phase is generally considered the most radioresistant phase, it was not surprising to observe little induction of p53 following irradiation. The rapid and high induction of p53 in G1 and G2/M following ionizing radiation is consistent with the observed p53-induced blocks in those cell cycle phases.

Interestingly, radiosensitization increased dramatically at the IC_{90} for dFdCyd, with an enhancement ratio of 2.7 compared to the enhancement ratios of ≤ 1.9 observed at the IC_{10} and IC_{50}[40]. We have demonstrated previously that radiosensitization with dFdCyd correlates with depletion of dATP pools and accumulation of cells in S-phase, but not with incorporation of dFdCMP into DNA. At the IC_{50} for dFdCyd, dATP was depleted >80% within 2 hr after drug addition and decreased to undetectable levels for the remainder of the 24 hr incubation. Thus, dATP depletion would be at least as great at the IC_{90}. Accumulation of

cells in S-phase was also similar at the IC_{50} and IC_{90} (78% vs. 84%, respectively). An important difference between these drug exposures is the persistence of the S-phase accumulation following irradiation. At the IC_{50} for dFdCyd, cells began to progress through the cell cycle after irradiation, with the percentage of cells in S-phase decreasing to 50% and 12% at 24 and 48 hr after washout, respectively. However, at the IC_{90}, cells persisted in S-phase longer, with 78% and 53% of cells remaining in S-phase 24 and 48 hr after irradiation, respectively. We have hypothesized that radiosensitization with dFdCyd is due to misincorporation events during DNA replication in the presence of low dATP pools [47]. Longer persistence of cells in S-phase with low dATP pools while DNA was slowly replicating would likely result in a greater number of misincorporation events and thus greater radiosensitization, as we have observed with dFdCyd at the IC_{90}.

The results presented here demonstrate that high dose dFdCyd induced rapid and high induction of p53 primarily in S-phase and G2/M, and thus did not prevent S-phase accumulation or radiosensitization. Furthermore, radiosensitization increased in MCF-7 cells with the higher and more physiologically relevant IC_{90} of dFdCyd. This is an important finding since the high concentrations of dFdCyd used in these studies is more similar to that achieved in clinical studies combining dFdCyd with radiotherapy [21,23,48]. Our results suggest that dFdCyd-mediated radiosensitization, at doses typically used in patients, should not be impeded by p53 status.

Footnotes to Chapter II

[1]The footnotes used are: dFdCyd, 2',2'-difluoro-2'-deoxycytidine; dCyd, deoxycytidine; dFdCMP, 5'-monophosphate of dFdCyd; dFdCDP, 5'-diphosphate of dFdCyd; dFdCTP, 5'-triphosphate of dFdCyd; dATP, deoxyadenosine triphosphate; dCTP, 5'-triphosphate of deoxycytidine; BrdUrd, bromodeoxyuridine.

References

1. Glimelius, B. Chemotherapy in the treatment of cancer of the pancreas. J.Hepatobiliary.Pancreat.Surg., *5*: 235-241, 1998.

2. Rothenberg, M. L., Moore, M. J., Cripps, M. C., Andersen, J. S., Portenoy, R. K., Burris, H. A., III, Green, M. R., Tarassoff, P. G., Brown, T. D., Casper, E. S., Storniolo, A. M., and Von Hoff, D. D. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. Ann.Oncol., *7*: 347-353, 1996.

3. Stephens, C. D. Gemcitabine: a new approach to treating pancreatic cancer. Oncol.Nurs.Forum, *25*: 87-93, 1998.

4. Stucky-Marshall, L. New agents in gastrointestinal malignancies: Part 2: Gemcitabine in clinical practice. Cancer Nurs., *22*: 290-296, 1999.

5. Crino, L., Mosconi, A. M., Scagliotti, G., Selvaggi, G., Novello, S., Rinaldi, M., Della, G. M., Gridelli, C., Rossi, A., Calandri, C., De Marinis, F., Noseda, M., and Tonato, M. Gemcitabine as second-line treatment for advanced non-small-cell lung cancer: A phase II trial. J.Clin.Oncol., *17*: 2081-2085, 1999.

6. Gridelli, C., Gallo, C., Shepherd, F. A., Illiano, A., Piantedosi, F., Robbiati, S. F., Manzione, L., Barbera, S., Frontini, L., Veltri, E., Findlay, B., Cigolari, S., Myers, R., Ianniello, G. P., Gebbia, V., Gasparini, G., Fava, S., Hirsh, V., Bezjak, A., Seymour, L., and Perrone, F. Gemcitabine plus vinorelbine compared with cisplatin plus vinorelbine or cisplatin plus gemcitabine for advanced non-small-cell lung cancer: a phase III trial of the Italian GEMVIN Investigators and the National Cancer Institute of Canada Clinical Trials Group. J.Clin.Oncol., *21*: 3025-3034, 2003.

7. Shepherd, F. A. Phase II trials of single-agent activity of gemcitabine in patients with advanced non-small cell lung cancer: an overview. Anticancer Drugs, *6 Suppl 6*: 19-25, 1995.

8. Takada, M., Negoro, S., Kudo, S., Furuse, K., Nishikawa, H., Takada, Y., Kamei, T., Niitani, H., and Fukuoka, M. Activity of gemcitabine in non-small-cell lung cancer: results of the Japan gemcitabine group (A) phase II study. Cancer Chemother.Pharmacol., *41*: 217-222, 1998.

9. Van Kooten, M., Traine, G., Cinat, G., Cazap, E., Comba, A. Z., Vicente, H., Sena, S., Nievas, O. R., and Orlando, M. Single-agent gemcitabine in pretreated patients with non-small-cell lung cancer: results of an Argentinean multicentre phase II trial. Br.J.Cancer, *81*: 846-849, 1999.

10. Zatloukal, P., Kanitz, E., Magyar, P., Jassem, J., Krzakowski, M., Pawlicki, M., Petruzelka, L., Chovan, L., Pesek, M., Janko, C., and Krejcy, K.

Gemcitabine in locally advanced and metastatic non-small cell lung cancer: the Central European phase II study. Lung Cancer, *22*: 243-250, 1998.

11. Lawrence, T. S., Chang, E. Y., Hahn, T. M., and Shewach, D. S. Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine). Clin.Cancer Res., *3*: 777-782, 1997.

12. McGinn, C. J., Shewach, D. S., and Lawrence, T. S. Radiosensitizing nucleosides. J.Natl.Cancer Inst., *88*: 1193-1203, 1996.

13. Rockwell, S. and Grindey, G. B. Effect of 2',2'-difluorodeoxycytidine on the viability and radiosensitivity of EMT6 cells in vitro. Oncol.Res., *4*: 151-155, 1992.

14. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W., and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells. Cancer Res., *54*: 3218-3223, 1994.

15. Fields, M. T., Eisbruch, A., Normolle, D., Orfali, A., Davis, M. A., Pu, A. T., and Lawrence, T. S. Radiosensitization produced in vivo by once- vs. twice-weekly 2'2'-difluoro-2'-deoxycytidine (gemcitabine). Int.J.Radiat.Oncol.Biol.Phys., *47*: 785-791, 2000.

16. Gregoire, V., Beauduin, M., Rosier, J. F., De Coster, B., Bruniaux, M., Octave-Prignot, M., and Scalliet, P. Kinetics of mouse jejunum radiosensitization by 2',2'-difluorodeoxycytidine (gemcitabine) and its relationship with pharmacodynamics of DNA synthesis inhibition and cell cycle redistribution in crypt cells. Br.J.Cancer, *76*: 1315-1321, 1997.

17. Gregoire, V., Cvilic, S., Beauduin, M., De Coster, B., Gueulette, J., Octave-Prignot, M., and Scalliet, P. Effect of gemcitabine on the tolerance of the lung to single-dose irradiation in C3H mice. Radiat.Res., *151*: 747-749, 1999.

18. Joschko, M. A., Webster, L. K., Groves, J., Yuen, K., Palatsides, M., Ball, D. L., and Millward, M. J. Enhancement of radiation-induced regrowth delay by gemcitabine in a human tumor xenograft model. Radiat.Oncol.Investig., *5*: 62-71, 1997.

19. Mason, K. A., Milas, L., Hunter, N. R., Elshaikh, M., Buchmiller, L., Kishi, K., Hittelman, K., and Ang, K. K. Maximizing therapeutic gain with gemcitabine and fractionated radiation. Int.J.Radiat.Oncol.Biol.Phys., *44*: 1125-1135, 1999.

20. Milas, L., Fujii, T., Hunter, N., Elshaikh, M., Mason, K., Plunkett, W., Ang, K. K., and Hittelman, W. Enhancement of tumor radioresponse in vivo by gemcitabine. Cancer Res., *59*: 107-114, 1999.

21. Blackstock, A. W., Bernard, S. A., Richards, F., Eagle, K. S., Case, L. D., Poole, M. E., Savage, P. D., and Tepper, J. E. Phase I trial of twice-weekly gemcitabine and concurrent radiation in patients with advanced pancreatic cancer. J.Clin.Oncol., *17*: 2208-2212, 1999.

22. de Lange, S. M., van Groeningen, C. J., Meijer, O. W., Cuesta, M. A., Langendijk, J. A., van Riel, J. M., Pinedo, H. M., Peters, G. J., Meijer, S., Slotman, B. J., and Giaccone, G. Gemcitabine-radiotherapy in patients with locally advanced pancreatic cancer. Eur.J.Cancer, *38*: 1212-1217, 2002.

23. Eisbruch, A., Shewach, D. S., Bradford, C. R., Littles, J. F., Teknos, T. N., Chepeha, D. B., Marentette, L. J., Terrell, J. E., Hogikyan, N. D., Dawson, L. A., Urba, S., Wolf, G. T., and Lawrence, T. S. Radiation concurrent with gemcitabine for locally advanced head and neck cancer: a phase I trial and intracellular drug incorporation study. J.Clin.Oncol., *19*: 792-799, 2001.

24. McGinn, C. J., Zalupski, M. M., Shureiqi, I., Robertson, J. M., Eckhauser, F. E., Smith, D. C., Brown, D., Hejna, G., Strawderman, M., Normolle, D., and Lawrence, T. S. Phase I trial of radiation dose escalation with concurrent weekly full-dose gemcitabine in patients with advanced pancreatic cancer. J.Clin.Oncol., *19*: 4202-4208, 2001.

25. Heinemann, V., Xu, Y. Z., Chubb, S., Sen, A., Hertel, L. W., Grindey, G. B., and Plunkett, W. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. Mol.Pharmacol., *38*: 567-572, 1990.

26. Baker, C. H., Banzon, J., Bollinger, J. M., Stubbe, J., Samano, V., Robins, M. J., Lippert, B., Jarvi, E., and Resvick, R. 2'-Deoxy-2'-methylenecytidine and 2'-deoxy-2',2'-difluorocytidine 5'-diphosphates: potent mechanism-based inhibitors of ribonucleotide reductase. J.Med.Chem., *34*: 1879-1884, 1991.

27. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W., and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine. Int.J.Radiat.Oncol.Biol.Phys., *34*: 867-872, 1996.

28. Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B., and Plunkett, W. Action of 2',2'-difluorodeoxycytidine on DNA synthesis. Cancer Res., *51*: 6110-6117, 1991.

29. Ruiz, v. H., V, Veerman, G., Vermorken, J. B., and Peters, G. J. 2',2'-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem.Pharmacol., *46*: 762-766, 1993.

30. Rosier, J. F., Michaux, L., Ameye, G., Cedervall, B., Libouton, J. M., Octave-Prignot, M., Verellen-Dumoulin, C., Scalliet, P., and Gregoire, V. The radioenhancement of two human head and neck squamous cell carcinomas by 2'-2' difluorodeoxycytidine (gemcitabine; dFdC) is mediated

by an increase in radiation-induced residual chromosome aberrations but not residual DNA DSBs. Mutat.Res., *527*: 15-26, 2003.

31. Shewach, D. S. and Lawrence, T. S. Radiosensitization of human tumor cells by gemcitabine in vitro. Semin.Oncol., *22*: 68-71, 1995.

32. Latz, D., Fleckenstein, K., Eble, M., Blatter, J., Wannenmacher, M., and Weber, K. J. Radiosensitizing potential of gemcitabine (2',2'-difluoro-2'-deoxycytidine) within the cell cycle in vitro. Int.J.Radiat.Oncol.Biol.Phys., *41*: 875-882, 1998.

33. Ostruszka, L. J. and Shewach, D. S. The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine. Cancer Res., *60*: 6080-6088, 2000.

34. Chen, M., Hough, A. M., and Lawrence, T. S. The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. Cancer Chemother.Pharmacol., *45*: 369-374, 2000.

35. Fechner, G., Perabo, F. G., Schmidt, D. H., Haase, L., Ludwig, E., Schueller, H., Blatter, J., Mller, S. C., and Albers, P. Preclinical evaluation of a radiosensitizing effect of gemcitabine in p53 mutant and p53 wild type bladder cancer cells. Urology, *61*: 468-473, 2003.

36. Galmarini, C. M., Clarke, M. L., Falette, N., Puisieux, A., Mackey, J. R., and Dumontet, C. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. Int.J.Cancer, *97*: 439-445, 2002.

37. Kielb, S. J., Shah, N. L., Rubin, M. A., and Sanda, M. G. Functional p53 mutation as a molecular determinant of paclitaxel and gemcitabine susceptibility in human bladder cancer. J.Urol., *166*: 482-487, 2001.

38. Lin, X., Ramamurthi, K., Mishima, M., Kondo, A., Christen, R. D., and Howell, S. B. P53 modulates the effect of loss of DNA mismatch repair on the sensitivity of human colon cancer cells to the cytotoxic and mutagenic effects of cisplatin. Cancer Res., *61*: 1508-1516, 2001.

39. Rieger, J., Durka, S., Streffer, J., Dichgans, J., and Weller, M. Gemcitabine cytotoxicity of human malignant glioma cells: modulation by antioxidants, BCL-2 and dexamethasone. Eur.J.Pharmacol., *365*: 301-308, 1999.

40. Robinson, B. W. and Shewach, D. S. Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines. Clin.Cancer Res., *7*: 2581-2589, 2001.

41. Tolis, C., Peters, G. J., Ferreira, C. G., Pinedo, H. M., and Giaccone, G. Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur.J.Cancer, *35*: 796-807, 1999.

42. Barber Jr, J, Robinson, B. W., Ostruszka, L., and Shewach, D. The Effects of Gemcitabine on p53 Expression, Cell Cycle Progression, and Radiosensitization in MCF-7 Breast Cancer Cells. Proceedings for the American Association for Cancer Research 43, 797-798. 2002.

43. Fertil, B., Dertinger, H., Courdi, A., and Malaise, E. P. Mean Inactivation Dose - A Useful Concept for Intercomparison of Human Cell-Survival Curves. Radiation Research, *99*: 73-84, 1984.

44. Hoy, C. A., Seamer, L. C., and Schimke, R. T. Thermal-Denaturation of Dna for Immunochemical Staining of Incorporated Bromodeoxyuridine (Brdurd) - Critical Factors That Affect the Amount of Fluorescence and the Shape of Brdurd Dna Histogram. Cytometry, *10*: 718-725, 1989.

45. Ljungman, M. Dial 9-1-1 for p53: mechanisms of p53 activation by cellular stress. Neoplasia., *2*: 208-225, 2000.

46. Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., Seno, T., Garrett, C., and Wataya, Y. Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. J.Biol.Chem., *262*: 8235-8241, 1987.

47. Flanagan S.A, Robinson, B. W., and Shewach, D. The presence and persistence of mismatched nucleotides in DNA correlate with gemcitabine radiosensitization. Proceedings of the American Association for Cancer Research 46, 395-396. 2005.

48. Blackstock, A. W., Lesser, G. J., Fletcher-Steede, J., Case, L. D., Tucker, R. W., Russo, S. M., White, D. R., and Miller, A. Phase I study of twice-weekly gemcitabine and concurrent thoracic radiation for patients with locally advanced non-small-cell lung cancer. Int.J.Radiat.Oncol.Biol.Phys., *51*: 1281-1289, 2001.

Chapter III

ROLE OF p53R2 IN RADIOSENSITIZATION WITH dFdCyd

Summary

Ribonucleotide reductase is a tetrameric protein consisting of two regulatory subunits (R1) and two catalytic subunits (R2). Expression of R1 is constant throughout the cell cycle, whereas R2 is expressed primarily in S-phase. Recently, a novel ribonucleotide reductase gene, p53R2, was discovered. p53R2 is a homologue of the ribonucleotide reductase R2 subunit, and it was shown to be induced in a p53-dependent fashion following DNA damage by ionizing radiation with a concomitant decrease in R2. However, little is known about the effect of RR inhibitors on p53R2 expression. In particular, we wished to evaluate the effect of 2',2'-difluoro-2'-deoxycytidine (dFdCyd), a nucleoside analog that can inhibit RR and be incorporated into DNA, on p53R2 expression. MCF-7 cells were incubated for 24 hr with dFdCyd at the IC_{10}, IC_{50} or IC_{90} or treated with 5 Gy radiation, and then evaluated for expression of p53, p53R2 and R2 by Western blot analysis and cell cycle distribution by dual parameter flow cytometry. p53R2 was expressed within 4 hr after dFdCyd addition at the IC_{10}, IC_{50} or IC_{90} or after irradiation with 5 Gy and persisted for at least 72 hr. Exposure to dFdCyd resulted in an increase in S-phase content and an increase in R2 expression, whereas exposure to ionizing radiation resulted in a decrease in S-phase content and downregulation of R2. We also wanted to evaluate

whether p53R2 expression altered dNTP depletion and radiosensitization in wt p53 cell lines compared to their p53-deficient counterparts. Although p53R2 was induced within 2 hr of dFdCyd exposure in wt p53 cell lines, dATP depletion and radiosensitization were similar to that seen in p53-deficient cell lines. Taken together, these results suggest that p53R2 and R2 can be expressed simultaneously, and that p53R2 induction does not alter dNTP depletion in response to dFdCyd exposure.

Introduction

Gemcitabine (dFdCyd, 2', 2'-difluoro-2'-deoxycytidine) is a nucleoside analog with activity against a wide variety of malignancies, including pancreatic [1,2] and non-small cell lung cancers [3-5]. dFdCyd has shown excellent activity both *in vitro* and *in vivo*, either as a single agent or in combination with other drugs such as cisplatin [6] and docetaxel [7]. In addition, many preclinical studies have revealed the ability of dFdCyd to sensitize cultured cells to the effects of radiation, a process known as radiosensitization [8-11]. Clinical studies indicate that this radiosensitizing effect can be observed *in vivo* as well [12-17].

While the radiosensitizing property of dFdCyd has been demonstrated in many different *in vitro* and *in vivo* studies, the mechanism by which this occurs has remained elusive. Distinguishing it from other radiosensitizers, dFdCyd does not enhance radiation-induced damage or alter the rate of repair [18,19]. Two major biologic effects occur with dFdCyd. Following intracellular phosphorylation, the diphosphate (dFdCDP) is a potent inhibitor of ribonucleotide reductase, the rate-limiting enzyme in the de novo biosynthesis of deoxynucleotides [20,21]. This

inhibition results in depletion of dNTP pools, particularly dATP, and inhibition of DNA synthesis [8,11]. The triphosphate (dFdCTP) is incorporated into DNA, and it is thought that this effect contributes to the majority of the cytotoxicity observed with dFdCyd [22]. Previous studies by this and other labs have shown that dATP depletion [11,23,24] and S-phase accumulation prior to irradiation correlate strongly with radiosensitization [8,11,18].

Ribonucleotide reductase is responsible for the conversion of ribonucleotide diphosphates (rNDPs) to their corresponding deoxyribonucleotide diphosphates (dNDPs) through radical-based chemistry, providing a balanced supply of precursors for both DNA synthesis and repair [25,26]. Ribonucleotide reductase exists as a heterotetramer ($\alpha2\beta2$) comprised of two catalytic subunits (R1) and two regulatory subunits (R2). Recently a p53-inducible RR subunit, p53R2, was discovered, and it has been shown to be activated following DNA damage caused by exposure to ultraviolet or ionizing radiation, or cytotoxic concentrations of certain drugs [27]. p53R2 has high sequence homology with R2 except in the NH_2-terminal region, and this difference has been postulated to be of regulatory importance [27]. Studies have demonstrated that p53R2 can bind with R1 to form an active heterotetramer [28]. The appearance of p53R2 following induction of p53 may be due in part to redistribution, since previous studies have shown that p53R2 is bound to p53 in undamaged cells and released following DNA damage [29]. p53R2 has also been proposed to have a role in development, as p53R2-null mice have several developmental deficiencies, and die within 14 weeks post-birth due to renal failure [30].

In view of the important role for ribonucleotide reductase in radiosensitization with dFdCyd, we wished to determine whether p53R2 affected this process. We and others have demonstrated that dFdCyd, at concentrations $\geq IC_{50}$, induces wild-type p53 expression. While it may be presumed that induction of p53 by dFdCyd was accompanied by induction of p53R2 at the IC_{50}, p53R2 expression was not measured. In addition, it is not clear whether the low amount of p53 expression at the IC_{10} would be sufficient to induce p53R2. We also wanted to evaluate if induction of p53R2 resulted in decreased dATP depletion and decreased radiosensitization in wt p53 cell lines compared to p53-deficient cell types. Preliminary accounts of a portion of these findings have been presented previously in abstract form [31,32].

Materials and Methods

Cell Culture and Drug Preparation. The MCF-7 and MCF-7/Adr breast carcinoma cells were cultured in RPMI medium (GIBCO Laboratories, Grand Island, NY), supplemented with 10% calf serum (GIBCO), and 2 mM L-glutamine (Fisher Scientific, Fair Lawn, NJ). The HCT116 and HCT116 p53^{-/-} colon carcinoma cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO), supplemented with 10% fetal bovine serum (GIBCO), and 2mM L-glutamine (Fisher Scientific). Cells were maintained in logarithmic growth as a monolayer in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. Gemcitabine was a generous gift from Eli Lilly and Co. (Indianapolis, IN), which was dissolved in PBS to obtain a stock solution of 10 mM and sterilized before diluting further with PBS to achieve final concentrations of 1, 10, and 100 μM.

65

Irradiation of Cells. Monolayer cultures of cells were irradiated using Co^{60} (AECL Theratron 80) at 1-2 Gy/min. Dosimetry was carried out using an ionizing chamber connected to an electrometer system that was directly traceable to a National Institute of Standards and Technology standard. All cells were irradiated at room temperature.

Flow Cytometry Analysis. Control or drug-treated and/or irradiated cells were incubated in the dark with 30 μM bromodeoxyuridine (BrdUrd) for 15 minutes prior to the conclusion of the incubation period or for 15 minutes prior to irradiation, and processed as described previously [33].

Western Blot Analysis. After harvesting, lysis, and collection, the supernatant was used for analysis after determination of protein concentration with a Bio-Rad (Bio-Rad Laboratories, Hercules, CA) protein assay. After electrophoresis for 2 hours at 150 V, the protein was transferred onto Immobilon-P transfer membrane (Millipore Corp., Bedford, MA) for 2 hours at 125 mA. After overnight blocking, the membrane was then incubated with a 1:1000 dilution of either p53R2 (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal goat, R2 (Santa Cruz) monoclonal goat, p53 (Ab-6) monoclonal mouse (Calbiochem), or Actin IgG antibody (Calbiochem) for 2 hours followed by incubation with a 1:10000 dilution of the corresponding secondary IgG horseradish peroxidase linked antibody for 1 hour at room temperature. After washing, proteins that bound the antibodies were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Nucleotide Pool Analysis. Cellular nucleotides were assayed as described previously [11]. Briefly, cells were harvested by trypsinization and nucleotides were extracted using 0.4 N perchloric acid. Neutralized extracts were stored at $-20°C$ until analysis. Ribonucleotides were removed from the extracts using a boronate affinity column [34]. Cellular dNTPs and dFdCTP were separated and quantified by strong anion exchange HPLC using a Waters Alliance (Milford, MA) gradient system equipped with a photodiode array detector and controlled by Millennium 2010 software. Samples were loaded onto a Partisphere 4.6x250 mm strong anion exchange column (Whatman, Hillshore, OR) and nucleotides were eluted at 2 ml/min with a linear gradient of ammonium phosphate buffer ranging in concentration from 0.15 M (pH 2.8) to 0.60 M (pH 2.8). Nucleotides were identified on the basis of their UV absorbance spectrum and quantified at either 254 or 281 nm by comparison to the absorbance of a known amount of authentic standard. All nucleotide pool measurements represent the average of at least four determinations except for the 16 hour point (duplicate determinations).

Cell Survival Assay. After treatment with dFdCyd and/or ionizing irradiation, cells were plated in 6-well dishes and allowed to grow for 10-14 days and colonies were stained with crystal violet. Cell survival was expressed as a percentage of the control survival, after correcting for plating efficiency. Radiation survival data from dFdCyd-treated cells were corrected for plating efficiency using an unirradiated plate treated with dFdCyd under the same conditions. Cell survival curves were fit using a linear-quadratic equation [35]. Radiation sensitivity is expressed in terms of the mean inactivation dose, which

represents the area under the cell survival curve. Radiosensitization is expressed as an enhancement ratio, which is defined as the mean inactivation dose (control)/mean inactivation dose (dFdCyd).

Results

Cell cycle distribution and protein expression following exposure to dFdCyd

We wished to determine whether induction of p53 by dFdCyd resulted in expression of p53R2, and the relationship to R2. Since R2 is a cell cycle-regulated protein and dFdCyd produces an S-phase block, we also determined cell cycle position at each time point. Consistent with our previous results [36], dFdCyd produced an S-phase block resulting in accumulation of 73%, 80% and 85% of viable cells in S-phase by 24 hr after addition of the IC_{10}, IC_{50} and IC_{90}, respectively (Fig 3.1). Following drug washout, the S-phase population declined at the IC_{10} and IC_{50}, achieving control values or lower within 24 – 36 hr, respectively (Figs. 3.1A, 3.1B). At the IC_{90} for dFdCyd (Fig 3.1C), S-phase content remained elevated for 24 hr post-washout but declined to 16% by 36 hr.

Western blot analysis demonstrated that exposure to IC_{10} dFdCyd resulted in a low but noticeable increase in p53 expression within 2 hr after drug addition (Fig. 3.2A). Expression of p53R2 was apparent within 4 – 6 hr after drug addition, and it continued to increase over the 24 hr drug incubation period. At the IC_{50} and IC_{90} for dFdCyd, expression of p53 and subsequently p53R2 was more rapid and intense (Figs. 3.2B, 3.2C). Expression of R2 also increased during the drug incubation period for each dFdCyd concentration tested.

68
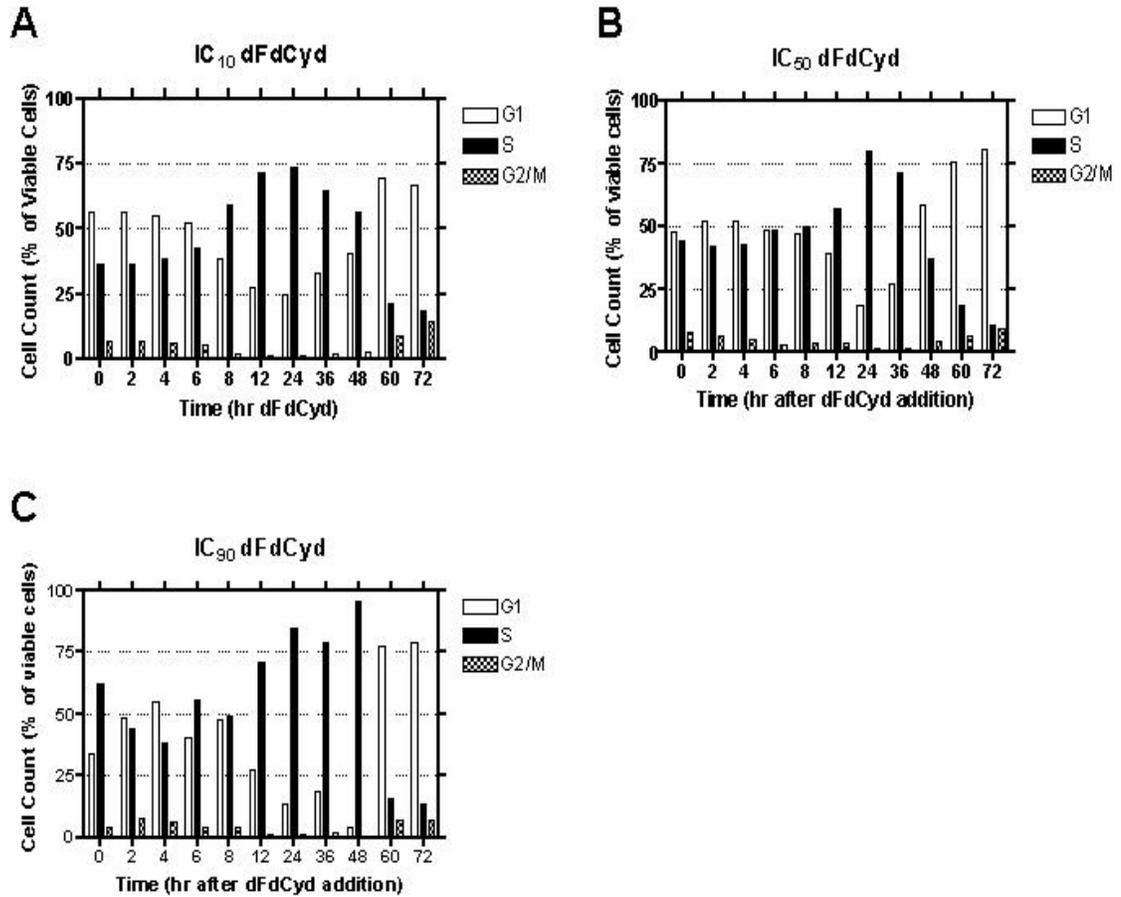
Figure 3.1. Cell Cycle Distribution Following Exposure to dFdCyd. MCF-7 cells were exposed to A) IC_{10}, B) IC_{50}, or C) IC_{90} dFdCyd for up to 24 hr, harvested and processed for flow cytometry at times indicated as stated in "Materials and Methods." Drug was removed after 24 hr, and measurements taken thereafter are during the period of recovery.
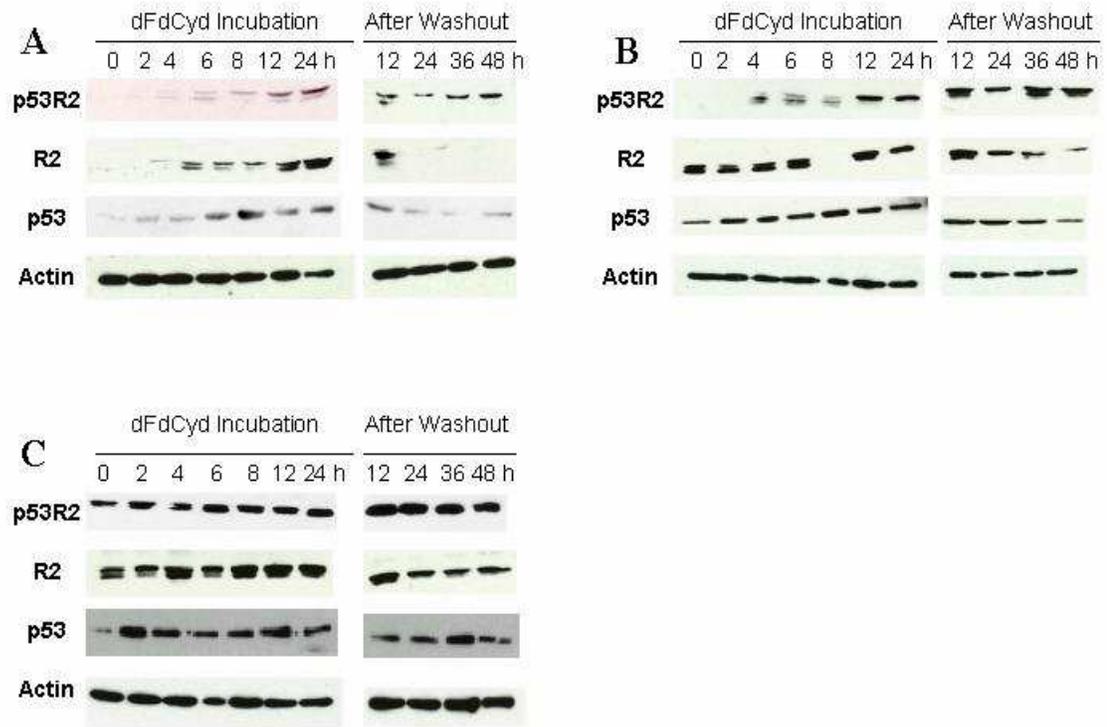
Figure 3.2. Protein Expression Following Exposure to dFdCyd. MCF-7 cells were exposed to A) IC_{10}, B) IC_{50}, or C) IC_{90} dFdCyd for up to 24 hr, harvested and processed for western blotting at times indicated as stated in "Materials and Methods." Actin was used as a loading control.

70

Following dFdCyd washout, there was a substantial decrease in p53 expression by 48 hr at the IC_{10} and IC_{50} (Figs. 3.2A, 3.2B), whereas p53 remained elevated after washout of the IC_{90} (Fig 3.2C). However, no decreases were observed in the expression of p53R2 after drug washout, as its expression was increased for at least 48 hr post-washout at all three concentrations of dFdCyd tested. R2 expression was apparent at 12 hr post-washout but decreased at later timepoints at the IC_{10}, IC_{50} and IC_{90} for dFdCyd (Fig 3.2).

Protein expression and cell cycle distribution following exposure to ionizing radiation

Following exposure of cells to 5 Gy ionizing radiation resulted in cells cycling out of S-phase and accumulating primarily in G1 and to a lesser extent in G2/M. By 6 hr post-irradiation, the S-phase fraction began to decrease from its initial level of approximately 35% (Fig 3.3). This change was accompanied by a transient increase in G2/M. The number of cells in G1 increased within 8 hr post-irradiation, and >85% of the cells remained in G1 through 60 hr. A moderate increase in S-phase cells (24%) at 72 hr post-irradiation indicated that cells were beginning to recover from irradiation. The G1 block is consistent with intact radiation-induced cell cycle checkpoints. Irradiation of MCF-7 cells with 5 Gy resulted in rapid and strong expression of p53 and p53R2 (Fig 3.4). Both proteins were expressed for at least 60 hr post-irradiation. In contrast, R2 expression was highest at 0 hr, became undetectable by 6 hr post-irradiation and was not visible again up to 72 hr post-irradiation. As expected for an S-phase
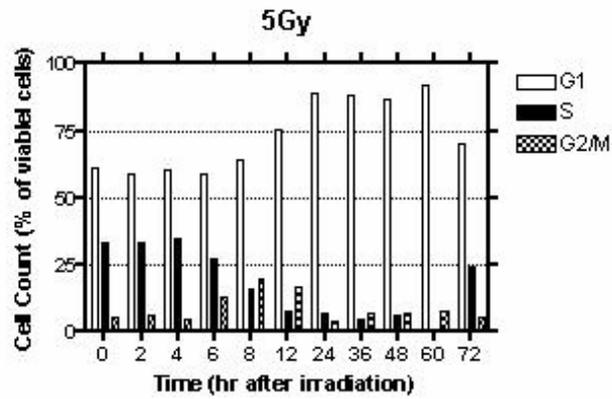
Figure 3.3. Cell Cycle Distribution Following Exposure to dFdCyd. MCF-7 cells were exposed to 5 Gy ionizing radiation, harvested and processed for flow cytometry at times indicated as stated in "Materials and Methods."
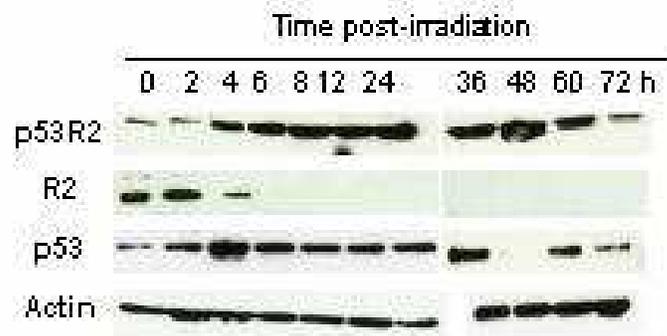
Figure 3.4. Protein Expression Following Exposure to dFdCyd. MCF-7 cells were exposed to 5 Gy ionizing radiation, harvested and processed for western blotting at times indicated as stated in "Materials and Methods." Actin was used as a loading control.

specific protein, this expression pattern for R2 corresponded to the change in cell cycle distribution.

Cell cycle distribution and protein expression following exposure to dFdCyd and radiation

In response to dFdCyd exposure, cells accumulated in S-phase, with approximately 25 % of cells remaining in G1 and G2/M at 24 hr dFdCyd exposure (Fig 3.5). After irradiation, the cell cycle appeared similar to that with radiation alone in which there was an increase in G1 cells within 24-48 hr after irradiation. Incubation of cells with dFdCyd at the IC_{10}, IC_{50} or IC_{90} for 24 hr followed by 5 Gy irradiation produced increases in p53 and p53R2 expression (Fig 3.6). While p53 appeared to decrease by 72 hr post-drug incubation and irradiation, p53R2 expression remained elevated for at least 72 hr. R2 expression increased during drug incubation as cells accumulated in S-phase, but decreases in R2 were observed by 36 hr post drug incubation and irradiation at all dFdCyd concentrations evaluated. The block of cells in G1 was maintained through 72 hr post-irradiation, while S-phase content remained depressed during this time.

p53R2 expression in matched cell lines

p53R2 expression was evaluated at the IC_{50} in two sets of cell lines matched for p53 status, MCF-7 (wt p53) and MCF-7/Adr (mt p53) breast cancer cells, and HCT116 (wt p53) and HCT116 p53$^{-/-}$ cells. Similar to Fig 3.1, p53R2 expression was induced within 2 hr of exposure to dFdCyd (Fig 3.7). Expression remained elevated for up to 24 hr of exposure. Expression of p53R2 was also induced

74
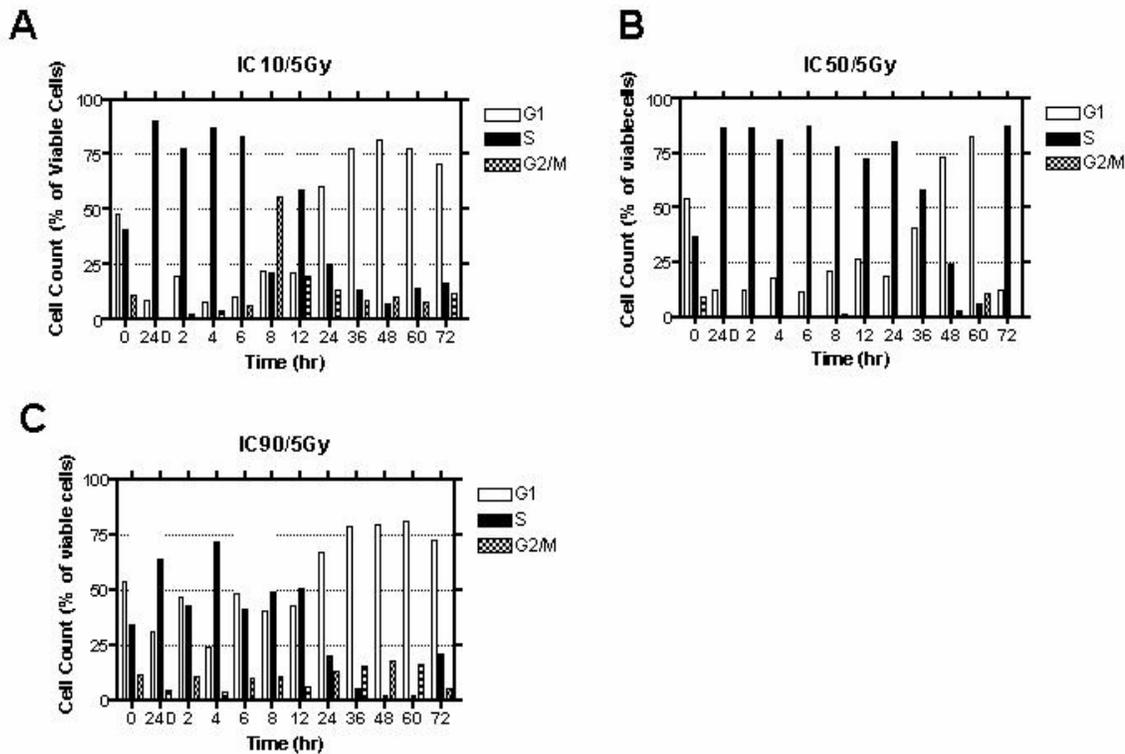
Figure 3.5. Cell Cycle Distribution Following Exposure to dFdCyd and Ionizing Radiation. MCF-7 cells were exposed to A) IC$_{10}$, B) IC$_{50}$, or C) IC$_{90}$ dFdCyd followed by 5 Gy ionizing radiation, harvested and processed for flow cytometry at times indicated as stated in "Materials and Methods."
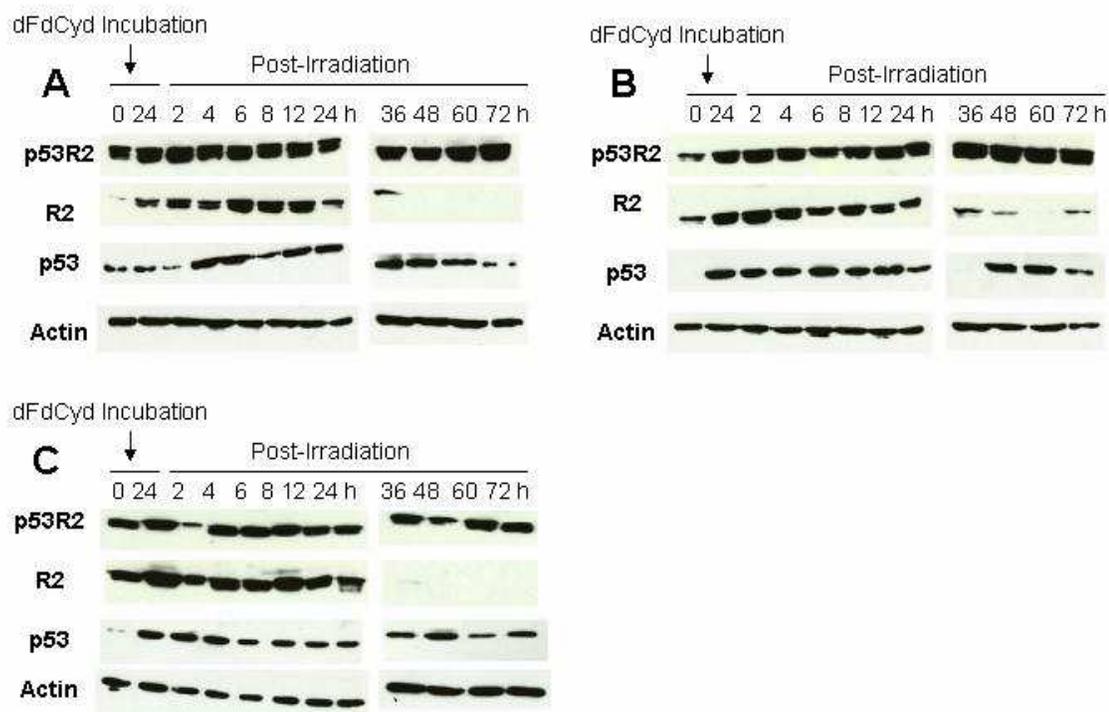
Figure 3.6. Protein Expression Following Exposure to dFdCyd and Ionizing Radiation. MCF-7 cells were exposed to A) IC_{10}, B) IC_{50}, or C) IC_{90} dFdCyd followed by 5 Gy ionizing radiation, harvested and processed for western blotting at times indicated as stated in "Materials and Methods." Actin was used as a loading control.
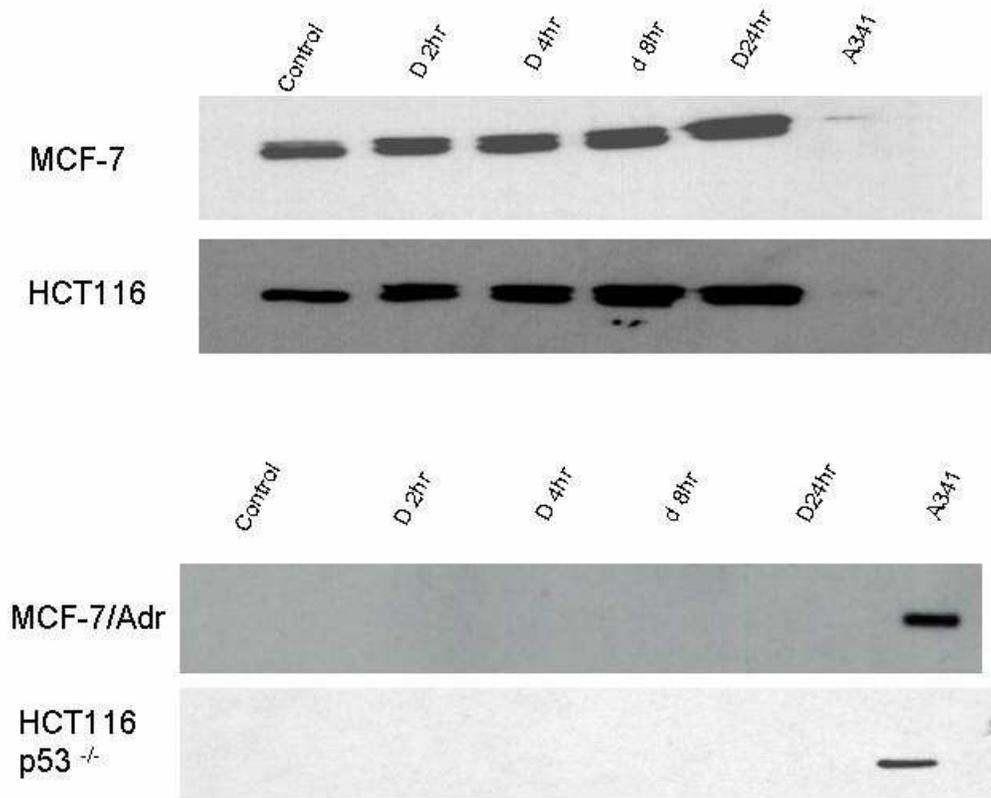
Figure 3.7. p53R2 Expression in Cell Lines Matched for p53 Status. Breast and colon cancer cell lines matched for p53 status were exposed to IC_{50} dFdCyd for up to 24 hr and assayed for p53R2 expression by western blotting. A341 lung cancer cells were used as a positive control for p53R2 expression.

within 2 hr of exposure to dFdCyd in the HCT116 cell line. No expression of

p53R2 was detected in the p53 deficient cell lines.

dATP depletion

After determining that p53R2 could be induced in response to dFdCyd in wt

p53 expressing cell lines, we next wanted to investigate whether induction of

p53R2altered dATP depletion. In response to IC_{10} dFdCyd, significant depletion

of dATP is observed within 4 hr of dFdCyd exposure in both breast cancer cell

lines (Fig 3.8A). By 8 hr of exposure, both cell lines exhibited depletion of dATP

to < 20% of control levels. dATP levels had recovered some at 24 hr of

exposure; however, the rebound effect was greater in the wt p53 cell line.

Exposure of the breast cancer cell lines to IC_{50} dFdCyd resulted in a more rapid

depletion of dATP. By 4 hr of exposure, dATP was depleted to approximately

10% of control values, and was near undetectable levels by 8 hr of exposure (Fig

3.8B). dATP remained depleted for the remainder of the 24 hr exposure. dATP

was also rapidly depleted in HCT116 cells after exposure to IC_{50} dFdCyd. By 4

hr of exposure, dATP had reached undetectable levels, and remained depleted

for the remainder of the exposure (Fig 3.8C). dATP was depleted much more

slowly in the HCT116 p53-/- cell line, most likely due to slower accumulation of

dFdCyd metabolites (data not shown).

Cytotoxicity and Radiosensitization

After determining that p53R2 was induced in response to dFdCyd in wild-type

p53 cell lines but dATP depletion was not affected, we next wanted to examine if

p53R2 induction altered cytotoxicity and radiosensitization. The breast cancer
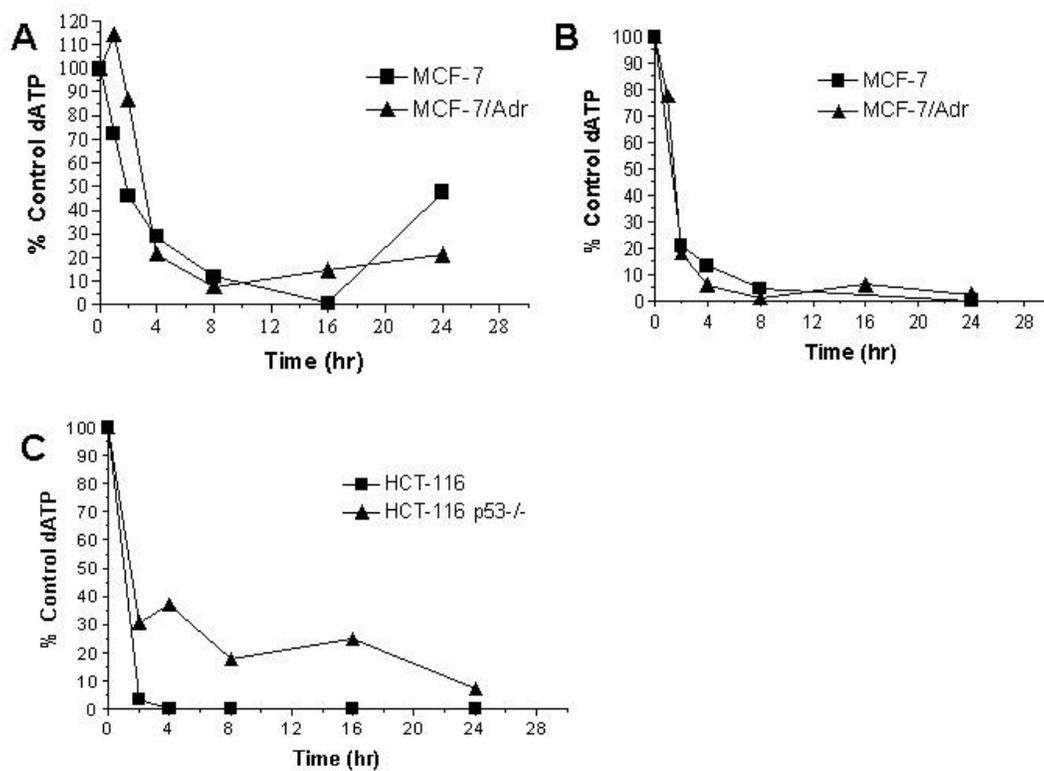
Figure 3.8. dATP depletion in response to dFdCyd exposure. Breast cancer cells were exposed to A) IC_{10} or B) IC_{50} dFdCyd, or C) colorectal cell lines were exposed to IC_{50} dFdCyd for up to 24 hr, and dATP levels were measured as described in "Materials and Methods."

Cell Line	[dFdCyd]	Radiation Enhancement Ratio	D-bar (no drug)
MCF-7 (p53+)	10 nM (IC10)	1.9 ± 0.1	2.6 ± 0.2
	80 nM (IC50)	1.7 ± 0.3	
MCF-7/Adr (p53-)	25 nM	1.6 ± 0.1	3.6 ± 0.2
	60 nM	1.6 ± 0.1	
HCT116 (p53+)	30 nM	1.3 ± 0.1	3.2 ± 0.2
	100nM	1.5 ± 0.1	
HCT116 p53 -/- (p53-)	300 nM	1.3	2.7 ± 0.1
	800 nM	1.5 ± 0.2	

Table 3.1. Cytotoxicity and Radiosensitization in Breast and Colon Cancer Cell Lines. Breast and colon cancer cells were assayed for cytotoxicity and radiosensitization as described in "Materials and Methods."

cell lines demonstrated similar sensitivities to dFdCyd as determined by the IC_{10} and IC_{50} (Table 3.1). However, MCF-7/Adr cells were less sensitive to radiation as determined by D-bar. Both cell lines exhibited excellent radiosensitization at the IC_{10} and IC_{50}, with radiation enhancement rations ≥ 1.6.

HCT116 cells were much more sensitive to dFdCyd compared to their p53 deficient counterparts. This again is probably due to the slower and lowered accumulation of dFdCyd metabolites in HCT116 p53$_{-/-}$ cells. HCT116 p53-/- cells were slightly more sensitive to ionizing radiation. Despite differences in sensitivity to dFdCyd and ionizing radiation when given alone, these cell lines exhibited a similar radiosensitization profile. Both colon cancer cell lines were only radiosensitized at $\geq IC_{50}$ dFdCyd concentrations.

Discussion

p53R2 is a novel homologue of the ribonucleotide reductase subunit R2 that is activated via p53 following DNA damage. p53R2 combines with R1 to form an active holoenzyme, and it has been proposed that this activation results in deoxynucleotide production for DNA repair [37]. It is likely that R1 is constitutively active so that it can form an active RR with p53R2 to provide dNTPs in any phase of the cell cycle, whereas R1 and R2 form the holoenzyme only active in S-phase. While p53R2 can be expressed in any cell cycle phase, R2 is activated in an S-phase dependent fashion and is rapidly degraded following progression out of S-phase. While previous studies demonstrated that expression of p53R2 was rapidly induced following DNA damage from ionizing radiation [27], this is the first study that has extensively examined the kinetics of p53R2 and R2

81

expression in response to a chemotherapeutic agent, either alone or in combination with ionizing radiation. Here we demonstrated that, after treating MCF-7 cells with dFdCyd which results in the accumulation of cells in S-phase, we were able to detect simultaneous expression of p53R2 and R2. Our results indicate that p53R2 and R2 can be induced simultaneously in response to S-phase directed agents. Our results also suggest that p53R2 induction does not alter dATP depletion or radiosensitization in wt p53 cell lines compared to their p53-deficient counterparts.

We have extensively studied the kinetics of expression of the two small ribonucleotide reductase proteins under radiosensitizing conditions. Surprisingly, we observed prolonged increases in p53R2 expression in response to non-cytotoxic concentrations of dFdCyd. p53R2 expression remained elevated for at least 48 hr post-washout, far longer than required for dNTP pools to stabilize. Expression of p53 R2 also remained elevated for at least 72 hr post-irradiation. This prolonged expression also was not dependent on p53, as p53 expression returned to control levels by 48 hr post-washout of low concentrations of dFdCyd. This finding implies that p53R2 may have other roles in the cell in addition to its role as a ribonucleotide reductase subunit. A previous study by Thelander *et al* suggests that p53R2 is involved in the G1/S transition, as their study found that p53R2 expression peaked when cells were at the G1/S border [38]. It is tempting to speculate that p53R2 may also be involved in repair induced by these insults, but previous studies have demonstrated that most radiation-induced damage is repaired within 4 hr post-irradiation.

Previous studies have demonstrated that, in wild-type p53 cells, both p53R2 and R2 associate with p53 in resting cells [29]. Following exposure to DNA damaging agents such as UV, both subunits translocate from the cytoplasm to the nucleus and this movement results in increased ribonucleotide reductase activity. We were able to detect increases in p53R2 expression within 4 hr after exposure to dFdCyd or ionizing radiation. These findings support the hypothesis of translocation, as it is unlikely that a transcriptional mechanism would result in rapid increases in expression. It is possible however that both translocation and transcription play a significant role in p53R2 upregulation. An interesting and perhaps unexpected result was the longevity of p53R2 upregulation following DNA damage, as we were able to detect p53R2 expression for at least 72 hr after exposure to DNA damaging agents. It is possible that early increases in p53R2 expression are due to translocation, and this activity is maintained via transcription.

Although p53R2 expression has been evaluated in a number of cell lines in response to DNA damage, very little work has been done to examine the exact effects of p53R2 expression on dNTP pools. Here we have studied dATP depletion in response to dFdCyd exposure in cell lines matched for p53 status, and subsequently the ability to induce p53R2 after DNA damage. Although p53R2 was induced within 2hr of dFdCyd exposure, dATP depletion was not altered in wt p53 cell lines compared to their p53-deficient counterparts. This suggests that p53R2 does not contribute significantly to dNTP pools. However, these cell lines have been cultured separately over a number of years and may

have acquired differences that altered cellular response to dFdCyd. It is also possible that cell lines have intrinsic differences that also can affect cellular response. It will be imperative to determine the exact role of p53R2 on maintaining dNTP pools in a single cell line.

By preparing samples for both western blotting and cell cycle analysis simultaneously, we have demonstrated that p53R2 and R2 can be induced simultaneously in response to a chemotherapeutic agent. Also, the data indicate that R2 correlates with the level of S-phase accumulation, whereas p53R2 expression is independent of cell cycle regulation. Our results also demonstrate that expression of p53R2 does not affect sensitivity to dFdCyd either as a single agent or in combination with ionizing radiation. This suggests that p53R2 activity does not significantly alter dNTP pools, as concluded by Thelander *et al* [38]. However, it is also possible that, in specific cell types, that p53R2 activity contributes sufficiently to the maintenance of dNTP pools following exposure to dFdCyd to prevent radiosensitization, and this hypothesis is currently the subject of ongoing investigation in this laboratory.

Footnotes to Chapter III

[1]The footnotes used are: dFdCyd, 2',2'-difluoro-2'-deoxycytidine; dCyd, deoxycytidine; dFdCMP, 5'-monophosphate of dFdCyd; dFdCDP, 5'-diphosphate of dFdCyd; dFdCTP, 5'-triphosphate of dFdCyd; dNTP, deoxynucleotide triphosphate; dATP, deoxyadenosine triphosphate, dCTP, deoxycytidine triphosphate; HPLC, high performance lipid chromatography; RR, ribonucleotide reductase.

References

1. Glimelius, B. Chemotherapy in the treatment of cancer of the pancreas. J.Hepatobiliary.Pancreat.Surg., *5*: 235-241, 1998.

2. Rothenberg, M. L., Moore, M. J., Cripps, M. C., Andersen, J. S., Portenoy, R. K., Burris, H. A., III, Green, M. R., Tarassoff, P. G., Brown, T. D., Casper, E. S., Storniolo, A. M., and Von Hoff, D. D. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer. Ann.Oncol., *7*: 347-353, 1996.

3. Shepherd, F. A. Phase II trials of single-agent activity of gemcitabine in patients with advanced non-small cell lung cancer: an overview. Anticancer Drugs, *6 Suppl 6*: 19-25, 1995.

4. Takada, M., Negoro, S., Kudo, S., Furuse, K., Nishikawa, H., Takada, Y., Kamei, T., Niitani, H., and Fukuoka, M. Activity of gemcitabine in non-small-cell lung cancer: results of the Japan gemcitabine group (A) phase II study. Cancer Chemother.Pharmacol., *41*: 217-222, 1998.

5. Zatloukal, P., Kanitz, E., Magyar, P., Jassem, J., Krzakowski, M., Pawlicki, M., Petruzelka, L., Chovan, L., Pesek, M., Janko, C., and Krejcy, K. Gemcitabine in locally advanced and metastatic non-small cell lung cancer: the Central European phase II study. Lung Cancer, *22*: 243-250, 1998.

6. Abratt, R. P., Bezwoda, W. R., Goedhals, L., and Hacking, D. J. Weekly gemcitabine with monthly cisplatin: effective chemotherapy for advanced non-small-cell lung cancer. J.Clin.Oncol., *15*: 744-749, 1997.

7. Sherman, W. H. and Fine, R. L. Combination gemcitabine and docetaxel therapy in advanced adenocarcinoma of the pancreas. Oncology, *60*: 316-321, 2001.

8. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W., and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine. Int.J.Radiat.Oncol.Biol.Phys., *34*: 867-872, 1996.

9. McGinn, C. J., Shewach, D. S., and Lawrence, T. S. Radiosensitizing nucleosides. J.Natl.Cancer Inst., *88*: 1193-1203, 1996.

10. Rockwell, S. and Grindey, G. B. Effect of 2',2'-difluorodeoxycytidine on the viability and radiosensitivity of EMT6 cells in vitro. Oncol.Res., *4*: 151-155, 1992.

11. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W., and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells. Cancer Res., *54*: 3218-3223, 1994.

12. Fields, M. T., Eisbruch, A., Normolle, D., Orfali, A., Davis, M. A., Pu, A. T., and Lawrence, T. S. Radiosensitization produced in vivo by once- vs. twice-weekly 2'2'-difluoro-2'-deoxycytidine (gemcitabine). Int.J.Radiat.Oncol.Biol.Phys., *47*: 785-791, 2000.

13. Gregoire, V., Beauduin, M., Rosier, J. F., De Coster, B., Bruniaux, M., Octave-Prignot, M., and Scalliet, P. Kinetics of mouse jejunum radiosensitization by 2',2'-difluorodeoxycytidine (gemcitabine) and its relationship with pharmacodynamics of DNA synthesis inhibition and cell cycle redistribution in crypt cells. Br.J.Cancer, *76*: 1315-1321, 1997.

14. Gregoire, V., Cvilic, S., Beauduin, M., De Coster, B., Gueulette, J., Octave-Prignot, M., and Scalliet, P. Effect of gemcitabine on the tolerance of the lung to single-dose irradiation in C3H mice. Radiat.Res., *151*: 747-749, 1999.

15. Joschko, M. A., Webster, L. K., Groves, J., Yuen, K., Palatsides, M., Ball, D. L., and Millward, M. J. Enhancement of radiation-induced regrowth delay by gemcitabine in a human tumor xenograft model. Radiat.Oncol.Investig., *5*: 62-71, 1997.

16. Mason, K. A., Milas, L., Hunter, N. R., Elshaikh, M., Buchmiller, L., Kishi, K., Hittelman, K., and Ang, K. K. Maximizing therapeutic gain with gemcitabine and fractionated radiation. Int.J.Radiat.Oncol.Biol.Phys., *44*: 1125-1135, 1999.

17. Milas, L., Fujii, T., Hunter, N., Elshaikh, M., Mason, K., Plunkett, W., Ang, K. K., and Hittelman, W. Enhancement of tumor radioresponse in vivo by gemcitabine. Cancer Res., *59*: 107-114, 1999.

18. Lawrence, T. S., Chang, E. Y., Hahn, T. M., and Shewach, D. S. Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine). Clin.Cancer Res., *3*: 777-782, 1997.

19. Rosier, J. F., Michaux, L., Ameye, G., Cedervall, B., Libouton, J. M., Octave-Prignot, M., Verellen-Dumoulin, C., Scalliet, P., and Gregoire, V. The radioenhancement of two human head and neck squamous cell carcinomas by 2'-2' difluorodeoxycytidine (gemcitabine; dFdC) is mediated by an increase in radiation-induced residual chromosome aberrations but not residual DNA DSBs. Mutat.Res., *527*: 15-26, 2003.

20. Baker, C. H., Banzon, J., Bollinger, J. M., Stubbe, J., Samano, V., Robins, M. J., Lippert, B., Jarvi, E., and Resvick, R. 2'-Deoxy-2'-methylenecytidine and 2'-deoxy-2',2'-difluorocytidine 5'-diphosphates: potent mechanism-based inhibitors of ribonucleotide reductase. J.Med.Chem., *34*: 1879-1884, 1991.

21. Heinemann, V., Xu, Y. Z., Chubb, S., Sen, A., Hertel, L. W., Grindey, G. B., and Plunkett, W. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. Mol.Pharmacol., *38*: 567-572, 1990.

22. Ruiz, v. H., V, Veerman, G., Vermorken, J. B., and Peters, G. J. 2',2'-Difluoro-deoxycytidine (gemcitabine) incorporation into RNA and DNA of tumour cell lines. Biochem.Pharmacol., *46*: 762-766, 1993.

23. Latz, D., Fleckenstein, K., Eble, M., Blatter, J., Wannenmacher, M., and Weber, K. J. Radiosensitizing potential of gemcitabine (2',2'-difluoro-2'-deoxycytidine) within the cell cycle in vitro. Int.J.Radiat.Oncol.Biol.Phys., *41*: 875-882, 1998.

24. Ostruszka, L. J. and Shewach, D. S. The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine. Cancer Res., *60*: 6080-6088, 2000.

25. Jordan, A. and Reichard, P. Ribonucleotide reductases. Annu.Rev.Biochem., *67*: 71-98, 1998.

26. Thelander, L. and Berg, P. Isolation and characterization of expressible cDNA clones encoding the M1 and M2 subunits of mouse ribonucleotide reductase. Mol.Cell Biol., *6*: 3433-3442, 1986.

27. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature, *404*: 42-49, 2000.

28. Zhou, B., Liu, X., Mo, X., Xue, L., Darwish, D., Qiu, W., Shih, J., Hwu, E. B., Luh, F., and Yen, Y. The human ribonucleotide reductase subunit hRRM2 complements p53R2 in response to UV-induced DNA repair in cells with mutant p53. Cancer Res., *63*: 6583-6594, 2003.

29. Xue, L., Zhou, B., Liu, X., Qiu, W., Jin, Z., and Yen, Y. Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p53R2 as well as hRRM2 subunits. Cancer Res., *63*: 980-986, 2003.

30. Kimura, T., Takeda, S., Sagiya, Y., Gotoh, M., Nakamura, Y., and Arakawa, H. Impaired function of p53R2 in Rrm2b-null mice causes severe renal failure through attenuation of dNTP pools. Nat.Genet., *34*: 440-445, 2003.

31. Barber Jr, J, Shewach, D., and Im, M. M. Induction of p53R2 by dFdCyd Does not Prevent Induction of R2. Proceedings for the American Association for Cancer Research, 2003.

32. Barber Jr, J and Shewach, D. S. siRNA for p53R2 Inhibits Increases in p53R2 Expression in Response to DNA Damaging Agents. Proceedings for the American Association for Cancer Research, 2006.

33. Hoy, C. A., Seamer, L. C., and Schimke, R. T. Thermal denaturation of DNA for immunochemical staining of incorporated bromodeoxyuridine (BrdUrd): critical factors that affect the amount of fluorescence and the shape of BrdUrd/DNA histogram. Cytometry, *10*: 718-725, 1989.

34. Shewach, D. S. Quantitation of deoxyribonucleoside 5'-triphosphates by a sequential boronate and anion-exchange high-pressure liquid chromatographic procedure. Anal.Biochem., *206*: 178-182, 1992.

35. Fertil, B., Dertinger, H., Courdi, A., and Malaise, E. P. Mean inactivation dose: a useful concept for intercomparison of human cell survival curves. Radiat.Res., *99*: 73-84, 1984.

36. Robinson, B. W. and Shewach, D. S. Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines. Clin.Cancer Res., *7*: 2581-2589, 2001.

37. Guittet, O., Hakansson, P., Voevodskaya, N., Fridd, S., Graslund, A., Arakawa, H., Nakamura, Y., and Thelander, L. Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. J.Biol.Chem., *276*: 40647-40651, 2001.

38. Hakansson, P., Hofer, A., and Thelander, L. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. J.Biol.Chem., 2006.

Chapter IV

p53R2 EXPRESSION IN RESPONSE TO dFdCyd EXPOSURE ACTS TO MAINTAIN dNTP POOLS AND ALTER RADIOSENSITIZATION

Summary

Ribonucleotide reductase (RR) is a tetrameric protein consisting of two regulatory subunits (R1) and two catalytic subunits (R2). Expression of R1 is constant throughout the cell cycle, whereas R2 is expressed primarily in S-phase. p53R2 is a homologue of the ribonucleotide reductase R2 subunit, and it was shown to be induced in a p53-dependent fashion following DNA damage by ionizing radiation and a few DNA-damaging drugs. Previously, we evaluated the effects of p53R2 induction on dFdCyd-mediated dATP depletion and radiosensitization in cell lines matched for p53 status. However, those cell lines have been cultured separately over a number of years and may have acquired differences that altered response to dFdCyd. Therefore, it was imperative to evaluate the effects of p53R2 induction in a single cell line. In A549 non-small cell lung cancer cells that were not radiosensitized at dFdCyd <IC_{50}, siRNA-mediated suppression of p53R2 produced excellent radiosensitization at the IC_{10}. Silencing of p53R2 expression in A549 cells also resulted in increased depletion of dNTP pools in response to dFdCyd exposure. Taken together, these results demonstrate that dFdCyd can induce p53R2 expression, and this expression may act to maintain dNTP pools.

90

Introduction

Gemcitabine (dFdCyd, 2', 2'-difluoro-2'-deoxycytidine) is a nucleoside analog with activity against a wide variety of malignancies, including pancreatic (1, 2) and non-small cell lung cancer (3-5). dFdCyd has shown excellent activity both *in vitro* and *in vivo*, either as a single agent or in combination with other drugs such as cisplatin (6) and docetaxel (7). Several preclinical studies have demonstrated the ability of dFdCyd to sensitize cultured cells or tumor xenografts in animals to the effects of radiation, a process known as radiosensitization (8-11). Clinical studies indicate that this radiosensitizing effect can be observed *in vivo* as well (12-17).

While the radiosensitizing property of dFdCyd has been demonstrated in many different *in vitro* and *in vivo* studies, the mechanism by which this occurs has yet to be elucidated. Most radiosensitizers work through either potentiation of radiation-induced DNA damage or through inhibition of its repair. dFdCyd is unique in that it does not enhance radiation-induced DNA damage or alter the rate of repair (18, 19). Radiosensitization has been previously found to correlate with two processes. Previous studies by this and other labs have shown that dATP depletion (20-22) and S-phase accumulation prior to irradiation correlate strongly with radiosensitization (23-25).

Recently a p53-inducible RR subunit, p53R2, was discovered, and it has been shown to be activated following DNA damage caused by exposure to ultraviolet or ionizing radiation, or cytotoxic concentrations of certain drugs (26). p53R2 has high sequence homology with R2 except in the NH_2-terminal region, and this

91

difference has been postulated to be of regulatory importance (27). Studies have demonstrated that p53R2 can bind with R1 to form an active heterotetramer (28). Previous studies have also demonstrated that p53R2, as well as R2, are regulated through interactions with wild-type p53 (29). Studies in mice have demonstrated that p53R2 is essential in development, as p53R2-null mice die within 14 weeks after birth (30, 31). In addition to its role in ribonucleotide reduction, p53R2 also has antioxidant (32) and metastasis-suppressing (33, 34) properties.

In view of the important role for ribonucleotide reductase in radiosensitization with dFdCyd, we wished to determine whether p53R2 affected this process. Previously, we have investigated induction of p53R2 following dFdCyd exposure, and found that p53R2 was induced in as little as 2 hr after dFdCyd addition. In cell lines matched for p53, and subsequently p53R2, status, induction of p53R2 did not affect dATP depletion or radiosensitization. However, these cell lines displayed differences in sensitivity to either dFdCyd or ionizing radiation as single agents. Also, these cell lines have been cultured separately over a number of years. As a result, these cell lines may have acquired differences that altered cellular response to dFdCyd and/or radiation. In order to more accurately examine the effects of p53R2 induction on dATP depletion and radiosensitization, we utilized siRNA-directed suppression of p53R2 expression to determine the contribution of this protein to cytotoxicity and radiosensitization with dFdCyd. Preliminary accounts of a portion of these findings have been presented previously in abstract form (35).

Materials and Methods

Cell Culture and Drug Preparation. The MCF-7 breast carcinoma cell line was cultured in RPMI medium (GIBCO Laboratories, Grand Island, NY), supplemented with 10% calf serum (GIBCO), and 2 mM L-glutamine (Fisher Scientific, Fair Lawn, NJ). The A549 lung carcinoma cell line was cultured in Dulbecco's Modified Eagle Medium (GIBCO), supplemented with 10% fetal bovine serum (GIBCO), and 2mM L-glutamine (Fisher Scientific). Cells were maintained in logarithmic growth as a monolayer in T-75 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. Gemcitabine was a generous gift from Eli Lilly and Co. (Indianapolis, IN), which was dissolved in PBS to obtain a stock solution of 10 mM and sterilized before diluting further with PBS to achieve final concentrations of 1, 10, and 100 μM.

Irradiation of Cells. Monolayer cultures of cells were irradiated using Co^{60} (AECL Theratron 80) at 1-2 Gy/min. Dosimetry was carried out using an ionizing chamber connected to an electrometer system that was directly traceable to a National Institute of Standards and Technology standard. All cells were irradiated at room temperature.

siRNA Silencing. Cells are plated in 12-well dishes at a concentration of 100,000 cells/well for each condition and allowed to grow for 48 hr. Cells were then transfected with siRNA (Dharmacon, Lafayette, CO) using Lipofectamine (Gibco) for 24 hr. Following this incubation, each well was washed and repleted with 1.5 ml media. Cells were allowed to grow for another 48 hr then processed for Western blotting or clonogenic survival as described below.

Western Blot Analysis. After harvesting, lysis, and collection, the supernatant was used for analysis after determination of protein concentration with a Bio-Rad (Bio-Rad Laboratories, Hercules, CA) protein assay. After electrophoresis for 2 hours at 150 V, the protein was transferred onto Immobilon-P transfer membrane (Millipore Corp., Bedford, MA) for 2 hours at 125 mA. After overnight blocking, the membrane was then incubated with a 1:1000 dilution of either p53R2 (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal goat, R2 (Santa Cruz) monoclonal goat, p53 (Ab-6) monoclonal mouse (Calbiochem), or Actin IgG antibody (Calbiochem) for 2 hours followed by incubation with a 1:10000 dilution of the corresponding secondary IgG horseradish peroxidase linked antibody for 1 hour at room temperature. After washing, proteins that bound the antibodies were visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

Cell Survival Assay. After treatment with dFdCyd and/or ionizing irradiation, cells were plated in 6-well dishes and allowed to grow for 10-14 days and colonies were stained with crystal violet. Cell survival was expressed as a percentage of the control survival, after correcting for plating efficiency. Radiation survival data from dFdCyd-treated cells were corrected for plating efficiency using an unirradiated plate treated with dFdCyd under the same conditions. Cell survival curves were fit using a linear-quadratic equation (36). Radiation sensitivity is expressed in terms of the mean inactivation dose, which represents the area under the cell survival curve.   Radiosensitization is

94

expressed as an enhancement ratio, which is defined as the mean inactivation dose (control)/mean inactivation dose (dFdCyd).

Nucleotide Pool Analysis. Cellular nucleotides were assayed as described previously (37). Briefly, cells were harvested by trypsinization and nucleotides were extracted using 0.4 N perchloric acid. Neutralized extracts were stored at –20°C until analysis. Ribonucleotides were removed from the extracts using a boronate affinity column (38). Cellular dNTPs and dFdCTP were separated and quantified by strong anion exchange HPLC using a Waters Alliance (Milford, MA) gradient system equipped with a photodiode array detector and controlled by Millennium 2010 software. Samples were loaded onto a Partisphere 4.6x250 mm strong anion exchange column (Whatman, Hillshore, OR) and nucleotides were eluted at 2 ml/min with a linear gradient of ammonium phosphate buffer ranging in concentration from 0.15 M (pH 2.8) to 0.60 M (pH 2.8). Nucleotides were identified on the basis of their UV absorbance spectrum and quantified at either 254 or 281 nm by comparison to the absorbance of a known amount of authentic standard. All nucleotide pool measurements represent the average of at least four determinations except for the 16 hour point (duplicate determinations).

Results

SiRNA Silencing of p53R2 Expression in MCF-7 Cells

The previous studies demonstrated that, even at the IC_{10} for dFdCyd, p53 and p53R2 could be induced. With the prolonged expression of p53R2 for several days following drug and radiation treatment, it seemed possible that p53R2 might

elevate the dATP pools sufficiently to thus lessen radiosensitization with dFdCyd.

Thus, we wished to determine whether decreasing the expression of p53R2 would prevent radiosensitization with dFdCyd. To accomplish this, MCF-7 cells were transfected with siRNA for p53R2 for 24 hr, then allowed to grow for various lengths of time before dFdCyd addition to determine the optimal timecourse for p53R2 suppression. Subsequent silencing of dFdCyd-mediated p53R2 expression was measured using Western blotting. dFdCyd-mediated p53R2 expression was inhibited by siRNA when cells were exposed to dFdCyd 24 hr post transfection (Fig 4.1A). Maximal inhibition of p53R2 expression was observed when cells were exposed to dFdCyd 48 hr post transfection, and p53R2 expression in response to dFdCyd was inhibited for at least another 48 hr. This was a specific response, since a non-specific siRNA sequence or siRNA for lamin did not alter p53R2 expression. At the time of maximal inhibition of p53R2 expression, expression of R2 and p53 were unaffected, demonstrating the specificity of the siRNA sequence and silencing technique (Fig 4.1B).

Cytotoxicity and Radiosensitization in MCF-7 Cells Following Suppression of p53R2

   After determining conditions under which siRNA could effectively inhibit p53R2 expression in response to dFdCyd exposure, the effects of this inhibition on dFdCyd sensitivity were evaluated. At 48 hr post-transfection, cells were exposed to a range of concentrations of 3 nM to 300 nM of dFdCyd for 24 hr, and cytotoxicity was evaluated using a colony-formation assay. Cells untreated with

siRNA demonstrated dose-dependent sensitivity to dFdCyd, with an observed

IC_{50} of 55 nM (Fig 4.2A). Pretreatment with siRNA for p53R2 did not decrease

sensitivity to dFdCyd (IC_{50} = 55 nM). Cells treated with non-specific siRNA

demonstrated similar sensitivity to dFdCyd (IC_{50} = 70 nM). Western blot analysis

indicated that p53R2 expression was significantly suppressed using siRNA (Fig

4.2B). Thus, inhibition of p53R2 expression did not alter sensitivity to dFdCyd in

the MCF-7 cells.

Since radiosensitization of MCF-7 cells with dFdCyd does not depend upon

cytotoxicity, the effect of siRNA suppression of p53R2 on radiation sensitization

was determined. At 48 hr post-transfection, cells were incubated for 24 hr with

dFdCyd at the IC_{10} followed by irradiation. MCF-7 cells not treated with siRNA

were radiosensitized by IC_{10} (10 nM) dFdCyd, with an observed radiation

enhancement ratio (RER) of 1.7$\pm$0.3 (Fig 4.3, Table 4.1). After 72 hr of exposure

to siRNA for p53R2, dFdCyd-mediated radiosensitization was not enhanced, as

the observed RER was 1.8$\pm$0.3. Treatment with a non-specific siRNA sequence

for 72 hr also did not significantly alter dFdCyd-mediated radiosensitization, as

the RER was 1.5$\pm$0.2. Western blot analysis indicated substantial silencing of

p53R2 expression in response to dFdCyd.

siRNA Silencing of p53R2 in the A549 Cell Line

After determining that p53R2 status did not appear to alter radiosensitization

in a cell line that was already highly radiosensitized by dFdCyd, we then wanted

to examine the effects of p53R2 expression in a cell line that was not

97
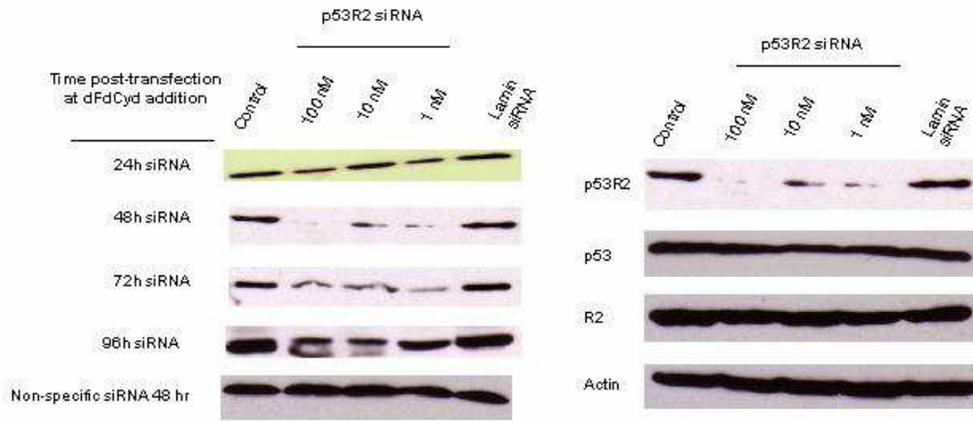
Figure 4.1. siRNA Suppression of dFdCyd-Mediated p53R2 Expression.
MCF-7 cells were treated to an siRNA transfection mixture for 24 hr. At various
timepoints thereafter, cells were exposed to dFdCyd for 24 hr and assayed for
protein expression by western blotting as described in "Materials and Methods".
Actin was used as a loading control.
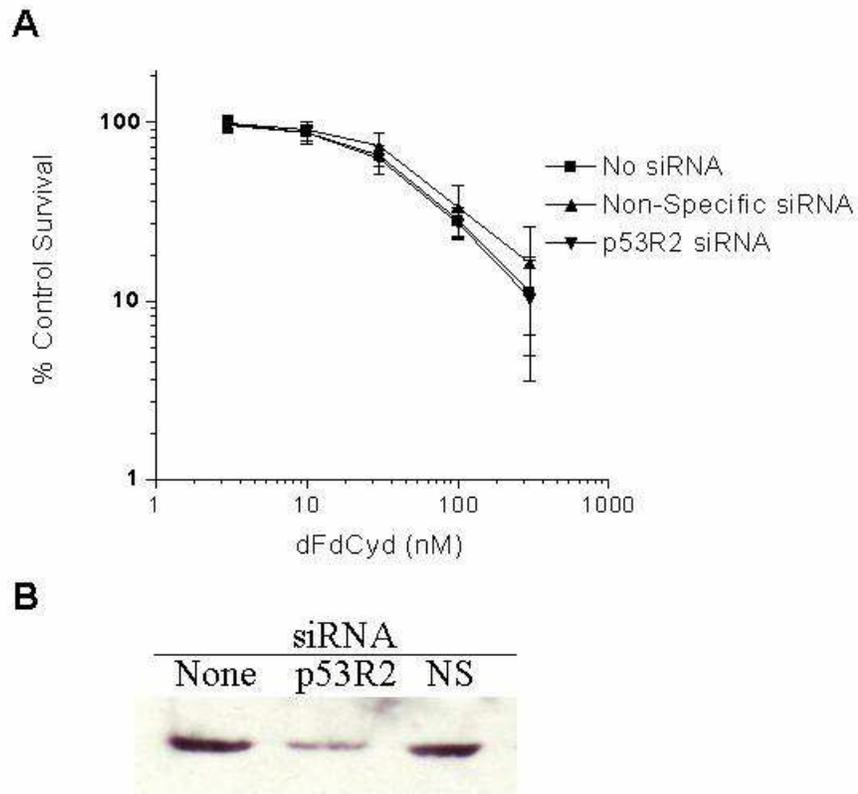
Figure 4.2. dFdCyd-Mediated Cytotoxicity Following siRNA Suppression of p53R2. A) MCF-7 cells were transfected for 24 hr, then allowed to grow for 48 hr. Cells were then exposed to 3nm-300nm dFdCyd for 24 hr, and evaluated for cell survival as described in "Materials and Methods". B) Suppression of p53R2 expression was evaluated by western blotting.
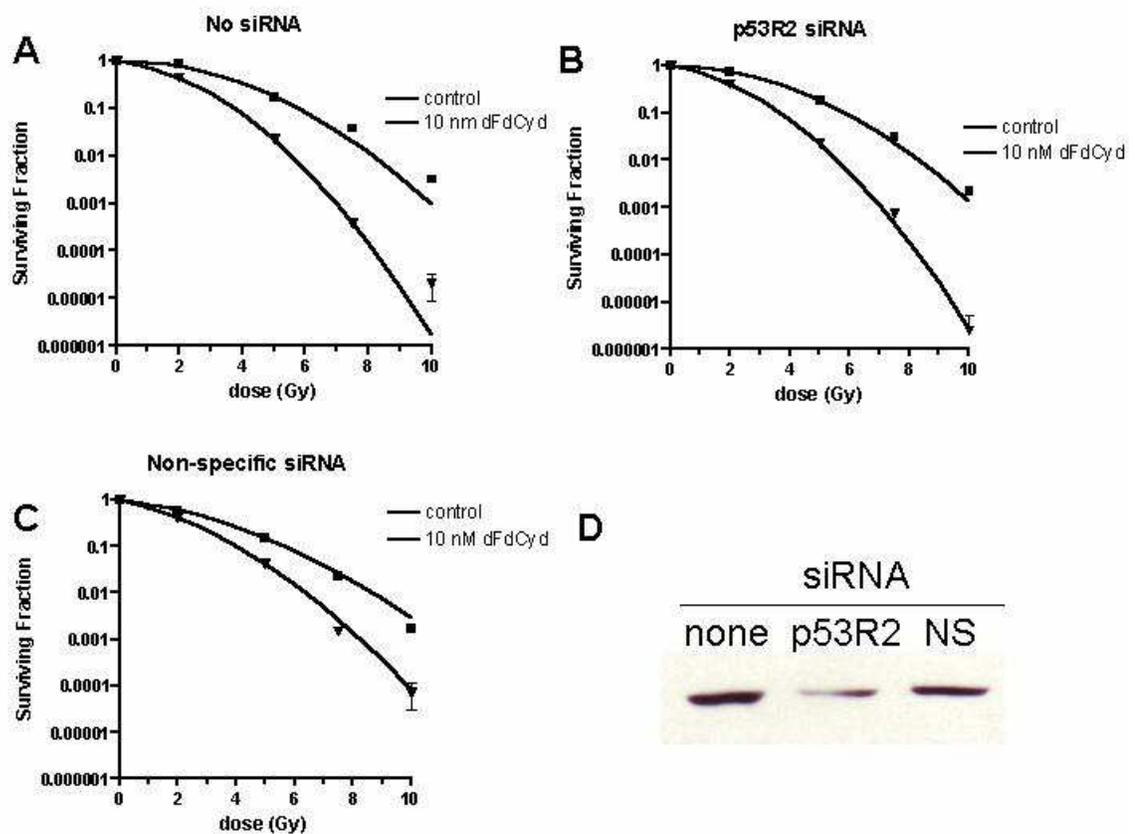
Figure 4.3. Radiosensitization in MCF-7 Cells Following Suppression of p53R2. In MCF-7 cells either A) not transfected, B)transfected with p53R2 siRNA, or C) transfected with non-specific siRNA, dFdCyd-mediated radiosensitization is evaluated. D) p53R2 silencing is confirmed using western blotting. Figures A-C are a representative experiment that was performed at three times

	dFdCyd IC_{10}	Enhancement Ratio	D-Bar
No siRNA	10 nM	1.7$\pm$0.3	3.0$\pm$0.3
p53R2 siRNA	10 nM	1.8$\pm$0.3	3.0$\pm$0.3
Non-Specific siRNA	10 nM	1.6$\pm$0.2	2.7$\pm$0.2

Table 4.1. dFdCyd-mediated Cytotoxicity and Radiosensitization Following siRNA Transfection in MCF-7 Cells. In MCF-7 breast cancer cells either not transfected, or transfected with p53R2 or non-specific siRNA, cytotoxicity, radiosensitization, and radiation sensitivity were analyzed.

radiosensitized by dFdCyd at non-cytotoxic concentrations. In previous work, the

A549 lung carcinoma (wild-type p53) cell line exhibited little to no

radiosensitization with< IC_{50} dFdCyd (non-published findings). p53R2

expression also had not been previously examined in this cell line. Western blot

analysis indicated that p53R2 was maintained at low levels in unstressed A549

cells, and induced within 24 hr of IC_{10} dFdCyd addition (Fig. 4.4). Similar to

findings in the MCF-7 cell line, expression of p53R2 remained elevated for at

least 96 hr post-washout. Using siRNA technology, we were able to effectively

silence dFdCyd-mediated increases in p53R2 expression (Fig. 4.4). To

characterize p53R2 silencing, A549 cells were transfected for 24 hr, and allowed

to grow for various lengths of time before a 24 hr exposure to dFdCyd. dFdCyd-

mediated p53R2 expression was completely suppressed for at least 120 hr post

transfection.

Cytotoxicity and Radiosensitization in the A549 Cell Line Following p53R2

Silencing

After determining that we could effectively silence increases in p53R2

expression in response to dFdCyd exposure in A549 cells, we next wanted to

examine the effects of this silencing on dFdCyd-mediated cytotoxicity and

radiosensitization. Similar to previous experiments in MCF-7 cells, cells were

treated with dFdCyd 48 hr post-transfection and assayed for cell survival.

Although p53R2 expression was significantly suppressed, no effect on dFdCyd

cytotoxicity was observed (Fig. 4.5). Cells not treated with siRNA demonstrated

dose-dependent sensitivity to dFdCyd, with an IC_{10} and IC_{50} of 10nM and 45 nM,

102
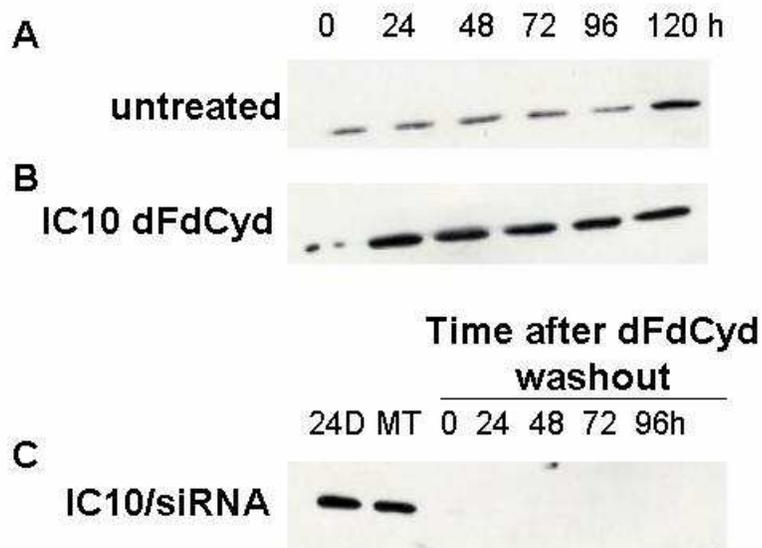
Figure 4.4. Silencing of p53R2 Expression in A549 Cells. A) p53R2 expression was evaluated in unstressed cells. B) p53R2 expression was evaluated in response to a 24 hr exposure to IC_{10} dFdCyd. C) Silencing of p53R2 expression was evaluated. Cells were exposed to the transfection mixture for 24 hr then allowed to grow for another 48 hr. Cells were then exposed to IC10 dFdCyd for 24 hr, then harvested at the times indicated and assayed for p53R2 expression. 24D indicates a 24 hr exposure to dFdCyd, and MT indicates a mock transfection, consisting of a 24 hr exposure to the transfection mixture without siRNA, and dFdCyd exposure 48 hr after transfection.
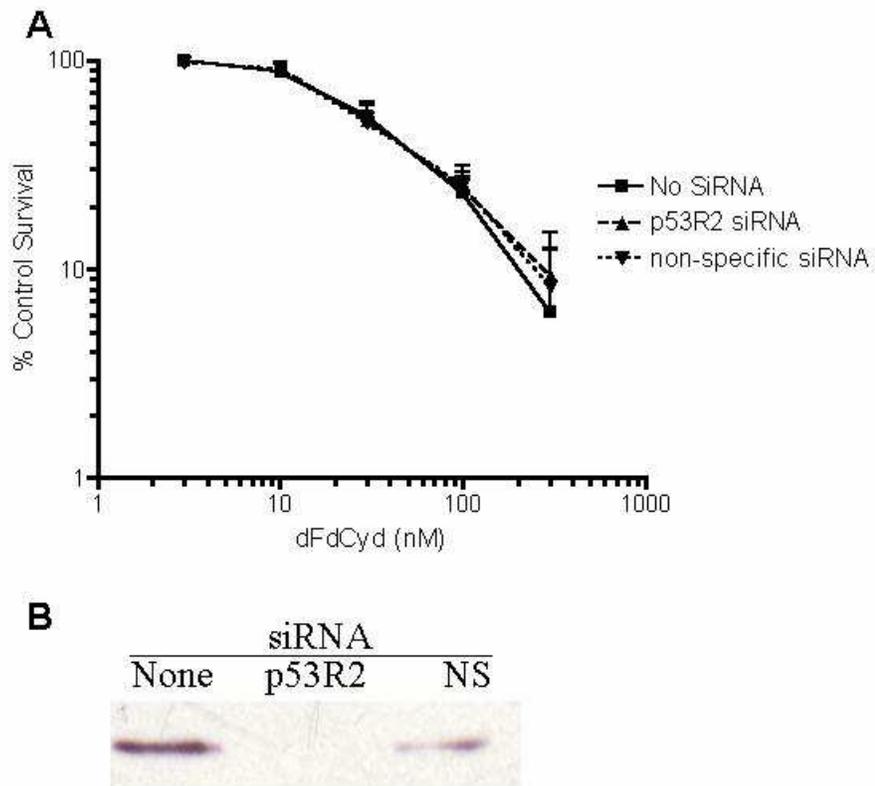
Figure 4.5. Cytotoxicity in A549 Cells Following Silencing of p53R2. A) dFdCyd- mediated cytotoxicity was evaluated following siRNA silencing of p53R2. Cells were transfected for 24 hr, then allowed to grow for 48 hr. Cells were then exposed to dFdCyd for 24 hr and assayed for cell survival. B) siRNA silencing of p53R2 was assayed using western blotting.

respectively. Following suppression of p53R2, dFdCyd sensitivity was slightly

but not significantly decreased at higher dFdCyd concentrations. Pretreatment

with non-specific siRNA did not change dFdCyd sensitivity from that observed in

cells not treated with siRNA.

We next evaluated the effects of p53R2 silencing on radiosensitization in

A549 cells. After 48 hr post-transfection, A549 cells were treated with IC_{10}

dFdCyd for 24 hr followed by 5 Gy irradiation. Cells that were not pretreated with

siRNA were marginally radiosensitized, with a RER of 1.3$\pm$0.1 (Fig 4.6).

Following silencing of p53R2, radiosensitization was increased, with an observed

RER of 1.8$\pm$0.1. Radiosensitization was not altered when cells were pretreated

with non-specific siRNA, with an observed RER of 1.3 $\pm$ 0.1.

DNTP Pools Following p53R2 Suppression

After observing that p53R2 silencing increased radiosensitization in A549

cells, we next wanted to determine if this was the result of greater dATP

depletion in response to dFdCyd exposure. In previous studies, dATP was

depleted to about 35% of control levels in response to IC_{10} dFdCyd (unpublished

results). At 48 hr post-transfection, A549 cells were treated with dFdCyd for up

to 24 hr, and dNTP pool levels were measured. In untransfected cells, dATP

was depleted to approximately 35% of control levels, at 4 hr of dFdCyd exposure

(Fig 4.7A). dATP levels recovered at 24 hr of exposure to near control levels.

Following suppression of p53R2, dATP levels were depleted to nearly 10% of

control levels at 4 hr of dFdCyd exposure, and this level of depletion was
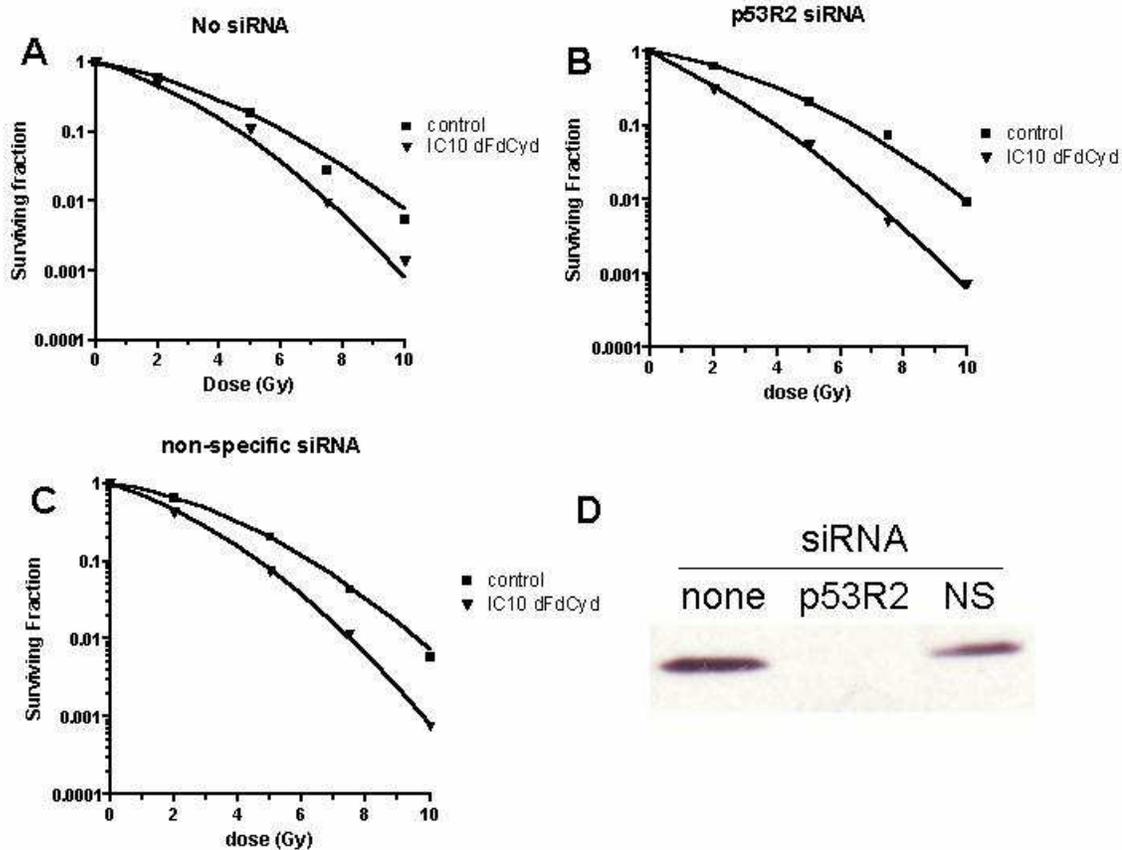
**Figure 4.6. dFdCyd-Mediated Radiosensitization Following Suppression of p53R2 Expression.** Radiosensitization was assayed in A549 cells that were either A)untransfected, B)transfected with p53R2 siRNA, or C) transfected with non-specific siRNA, as described in "Materials and Methods". D) Silencing of p53R2 was confirmed using western blotting. Figures A-C are representative of an experiment that was performed at least three times.

	dFdCyd IC_{10}	Enhancement Ratio	D-Bar
No siRNA	10 nM	1.3 ± 0.1	2.9 ± 0.1
p53R2 siRNA	10 nM	1.8 ± 0.1	3.1 ± 0.1
Non-Specific siRNA	10 nM	1.3 ± 0.1	3.2 ± 0.1

Table 4.2. dFdCyd-mediated Cytotoxicity and Radiosensitization Following siRNA Transfection in A549 Cells. In A549 lung cancer cells either not transfected, or transfected with p53R2 or non-specific siRNA, cytotoxicity, radiosensitization, and radiation sensitivity were analyzed.

maintained at 24 hr of exposure. Following transfection with non-specific siRNA, dATP depletion was similar to that observed in untransfected cells.

Furthermore, following suppression of p53R2 expression, dGTP, TTP, and dCTP were depleted in response to dFdCyd. Conversely, in untransfected cells or cells treated with non-specific siRNA, dNTPs were not significantly depleted (Fig 4.7). TTP levels were 200% of control levels at 24 hr of dFdCyd exposure, compared to 30% in cells in which p53R2 had been silenced. While dCTP and dGTP were depleted in cells treated with p53R2 siRNA, the levels of these dNTPs were at near control levels at 24 hr of dFdCyd exposure.

Discussion

p53R2 is a p53-inducible homologue of the small subunit of ribonucleotide reductase, and it has been proposed to be activated in response to DNA damage to provide dNTPs for DNA repair (39). Previous research has demonstrated that p53R2 can pair with R1 and produce ribonucleotide reductase activity (40). Although much work has been dedicated to observing p53R2 expression and ribonucleotide reductase activity in response to radiation, little has been done to evaluate p53R2 expression in response to chemotherapeutics. In particular, no study has examined the effects of p53R2 expression in response to chemotherapeutics on dNTP pool levels. Here we have examined p53R2 expression in response to dFdCyd on dNTP pool levels and radiosensitization through the use of siRNA silencing. We have demonstrated that p53R2 expression actively maintains dNTP pools in response to dFdCyd, and in certain cell lines this activity can decrease radiosensitization.

108
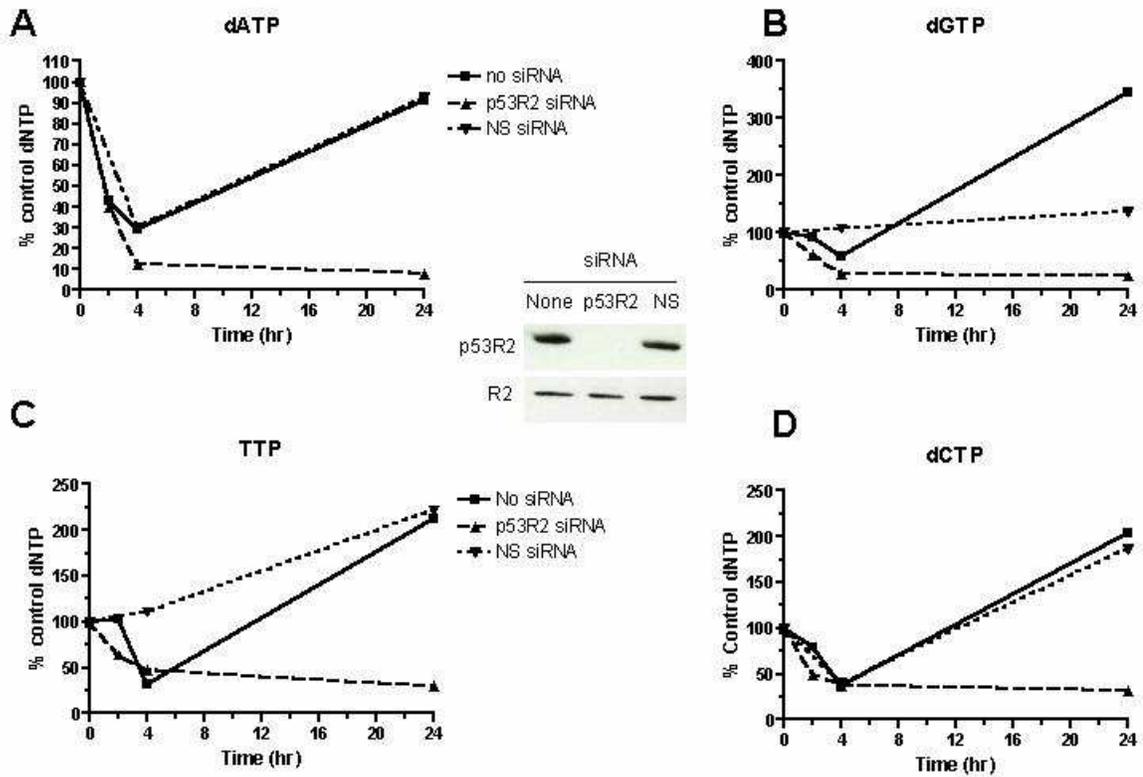
Figure 4.7. dNTP pools depletion in response to dFdCyd Exposure. In cells that were either untransfected (squares), transfected with p53R2 siRNA (triangles), or transfected with non-specific siRNA (upside down triangles), depletion of A) dATP, B) dGTP, C) TTP, or D) dCTP was evaluated. p53R2 and R2 expression was evaluated using western blotting (inset).

We examined the effects of p53R2 silencing on dFdCyd-mediated cytotoxicity in two wt p53 cell lines. Suppression of p53R2 expression did not alter sensitivity to dFdCyd as a single agent in either cell line. This finding contrasts with a previous study that reported that silencing of p53R2 increased the cytotoxic effects of 5-fluorouracil (41). These differences may be attributable to the different targets of these two agents. Another study found that the R1/p53R2 pairing demonstrated differing sensitivities to ribonucleotide reductase inhibitors compared to the R1/R2 pairing in a cell free system (42). In that study, the R1/p53R2 pairing was more sensitive to the iron chelator DFO, less sensitive to the radical scavenger hydroxyurea, and equally sensitive to triapine, which is an iron chelator and a radical scavenger, compared to the R1/R2 pairing. It will be important to determine if the effects observed in a cell-free system will translate to intact cells, and if silencing of individual subunits can potentiate the cytotoxicity of these agents.

Although we did not observe an increase in dFdCyd-mediated radiosensitization in MCF-7 cells, radiosensitization was increased in A549 cells following p53R2 suppression. We previously observed that exposure to dFdCyd resulted to dATP depletion in MCF-7 to near undetectable levels, although dATP levels recovered slightly at later timepoints (43), whereas exposure to dFdCyd in A549 cells only resulted in 65% depletion of dATP compared to untreated cells (unpublished results). It is possible that, once the 80% threshold required for radiosensitization is reached, increased dATP depletion alone does not increase radiosensitization. This is supported by the finding that increased dATP

depletion in MCF-7 cells, achieved by increasing dFdCyd concentration the IC_{10} to the IC_{50}, did not result in increased radiosensitization (44). However, we also observed near complete p53R2 suppression in A549 cells, compared to approximately 70% suppression on p53R2 expression in MCF-7 cells. It is also possible that this low level of p53R2 expression is sufficient to maintain dATP pools in response to dFdCyd, thus preventing increased radiosensitization.

In this study, we found that suppression of p53R2 resulted in increased dATP depletion in response to dFdCyd exposure compared to untransfected A549 cells. In addition, we also observed increased depletion of the other dNTPs. Previous studies have demonstrated that only dATP is significantly depleted in response to low concentrations of dFdCyd (45). Here we have demonstrated that following suppression of p53R2, all dNTPs are depleted to < 40 % of control levels in response to dFdCyd exposure. This strongly suggests that the activity of p53R2, not salvage pathways, is the major contributor in maintaining dNTPs in response to dFdCyd in wt p53 A549 cells. In mt p53 cells, salvage pathways are likely the major contributor to maintenance of dNTPs following dFdCyd exposure.

The results indicate that p53R2 actively maintains dNTP pools in response to dFdCyd exposure in A549 cells, and this activity can decrease radiosensitization in certain cell lines. As p53R2 suppression resulted in increased dATP depletion following dFdCyd exposure in A549 cells, it is possible that dFdCyd is a more potent inhibitor of R1/R2 compared to R1/p53R2. However, it is also possible that dFdCDP did not accumulate to a high enough level to inhibit ribonucleotide reductase activity in A549 cells. In either case, our findings suggest that p53R2

111

should be further evaluated as a target to manipulate in increasing

radiosensitization with dFdCyd and other ribonucleotide reductase inhibitors.

Footnotes to Chapter IV

[1]The footnotes used are: dFdCyd, 2',2'-difluoro-2'-deoxycytidine; dCyd, deoxycytidine; dFdCMP, 5'-monophosphate of dFdCyd; dFdCDP, 5'-diphosphate of dFdCyd; dFdCTP, 5'-triphosphate of dFdCyd; dNTP, deoxynucleotide triphosphate; dATP, deoxyadenosine triphosphate, dCTP, deoxycytidine triphosphate; TTP, thymidine triphosphate; dGTP, deoxyguanosine triphosphate; HPLC, high performance lipid chromatography; RR, ribonucleotide reductase.

References

1. Glimelius, B. Chemotherapy in the treatment of cancer of the pancreas, J.Hepatobiliary.Pancreat.Surg., *5:* 235-241, 1998.

2. Rothenberg, M. L., Moore, M. J., Cripps, M. C., Andersen, J. S., Portenoy, R. K., Burris, H. A., III, Green, M. R., Tarassoff, P. G., Brown, T. D., Casper, E. S., Storniolo, A. M. and Von Hoff, D. D. A phase II trial of gemcitabine in patients with 5-FU-refractory pancreas cancer, Ann.Oncol., *7:* 347-353, 1996.

3. Shepherd, F. A. Phase II trials of single-agent activity of gemcitabine in patients with advanced non-small cell lung cancer: an overview, Anticancer Drugs, *6 Suppl 6:* 19-25, 1995.

4. Takada, M., Negoro, S., Kudo, S., Furuse, K., Nishikawa, H., Takada, Y., Kamei, T., Niitani, H. and Fukuoka, M. Activity of gemcitabine in non-small-cell lung cancer: results of the Japan gemcitabine group (A) phase II study, Cancer Chemother.Pharmacol., *41:* 217-222, 1998.

5. Zatloukal, P., Kanitz, E., Magyar, P., Jassem, J., Krzakowski, M., Pawlicki, M., Petruzelka, L., Chovan, L., Pesek, M., Janko, C. and Krejcy, K. Gemcitabine in locally advanced and metastatic non-small cell lung cancer: the Central European phase II study, Lung Cancer, *22:* 243-250, 1998.

6. Abratt, R. P., Bezwoda, W. R., Goedhals, L. and Hacking, D. J. Weekly gemcitabine with monthly cisplatin: effective chemotherapy for advanced non-small-cell lung cancer, J.Clin.Oncol., *15:* 744-749, 1997.

7. Sherman, W. H. and Fine, R. L. Combination gemcitabine and docetaxel therapy in advanced adenocarcinoma of the pancreas, Oncology, *60:* 316-321, 2001.

8. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W. and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine, Int.J.Radiat.Oncol.Biol.Phys., *34:* 867-872, 1996.

9. McGinn, C. J., Shewach, D. S. and Lawrence, T. S. Radiosensitizing nucleosides, J.Natl.Cancer Inst., *88:* 1193-1203, 1996.

10. Rockwell, S. and Grindey, G. B. Effect of 2',2'-difluorodeoxycytidine on the viability and radiosensitivity of EMT6 cells in vitro, Oncol.Res., *4:* 151-155, 1992.

11. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W. and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells, Cancer Res., *54:* 3218-3223, 1994.

12. Fields, M. T., Eisbruch, A., Normolle, D., Orfali, A., Davis, M. A., Pu, A. T. and Lawrence, T. S. Radiosensitization produced in vivo by once- vs. twice-weekly 2'2'-difluoro-2'-deoxycytidine (gemcitabine), Int.J.Radiat.Oncol.Biol.Phys., *47:* 785-791, 2000.

13. Gregoire, V., Beauduin, M., Rosier, J. F., De Coster, B., Bruniaux, M., Octave-Prignot, M. and Scalliet, P. Kinetics of mouse jejunum radiosensitization by 2',2'-difluorodeoxycytidine (gemcitabine) and its relationship with pharmacodynamics of DNA synthesis inhibition and cell cycle redistribution in crypt cells, Br.J.Cancer, *76:* 1315-1321, 1997.

14. Gregoire, V., Cvilic, S., Beauduin, M., De Coster, B., Gueulette, J., Octave-Prignot, M. and Scalliet, P. Effect of gemcitabine on the tolerance of the lung to single-dose irradiation in C3H mice, Radiat.Res., *151:* 747-749, 1999.

15. Joschko, M. A., Webster, L. K., Groves, J., Yuen, K., Palatsides, M., Ball, D. L. and Millward, M. J. Enhancement of radiation-induced regrowth delay by gemcitabine in a human tumor xenograft model, Radiat.Oncol.Investig., *5:* 62-71, 1997.

16. Mason, K. A., Milas, L., Hunter, N. R., Elshaikh, M., Buchmiller, L., Kishi, K., Hittelman, K. and Ang, K. K. Maximizing therapeutic gain with gemcitabine and fractionated radiation, Int.J.Radiat.Oncol.Biol.Phys., *44:* 1125-1135, 1999.

17. Milas, L., Fujii, T., Hunter, N., Elshaikh, M., Mason, K., Plunkett, W., Ang, K. K. and Hittelman, W. Enhancement of tumor radioresponse in vivo by gemcitabine, Cancer Res., *59:* 107-114, 1999.

18. Lawrence, T. S., Chang, E. Y., Hahn, T. M. and Shewach, D. S. Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine), Clin.Cancer Res., *3:* 777-782, 1997.

19. Rosier, J. F., Michaux, L., Ameye, G., Cedervall, B., Libouton, J. M., Octave-Prignot, M., Verellen-Dumoulin, C., Scalliet, . and Gregoire, V. The radioenhancement of two human head and neck squamous cell carcinomas by 2'-2' difluorodeoxycytidine (gemcitabine; dFdC) is mediated by an increase in radiation-induced residual chromosome aberrations but not residual DNA DSBs, Mutat.Res., *527:* 15-26, 2003.

20. Latz, D., Fleckenstein, K., Eble, ., Blatter, J., Wannenmacher, M. and Weber, K. J. Radiosensitizing potential of gemcitabine (2',2'-difluoro-2'-deoxycytidine) within the cell cycle in vitro, Int.J.Radiat.Oncol.Biol.Phys., *41:* 875-882, 1998.

21. Ostruszka, L. J. and Shewach, D. S. The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine, Cancer Res., *60:* 6080-6088, 2000.

22. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W. and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells, Cancer Res., *54:* 3218-3223, 1994.

23. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W. and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine, Int.J.Radiat.Oncol.Biol.Phys., *34:* 867-872, 1996.

24. Lawrence, T. S., Chang, E. Y., Hahn, T. M. and Shewach, D. S. Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine), Clin.Cancer Res., *3:* 777-782, 1997.

25. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W. and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells, Cancer Res., *54:* 3218-3223, 1994.

26. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y. and Nakamura, Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage, Nature, *404:* 42-49, 2000.

27. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y. and Nakamura, Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage, Nature, *404:* 42-49, 2000.

28. Zhou, B., Liu, X., Mo, X., Xue, L., Darwish, D., Qiu, W., Shih, J., Hwu, E. B., Luh, F. and Yen, Y. The human ribonucleotide reductase subunit hRRM2 complements p53R2 in response to UV-induced DNA repair in cells with mutant p53, Cancer Res., *63:* 6583-6594, 2003.

29. Xue, L., Zhou, B., Liu, X., Qiu, W., Jin, Z. and Yen, Y. Wild-type p53 regulates human ribonucleotide reductase by protein-protein interaction with p53R2 as well as hRRM2 subunits, Cancer Res., *63:* 980-986, 2003.

30. Kimura, K., Sawada, T., Komatsu, M., Inoue, M., Muguruma, K., Nishihara, T., Yamashita, Y., Yamada, N., Ohira, M. and Hirakawa, K. Antitumor effect of trastuzumab for pancreatic cancer with high HER-2 expression and enhancement of effect by combined therapy with gemcitabine, Clin.Cancer Res., *12:* 4925-4932, 2006.

31. Powell, D. R., Desai, U., Sparks, M. J., Hansen, G., Gay, J., Schrick, J., Shi,Z.Z., Hicks,J. and Vogel,P. Rapid development of glomerular injury and renal failure in mice lacking p53R2, Pediatr.Nephrol., *20:* 432-440, 2005.

32. Xue, L., Zhou, B., Liu, X., Wang, T., Shih, J., Qi, C., Heung, Y. and Yen, Y. Structurally dependent redox property of ribonucleotide reductase subunit p53R2, Cancer Res., *66:* 1900-1905, 2006.

33. Liu, X., Zhou, B., Xue, L., Shih, J., Tye, K., Lin, W., Qi, C., Chu, P., Un, F., Wen, W. and Yen, Y. Metastasis-suppressing potential of ribonucleotide reductase small subunit p53R2 in human cancer cells, Clin.Cancer Res., *12:* 6337-6344, 2006.

34. Liu, X., Zhou, B., Xue, L., Yen, F., Chu, P., Un, F. and Yen, Y. Ribonucleotide reductase subunits M2 and p53R2 are potential biomarkers for metastasis of colon cancer, Clin.Colorectal Cancer, *6:* 374-381, 2007.

35. Barber Jr, J. and Shewach, D. S. siRNA for p53R2 Inhibits Increases in p53R2 Expression in Response to DNA Damaging Agents, Proceedings for the American Association for Cancer Research, 2006.

36. Fertil, B., Dertinger, H., Courdi, A. and Malaise, E. P. Mean inactivation dose: a useful concept for intercomparison of human cell survival curves, Radiat.Res., *99:* 73-84, 1984.

37. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W. and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells, Cancer Res., *54:* 3218-3223, 1994.

38. Shewach, D. S. Quantitation of deoxyribonucleoside 5'-triphosphates by a sequential boronate and anion-exchange high-pressure liquid chromatographic procedure, Anal.Biochem., *206:* 178-182, 1992.

39. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y. and Nakamura, Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage, Nature, *404:* 42-49, 2000.

40. Zhou, B., Liu, X., Mo, X., Xue, L., Darwish, D., Qiu, W., Shih, J., Hwu, E. B., Luh, F. and Yen, Y. The human ribonucleotide reductase subunit hRRM2 complements p53R2 in response to UV-induced DNA repair in cells with mutant p53, Cancer Res., *63:* 6583-6594, 2003.

41. Yanamoto, S., Iwamoto, T., Kawasaki, G., Yoshitomi, I., Baba, N. and Mizuno, A. Silencing of the p53R2 gene by RNA interference inhibits growth and enhances 5-fluorouracil sensitivity of oral cancer cells, Cancer Lett., *223:* 67-76, 2005.

42. Shao, J., Zhou, B., Zhu, L., Qiu, W., Yuan, Y. C., Xi, B. and Yen, Y. In vitro characterization of enzymatic properties and inhibition of the p53R2 subunit of human ribonucleotide reductase, Cancer Res., *64:* 1-6, 2004.

43. Robinson, B. W. and Shewach, D. S. Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines, Clin.Cancer Res., *7:* 2581-2589, 2001.

44. Robinson, B. W. and Shewach, D. S. Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines, Clin.Cancer Res., *7:* 2581-2589, 2001.

45. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W. and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells, Cancer Res., *54:* 3218-3223, 1994.

Chapter V

CONCLUSIONS

The major goal of this dissertation was to better understand the mechanisms by which dFdCyd potentiates radiation-induced cell killing. Previous work has demonstrated that radiosensitization with dFdCyd correlated with dATP depletion [1-3] and S-phase accumulation [3-5] prior to radiation exposure. We evaluated the roles of p53, and p53R2, a ribonucleotide reductase small subunit homologue, in the radiosensitizing process. Whereas previous studies focused on low concentrations of dFdCyd in radiosensitization [2], it was important to determine the effect of high, clinically relevant concentrations on induction of the cell cycle regulatory protein p53 and subsequent radiosensitization. Studies in Chapter II demonstrated that although we were able to rapidly induce p53 expression in response to clinically relevant concentrations of dFdCyd, excellent radiosensitization was observed in MCF-7 cells. As ribonucleotide reductase is a target of dFdCyd, it was a natural progression to investigate potential roles of p53R2, a newly discovered ribonucleotide reductase subunit, in our radiosensitizing model. Also the accumulated work in this area has not defined a role for p53 in dFdCyd-mediated radiosensitization [6]. Since p53 promotes both pathways of survival and cell death, the survival pathways could nullify the effects of the death pathways. By targeting a component of the survival pathway, it may be possible to shift the effects of p53 toward cell death. Previous studies

had reported a slow increase in p53R2 expression in response to large doses of ionizing radiation [6]. Therefore it was necessary to first determine if p53R2 would be induced by dFdCyd quickly enough to protect dNTP pools from depletion. In Chapter III we evaluated the kinetics of p53R2 expression in response to dFdCyd and/or ionizing radiation and observed a dose-dependent effect on induction. However, induction of p53R2 did not affect dATP depletion or radiosensitization in cell lines isogenic for p53 status. In Chapter IV we more closely evaluated the effects of p53R2 expression on radiosensitization by silencing p53R2 in MCF-7 and A549 cells using siRNA. We observed an increase in radiosensitization in A549 cells following silencing of p53R2, which corresponded to an increase in dATP depletion.

Much work has been dedicated to evaluating the effects of p53 on dFdCyd-mediated cytotoxicity and radiosensitization [7-14]. In particular, work in D54 glioblastoma cells appeared to indicate that p53 expression resulted in G1 accumulation in response to dFdCyd, perhaps due to damaged cells progressing from S-phase into G2/M and ultimately G1. This suggested that p53 could alter clinical response by preventing the S-phase accumulation necessary for radiosensitization. Since tumors are generally a mixture of p53 wt and mt cells, this would be a major clinical issue. Our work here confirmed the opposite, that clinically relevant concentrations of dFdCyd combined with ionizing radiation resulted in increased radiosensitization compared to lower dFdCyd concentrations in MCF-7 cells, a finding that bodes well for clinical outcome. The D54 cell line has been unique in its resistance to radiosensitization. Closer

examination of this cell line could provide information on which cellular characteristics result in resistance to dFdCyd-mediated radiosensitization, giving clinicians more tools to enhance clinical outcome. Overall, the mounting evidence suggests that the effects of p53 on dFdCyd-mediated cytotoxicity and radiosensitization are cell line specific.

We initiated a study to closely examine the kinetics of p53R2 expression in response to a chemotherapeutic agent, either alone or in combination with radiation. Interestingly, p53R2 expression was induced within 4 hr of dFdCyd addition in MCF-7 cells, even at low, non-cytotoxic concentrations. Our lab has subsequently demonstrated that non-cytotoxic concentrations of dFdCyd can cause an increase in DNA damage or as measured by H2AX phosphorylation (Michael Im, unpublished results). DNA damage as measured by phosphorylated H2AX returned to near-control levels within 6 hr after dFdCyd removal. Previous work has demonstrated that most radiation-induced DNA damage is repaired within this time frame as well [15]. However, p53R2 expression remained elevated for at least 48 hr after drug removal and for at least 72 hr post-irradiation in the MCF-7 cell line, and data also indicated that p53R2 remained elevated for at least 96 hr after dFdCyd washout in the A549 cell line. Our study also demonstrated that p53 expression returned to near control levels by 48 hr post-washout following exposure to low or moderate concentrations of dFdCyd, although expression of p53 remained elevated at this timepoint after exposure to IC_{90} dFdCyd or ionizing radiation in MCF-7 cells. This begs the question, why does p53R2 expression remain elevated long after DNA damage

121

is expected to have been repaired? One possible answer is that p53R2, once activated, remains upregulated as a safeguard against future insults. It is also possible that p53R2 remains upregulated due to the occurrence of secondary lesions that previous studies were not designed to detect such as persistent DNA mismatches. One can also speculate that continued persistence of p53R2 expression serves in signaling programmed cell death, although little to no cell death would be expected following exposure to IC_{10} dFdCyd.

Despite observing significant inhibition of p53R2 using siRNA silencing in both cell lines, we only observed an increase in radiosensitization in the A549 cell line. Our data indicate that silencing of p53R2 in A549 cells increases dFdCyd-mediated radiosensitization as measured by an increase in the radiation enhancement ratio (RER) from 1.3 to 1.8. A recent report by Yen *et al* has demonstrated that suppression of p53R2 results in depleted dNTP pools [16]. However, in the MCF-7 cell line, we were not able to increase radiosensitization with dFdCyd by suppression of p53R2. It has been long established that dFdCyd significantly alters dNTP pools, most importantly a drastic reduction in dATP levels in solid tumor cells, and this reduction of dATP correlates with radiosensitization [3,17]. In the MCF-7 cell line, it is likely that addition of dFdCyd results in dNTP depletion to such an extent that suppression of p53R2 has little added benefit to dFdCyd-mediated radiosensitization. However, previous unpublished data from our lab indicates that addition of dFdCyd only results in a moderate decrease in dNTP pools in A549 cells. Since we did not observe complete silencing of p53R2 in MCF-7 cells, it is possible that the remaining level

of p53R2 was sufficient to maintain dNTP pools in response to dFdCyd. However, previous work in our lab demonstrated no change in dFdCyd-mediated radiosensitization in MCF-7 cells at the IC_{50} compared to the IC_{10}, despite a more rapid and complete depletion of dATP [13], suggesting that further suppression of p53R2 will likely not increase radiosensitization. Western blot analysis also indicated two discrete bands corresponding to p53R2 in MCF-7 cells, opposed to a singular band in A549 cells. It is possible that the second observed band in MCF-7 cells represents partially degraded p53R2 or a dominant negative version of this protein. Either of these scenarios could presumably result in lowered p53R2 activity in this cell line, which is supported by increased dATP depletion observed in MCF-7 cells at the IC_{10} compared to A549 cells, a hypothesis requiring further laboratory investigation.

Our work here suggests that upregulation of p53R2 results in dNTP pool maintenance in A549 cells, in contrast to a report by Thelander *et al* suggesting that upregulation of p53R2 does not significantly alter dNTP pools following exposure to adriamycin or ultraviolet radiation [18]. However, that study reported a markedly slower and more modest increase in p53R2 expression than we observed. The Thelander study utilized cells derived from normal tissues, and it is possible that p53R2 is induced at much lower levels in normal tissue compared to cancer cells. These findings suggest that the role of p53R2 may shift once a tissue becomes cancerous, or perhaps play a role in tumorigenesis. In this scenario, in normal tissues, p53R2 is maintained at low levels and acts primarily to maintain a basal level of dNTPs for DNA repair in non-cycling cells once

123

paired with R1. Conversely, in cancerous tissues, p53R2 can be upregulated quickly to respond to chemotherapeutics and other insults that could result in tumor death if left unchecked, allowing for the continued progression of cancerous lesions. This hypothesis is supported by a number of histological studies that found p53R2 to be undetectable in normal tissues, but increasingly expressed with increased tumor progression [19-21]. This scenario, while intriguing, would require further investigation to be confirmed.

The increasing research in this field indicates that p53R2 is an attractive target for gene manipulation in cancer therapy. As previously mentioned, silencing of p53R2 does result in a moderate increase in 5-fluorouracil-mediated cytotoxicity [22]. Inhibition of p53R2 could result in both a decreased rate and fidelity of DNA repair after exposure to DNA damaging agents, due to perturbations in the dNTP pools. The effects of p53R2 suppression are most likely to be of benefit with agents that create or potentiate oxidative stress, such as radiation and radiomimetics. It has been previously demonstrated that p53R2-null mice die within 14 weeks post-birth due to kidney failure caused by oxidative stress [23,24], and p53R2 has been reported to possess antioxidant activity [25]. Silencing or suppression of p53R2 may increase the therapeutic benefits of these agents by increasing their cytotoxic potential within the tumor. As a result, these agents could be administered at lower doses, thereby diminishing death to normal tissue and improving the quality of life for patients. It must be noted that we did not observe a difference in radiation sensitivity in our studies following suppression of p53R2. However, our studies were designed to

achieve optimal silencing of p53R2 at the time of dFdCyd exposure. A study in which p53R2 silencing was optimal at the time of irradiation could possibly produce a different result.

On the other hand, manipulation of p53R2 may not occur without cost. Recent studies have indicated that p53R2 prevents metastasis, counteracting the pro-metastatic properties of R2 [26,27]. Long-term suppression of p53R2 in wt p53 tumors may result in increased cytotoxicity locally, but produce deleterious effects globally. However, p53R2 will remain an exciting gene therapy target, particularly in combination with current ribonucleotide reductase inhibitors that appear to inhibit R1/R2 more effectively than R1/p53R2. Scientists and clinicians alike must move cautiously in investigation and incorporating this strategy into treatment.

In our studies, suppression of p53R2 led to increased radiosensitization in A549 cells. However, other studies did not demonstrate a difference in radiosensitization when p53 was disrupted in other wt p53 cell lines [7,28]. Since p53R2 is directly induced by p53, it seems puzzling on the surface that disruption of p53 did not alter radiosensitization in the same manner as silencing of p53R2. Although those studies did not examine dNTP pool depletion, it is likely that dNTP pools were depleted in response to dFdCyd similarly following p53 disruption as we observed following p53R2 silencing. It is possible that, following disruption of p53, cells can accumulate DNA damage without being targeted for programmed cell death, thus leaving damaged cells free to propagate.
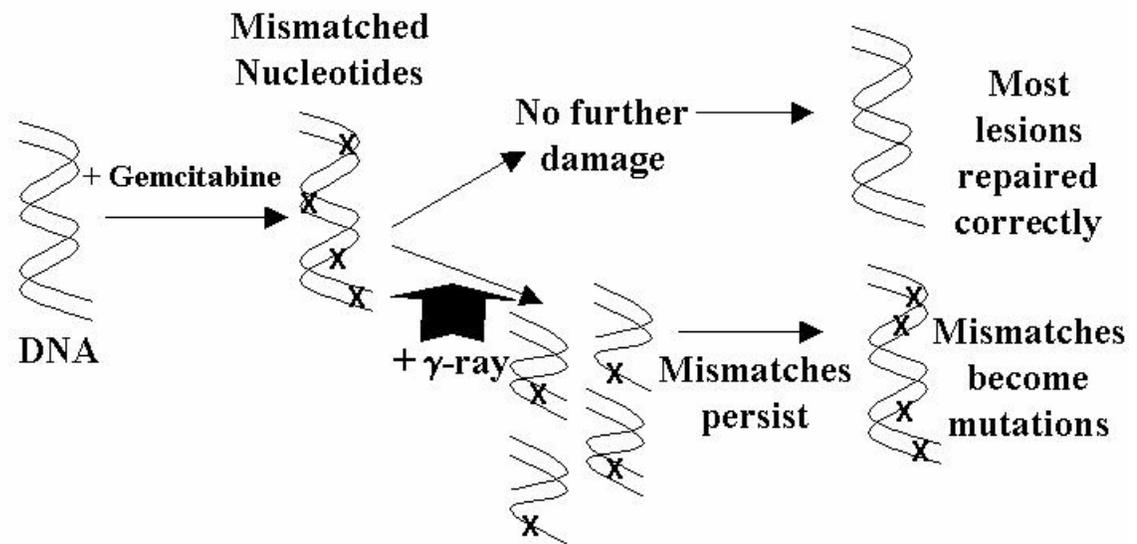
Figure 5.1. Proposed Mechanism of dFdCyd-Mediated Radiosensitization.

Conversely, when p53R2 is inhibited in p53-expressing cells, p53 can induce programmed cell death and damaged cells are eliminated.

Much work in this laboratory and others has helped to form our current model of dFdCyd-mediated radiosensitization (Fig 5.1). Following exposure to dFdCyd alone, the dFdCDP-mediated imbalance in dNTP pools will result in DNA mismatches. Without further insults, most mismatches can be repaired and most cells can recover. However, if ionizing radiation follows dFdCyd exposure, increased DNA damage in the face of depleted dNTP pools results in increased persisting mismatches which eventually become mutations, resulting in cell death. This hypothesis is supported by previous studies in this laboratory [29]. In wt p53 cells, upregulation of p53R2 in response to dFdCyd and ionizing radiation may result in dNTP pool maintenance and fewer persisting mutations, thus reducing radiosensitization. A future project in this laboratory will evaluate the effects of p53R2 silencing on mutation frequency in response to dFdCyd and radiation in an attempt to correlate these findings into a more comprehensive theory. If our current hypothesis is correct, then silencing of p53R2 in A549 cells should result in increased mutation frequency to correspond to increased radiosensitization.

The discovery of p53R2 by Tanaka *et al* was considered a major development for the ribonucleotide reductase field, as it potentially answered the longstanding question of how dNTPs were produced for DNA repair outside of S-phase. Further research on this novel protein has uncovered new and unexpected roles, so it appears that p53R2 has an even greater role than initially anticipated. As

we were able to increase dFdCyd-mediated radiosensitization through silencing of p53R2 in the A549 cell line, p53R2 appears to be an attractive target for gene manipulation for select treatments and cancer types. This manipulation by no means should be viewed as a cure-all, and much work is still required in identifying both the treatments and cancer types that will benefit most from inhibition of p53R2. As the field moves closer toward the individualization of cancer treatment, identifying targets such as p53R2 will be of great importance in further understanding cancer progression and achieving greater clinical success.

References

1. Latz, D., Fleckenstein, K., Eble, M., Blatter, J., Wannenmacher, M., and Weber, K. J. Radiosensitizing potential of gemcitabine (2',2'-difluoro-2'-deoxycytidine) within the cell cycle in vitro. Int.J.Radiat.Oncol.Biol.Phys., *41*: 875-882, 1998.

2. Ostruszka, L. J. and Shewach, D. S. The role of cell cycle progression in radiosensitization by 2',2'-difluoro-2'-deoxycytidine. Cancer Res., *60*: 6080-6088, 2000.

3. Shewach, D. S., Hahn, T. M., Chang, E., Hertel, L. W., and Lawrence, T. S. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells. Cancer Res., *54*: 3218-3223, 1994.

4. Lawrence, T. S., Chang, E. Y., Hahn, T. M., Hertel, L. W., and Shewach, D. S. Radiosensitization of pancreatic cancer cells by 2',2'-difluoro-2'-deoxycytidine. Int.J.Radiat.Oncol.Biol.Phys., *34*: 867-872, 1996.

5. Lawrence, T. S., Chang, E. Y., Hahn, T. M., and Shewach, D. S. Delayed radiosensitization of human colon carcinoma cells after a brief exposure to 2',2'-difluoro-2'-deoxycytidine (Gemcitabine). Clin.Cancer Res., *3*: 777-782, 1997.

6. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. Nature, *404*: 42-49, 2000.

7. Chen, M., Hough, A. M., and Lawrence, T. S. The role of p53 in gemcitabine-mediated cytotoxicity and radiosensitization. Cancer Chemother.Pharmacol., *45*: 369-374, 2000.

8. Fechner, G., Perabo, F. G., Schmidt, D. H., Haase, L., Ludwig, E., Schueller, H., Blatter, J., Mller, S. C., and Albers, P. Preclinical evaluation of a radiosensitizing effect of gemcitabine in p53 mutant and p53 wild type bladder cancer cells. Urology, *61*: 468-473, 2003.

9. Galmarini, C. M., Clarke, M. L., Falette, N., Puisieux, A., Mackey, J. R., and Dumontet, C. Expression of a non-functional p53 affects the sensitivity of cancer cells to gemcitabine. Int.J.Cancer, *97*: 439-445, 2002.

10. Kielb, S. J., Shah, N. L., Rubin, M. A., and Sanda, M. G. Functional p53 mutation as a molecular determinant of paclitaxel and gemcitabine susceptibility in human bladder cancer. J.Urol., *166*: 482-487, 2001.

11. Lin, X., Ramamurthi, K., Mishima, M., Kondo, A., Christen, R. D., and Howell, S. B. P53 modulates the effect of loss of DNA mismatch repair on

the sensitivity of human colon cancer cells to the cytotoxic and mutagenic effects of cisplatin. Cancer Res., *61*: 1508-1516, 2001.

12. Rieger, J., Durka, S., Streffer, J., Dichgans, J., and Weller, M. Gemcitabine cytotoxicity of human malignant glioma cells: modulation by antioxidants, BCL-2 and dexamethasone. Eur.J.Pharmacol., *365*: 301-308, 1999.

13. Robinson, B. W. and Shewach, D. S. Radiosensitization by gemcitabine in p53 wild-type and mutant MCF-7 breast carcinoma cell lines. Clin.Cancer Res., *7*: 2581-2589, 2001.

14. Tolis, C., Peters, G. J., Ferreira, C. G., Pinedo, H. M., and Giaccone, G. Cell cycle disturbances and apoptosis induced by topotecan and gemcitabine on human lung cancer cell lines. Eur.J.Cancer, *35*: 796-807, 1999.

15. Iliakis, G. The role of DNA double strand breaks in ionizing radiation-induced killing of eukaryotic cells. Bioessays, *13*: 641-648, 1991.

16. Liu, X., Zhou, B., Xue, L., Shih, J., Tye, K., Qi, C., and Yen, Y. The ribonucleotide reductase subunit M2B subcellular localization and functional importance for DNA replication in physiological growth of KB cells. Biochem.Pharmacol., *70*: 1288-1297, 2005.

17. Shewach, D. S. and Lawrence, T. S. Radiosensitization of human tumor cells by gemcitabine in vitro. Semin.Oncol., *22*: 68-71, 1995.

18. Hakansson, P., Hofer, A., and Thelander, L. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. J.Biol.Chem., *281*: 7834-7841, 2006.

19. Okumura, H., Natsugoe, S., Yokomakura, N., Kita, Y., Matsumoto, M., Uchikado, Y., Setoyama, T., Owaki, T., Ishigami, S., and Aikou, T. Expression of p53R2 is related to prognosis in patients with esophageal squamous cell carcinoma. Clin.Cancer Res., *12*: 3740-3745, 2006.

20. Uramoto, H., Sugio, K., Oyama, T., Hanagiri, T., and Yasumoto, K. P53R2, p53 inducible ribonucleotide reductase gene, correlated with tumor progression of non-small cell lung cancer. Anticancer Res., *26*: 983-988, 2006.

21. Yanamoto, S., Kawasaki, G., Yoshitomi, I., and Mizuno, A. Expression of p53R2, newly p53 target in oral normal epithelium, epithelial dysplasia and squamous cell carcinoma. Cancer Lett., *190*: 233-243, 2003.

22. Yanamoto, S., Iwamoto, T., Kawasaki, G., Yoshitomi, I., Baba, N., and Mizuno, A. Silencing of the p53R2 gene by RNA interference inhibits growth and enhances 5-fluorouracil sensitivity of oral cancer cells. Cancer Lett., *223*: 67-76, 2005.

23. Kimura, T., Takeda, S., Sagiya, Y., Gotoh, M., Nakamura, Y., and Arakawa, H. Impaired function of p53R2 in Rrm2b-null mice causes severe renal failure through attenuation of dNTP pools. Nat.Genet., *34*: 440-445, 2003.

24. Powell, D. R., Desai, U., Sparks, M. J., Hansen, G., Gay, J., Schrick, J., Shi, Z. Z., Hicks, J., and Vogel, P. Rapid development of glomerular injury and renal failure in mice lacking p53R2. Pediatr.Nephrol., *20*: 432-440, 2005.

25. Xue, L., Zhou, B., Liu, X., Wang, T., Shih, J., Qi, C., Heung, Y., and Yen, Y. Structurally dependent redox property of ribonucleotide reductase subunit p53R2. Cancer Res., *66*: 1900-1905, 2006.

26. Liu, X., Zhou, B., Xue, L., Shih, J., Tye, K., Lin, W., Qi, C., Chu, P., Un, F., Wen, W., and Yen, Y. Metastasis-suppressing potential of ribonucleotide reductase small subunit p53R2 in human cancer cells. Clin.Cancer Res., *12*: 6337-6344, 2006.

27. Liu, X., Zhou, B., Xue, L., Yen, F., Chu, P., Un, F., and Yen, Y. Ribonucleotide reductase subunits M2 and p53R2 are potential biomarkers for metastasis of colon cancer. Clin.Colorectal Cancer, *6*: 374-381, 2007.

28. Pauwels, B., Korst, A. E., Andriessen, V., Baay, M. F., Pattyn, G. G., Lambrechts, H. A., Pooter, C. M., Lardon, F., and Vermorken, J. B. Unraveling the mechanism of radiosensitization by gemcitabine: the role of TP53. Radiat.Res., *164*: 642-650, 2005.

29. Flanagan, S. A., Robinson, B. W., Krokosky, C. M., and Shewach, D. S. Mismatched nucleotides as the lesions responsible for radiosensitization with gemcitabine: a new paradigm for antimetabolite radiosensitizers. Mol.Cancer Ther., *6*: 1858-1868, 2007.