Stable Suppression of the R2 Subunit of Ribonucleotide Reductase by R2-targeted Short Interference RNA Sensitizes p53(−/−) HCT-116 Colon Cancer Cells to DNA-damaging Agents and Ribonucleotide Reductase Inhibitors*

Received for publication, February 25, 2004, and in revised form, March 25, 2004
Published, JBC Papers in Press, April 19, 2004, DOI 10.1074/jbc.M402056200

Z. Ping Lin‡, Michael F. Belcourt§, Joseph G. Cory¶, and Alan C. Sartorelli||

From the ‡Department of Pharmacology and Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06520, §Vion Pharmaceuticals Incorporated, New Haven, Connecticut 06511, and the ¶Department of Biochemistry, East Carolina University School of Medicine, Greenville, North Carolina 27834

Ribonucleotide reductase catalyzes the production of deoxyribonucleoside diphosphates, the precursors of deoxyribonucleoside triphosphates for DNA synthesis. Mammalian ribonucleotide reductase (RNR) is a tetramer consisting of two non-identical homodimers, R1 and either R2 or p53R2, which are considered to be involved in DNA replication and repair, respectively. We have demonstrated that DNA damage by doxorubicin and cisplatin caused a steady elevation of the R2 protein in p53(−/−) HCT-116 human colon carcinoma cells but induced degradation of the protein in p53(+/+). To evaluate the involvement of R2 in response to DNA damage, p53(−/−) HCT-116 cells were stably transfected with an expression vector transcribing short hairpin/short interference RNA directed against R2 mRNA. Stably transfected clones exhibited a pronounced reduction of the R2 protein with no change in the cellular growth rate. Furthermore, short interference RNA-mediated reduction of the R2 protein caused a marked increase in sensitivity to the DNA-damaging agent cisplatin as well as to the RNR inhibitors Triapine® and hydroxyurea. Ectopic expression of p53R2 partially reversed the cytotoxicity of cisplatin but not that of RNR inhibitors to R2 knockout cells. The increase in sensitivity to cisplatin and RNR inhibitors was correlated with the suppression of dATP and dCTP levels caused by stable expression of R2-targeted short interference RNA. These results indicated that DNA damage resulted in elevated levels of the R2 protein and dNTPs and, consequently, enhanced the survival of p53(−/−) HCT-116 cells. The findings provide evidence that R2-RNR can be employed to supply dNTPs for the repair of DNA damage in cells with an impaired p53-dependent induction of p53R2.

Ribonucleotide reductase (RNR) catalyzes a rate-limiting reaction in which ribonucleoside diphosphates are converted to their corresponding deoxyribonucleoside diphosphates, the precursors of deoxyribonucleoside triphosphates (dNTPs) required for DNA synthesis and repair (1). In mammalian cells, the catalytically active RNR is considered to be an αβ heterotetramer consisting of two large R1 subunits and two small R2 subunits. To maintain a balanced size of dNTP pools (2), the enzymatic activity of RNR is tightly regulated by the binding of nucleoside triphosphates (ATP, dATP, dGTP, and dTTP) to allosteric sites in the R1 subunit (1). In proliferating cells, the level of the R2 protein is low in the G1 phase of the cell cycle but accumulates and reaches maximal levels during the S phase, followed by degradation when passing through the G2/M phase (3–6). Thus, the activity of RNR is additionally controlled by cell cycle-specific availability of the R2 subunit, whereas the R1 protein level remains relatively constant throughout the cell cycle (3, 4). When cells undergo G1 arrest following DNA damage, the supply of dNTPs for DNA repair is, in turn, provided by transcriptional activation of the recently identified R2 homologue, p53R2 (7, 8). Thus, DNA damage causes p53-dependent induction of p53R2 that complexes with R1 to form active RNR in arrested cells (9).

The discovery of p53R2 by Tanaka et al. (7) provides an explanation of how cells with damaged DNA can acquire a supply of dNTPs for DNA repair when the R2 level is repressed during G1 arrest; they proposed two independent pathways that lead to the supply of dNTPs in mammalian cells. One is mediated by the R2 subunit of RNR in maintaining dNTP pools for DNA replication during the S phase of the cell cycle. The other involves p53R2 to supply dNTPs for DNA repair when DNA damage occurs (7, 10). However, with >50% of human cancers containing mutated or deleted p53, it is unclear whether malignant cells that lack inducible p53R2 are capable of supplying sufficient dNTPs through this pathway for DNA repair.

Early studies have demonstrated a positive correlation between the enzymatic activity of RNR and the rate of proliferation of transplanted Novikoff carcinomas (11, 12). Thus, because of its critical role in the de novo synthesis of DNA, RNR is considered to be a potential target for the development of cancer chemotherapeutic agents. At present, hydroxyurea is the only clinically used RNR inhibitor; however, several more potent and specific inhibitors of RNR have been developed and are currently being evaluated clinically (13–17). We have shown previously that the RNR inhibitor Triapine, in conjunction with a variety of DNA-damaging agents, produces synergistic inhibition of transplanted L1210 leukemia cells, presumably by inhibiting the ability of cancer cells to repair drug-induced damage to DNA (18). Thus, it is important to fully
understand the regulation of RNR and its role in DNA repair, especially for cancer cells with mutated p53.

In the present study, we have examined the regulation of the R2 and p53R2 subunits of RNR in response to DNA damage in p53<sup>−/−</sup> and p53<sup>+/−</sup> cells. The studies were derived from early passaged cells stored in liquid nitrogen and expanded into cell lines. The empty vector-transfected cell lines were trypsinized and plated in a medium containing 50 μg/ml ganciclovir resistance marker (20) were purchased from Invitrogen. pmU6 p53R2 containing N-terminal FLAG-tagged p53R2 cDNA (8) were obtained from Dr. Karen Vousden (NCI, National Institutes of Health, Bethesda, MD). The plasmid pCMV/βd expressing the basic leucine zipper (19) and the plasmid pcDNA3.1/Zeoc expressing the Zeocin resistance marker (20) were purchased from Invitrogen. pmU6 (21) containing the mouse U6 promoter (−315/+1) was provided by Dr. Sydney Altman (Yale University; New Haven, CT). The DNA sequences of the mouse U6 promoter alone and the mouse U6 promoter adjacent upstream to R2-targeted sh/siRNA were amplified by PCR using the pmU6 plasmid as a template. The sh/siRNA sequence contains a 19-nucleotide sense sequence of human R2 cDNA (696−714; GenBank® accession number BC001886) separated by a nine-nucleotide spacer, TACCTATGGTGAACTCTCTAGAAGTTCACCATAGGTAGCCTCAAA−3′ (TTCTTTCTTTCTTTCTTTCGGCGGTGGAGGCGG−3′). Following amplification, the PCR products were subcloned into the TA cloning vector pCR-XL-TOPO (Invitrogen). The orientation and DNA sequencing. The fragments were released from the cloning vector by digestion with MluI and NotI. To create the R2 siRNA expression cassette, the 19-nucleotide antisense sequence complementary to the sense sequence and followed by five consecutive Ts as the transcriptional terminator. The PCR primers used are the forward (5′-GATCAGGACGGCGCCATCTC-3′) and the reverse (5′-AAAAAGAGCG-TACCTATGGTGAACTCTCTAGAAGTTCACCATAGGTAGCCTCAAA-GG-3′). Following amplification, the PCR products were subcloned into the TA cloning vector pCR-XL-TOPO (Invitrogen). The orientation and sequence of the inserts were confirmed by restriction digestion and DNA sequencing. The fragments were released from the cloning vector by digestion with MluI and NotI. To create the R2 siRNA expression vector pZeo-R2-siRNA696 or the empty vector pZeo/βd, the fragment was ligated into pcDNA3.1/Zeo from which the CMV promoter was removed by digestion with MluI and NotI.

Stable Transfection—p53<sup>−/−</sup> HCT-116 cells were seeded in T25 flasks for 24 h prior to transfection. Cells were co-transfected with 5 μg of the empty vector pCMVneo or pCMV-FLAG-p53R2 with 0.5 μg of pCMV/βd using the TransFast transfection reagent (Promega, Madison, WI) according to the manufacturer’s instructions. After 48 h, cells were trypsinized and plated in a medium containing 5% FCS and 5 μg/ml G418. Following selection for 2 weeks, stable populations of p53R2-resistant cells were pooled and single-cell sorted into 96-well plates with a growth medium containing 8 μg/ml G418. Sorted single cells were grown under selection for an additional 2 weeks and expanded into stable cell lines. Cells stably expressing FLAG-p53R2 were screened and identified by reverse transcription PCR and Western blotting analyses. For stable silencing of R2, previous generated stable p53<sup>−/−</sup> HCT116 cell lines were transfected with 6 μg of the empty vector pZeo/βd or pZeo-R2-siRNA696 as described above. After 48 h, cells were trypsinized and plated in a medium containing 50 μg/ml Zeocin. Resistant colonies of R2-silenced cells were individually isolated and expanded into cell lines. The empty vector-transfected cell lines and were total populations of resistant colonies. All stable cell lines used in the studies were derived from early passaged cells stored in liquid nitrogen immediately upon verification of their expression status.

Western Blotting and Immunoprecipitation—Cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4, and 1% SDS), boiled, and passed through a Qiashredder (Qagen; Valencia, CA) to shear DNA. Protein concentrations were determined by the Bio-Rad D<sub>2</sub> protein assay according to the manufacturer’s instructions. Forty micrograms of protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% milk in TBS (10 mM Tris-HCl, pH 8, 150 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature and incubated with primary antibody in the blocking solution at 4 °C overnight. The membrane was subsequently washed with TBS, incubated with a horse-radish peroxidase-conjugated secondary antibody in blocking solution at room temperature for 1 h, and washed again. The target protein was visualized by the enhanced chemiluminescence method (Amersham Biosciences). For immunoprecipitations, cells were dissolved in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.5% Nonidet P-40) with Complete Mini protease inhibitor mixture (Roche Applied Science). The lysate was cleared and incubated with goat polyclonal anti-R1 antibody (Santa Cruz Biotechnology) at 4 °C for 1 h and then incubated with protein A/G Plus agarose beads (Santa Cruz Biotechnology) with constant agitation at 4 °C overnight. The agarose beads were collected, washed, and resuspended in 1× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 0.006% b-mercaptoethanol, and 1% w/v sodium deoxycholate) and boiled for 5 min. The supernatant cleared from the agarose beads was then collected for analysis by Western blotting.

Cell Viability Assay—Cells were seeded into 96-well plates at 3000 cells/well in complete McCoy’s medium and incubated for 24 h. Each drug was then added at various concentrations to quadruplicate wells in a final volume of 100 μl of medium. After 72 h of incubation, 20 μl of MTS tetrazolium reagent (CellTiter 96 One Solution; Promega) was added, and the incubation was continued for an additional 2 h at 37 °C. The absorbance of soluble MTS tetrazolium formazan produced by viable cells was measured at 490 nm using a microplate reader. The sensitivity of cells to each drug was expressed as a percentage of the vehicle-treated control.

Quantification of dNTP Levels—Cellular deoxyribonucleoside triphosphates were quantified as described by Sherman and Fyfe (22) and modified by Gao et al. (23). Approximately 1 × 10<sup>6</sup> exponentially growing cells were untreated or treated with 20 μl of cisplatin or 0.5 μl Triapine, washed twice with phosphate-buffered saline, and then extracted with 60% methanol. The extracts were heated to 95 °C for 5 min, centrifuged briefly to remove cell debris, and frozen at −70 °C until analyzed. The following oligonucleotides were used in the procedure: for dCTP determination, 5′-TTCCTCTTCCTTCTTCTTCTGCGGGTTAGGCGCGGC-3′; for dGTP determination, 5′-TTCTTTCTCTTCTTCTTCTTGGGCGTTAGGCGCGGC-3′; for dATP determination, 5′-ATATTATTATTATTATTAGGGCGGTTAGGCGCGGC-3′; for dTTP determination, 5′-TAAGTGGGTTAGGCGTTAGGCGCGGC-3′; for dTTP determination, 5′-AATAAAATATATATATATATATATGCCTCAGGC-3′; and for dGTP determination, 5′-ATATTATTATTATTATTATTATTATTATTATGAGCGCGGC-3′. The reaction mixture contained 0.05 μl of Taq DNA polymerase (Qua- gen), 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.25 μM template primer, and 2.5 μM [3H]dATP (15 Ci/mmol, for dCTP, dTTP, and dGTP determinations) or 2.5 μM [3H]dTTP (15 Ci/mmol for dATP determination). Five microliters of extract from ~1 × 10<sup>5</sup> cells was then added to 45 μl of the reaction mix and incubated for 30 min at 37 °C. Forty microliters of the mixture was applied onto Whatman DE51 paper, which was washed three times with 5% Na<sub>2</sub>PO<sub>4</sub> (10 min/wash), rinsed with distilled water and then with 95% ethanol, and dried. The radioactivity on the filters was quantified by liquid scintillation spectrometry. The amount of radioactivity incorporated into DNA was proportional to the concentration of the dNTP being quantified.

RESULTS

DNA Damage by Doxorubicin- and Cisplatin-induced p53R2 and R2 Expression in p53<sup>−/−</sup> and p53<sup>+/−</sup> HCT-116 Cells—The induction of p53R2 in response to DNA damage has been shown to depend upon functional wild-type p53 (7, 8). To determine the effects of p53 on changes in the expression of p53R2 and R2 following DNA damage, protein levels of these subunits in p53<sup>−/−</sup> and p53<sup>+/−</sup> HCT-116 human colon cancer cells were evaluated by Western blot analysis. As shown in Fig. 1, treatment with doxorubicin, a potent DNA-damaging agent, caused a concentration- and time-dependent induction of p53R2 in p53<sup>−/−</sup> but not in p53<sup>+/−</sup> cells. The other p53-inducible protein measured, PIG3 (24), also exhibited a similar response to doxorubicin. The R2 subunit displayed an
expression profile distinct from that of p53R2. Thus, exposure to doxorubicin for 48 h resulted in the disappearance of R2 from p53
(H11001/H11001) cells, presumably by proteolytic degradation (Fig. 1B). In contrast, treatment of p53(H11002/H11002) cells with doxorubicin caused a small but steady increase in R2 without undergoing degradation. Treatment of p53(+/-) cells with cisplatin resulted in a pattern of R2 degradation similar to that caused by doxorubicin (Fig. 1C). The apparent disappearance of the R2 protein caused by cisplatin occurred relatively later at 72 h. These results indicate that R2 protein levels become steadily elevated by DNA damage in p53(-/-) cells in which both p53-dependent p53R2 induction and R2 degradation pathways are defective.

Sensitivity of p53(+/-) and p53(-/-) HCT-116 Cells to Doxorubicin and Other Chemotherapeutic Agents—The sensitivity of p53(+/-) and p53(-/-) HCT-116 cells to various
concentrations of the DNA-damaging chemotherapeutic agents doxorubicin, etoposide, and cisplatin were compared with a measurement of cytotoxicity by the MTS assay. Vincristine, an anti-microtubule agent that induces p53 (25, 26) but does not damage DNA, was also included in the study. As demonstrated in Fig. 2, \( p53(+/+) \) cells exhibited greater sensitivity to doxorubicin, cisplatin, etoposide, and vincristine than did \( p53(-/-) \) HCT-116 cells. Thus, a lack of p53 function in \( p53(-/-) \) cells resulted in a decrease in the sensitivity to both the DNA-damaging and anti-microtubule agents. This finding implies that the increased level of p53R2 measured in response to DNA damage may not be functional in \( p53(+/+) \) HCT-116 cells or may not produce sufficient levels of dNTPs for DNA repair. Because a decrease in sensitivity to microtubule inhibition by vincristine was observed, it is also suggested that p53 activation by the agents employed may predominantly initiate apoptosis and/or cell cycle arrest that overrides the repair of DNA.

Stable Ectopic Expression of \( p53R2 \) in \( p53(-/-) \) HCT-116 Cells—To gain information on the role of p53R2 in DNA repair and sensitivity to DNA-damaging agents, it was important to ascertain the impact of other p53-dependent events such as apoptosis on the survival of HCT-116 cells. To accomplish this goal, stable \( p53(-/-) \) HCT-116 cell lines transfected with the \( p53R2 \) expression vector and selected for ectopic expression of \( p53R2 \) were developed. Western blot analyses of an empty vector (henceforth known as "Vector") and \( p53R2 \) expression vector-transfected stable clones are shown in Fig. 3. A pronounced, relatively slow migrating band of FLAG-tagged \( p53R2 \) was detected in clones 6, 8, 11, and 18; the identity of this ectopic expression was further confirmed using an anti-FLAG antibody. All clones, including the Vector, exhibited a low basal level of endogenous \( p53R2 \). The levels of ectopic \( p53R2 \) protein in \( p53(-/-) \) HCT-116 cells were comparable with those of the doxorubicin-induced endogenous \( p53R2 \) observed in \( p53(+/+) \) HCT-116 cells (Fig. 1). Furthermore, ectopic expression of \( p53R2 \) did not cause apparent changes in the levels of R2, suggesting that the expression of both proteins is independently regulated in the absence of DNA damage.

Sensitization to DNA Damage by R2-targeted siRNA

Fig. 2. Sensitivity of \( p53(+/+) \) and \( p53(-/-) \) HCT116 cells to doxorubicin (A), cisplatin (B), etoposide (C), and vincristine (D). Cells were exposed to various concentrations of each agent for 72 h, and survival was subsequently determined by MTS cytotoxicity assays. Data are the means ± S.E. from three independent experiments.

Fig. 3. Stable expression of \( p53R2 \) in \( p53(-/-) \) HCT116 cells. Total protein from stable clones transfected with empty vector and pCMV-FLAG-p53R2 were analyzed by Western blotting using anti-p53R2, anti-FLAG, anti-R2, and anti-actin antibodies. A representative Western blot is shown.
Sensitization to DNA Damage by R2-targeted siRNA

As well as p53R2-expressing clone 6 and clone 8 cells were stably transfected with either the mouse U6 promoter-only vector or the R2-sh/siRNA expression vector. Ten to fifteen stable transfected clones were established from each Vector, clone 6, and clone 8 cell line and screened for suppression of R2 levels by Western blot analysis. About 65% of all clones showed a >50% reduction in R2 protein levels (data not shown). An R2-silenced clone corresponding to Vector, clone 6, or clone 8, each exhibiting an 80–90% reduction in the R2 protein, was chosen and is shown in Fig. 4A. The knockdown of R2 did not affect ectopic p53R2 levels, and these clones exhibited no apparent differences in growth rate as compared with their counterparts that have normal levels of the R2 subunit.

To determine whether differences occurred in the composition of RNR subunits in ectopic p53R2-expressing R2-silenced cells, co-immunoprecipitation experiments were performed. Native protein from Vector, clone 6, and their corresponding R2-silenced clones was immunoprecipitated with an anti-R1 antibody. Co-immunoprecipitates were analyzed by Western blot using anti-p53R2 and anti-R2 antibodies. As shown in Fig. 4B, p53R2 co-immunoprecipitated with R1 was detected only in clone 6 but not in Vector cells. This finding indicates that ectopically expressed p53R2 in p53(−/−) cells forms RNR with an endogenous R1 subunit. As expected, R2 co-immunoprecipitated with R1 was observed in both Vector and clone 6 cells. Silencing of R2 in Vector and clone 6 cells caused a decrease in the levels of R2 co-immunoprecipitated with R1. This observation is consistent with a decrease in the total level of R2 in R2-silenced cells detected by Western blot analysis (Fig. 4A).

Because DNA damage caused by doxorubicin up-regulated R2 in p53(−/−) HCT-116 cells (Fig. 1), we determined whether stable knockdown of R2 suppressed the induction of R2 produced by treatment with DNA-damaging agents. Vector, clone 6, and clone 8, as well as their corresponding R2-silenced counterparts, were exposed to 20 μM cisplatin, 0.25 μM doxorubicin, or 10 nM vincristine for 24 h, and the expression of R2 protein was measured by Western blot analysis. Fig. 4C demonstrates that treatment with cisplatin and doxorubicin, but not with vincristine, resulted in the induction of R2 in Vector, clone 6, and clone 8 cells. DNA damage by cisplatin and doxorubicin also caused the induction of R2 in all R2-silenced clones, but the induced levels of R2 were markedly less than that occurring in R2 non-silenced cells.

Sensitization of p53(−/−) HCT-116 Cells to the DNA-damaging Agent Cisplatin and the RNR Inhibitors Triapine and Hydroxyurea by Knockdown of the R2 Subunit—The effects of R2 knockdown and ectopic p53R2 expression on the sensitivity of p53(−/−) HCT-116 cells to DNA damage and RNR inhibition was evaluated in Vector, clone 6, clone 8, and their corresponding R2-silenced cells. Following exposure to various concentrations of cisplatin, doxorubicin, Triapine, hydroxyurea, and vincristine for 72 h, cytotoxicity was determined by MTS assays. As shown in Fig. 5A, suppression of R2 levels by stable silencing markedly sensitized Vector cells to cisplatin. In contrast, clone 6 and clone 8 cells with suppressed levels of the R2 subunit exhibited only a slight increase in sensitivity to cisplatin. Without the knockdown of R2, clone 6 and clone 8 cells exhibited similar but slightly more resistance to cisplatin than did Vector cells. The profile of sensitivity to doxorubicin was analogous to that of the sensitivity to cisplatin with these cells (data not shown). These results suggest that the sensitivity of p53(−/−) HCT-116 cells to DNA damage caused by cisplatin and doxorubicin is determined by the levels of R2. However, R2 silencing only produced partial sensitization to DNA damage in cells ectopically expressing p53R2.

Stable knockdown of R2 in Vector, clone 6, and clone 8 cells...
also caused a pronounced increase in sensitivity to the RNR inhibitors Triapine and hydroxyurea (Fig. 5, B and C). In this situation, all clones with suppressed levels of R2 exhibited similar enhanced sensitivity to these agents. Thus, ectopic expression of p53R2 in clones 6 and clone 8 cells did not prevent the cytotoxic effects of the RNR inhibitors when the level of R2 is suppressed. These findings demonstrate that the level of R2 critically determines the sensitivity of p53(H11002/H11002/H11002) HCT-116 cells to the inhibitors of RNR. In addition, ectopic expression of p53R2 does not appear to compensate for the decreased levels of R2 caused by stable knockdown. No significant differences occurred among Vector, clone 6, clone 8, and their corresponding R2-silenced clones in their sensitivity to the anti-microtubule agent vincristine (Fig. 5D).

**Suppression of DNA Damage-induced Elevation of dNTPs by Stable Expression of R2-targeted siRNA in p53(H11002/H11002/H11002) HCT-116 Cells**—Changes in dNTP pools associated with R2 silencing and ectopic p53R2 expression were quantified in Vector and clone 6 and their corresponding R2-silenced cells treated with cisplatin. The levels of dNTP were determined in cells exposed to 20 μM cisplatin for 24 h, a time and drug concentration at which substantial induction of the R2 protein was observed (Fig. 4C). As demonstrated in Fig. 6, treatment with cisplatin caused a marked increase in the levels of all four dNTPs in all of the cell lines. R2 knockdown resulted in considerable repression of elevated dNTP levels in cisplatin-treated Vector cells. In contrast, the levels of dNTPs were relatively less affected by R2 silencing in clone 6 cells treated with cisplatin. These results indicate that R2 knockdown leads to the suppression of the DNA damage-induced elevation of dNTP levels in Vector cells. Ectopic expression of p53R2 in clone 6 cells may be capable of partially reversing the decrease in dNTP levels caused by R2 silencing.

**Reduction of dATP and dGTP Levels in p53(H11002/H11002/H11002) HCT-116 Cells Caused by the Inhibition of RNR by Triapine**—The RNR inhibitor Triapine produced greater cytotoxicity toward R2-silenced cells than toward R2 non-silenced cells regardless of the presence of ectopic expression of p53R2 (Fig. 5B). To determine whether this differential sensitivity correlated with changes in RNR activity, the levels of the dNTPs were determined in the presence and absence of Triapine. Vector and clone 6 and their corresponding R2-silenced cells were exposed to 0.5 μM Triapine for 1 h; this treatment resulted in a pronounced decrease in dATP levels and a moderate reduction in dGTP levels. In contrast, the levels of dCTP and dTTP were minimally affected by Triapine treatment in all of the cell lines (Fig. 7). Furthermore, R2 silencing in both Vector and clone 6 cells led to a greater reduction in the levels of dATP (7.9- and 30.3-fold, respectively) than that occurring in cells without R2 silencing (4.4- and 7.8-fold, respectively) in the presence of Triapine (Fig. 7A). By comparison, the effects of R2 silencing on Triapine-suppressed dGTP levels were relatively small (Fig. 7B). These results indicate that the

---

**Fig. 5. Sensitivity of control and R2-silenced p53(−/−) HCT-116 cells (Vector, clone 6, and clone 8) to cisplatin (A), Triapine (B), hydroxyurea (C), and vincristine (D).** Cells were exposed to various concentrations of each agent for 72 h. Cell survival was subsequently determined by the MTS assay. For panels A and D, data are the means ± S.E. from three independent experiments. Panels B and C show representative results performed in triplicate; data are the means ± S.D.
knockdown of R2 enhances the effects of Triapine on the suppression of dATP levels. Clone 6 cells exhibited a pattern of decrease in dATP and dGTP levels similar to that occurring in Vector cells in response to Triapine treatment. This observation is consistent with the cytotoxicity data, suggesting that ectopic expression of p53R2 cannot compensate for the further decrease in dATP pools caused by the knockdown of the R2 subunit following treatment with Triapine.

DISCUSSION

The relatively recent discovery of p53R2 not only provides direct evidence of the involvement of p53 in DNA repair but also has a significant impact on the current knowledge regarding the complex regulation of RNR in both the synthesis of DNA and the response to DNA damage. Our results demonstrate that R2 becomes down-regulated as p53R2 progressively increases following drug-induced damage to DNA in p53(+/+) HCT-116 cells. This phenomenon was also observed with other cell types harboring wild-type p53 (7, 10). These findings imply that, during DNA damage-induced G1 arrest, p53R2 replaces R2 in forming an active RNR with the R1 subunit in a p53-dependent manner. Thus, it is conceivable that cells with wild-type p53 are quite proficient in repairing damaged DNA and, thereby, less sensitive to DNA-damaging agents. This notion is supported by studies showing that abrogation of p53 function sensitizes certain types of cells to DNA damage by cancer chemotherapeutic agents (27, 28).

In contrast, however, our data demonstrate that p53(+/+) HCT-116 cells exhibit greater sensitivity to DNA-damaging and anti-microtubule agents than do p53(−/−) cells. Yamaguchi et al. (10) found that HCT-116 cells contain a point mutation at codon 115 in the p53R2 subunit. This mutation results in a substitution of the amino acid valine by leucine and a loss of RNR activity in these cells. Therefore, these authors concluded that a defect in the function of p53R2 might lead to the activation of the p53-mediated apoptotic pathway in HCT-116 cells. Thus, if p53-dependent apoptosis prevails or predominates over p53R2-mediated DNA repair, it is possible that a lack of p53 function in p53-deficient cells would cause a decrease in sensitivity to chemotherapeutic agent-induced DNA damage.

The involvement of the R2 subunit in DNA repair remains elusive. Studies have shown that DNA damage induces the expression of yeast RNR2, the homologue of mammalian R2, and that mutation of the RNR2 gene results in increased sensitivity to DNA damage (29, 30). Evidence is also available to show that R2 is induced by DNA damage in mammalian cells (31–33). However, the direct contribution of the mammalian R2 subunit to DNA repair is still uncertain. In normal cells, R2 is tightly regulated in a cell-cycle dependent manner; an increased level of the R2 protein occurs during the S phase or during a DNA-damage/replication block, which has been attributed to an increased stability of the protein but not to the elevated transcription of the gene (34, 35). Upon entrance of cells into mitosis, the R2 protein is rapidly degraded by the proteasome-dependent pathway, presumably to prevent unscheduled DNA synthesis (34, 35). These results support our findings in p53(+/+) HCT-116 cells, because these cells exhibit R2 degradation following 48 and 72 h of exposure to...
doxorubicin and cisplatin, respectively (Fig. 1), probably as a result of the cells undergoing p53-dependent G1 arrest after passing through the G2/M phase.

Although the p53R2 subunit has been identified as a DNA damage-inducible RNR subunit in mammalian cells, the majority of human cancers with p53 mutations or deletions would be expected to be devoid of this response, which leads to the repair of damaged DNA. We hypothesize that these neoplastic cells may circumvent this apparent disadvantage by utilizing the R2 subunit as a substitute for p53R2 to supply dNTPs for DNA repair. Thus, our findings demonstrate that p53(H11002/H11002/H11002) HCT-116 cells that do not undergo G1 arrest by DNA damage display no degradation but instead exhibit a steady-state increase in the level of the R2 protein (Fig. 1). This increase in the level of the R2 subunit is strongly correlated with the loss of G1 arrest and a concomitant increase in the number of cells in the S and G2/M phases (data not shown), where the R2 protein is highly stabilized (34).

The induction of the R2 protein following DNA damage may have a strong influence on the survival of p53(H11002/H11002/H11002) HCT-116 cells. Cisplatin-mediated cytotoxicity is largely attributed to the formation of DNA intrastrand crosslinks (36). Thus, the sensitivity to cisplatin may depend upon the ability of cells to remove this DNA lesion primarily through nucleotide excision repair (37, 38). A pronounced increase in the R2 protein in p53(H11002/H11002/H11002) cells following drug-induced DNA damage suggests that an elevation in the activity of RNR occurs concurrently with an enhanced ability to repair damaged DNA. This speculation is supported by our finding that DNA damage by cisplatin causes a marked increase in dNTP levels. Similar findings by others have shown that, in yeast and other cell types, DNA damage by a variety of agents causes an increase in dNTP levels that result in enhanced survival (2, 39). Using the approach of RNA interference to specifically down-regulate the R2 subunit, our results further demonstrate that the level of the R2 protein is strongly correlated with the levels of dNTPs and the sensitivity of cells to cisplatin in p53(H11002/H11002/H11002) HCT-116 cells. Collectively, these findings support the contention that the level of the R2 protein is a reflection of the ability of the cells to participate in DNA repair and, therefore, is a determinant of the sensitivity to DNA damage.

Ectopic expression of p53R2 partially reversed the R2-siRNA-mediated sensitization of p53(H11002/H11002/H11002) HCT-116 cells to DNA damage caused by cisplatin. This observation further demonstrates that R2-RNR and p53R2-RNR can be interchangeable for supplying dNTPs in the process of DNA repair. However, the cytotoxic effect of the RNR inhibitors on R2-silenced cells could not be rescued by the ectopic expression of p53R2. This discrepancy in the ability of p53R2 to reverse the cytotoxicity of these agents may be ascribed, in part, to two possibilities. First, p53R2 may not be fully capable of supplying the dNTPs for DNA replication, even though our results suggest that R2 can substitute for p53R2 in DNA repair. Second, it is possible that ectopically expressed p53R2 is also subjected to inhibition by Triapine and hydroxyurea. Both R2 and p53R2 share >80% homology in their amino acid sequence (9), and both subunits contain non-heme iron and a tyrosyl-free radical that is quenched by inhibitors of RNR (40). A recent study has indeed shown that p53R2-RNR and R2-RNR are equally sen-

---

**Fig. 7.** Suppression of dNTP levels in p53(H11002/H11002/H11002) HCT-116 cells (Vector and clone 6) by Triapine. Cells were exposed to 0.5 μM Triapine for 1 h. The levels of dATP (A), dGTP (B), dCTP (C), and dTTP (D) were quantified as described under “Experimental Procedures.” Data are representative results performed in triplicate and expressed as the means ± S.D.
sitive to Triapine, whereas R2-RNR is 2.5-fold more sensitive to hydroxyurea than p53R2-RNR in vitro (41).

The important role of RNR in DNA synthesis and repair has made it a particularly good target for anticancer agents and gene therapy. Our findings suggest that p53-deficient cancer cells with relatively low levels of the R2 protein are more vulnerable to DNA-damaging agents and to inhibitors of RNR. Thus, down-regulation of the R2 protein by pharmacological or molecular means should enhance the effectiveness of these two classes of agents. In keeping with this probability, a recent study by Zhou et al. (42) has shown that the expression of antisense R2 sensitizes p53-mutant PC3 cells to UV irradiation. Using RNA interference, our results further demonstrate a positive correlation between the levels of R2 and the sensitivity to DNA damage in cells with deficient p53 pathways. These findings highlight the rationale of employing RNR inhibitors in conjunction with DNA-damaging agents for cancer chemotherapy. The results also support the possibility of developing R2-targeted gene therapy (43). Such an approach would avoid the nonspecific toxicity of RNR inhibitors while still achieving the goal of improving the efficacy of DNA-damaging agents.

Acknowledgments—We thank Rocco Carbone for assistance with cell sorting. We also thank Hao Zeng for an initial contribution to the project.

REFERENCES
1. Thelander, L., and Reichard, P. (1979) Annu. Rev. Biochem. 48, 133–158
2. Bunz, L., Kohalmi, S. E., Kunz, T. A., Mathews, C. K., McIntosh, E. M., and Reidy, J. A. (1994) Mutat. Res. 318, 1–64
3. Eriksson, S., Graslund, A., Skog, S., Thelander, L., and Tribukait, B. (1984) J. Biol. Chem. 259, 11695–11700
4. Engstrom, Y., Eriksson, S., Aldeivik, I., Skog, S., Thelander, L., and Tribukait, B. (1985) J. Biol. Chem. 260, 9114–9116
5. Bjorklund, S., Skog, S., Tribukait, B., and Thelander, L. (1990) Biochemistry 29, 5452–5458
6. Mann, G. J., Musgrove, E. A., Fox, R. M., and Thelander, L. (1988) Cancer Res. 48, 5151–5156
7. Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y., and Nakamura, Y. (2000) Nature 404, 42–49
8. Nakano, K., Balint, E., Ashcroft, M., and Vousden, K. H. (2000) Oncogene 19, 4283–4289
9. Guitet, O., Hakansson, P., Voevodskaya, N., Fridd, S., Graslund, A., Arakawa, H., Nakamura, Y., and Thelander, L. (2001) J. Biol. Chem. 276, 40647–40651
10. Yamaguchi, T., Matsuda, K., Sagiya, Y., Iwadate, M., Fujino, M. A., Nakamura, Y., and Arakawa, H. (2001) Cancer Res. 61, 8256–8262
11. Elford, H. L., Freese, M., Passamani, E., and Morris, H. P. (1970) J. Biol. Chem. 245, 5228–5233
12. Weber, G. (1980) Oncology 37, 19–24
13. Cory, J. G., Cory, A. H., Rappa, G., Larice, A., Liu, M. C., Lin, T. S., and Sartorelli, A. C. (1994) Biochem. Pharmacol. 48, 335–344
14. Finch, R. A., Liu, M. C., Cory, A. H., Cory, J. G., and Sartorelli, A. C. (1999) Adv. Enzyme Regul. 39, 3–12
15. Fritzler-Szekeres, M., Salamon, A., Grusch, M., Horvath, Z., Hochtl, T., Steinbrugger, R., Jager, W., Kropitz, G., Elford, H. L., and Szekeres, T. (2002) Biochem. Pharmacol. 64, 481–485
16. Inayat, M. S., Cheng, D., Mohiuddin, M., Elford, H. L., Gallicchio, V., and Ahmed, M. M. (2002) Cancer Biol. Ther. 1, 539–545
17. Liu, M. C., Lin, T. S., and Sartorelli, A. C. (1992) J. Med. Chem. 35, 3672–3677
18. Finch, R. A., Liu, M., Grill, S. P., Rose, W. C., Loomis, R., Vasquez, K. M., Cheng, Y., and Sartorelli, A. C. (2000) Biochem. Pharmacol. 59, 983–991
19. Kimura, M., Takatsuaki, A., and Yamaguchi, I. (1994) Biochim. Biophys. Acta 1219, 653–659
20. Mulsant, P., Gatignol, A., Dalens, M., and Trirabgy, H. (1988) Somat. Cell Mol. Genet. 14, 243–252
21. Das, G., Henning, D., Wright, D., and Reddy, R. (1988) EMBO J. 7, 503–512
22. Sherman, P. A., and Pyfe, J. A. (1989) Anal. Biochem. 180, 222–226
23. Gao, W. Y., Johns, D. G., and Mitsuya, H. (1994) Anal. Biochem. 223, 116–122
24. Polya, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1997) Nature 380, 300–305
25. Blagosklonny, M. V., Schulte, T. W., Nguyen, P., Minnaugh, E. G., Trepel, J., and Neckers, L. (1995) Cancer Res. 55, 4623–4626
26. Sablin, A. A., Chamakov, P. M., Levine, A. J., and Kopnin, B. P. (2001) Oncogene 20, 889–896
27. Pan, S., Smith, M. L., Rivet, D. J., Lu, D., Zhan, Q., Kohn, K. W., Fornace, A. J., Jr., and O’Connor, P. M. (1995) Cancer Res. 55, 1649–1654
28. Hawkins, D. S., Demers, G. W., and Galloway, D. A. (1996) Cancer Res. 56, 892–898
29. Elledge, S. J., and Davis, R. W. (1987) Mol. Cell. Biol. 7, 2783–2793
30. Elledge, S. J., and Davis, R. W. (1989) Mol. Cell. Biol. 9, 4932–4940
31. Hurta, R. A., and Wright, A. J. (1992) J. Biol. Chem. 267, 7066–7071
32. Filatov, D., Bjorklund, S., Johansson, E., and Thelander, L. (1996) J. Biol. Chem. 271, 23698–23704
33. Kuo, M. L., and Kinsella, T. J. (1998) Cancer Res. 58, 2245–2252
34. Chabes, A., and Thelander, L. (2000) J. Biol. Chem. 275, 17747–17753
35. Chabes, A. L., Pfleger, C. M., Kirschner, M. W., and Thelander, L. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3925–3929
36. Pinto, A. L., and Lipard, S. J. (1985) Biochim. Biophys. Acta 780, 167–180
37. Chaney, S. G., and Sancar, A. (1996) J. Natl. Cancer Inst. 88, 1346–1360
38. Furuta, T., Ueda, T., Aune, G., Sarasin, A., Kraemer, K. H., and Pommier, Y. (2002) Cancer Res. 62, 4899–4902
39. Chabes, A., Georgiev, B., Donkina, V., Zhao, X., Rothstein, R., and Thelander, L. (2003) Cell 112, 391–401
40. Thelander, M., Graslund, A., and Thelander, L. (1985) J. Biol. Chem. 260, 2737–2741
41. Shao, J., Zhou, R., Zhu, L., Qu, W., Yuan, Y. C., Xi, B., and Yen, Y. (2004) Cancer Res. 64, 1–6
42. Zhou, B., Liu, X., Mo, X., Xue, L., Darwish, D., Qu, W., Shih, J., Hwu, E. B., Lah, F., and Yen, Y. (2003) Cancer Res. 63, 6583–6584
43. Lee, Y., Vasieliakos, A., Feng, N., Lam, V., Xie, H., Wang, M., Jin, H., Xiong, K., Liu, C., Wright, J., and Young, A. (2003) Cancer Res. 63, 2802–2811