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Triapine (3-Aminopyridine-2-carboxaldehyde-thiosemicarbazone): A Potent Inhibitor of Ribonucleotide Reductase Activity with Broad Spectrum Antitumor Activity

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ABSTRACT. Previous studies from our laboratories have shown that (a) Triapine™ is a potent inhibitor of ribonucleotide reductase activity and (b) hydroxyurea-resistant L1210 leukemia cells are fully sensitive to Triapine. In an analogous manner, Triapine was similarly active against the wild-type and a hydroxyurea-resistant subline of the human KB nasopharyngeal carcinoma. Triapine was active *in vivo* against the L1210 leukemia over a broad range of dosages and was curative for some mice. This agent also caused pronounced inhibition of the growth of the murine M109 lung carcinoma and human A2780 ovarian carcinoma xenografts in mice. Optimum anticancer activity required twice daily dosing due to the duration of inhibition of DNA synthesis which lasted about 10 hr in L1210 cells treated with Triapine *in vivo*. DNA synthesis in normal mouse tissues (i.e. duodenum and bone marrow) uniformly recovered faster than that in L1210 leukemia cells, demonstrating a pharmacological basis for the therapeutic index of this agent. Triapine was more potent than hydroxyurea in inhibiting DNA synthesis in L1210 cells *in vivo*, and the effects of Triapine were more pronounced. In addition, the duration of the inhibition of DNA synthesis in leukemia cells from mice treated with Triapine was considerably longer than in those from animals treated with hydroxyurea. Combination of Triapine with various classes of agents that damage DNA (e.g. etoposide, cisplatin, doxorubicin, and 1-acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine) resulted in synergistic inhibition of the L1210 leukemia, producing long-term survivors of tumor-bearing mice treated with several dosage levels of the combinations, whereas no enhancement of survival was found when Triapine was combined with gemcitabine or cytosine arabinoside. The findings demonstrate the superiority of Triapine over hydroxyurea as an anticancer agent and further suggest that prevention by Triapine of repair of DNA lesions created by agents that damage DNA may result in efficacious drug combinations for the treatment of cancer. BIOCHEM PHARMACOL **59**;8: 983–991, 2000. © 2000 Elsevier Science Inc.

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Ribonucleotide reductase (EC 1.17.4.1; RR‖) catalyzes the synthesis of deoxyribonucleotides from their ribonucleotide precursors and as such is responsible for maintaining a balanced supply of the deoxyribonucleotides required for DNA synthesis and repair [1, 2]. The regulation of this multisubunit enzyme (two identical R1 subunits and two identical R2 subunits) is complex. The R1 subunit contains the binding sites for the ribonucleotide substrates, as well as for allosteric effectors. The R2 subunit contains a tyrosyl free radical that is stabilized by a non-heme iron center [3], and both the iron and the tyrosyl radical are essential for catalytic activity. The R1 and R2 subunits together form the catalytically active enzyme.

Strong positive correlations have been established between RR activity and the rate of replication of cancer cells [4, 5]. RR, therefore, can be considered to be a site of vulnerability to be targeted by antitumor agents. Since the catalytic core of the R2 subunit requires an iron-stabilized free radical, destabilization of the iron center inactivates the enzyme. A number of compounds have been identified which inhibit RR by this mechanism [6–8]. Currently, HU

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‖ *Abbreviations:* RR, ribonucleotide reductase; HU, hydroxyurea; HCTs, α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones; 5-HP, 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone; AC, 1-acetyl-1,2-bis-(methylsulfonyl)-2-(2-chloroethyl)hydrazine; and CDP, cytidine diphosphate.

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is the only one of these agents used clinically to treat cancer. HU, however, has limited effectiveness as an anticancer drug, since it has a relatively low affinity for RR and a short half-life in humans [9, 10]. Another limitation is the relative ease with which resistance to HU develops in cancer cells, which may involve both quantitative and qualitative changes in RR (see [6] and [11] for references). Nonetheless, the clinical activity of HU suggests that the development of more effective inhibitors of RR could contribute to improved cancer chemotherapy regimens.

The HCTs are among the most potent known inhibitors of RR activity [12]. These agents are powerful iron chelators [13]. Studies have shown that the presence of iron is required for effective enzyme inhibition by agents of this class (see Ref. 12 for a discussion), suggesting that the iron–HCT complex itself was the inhibitory species [14]. That the iron chelate is the active inhibitory species was shown by Thelander and Gräslund [15], and preformed iron chelates of HCTs are considerably more potent than the uncoordinated HCTs as inhibitors of RR [16–18]. It is worth noting that there are several HCTs that effectively inhibit RR activity at concentrations more than 1000-fold lower than that required for HU.

The only HCT to be evaluated clinically is 5-HP [19, 20]. In spite of significant antitumor activity in animal models [21], clinical results with 5-HP were disappointing. This was due in large measure to its rapid inactivation through glucuronidation and elimination in human patients [18]. New HCTs that are not subject to such metabolic inactivation have been synthesized and evaluated for antitumor activity [22]. One of the most promising of these is 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP; Triapine™), which is currently in phase 1 clinical trial. We now report additional preclinical data which suggest that pharmacokinetic measurements of Triapine's inhibition of RR activity in cancer cells can be useful in designing optimum schedules of administration of Triapine, and that combinations of Triapine with agents that damage DNA produce synergistic inhibition of tumor growth and may lead to improvements in the effectiveness of cancer chemotherapy regimens.

MATERIALS AND METHODS

Drugs and Mice

Triapine was synthesized according to published methodology [22] and AC was synthesized as described [23]. Etoposide, cisplatin, doxorubicin, and HU were purchased from Sigma Chemical Co. BALB/c × DBA/2 (CD_2F_1) mice of 8–10 weeks of age were used with the L1210 leukemia and the M109 lung carcinoma. Athymic nu/nu mice were used with the human A2780 ovarian carcinoma xenograft. Mice were treated with a fine suspension of Triapine in 0.9% NaCl or water by i.p. or i.v. bolus injection (0.01 mL/g of mouse body weight). Dimethyl sulfoxide solutions of etoposide and AC were delivered at a rate of 0.001 mL/g of mouse body weight. Cisplatin, HU, and doxorubicin were given as aqueous solutions (0.01 mL/g mouse body weight).

Leukemia Studies

Female CD_2F_1 mice were inoculated with 1×10^5 murine L1210 leukemia cells. Twenty-four hours later, treatment was initiated and continued twice daily for 6 consecutive days. Post-inoculation life span was monitored for 60 days. To determine the effects of Triapine on leukemia cells in the brain, a bioassay procedure developed by Skipper *et al.* [24] was employed. Briefly, CD_2F_1 mice were given injections of drug or of a 0.9% solution of NaCl 24 hr after intracerebral inoculation of 1×10^5 L1210 cells. Approximately 24 hr later, the mice were killed and the brains of 3 mice/experimental group were collected. A brei was prepared from the 3 pooled brains, and the equivalent of one-half of one brain was injected i.p. into each of 5 mice of the same strain. Mean post-inoculation life spans were then determined, and viable L1210 cells/brain were estimated by using concomitantly derived inoculum response data that defined the relationship between life span and inoculum size [24].

Solid Tumor Studies

Two solid tumors were tested for responsiveness to treatment with Triapine. For the murine M109 lung carcinoma, breis of the tumor were implanted s.c. into CD_2F_1 mice, and therapy was initiated 4 days later and continued once or twice daily for 5 consecutive days. Paclitaxel, which is highly active against the M109 lung carcinoma [25], was used as a positive control and to provide some gauge of the relative effectiveness of Triapine in this model. In studies with the human A2780 ovarian carcinoma, fragments of the tumor were implanted s.c. in athymic mice, and therapy was initiated 11 days later and continued twice daily for 5 consecutive days. Tumor measurements were recorded on the initial day of treatment and at various times thereafter. Evaluation of data was performed using Gehan's generalized Wilcoxon test for medians [26].

Cell Culture Studies with the Human KB Nasopharyngeal Carcinoma

KB cells (American Type Culture Collection, Rockville, MD) were maintained at 37° in a humidified atmosphere containing 5% CO_2. The cells were grown in RPMI-1640 medium supplemented with 10% dialyzed fetal bovine serum and 100 μg/mL of kanamycin. KB cells resistant to HU were developed as described by Yen *et al.* [27].

Cytotoxicity Studies

Cells were plated at a density of 1×10^4 cells/mL per well in 24-well plates. Drugs were added to cells and incubations were continued for a period of 3 generations (untreated

control cells), followed by assessment of cell growth by the methylene blue assay described by Finlay *et al.* [28].

Ribonucleotide Reductase Assay

CDP reductase was assayed by the method of Steeper and Steuart [29] using Dowex 1-borate ion-exchange chromatography. The assay mixture contained 0.02 μCi of [^{14}C]CDP (52.9 mCi/mmol), 3 mM dithiothreitol, 6 mM $MgCl_2$, 30 mM HEPES, 5 mM ATP, 0.15 mM unlabeled CDP, and 10 μL of cellular extract in a final volume of 0.02 mL. The incubation time for the reaction was 60 min, during which time the reaction was linear.

Determination of DNA Synthesis in Mouse Tissues

CD_2F_1 mice were treated with an i.p. bolus dosage of 5 mg/kg of Triapine. Control mice were given an equivalent volume of 0.9% NaCl. At various times thereafter, mice were injected i.p. with 10 μCi of [^{3}H]thymidine (specific activity 67 Ci/mmol, 0.20 mL/mouse, 50 μCi/mL). Thirty minutes later, mice were killed by cervical dislocation, and the femoral bone marrow and approximately 3 cm of the duodenum proximal to the stomach were collected for measurement of DNA synthesis as determined by [^{3}H]thymidine incorporation as described below. For assessment of DNA synthesis in leukemia cells, mice were inoculated with 5×10^6 L1210 leukemia cells. Five days later, mice were treated with 5 mg/kg of Triapine or 70 mg/kg of HU and at various times thereafter, DNA synthesis was determined in the leukemia cells using [^{3}H]thymidine as described below.

Immediately after collection, tissues or leukemia cells were placed in lysis buffer (0.1 M Tris–HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl, 0.1 mg/mL of proteinase K) and incubated overnight at 50° in a shaking water bath. Samples were then vigorously vortexed and extracted with phenol/chloroform. The aqueous layer was collected, incubated with 10 mg/mL of RNase A (Boehringer Mannheim) for 2 hr at 37° and extracted again with phenol/chloroform. The DNA was precipitated with ethanol and redissolved in 10 mM Tris–HCl, pH 8.5, 1 mM EDTA. The DNA concentration was determined by UV absorbance at 260 nm and radioactivity therein was measured by liquid scintillation spectrometry.

RESULTS

Effectiveness of Triapine as an Antileukemic Agent

We have shown previously that Triapine was effective in prolonging the life span of mice bearing the L1210 leukemia and that the schedule of administration was important for the success of the treatment [22]. In the course of the current studies, we have confirmed these findings and have also demonstrated the effectiveness of this agent over a wide range of dosages. Data presented in Table 1 show that when given twice daily for 6 consecutive days after tumor inoculation, Triapine at doses ranging from 1.25 to 20 mg/kg per injection produced % T/C values in L1210 leukemia-bearing mice of 170 to 300% without lethal toxicity and cured some mice at each dosage of 2.5 mg/kg per injection and above. Treatment with 20 mg/kg per injection cured 27% of the mice.

TABLE 1. Effects of Triapine on the survival time of mice bearing the L1210 leukemia

Dosage* (mg/kg)	Av Δ wt† (%)	T/C × 100‡	Long-term survivors§
1.25	−0.8	170	0/10
2.5	−1.3	182	1/25
5.0	−1.1	224	3/30
7.5	−5.0	228	3/15
10.0	−4.5	215	4/20
20.0	−6.1	300	4/15

*Twice daily i.p. treatments, approximately 8 hr apart, with various doses of Triapine were initiated 24 hr after tumor implantation and continued for 6 consecutive days.

†Average change in body weight from onset to termination of therapy.

‡T/C × 100 represents the ratio of the survival time of treated to control animals × 100. T/C values were calculated only for those mice that died.

§Mice alive with no evidence of tumor 60 days after tumor implantation.

Effects of Triapine on the Growth of HU-Resistant Cells

The only primary RR inhibitor currently in clinical use is HU. Since one of the limitations of cancer chemotherapy with HU is the development of drug resistance, it is of considerable importance to seek other RR inhibitors with activity against cells resistant to HU. We have previously demonstrated that Triapine had activity against an HU-resistant subline of the murine L1210 leukemia [30]. In the present study, Triapine was also found to be active against a subline of the human KB nasopharyngeal carcinoma that exhibits 15-fold resistance to HU [27]. The comparative sensitivities of wild-type KB and HU-resistant KB cells to HU and Triapine are shown in Fig. 1. Triapine was considerably more potent than HU against wild-type cells, with an IC_{50} approximately 2 logs lower than HU. More importantly, there was little difference in the sensitivity of the wild-type and the HU-resistant cells to Triapine.

Inhibition of RR Activity in KB Cell Lines

To gain information on the basis for the different sensitivity profiles of wild-type and HU-resistant KB cells to HU and Triapine, we measured the degree of inhibition of RR activity in these two cell lines by these agents (Fig. 2). Triapine was found to be a much more potent inhibitor of the enzyme than HU regardless of the cellular source of the enzyme, with a comparable inhibition at roughly a 1000-fold lower concentration of Triapine than HU. The sensitivity of the RR from KB/HU cells and parental KB cells was not markedly different for either drug, however.

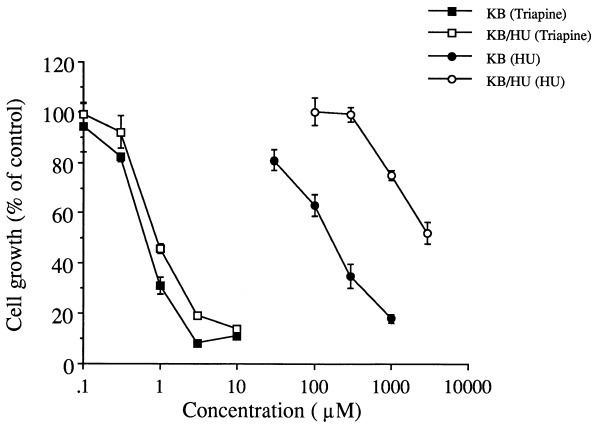

FIG. 1. Cytotoxicity of Triapine and HU to wild-type (KB) and HU-resistant (KB/HU) human KB nasopharyngeal carcinoma cells. Cells were plated at a density of 1 × 10^4 cells/mL per well in 24-well plates. Drugs were added to cells and incubations were continued for a period of 3 generations (untreated control cells), followed by assessment of cell growth by the methylene blue assay [28]. Data are the average ± SD of 3 separate determinations.

Effects of Triapine on the Growth of Solid Tumors in Mice

Since the potential of Triapine as a treatment for solid tumors had not been examined previously, in this study we evaluated the effectiveness of this agent against the murine M109 lung carcinoma and the human A2780 ovarian carcinoma. As shown in Fig. 3, Triapine significantly inhibited the growth in mice of the M109 lung carcinoma. The twice daily schedule was found to be more effective than a once daily schedule of administration and produced tumor growth delays (time to reach a mass of 1 gram) of 10 days compared to untreated control animals. On the twice daily schedule, Triapine was roughly comparable in effectiveness to paclitaxel. Growth of the human A2780 ovarian carcinoma xenograft in nude mice was also significantly inhibited by Triapine, with delays in time for tumors to attain a 1-gram mass of 10 and 19 days, respectively, at 8 and 10 mg/kg given on a twice daily schedule (Fig. 4).

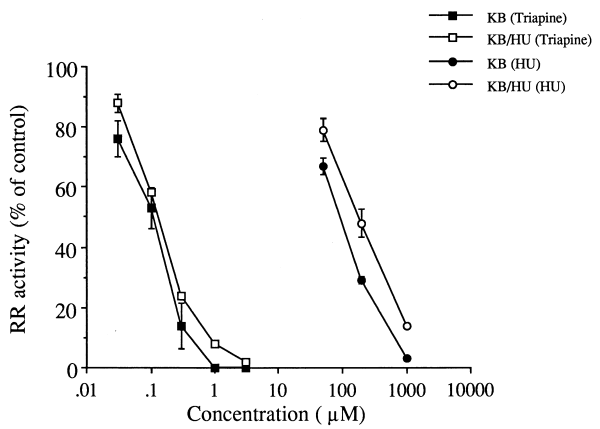

FIG. 2. Effects of Triapine and HU on RR activity in wild-type (KB) and HU-resistant (KB/HU) human KB nasopharyngeal carcinoma cells. Data are the average ± SD of three separate determinations.

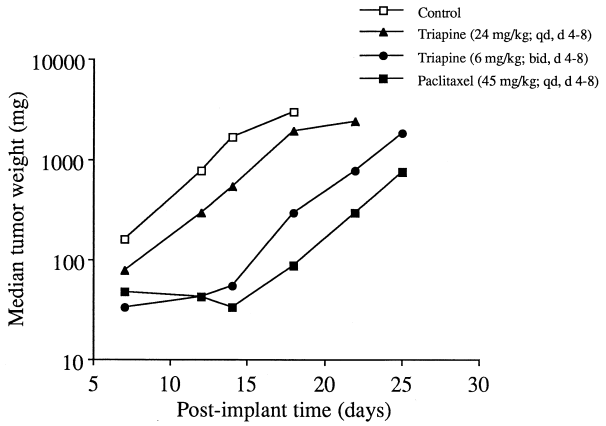

FIG. 3. The effects of Triapine on the murine M109 lung carcinoma. Breis of the M109 tumor were implanted s.c. into CD_2F_1 mice and therapy was initiated 4 days later. Various dosages of Triapine were injected i.v. either once daily (q.d.) or twice daily (b.i.d.) approximately 8 hr apart for 5 consecutive days, with 8 mice per group. Paclitaxel was included as a positive control. Data were analyzed using Gehan's generalized Wilcoxon test for medians [26]. Inhibition of tumor growth in mice treated on the twice daily schedule at 6 mg/kg was highly significant ($P < 0.0002$) compared to control.

DNA Synthesis in L1210 Leukemia Cells and Normal Mouse Tissues after Triapine Treatment

Agents designed to attack rapidly replicating cancer cells are also toxic to actively proliferating cells of the host, such as those of the bone marrow and the villi of the small intestine. As shown in Fig. 5, DNA synthesis was inhibited in the bone marrow and intestinal mucosa following treat-

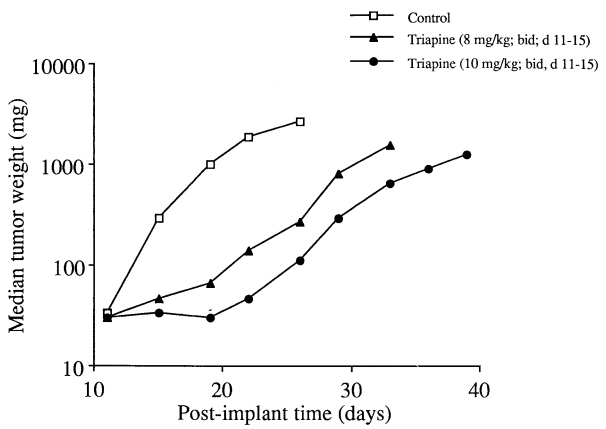

FIG. 4. The effects of Triapine on the human A2780 ovarian carcinoma. Fragments of the A2780 tumor were implanted s.c. into athymic mice and therapy was initiated 11 days later. Various dosages of Triapine were injected i.v. twice daily (b.i.d.) approximately 8 hr apart for 5 consecutive days, with 8 mice per group. Data were analyzed using Gehan's generalized Wilcoxon test for medians [26]. Inhibition of tumor growth in mice treated with both levels of Triapine was highly significant (8 mg/kg, $P < 0.0011$; 10 mg/kg, $P < 0.0008$).

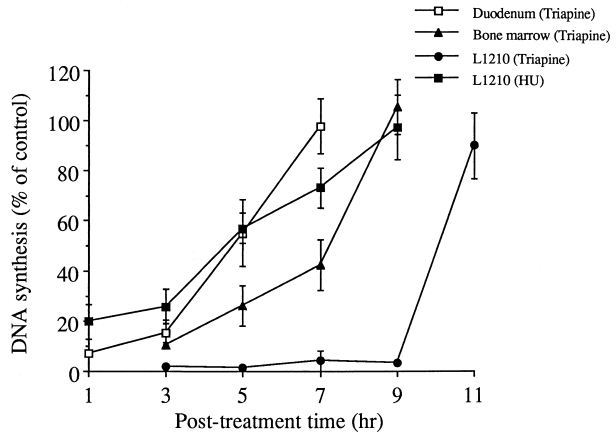

FIG. 5. Recovery of DNA synthesis in L1210 leukemia cells and normal mouse tissues after treatment with Triapine. L1210-bearing mice were injected i.p. with 5 mg/kg of Triapine. At the indicated times thereafter, mice were killed and DNA synthesis in the duodenum, bone marrow, and leukemia cells was determined. Recovery of DNA synthesis in L1210 cells from mice treated i.p. with 70 mg/kg of HU was included for comparison. Each point represents the average value ± SD of at least 3 mice.

ment of mice with the therapeutic dose of 5 mg/kg of Triapine. Complete recovery of DNA synthesis occurred in the intestinal mucosa and bone marrow 7 and 9 hr, respectively, after treatment, while in L1210 cells, DNA synthesis remained depressed by this dose of drug by more than 90% for up to 9 hr, then recovered to a normal rate by 11 hr. In this study, we also measured the comparative ability of HU to inhibit DNA synthesis in leukemia cells growing in mice. Figure 5 shows that the relatively high dose of 70 mg/kg of HU was not as potent or as effective as Triapine in this regard, producing only 80% inhibition of DNA biosynthesis and complete recovery by 8 hr.

Effects of Triapine on Leukemia Cells in the Brain

To achieve curative therapy, cancer cells must be eradicated throughout the body. Due to the blood/brain barrier, the CNS provides a pharmacological sanctuary which limits access to many drugs used in cancer chemotherapy. Because of this, we were interested in determining whether Triapine would be effective against tumor cells present in the brain. As shown in Table 2, Triapine penetrated the CNS and effectively killed greater than 95% of L1210 cells in the brain when administered as two doses of 40 mg/kg given 8 hr apart. Since i.p. injected L1210 cells rapidly become widely disseminated, the ability of Triapine to produce cures of mice bearing the L1210 leukemia indicated that it was effective against widely disseminated neoplastic cells.

Combination Therapy with Triapine and DNA-Damaging Agents

If neoplastic cells are deprived of the deoxyribonucleotide precursors needed for DNA repair, the tumor-inhibitory effects of anticancer agents that produce damage to DNA might be enhanced when employed in combination with an effective inhibitor of RR. We tested this concept by combining Triapine with a variety of agents that produce damage to DNA by various mechanisms and assessed the benefit derived from these combinations using the i.p. implanted L1210 leukemia system. We evaluated Triapine in combination with a representative of the epipodophylotoxins, etoposide, a topoisomerase II inhibitor; an anthracycline, doxorubicin; cisplatin, a platinum coordination complex; and AC, an alkylating agent. The results obtained are presented in Tables 3 to 6. Synergism was obtained when Triapine was combined with all four of the DNA-damaging agents tested, with several dosage levels of each combination producing long-term survivors. In contrast, cytosine arabinoside and gemcitabine did not produce enhanced antileukemic activity when used in admixture with Triapine (data not shown).

TABLE 2. Effectiveness of Triapine administered to mice i.p. against L1210 leukemia cells in the brain

Dosage* (mg/kg)	Log kill of leukemia cells in the brain†
10 × 2	0.80
20 × 2	1.26
40 × 2	1.49

*CD_2F_1 mice were given 2 i.p. injections of Triapine approximately 8 hr apart one day after intracerebral inoculation with 1×10^5 L1210 cells. Twenty-four hours after the last injection, the mice were killed by cervical dislocation and their brains collected for bioassay. A brei of brain tissue was prepared, and a volume of the resulting suspension equivalent to one-half of one brain was injected into each of 5 mice/group.

†Mean post-inoculation life spans were determined for the brain-inoculated mice, and viable L1210 cells per brain were estimated from inoculum response data that defined the relationship between life span and inoculum size.

DISCUSSION

The centrality of RR to cellular proliferation emphasizes the importance of developing more effective inhibitors of this enzyme in order to exploit the requirement of rapidly proliferating cancer cells for deoxyribonucleotide triphosphates. HU is the only significant RR inhibitor to have gained widespread clinical use. However, its relatively low affinity for RR, relatively short half-life in humans, susceptibility to metabolic inactivation, and the ease of the attainment of resistance to HU limit its effectiveness as an anticancer agent. Triapine is a much more potent inhibitor of RR activity than HU and also shows activity against HU-resistant tumors. Thus, in previous studies, Triapine was found to be fully inhibitory to a subline of the L1210 leukemia exhibiting 20-fold resistance to HU [30] and in the present study, a KB nasopharyngeal carcinoma resistant to HU was completely sensitive to Triapine. The relative resistance of the KB/HU subline to HU was due, at least in part, to a 3-fold increase in RR activity over the parent line [24]. As shown in Fig. 2, in which RR activity is normalized

TABLE 3. Effects of combinations of Triapine and etoposide on the survival time of mice bearing the L1210 leukemia

Compound	Dosage* (mg/kg)	Av Δ wt† (%)	T/C × 100‡	Long-term survivors§
Triapine	2.5	+2.0	192	0/5
	5.0	±0.0	219	0/5
	7.5	−6.2	275	0/5
Etoposide	2.5	+2.2	176	0/5
	5.0	−1.1	205	0/5
	10.0	−2.2	233	1/5
Triapine + etoposide	2.5 + 2.5	+2.2	181	0/5
	5.0	−1.1	187	0/5
	10.0	−2.2	243	0/5
	5.0 + 2.5	−2.2	197	0/5
	5.0	−2.2	325	0/5
	10.0	−7.7	373	4/5
	7.5 + 2.5	−6.6	364	2/5
	5.0	−7.6	378	2/5
	10.0	−11.2	—	5/5

*Twice daily i.p. treatments, approximately 8 hr apart, with various doses of Triapine were initiated 24 hr after tumor implantation and continued for 6 consecutive days. Various doses of etoposide were injected i.p. once daily for 6 consecutive days at the time of the first daily Triapine treatment.

†Average change in body weight from onset to termination of therapy.

‡T/C × 100 represents the ratio of the survival time of treated to control animals × 100. T/C values were calculated only for those mice that died.

§Mice alive with no evidence of tumor 60 days after tumor implantation. The average survival time of the untreated tumor-bearing control animals was 7.5 days.

as a percentage of that in untreated control cells, the RR from the KB/HU cells was minimally less sensitive to HU (and Triapine) inhibition (note the slight shift in the concentration/inhibition curves of RR from parental KB cells compared to KB/HU cells for both drugs) than the enzyme from wild-type cells. This suggests that a qualitative change in RR may also have occurred during the development of resistance to HU. To date, as cancer cells have not been selected for resistance to Triapine either in culture or *in vivo*, it is not known whether Triapine-resistant cells would be cross-resistant to HU.

We have recently shown that L1210 cells transfected with human MDR1 and MRP cDNAs were 2- to 3-fold resistant to the cytotoxic effects of Triapine and accumulated less radiolabeled Triapine than the parental vector-transfected control cells [31]. The cytotoxicity of Triapine was also shown to be significantly greater in *mrp* (multidrug resistance protein) gene knockout embryonic stem (ES) cells than in parental ES cells. These studies suggest that Triapine is a substrate for the P-glycoprotein and mrp and that if Triapine resistance evolves, increased drug efflux may be a contributing factor.

Although quenching of the iron-stabilized tyrosyl radical is involved in the action of both HU and Triapine on RR,

TABLE 4. Effects of combinations of Triapine and doxorubicin on the survival time of mice bearing the L1210 leukemia

Compound	Dosage* (mg/kg)	Av Δ wt† (%)	TC × 100‡	Long-term survivors§
Triapine	2.5	+2.0	194	0/5
	5.0	−1.8	250	0/5
	7.5	−5.7	256	0/5
Doxorubicin	0.5	+2.0	205	0/5
	1.0	+3.0	242	0/5
	1.5	+2.0	264	0/5
Triapine + doxorubicin	2.5 + 0.5	+4.0	208	1/5
	1.0	−3.0	199	2/5
	1.5	+1.0	222	3/5
	5.0 + 0.5	+1.0	216	3/5
	1.0	−4.0	190	2/5
	1.5	−3.2	236	3/5
	7.5 + 0.5	−6.9	181	4/5
	1.0	−8.9	—	5/5
	1.5	−9.8	—	5/5

*Twice daily i.p. treatments, approximately 8 hr apart, with various doses of Triapine were initiated 24 hr after tumor implantation and continued for 6 consecutive days. Various doses of doxorubicin were injected i.p. once daily for 6 consecutive days at the time of the first daily Triapine treatment.

†Average change in body weight from onset to termination of therapy.

‡T/C × 100 represents the ratio of the survival time of treated to control animals × 100. T/C values were calculated only for those mice that died.

§Mice alive with no evidence of tumor 60 days after tumor implantation. The average survival time of the untreated tumor-bearing control animals was 7.2 days.

TABLE 5. Effects of combinations of Triapine and cisplatin on the survival time of mice bearing the L1210 leukemia

Compound	Dosage* (mg/kg)	Av Δ wt† (%)	T/C × 100‡	Long-term survivors§
Triapine	1.25	+1.1	178	0/5
	2.5	−2.9	219	1/5
	5.0	−5.1	370	0/5
Cisplatin	0.5	+4.1	133	0/5
	1.0	−1.0	143	0/5
	2.0	−10.1	183	0/5
Triapine + cisplatin	1.25 + 0.5	±0.0	188	0/5
	1.0	−4.9	235	1/5
	2.0	−10.4	296	3/5
	2.5 + 0.5	+1.0	180	0/5
	1.0	−3.9	179	1/5
	2.0	−11.0	185	4/5
	5.0 + 0.5	−2.0	201	1/5
	1.0	−7.1	290	3/5
	2.0	−12.4	241	3/5

*Twice daily i.p. treatments, approximately 8 hr apart, with various doses of Triapine were initiated 24 hr after tumor implantation and continued for 6 consecutive days. Various doses of cisplatin were injected i.p. once daily for 6 consecutive days at the time of the first daily Triapine treatment.

†Average change in body weight from onset to termination of therapy.

‡T/C × 100 represents the ratio of the survival time of treated to control animals × 100. T/C values were calculated only for those mice that died.

§Mice alive with no evidence of tumor 60 days after tumor implantation. The average survival time of the untreated tumor-bearing control animals was 8.1 days.

the exact mechanisms involved do not appear to be identical. This is evidenced by the failure of the iron chelator desferoxamine to augment Triapine-induced inhibition of RR [32], whereas desferoxamine increased the inhibition of RR activity produced by HU [33]. Thus, the superior effectiveness of Triapine, compared to HU, as an inhibitor of RR may not simply be due to its greater potency.

DNA synthesis was inhibited in the two normal tissues examined after mice were treated with Triapine. However, the timing of the recovery of DNA synthesis in normal tissues differed from that in tumor cells. Thus, complete recovery of DNA synthesis occurred in the intestinal mucosa and bone marrow 7 and 9 hr, respectively, after treatment, while in L1210 cells, DNA synthesis remained depressed by more than 90% at 9 hr, then recovered to a normal rate by 11 hr. The differential recovery of normal tissue compared to tumor tissue is an important therapeutic principle underlying the success of cancer chemotherapy wherein rest periods during courses of therapy often permit the preferential recovery of sensitive normal tissues. In the case of Triapine, the more rapid recovery of normal tissues compared to tumor tissue demonstrates a possible pharmacological basis for the therapeutic index of the drug in

TABLE 6. Effects of combinations of Triapine and 1-acetyl-1,2-bis(methylsulfonyl)-2-(chloroethyl) hydrazine (AC) on the survival time of mice bearing the L1210 leukemia

Compound	Dosage* (mg/kg)	Av Δ wt† (%)	T/C × 100‡	Long-term survivors§
Triapine	2.5	+2.0	192	0/5
	5.0	±0.0	219	0/5
	7.5	−6.2	275	0/5
AC	2.5	+4.2	165	0/5
	5.0	−1.1	248	0/5
	10.0	−6.3	626	4/5
Triapine + AC	2.5 + 2.5	−1.0	227	3/5
	5.0	+3.2	280	4/5
	10.0	−1.0	293	4/5
	5.0 + 2.5	−2.1	253	4/5
	5.0	−3.1	—	5/5
	10.0	−2.2	—	5/5
	7.5 + 2.5	−3.2	387	4/5
	5.0	−4.2	—	5/5
	10.0	−11.8	—	5/5

*Twice daily i.p. treatments, approximately 8 hr apart, with various doses of Triapine were initiated 24 hr after tumor implantation and continued for 6 consecutive days. Various doses of AC were injected i.p. once daily for 6 consecutive days at the time of the first daily Triapine treatment.

†Average change in body weight from onset to termination of therapy.

‡T/C × 100 represents the ratio of the survival time of treated to control animals × 100. T/C values were calculated only for those mice that died.

§Mice alive with no evidence of tumor 60 days after tumor implantation. The average survival time of the untreated tumor-bearing control animals was 7.5 days.

animal tumor models. The timing of the recovery of DNA synthesis in the leukemia cells is also consistent with the twice daily optimum schedule of administration reported earlier [22]. Similar information from human trials may aid in the design of optimal schedules of Triapine administration.

Triapine was capable of crossing the blood/brain barrier and effectively killing >95% of leukemia cells in the brain, an effect comparable to that previously reported for cyclophosphamide [34]. This property of Triapine may have favorable implications for inclusion of this agent in combination chemotherapy regimens, particularly when other drugs in the regimen are not capable of crossing the blood/brain barrier.

Like HU, the effects of Triapine on cell proliferation are most likely restricted to the S-phase of the cell cycle. Thus, the most sensitive cancers to the cytotoxic action of this agent when used alone most likely are those with high growth fractions such as the acute leukemias or chronic leukemias in blast crisis. It is therefore noteworthy that the growth in mice of the two solid tumors examined in these studies, the murine M109 lung carcinoma and the human A2780 ovarian carcinoma xenograft, were significantly inhibited by Triapine as a single agent. However, to maximize the effectiveness of Triapine in solid tumors, it will likely be necessary to use combinations of chemotherapeutic agents. The current study demonstrates that combinations of Triapine with a variety of drugs that represent different classes of anticancer agents that function by damaging DNA are exceedingly effective in treating mice bearing the L1210 leukemia. This synergy is presumably due to the capacity of Triapine to inhibit the repair of lesions in the DNA induced by the drugs that damage DNA directly. Knowledge of the timing of the attempt by cancer cells to repair the DNA after damage might be useful in determining when to best use the RR inhibitor relative to the DNA-damaging agent. Sequential administration of the two agents might well prove superior to concomitant delivery. Detailed studies designed to optimize the treatment regimen/schedule of these drug combinations should be addressed.

As a class, the HCTs are among the most effective known inhibitors of RR. HCTs such as Triapine, with insensitivity to inactivation by *O*-glucuronidation, which was a major factor in the inactivity as an anticancer agent in humans of the only other HCT to reach clinical trial, 5-HP, may prove to have therapeutic efficacy. In preclinical studies in mice, Triapine, as a single agent, demonstrated a wide range of therapeutic utility, exhibiting significant activity against the L1210 leukemia over a broad range of dosages, as well as activity against the M109 and A2780 carcinomas without imposing intolerable host toxicity. Effective cancer chemotherapy most often requires inclusion of multiple agents in combination. The synergism exhibited between Triapine and various DNA-damaging agents further increases the attractiveness of this RR inhibitor for inclusion in combination chemotherapy regimens. Taken together, with the superiority of Triapine exhibited over HU, the findings suggest that, in addition to Triapine's activity as a single agent, prevention of repair of DNA lesions by Triapine will result in efficacious drug combinations for the treatment of several different forms of cancer.

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References

1. Thelander L and Reichard P, Reduction of ribonucleotides. *Annu Rev Biochem* **48:** 133–158, 1979.
2. Cory JG and Sato A, Regulation of ribonucleotide reductase activity in mammalian cells. *Mol Cell Biochem* **53:** 257–266, 1983.
3. Thelander M, Gräslund 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.
4. Elford HL, Freese M, Passamani E and Morris HP, Ribonucleotide reductase and cell proliferation. 1. Variations of ribonucleotide reductase activity with tumor growth in a series of rat hepatomas. *J Biol Chem* **245:** 5228–5233, 1970.
5. Weber G, Biochemical strategy of cancer cells and the design of chemotherapy. *Cancer Res* **43:** 3466–3492, 1983.
6. Moore EC and Hurlbert RB, The inhibition of ribonucleoside diphosphate reductase by hydroxyurea, guanazole and pyrazoloimidazole (IMPY). In: *Inhibitors of Ribonucleoside Diphosphate Reductase Activity* (Eds. Cory JG and Cory AH), pp. 165–201. Pergamon Press, Oxford, 1989.
7. Cory JG and Carter GL, Drug action on ribonucleotide reductase. *Adv Enzyme Regul* **24:** 385–401, 1985.
8. Kwon NS, Stuehr DJ and Nathan CF, Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. *J Exp Med* **174:** 761–767, 1991.
9. Beckloff GL, Lerner HJ, Frost D, Russo-Alesi FM and Gitomer S, Hydroxyurea (NSC-32065) in biologic fluids: Dose–concentration relationship. *Cancer Chemother Rep* **48:** 57–58, 1965.
10. Gwilt PR and Tracewell WG, Pharmacokinetics and pharmacodynamics of hydroxyurea. *Clin Pharmacokinet* **34:** 347–358, 1998.
11. Donehower RC, Hydroxyurea. In: *Cancer Chemotherapy and Biotherapy* (Eds. Chabner BA and Longo CL), pp. 253–260. Lippincott-Raven, Philadelphia, 1996.
12. Moore EC and Sartorelli AC, The inhibition of ribonucleotide reductase by α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones. In: *Inhibitors of Ribonucleoside Diphosphate Reductase Activity* (Eds. Cory JG and Cory AH), pp. 203–215. Pergamon Press, Oxford, 1989.
13. Antholine W, Knight J, Whelan H and Petering DH, Studies of the reaction of 2-formylpyridine thiosemicarbazone and its iron and copper complexes with biological systems. *Mol Pharmacol* **13:** 89–98, 1977.
14. Sartorelli AC, Agrawal KC, Tsiftsoglou AS and Moore EC, Characterization of the biochemical mechanism of action of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones. *Adv Enzyme Regul* **15:** 117–139, 1977.
15. Thelander L and Gräslund A, Mechanism of inhibition of mammalian ribonucleotide reductase by the iron chelate of 1-formylisoquinoline thiosemicarbazone. Destruction of the tyrosine free radical of the enzyme in an oxygen-requiring reaction. *J Biol Chem* **258:** 4063–4066, 1983.
16. Saryan LA, Ankel E, Krishnamurti C and Petering DH, Comparative cytotoxic and biochemical effects of ligands and

metal complexes of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones. *J Med Chem* **22:** 1218–1221, 1979.
17. Preidecker PJ, Agrawal KC, Sartorelli AC and Moore EC, Effects of the ferrous chelate of 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone (MAIQ-1) on the kinetics of reduction of CDP by ribonucleotide reductase of the Novikoff tumor. *Mol Pharmacol* **18:** 507–512, 1980.
18. Finch RA, Liu MC, Cory AH, Cory JG and Sartorelli AC, Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 3-AP): An inhibitor of ribonucleotide reductase with antineoplastic activity. *Adv Enzyme Regul* **39:** 3–12, 1999.
19. DeConti RC, Toftness BR, Agrawal KC, Tomchik R, Mead JA, Bertino JR, Sartorelli AC and Creasey WA, Clinical and pharmacological studies with 5-hydroxy-2-formylpyridine thiosemicarbazone. *Cancer Res* **32:** 1455–1462, 1972.
20. Krakoff IH, Etcubanas E, Tan C, Mayer K, Bethune V and Burchenal JH, Clinical trial of 5-hydroxypicolinaldehyde thiosemicarbazone (5-HP; NSC-107392), with special reference to its iron-chelating properties. *Cancer Chemother Rep* **58:** 207–212, 1974.
21. Creasey WA, Agrawal KC, Capizzi RL, Stinson KK and Sartorelli AC, Studies of the antineoplastic activity and metabolism of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones in dogs and mice. *Cancer Res* **32:** 565–572, 1972.
22. Liu MC, Lin TS and Sartorelli AC, Synthesis and antitumor evaluation of amino derivatives of pyridine-2-carboxaldehyde thiosemicarbazone. *J Med Chem* **35:** 3672–3677, 1992.
23. Shyam K, Penketh PG, Divo AA, Loomis RH, Rose WC and Sartorelli AC, Synthesis and evaluation of 1-acyl-1,2-bis-(methylsulfonyl)-2-(2-chloroethyl)hydrazines as antineoplastic agents. *J Med Chem* **36:** 3496–3502, 1993.
24. Skipper HE, Schabel FM, Trader MW and Thompson SA, Experimental evaluation of potential anticancer agents. XVIII. Effects of therapy on viability and rate of proliferation of leukemic cells in various anatomic sites. *Cancer Chemother Rep* **47:** 41–64, 1965.
25. Rose WC. Taxol: A review of its preclinical *in vivo* antitumor activity. *Anticancer Drugs* **3:** 311–321, 1992.
26. Gehan EA, A generalized Wilcoxon test for comparing arbitrarily singly censored samples. *Biometrika* **52:** 203–223, 1965.
27. Yen Y, Grill SP, Dutschman GE, Chang CN, Zhou BS and Cheng YC, Characterization of a hydroxyurea-resistant human KB cell line with supersensitivity to 6-thioguanosine. *Cancer Res* **54:** 3686–3691, 1994.
28. Finlay GJ, Baguley BC and Wilson WR, A semiautomated microculture method for investigating growth inhibitory effects of cytotoxic compounds on exponentially growing carcinoma cells. *Anal Biochem* **139:** 272–277, 1984.
29. Steeper JR and Steuart CD, A rapid assay for CDP reductase activity in mammalian cell extracts. *Anal Biochem* **34:** 123–130, 1970.
30. Cory JG, Cory AH, Rappa G, Lorico A, Liu MC, Lin TS and Sartorelli AC, Inhibitors of ribonucleotide reductase. Comparative effects of amino- and hydroxy-substituted pyridine-2-carboxaldehyde thiosemicarbazones. *Biochem Pharmacol* **48:** 335–344, 1994.
31. Rappa G, Lorico A, Liu MC, Kruh GD, Cory AH, Cory JG and Sartorelli AC, Overexpression of the multidrug resistance genes *mdr1*, *mdr3*, and *mrp* in L1210 cells resistant to inhibitors of ribonucleotide reductase. *Biochem Pharmacol* **54:** 649–655, 1997.
32. Cory JG, Cory AH, Rappa G, Lorico A, Liu MC, Lin TS and Sartorelli AC, Structure–function relationships for a new series of pyridine-2-carboxaldehyde thiosemicarbazones on ribonucleotide reductase activity and tumor cell growth in culture and *in vivo*. *Adv Enzyme Regul* **35:** 55–68, 1995.
33. Cory JG, Lasater L and Sato A, Effect of iron-chelating agents on inhibitors of ribonucleotide reductase. *Biochem Pharmacol* **30:** 979–984, 1981.
34. Avery TL, Finch RA, Vasquez KM, Radparvar S, Hanna NB, Revankar GR and Robins RK, Chemotherapeutic characterization in mice of 2-amino-9-β-D-ribofuranosylpurine-6-sulfinamide (sulfinosine), a novel purine nucleoside with unique antitumor properties. *Cancer Res* **50:** 2625–2630, 1990.