Thiazolidine Prodrugs as Protective Agents against γ-Radiation-Induced Toxicity and Mutagenesis in V79 Cells

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Representatives of two classes of thiazolidine prodrug forms of the well-known radioprotective agents L-cysteine, cysteamine, and 2-[(aminopropyl)amino]ethanethiol (WR-1065) were synthesized by condensing the parent thiolamine with an appropriate carbonyl donor. Inherent toxicity of the prodrugs was assessed in V79 cells using a clonogenic survival assay. Protection against radiation-induced cell death was measured similarly after exposure to 0−8 Gy γ (^{137}Cs) radiation. Antimutagenic activity was determined at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus. All thiazolidine prodrugs exhibited less toxicity than their parent thiolamines, sometimes dramatically so. Protection against radiation-induced cell death was observed for the 2-alkylthiazolidine, 2(*R*,*S*)-D-*ribo*-(1′,2′,3′,4′-tetrahydroxybutyl)thiazolidine (RibCyst), which produced a protection factor at 8 Gy of 1.8; the cysteine analogue, 2(*R*,*S*)-D-*ribo*-(1′,2′,3′,4′-tetrahydroxybutyl)thiazolidine-4(*R*)-carboxylic acid (RibCys), was less active. RibCyst also exhibited excellent antimutational activity, rivaling that of WR-1065. The 2-oxothiazolidine analogues showed little activity in either determination under the conditions tested, perhaps due to their enhanced chemical and biochemical stability.

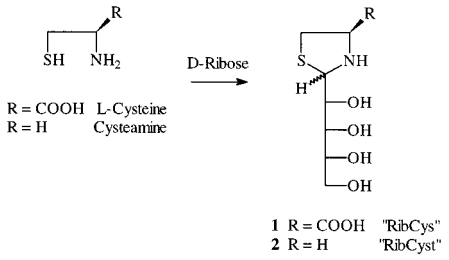
Introduction

Cysteine and cysteamine have been recognized as radioprotective agents for decades.[1,2] To extend the original observations, an extensive survey of compounds, mainly thiolamines and their derivatives, was initiated by the Walter Reed Army Hospital in the 1950s.[3] A promising radioprotective agent that emerged from this effort was WR-1065 (2-[(aminopropyl)amino]ethanethiol), a derivative of cysteamine.

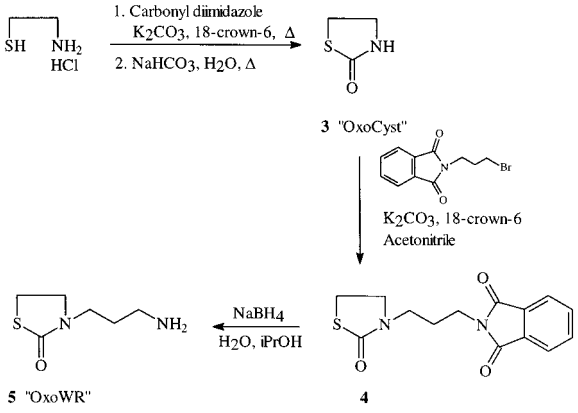
Unfortunately, the use of many thiolamines as radioprotectors is limited by the undesired side effects observed at protective doses, as well as limitations in oral activity.[4−9] To avoid these problems, we applied a prodrug approach, wherein the parent agent is chemically modified to overcome a biological barrier to its effective use.[10,11]

Our prodrug strategy involves incorporating the parent thiolamine into a thiazolidine ring structure, which effectively masks the reactive thiol group, reduces potential side effects, and alters key physicochemical properties.[12,13] For the current studies, representatives of two classes of thiazolidine analogues were synthesized and evaluated. The 2-alkylthiazolidines (Scheme 1) were designed to release the free thiolamine by nonenzymatic ring opening and hydrolysis.[12,14,15] Preliminary investigation of such radioprotectors has been promising.[16,17] In contrast, the 2-oxothiazolidines (Scheme 2) were designed to be inherently more stable, releasing the parent thiolamine only upon enzymatic cleavage, if at all. This category was modeled after the cysteine prodrug, 2-oxothiazolidine-4(*R*)-carboxylic acid (OTZ, Procysteine, "OxoCys"), which is known to release cysteine after biotransformation by the enzyme, 5-oxoprolinase.[18−20]

Scheme 1



Scheme 2



We constructed thiazolidine prodrug forms of cysteine, cysteamine, and WR-1065 to explore the radioprotective and antimutagenic activity of these derivatives. The thiazolidine analogues were tested in cultured V79 cells for (1) their inherent toxicity, (2) the ability to protect cells from radiation-induced cell death, and (3) the ability of selected agents to reduce radiation-induced

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mutagenesis at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus. Antimutagenic activity may be particularly advantageous for radioprotectors employed during the radiotherapy of cancer. Patients can develop secondary tumors as a result of mutations brought on by the radiation treatment, a complication becoming more prevalent as patients survive for longer periods of time after treatment. The ability to decrease therapy-induced mutagenesis in normal tissue, and hence treatment-derived secondary neoplasias, could significantly enhance the long-term clinical success of radiation treatments.

Results and Discussion

Chemistry. The synthesis of the 2-alkylthiazolidine analogues of cysteine (RibCys, **1**) and cysteamine (RibCyst, **2**), depicted in Scheme 1, involves the condensation of the aminothiol with D-ribose, which proceeds in good yield in either water or methanol. The chemistry of this reaction was originally investigated in the 1930s and is well documented.[12,15,21−24] Various synthetic methods were employed in the attempted synthesis of the corresponding alkylthiazolidine analogue of WR-1065, but none was successful, presumably due to the proximity of the nucleophilic primary amine and its effect on thiazolidine ring stability.

Upon nonenzymatic ring opening and hydrolysis, the 2-alkylthiazolidines release the parent thiolamine, along with an equimolar amount of the aldehyde used in prodrug construction.[14,15] Ribose was chosen as the carbonyl donor to eliminate possible toxicity associated with the release of the aldehyde component.[12]

The synthesis of the 2-oxothiazolidine analogues of cysteamine and WR-1065 is outlined in Scheme 2. OxoCyst (**3**) was prepared by the reaction of cysteamine with carbonyl diimidazole and also served as an intermediate in the synthesis of the WR-1065 analogue, OxoWR (**5**). The preparation of the latter involved alkylation of OxoCyst with 3-bromopropylphthalimide, followed by deprotection of the primary amine with $NaBH_4$.

The 2-oxothiazolidines were designed to be enzymatically cleaved by 5-oxoprolinase, similar to a corresponding prodrug of cysteine, OxoCys.[18,19] However, compounds with the molecular features present in the novel analogues (lack of the carboxyl group, N-alkylation) may not be substrates for this enzyme.[20] Agents that are not subject to either enzymatic or nonenzymatic biotransformation may provide further information regarding the ability of intact thiazolidines themselves to act as radioprotectors without the prerequisite release of thiolamine. This property has been suggested in previous reports.[25,26] V79 cells are known to contain 5-oxoprolinase.[27]

Biological Evaluation. As seen in Figure 1, each thiazolidine was less toxic to the V79 cells than its parent thiolamine, sometimes dramatically so. The thiazolidines of cysteine and cysteamine exhibited essentially no toxicity in these studies (Figure 1A,B), whereas the thiazolidine analogue of WR-1065 produced noticeable toxic effects, with a decrease in surviving fraction (SF) to <0.8 at 25 mM. In addition, the dose−response curve of the prodrug was very different from that of the parent drug (Figure 1C). WR-1065 was very toxic at or above 10 mM. The dramatic toxicity at lower concentrations of WR-1065 (~0.5 mM), the characteristic so-called "paradoxical toxicity" of thiol compounds,[4,5,16] was also observed.

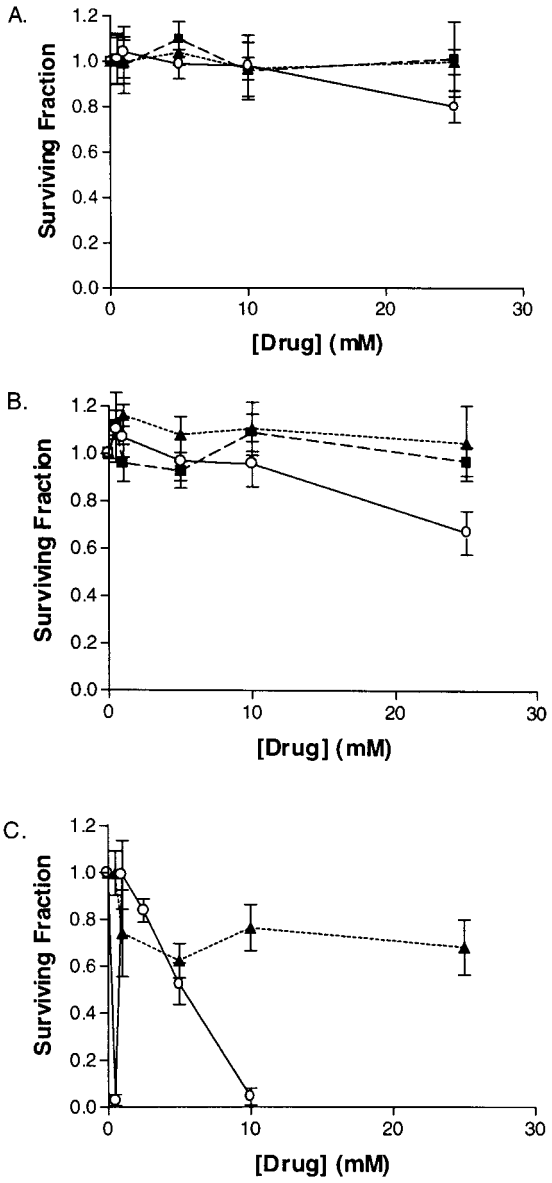

Figure 1. Effect of parent thiolamines and their thiazolidine prodrugs on clonogenic survival of V79 cells. The average of at least three separate experiments, each run in triplicate, is depicted; error bars represent standard deviation. (A) Toxicity of cysteine (○), RibCys (**1**, ■), and OxoCys (▲). (B) Toxicity of cysteamine (○), RibCyst (**2**, ■), and OxoCyst (**3**, ▲). (C) Toxicity of WR-1065 (○) and OxoWR (**5**, ▲).

The agents were then studied for their ability to protect V79 cells from radiation-induced cell death, again using a clonogenic survival assay. Figure 2 provides the results of these assays, and Table 1 lists the protection factors (PF) and dose modification factors (DMF) derived from these experiments. None of the thiazolidines in either the cysteine series (Figure 2A), the cysteamine series (Figure 2B), or the WR-1065 series (Figure 2C) provided the level of radioprotection observed upon treatment with the parent thiolamines. However, RibCyst (**2**) exhibited the greatest radioprotective activity under the conditions tested (PF = 1.8 at 8 Gy).

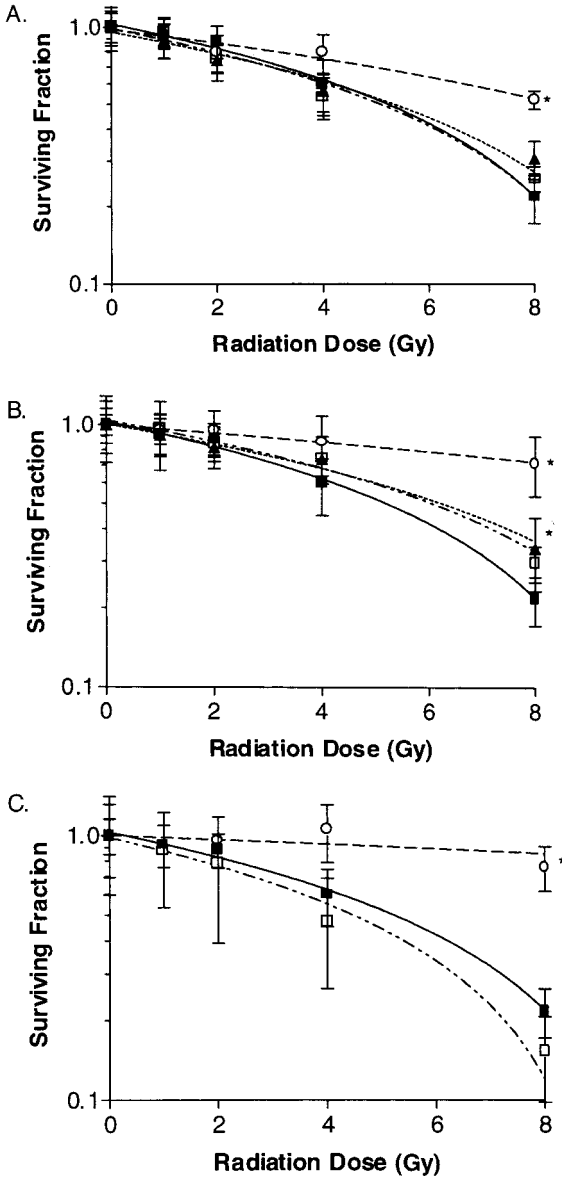

Figure 2. Radioprotection by the parent thiolamines and their thiazolidine prodrugs in V79 cells. Each point represents the average of at least three experiments, each run in triplicate; error bars represent standard deviation. ANOVA analysis was used to identify results which differ significantly ($p < 0.05$) from the control samples (at 8 Gy), indicated by the asterisk (*) symbol. The media control in each panel is represented by ■. (A) Protection by 10 mM cysteine (○), 25 mM RibCys (**1**, ▲), and 25 mM OxoCys (□). (B) Protection by 10 mM cysteamine (○), 25 mM RibCyst (**2**, ▲), and 25 mM OxoCyst (**3**, □). (C) Protection by 4 mM WR-1065 (○) and 10 mM OxoWR (**5**, □).

Due to the superior performance of RibCyst in the radioprotection experiments, the cysteamine series was tested for the ability to protect cells from radiation-induced mutagenesis. The hypoxanthine-guanine phosphoribosyltransferase (HGPRT) system monitors the ability of the cells to utilize purine salvage pathways and can be used to detect the induction of point mutations and small deletions.[28,29] The results of the HGPRT antimutation studies are shown in Figure 3. Irradiation at 4 Gy increased the frequency of mutation by approximately 30 mutants per 10^6 cells over background levels. The 2-oxothiazolidine, OxoCyst (**3**), exhibited no protection against radiation-induced mutation. However, the 2-alkylthiazolidine analogue, RibCyst (**2**), displayed excellent activity, providing protection similar to WR-1065. The parent thiolamine, cysteamine, produced intermediate results, which were statistically indistinguishable from controls. None of the agents themselves increased mutation rates above background (data not shown).

Table 1. Protection Factors (PF) and Dose Modification Factors (DMF) of the Parent Thiolamines and Their Thiazolidine Prodrugs

agent (mM)	PF[a] at 4 Gy	PF[a] at 8 Gy	DMF[b]
cysteine (10)	1.4	2.7	2.2
RibCys, **1** (25)	1.0	1.6	1.5
OxoCys (25)	0.9	1.4	1.3
cysteamine (10)	1.5	3.8	2.9
RibCyst, **2** (25)	1.3	1.8	1.3
OxoCyst, **3** (25)	1.3	1.3	1.1
WR-1065 (4)	1.8	4.1	4.4
OxoWR, **5** (10)	0.8	0.8	0.9

[a] PFs are calculated as the ratio of surviving fraction of cells in the presence of the agent to that in its absence at the respective radiation doses. [b] DMFs are calculated as the ratio of the terminal slope of the survival curve of the cells in the presence of the agent to that in its absence.

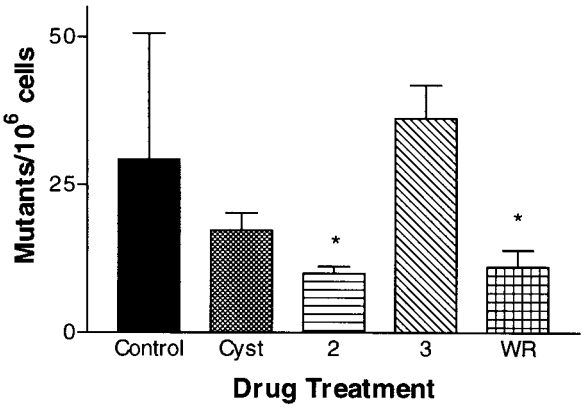

Figure 3. HGPRT mutation frequency in V79 cells receiving radiation alone (control) or radiation plus treatment with 10 mM cysteamine (Cyst), 25 mM RibCyst (**2**), 25 mM OxoCyst (**3**), or 4 mM WR-1065 (WR). Each point represents the average of two experiments with five data points per experiment; error bars represent standard deviation. ANOVA analysis was used to identify results which differ significantly ($p < 0.05$) from the control samples, indicated by the asterisk (*) symbol.

Conclusions

The studies discussed here illustrate that thiazolidines are less toxic in vitro than their parent thiolamines. The 2-oxothiazolidines appeared to be largely inactive as radioprotective agents under the conditions examined. In contrast, the 2-alkylthiazolidines, particularly RibCyst, were much more promising. RibCyst also displayed excellent antimutagenic activity, which rivaled that of WR-1065, a well-accepted standard of radioprotection and antimutagenesis;[30,31] OxoCyst was again inactive. Such antimutagenic activity may be beneficial in cancer radiotherapy, suppressing the formation of treatment-derived secondary tumors. Coupled with the reduced inherent toxicity, a significant enhancement in the therapeutic index of radiation treatment may result if an agent such as RibCyst is administered in conjunction with radiation therapy.

The activity associated with the 2-alkylthiazolidine analogues suggests that a similar prodrug form of WR-

1065 may also provide radioprotection and/or antimutation with reduced toxicity. Alternative schemes are being pursued to synthetically access this potentially interesting prodrug form.

Experimental Section

Chemistry. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Aldrich Chemical Co. (Milwaukee, WI), or Fisher Scientific (Pittsburgh, PA). WR-1065 was a gift from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. Melting points were determined on a Laboratory Devices USA Mel-Temp II melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were obtained on either an IBM Instruments 200 MHz or a Varian Unity 500 MHz FT-NMR spectrometer, as indicated. High-resolution mass spectral (HRMS) analyses were carried out on a Finnegan MAT 95 in the Department of Chemistry at the University of Utah. Elemental analysis was conducted by Galbraith Laboratories (Knoxville, TN). Thin-layer chromatography (TLC) was carried out using Whatman (Clifton, NJ) flexible backed 60 Å silica gel plates, with a layer thickness of 0.25 mm. Whatman silica gel (60 Å, 230−400 mesh) was used for sample purification using column chromatography.

2(*R,S*)-D-ribo-(1′,2′,3′,4′-Tetrahydroxybutyl)thiazolidine-4(*R*)-carboxylic Acid (RibCys, 1) and 2(*R,S*)-D-ribo-(1′,2′,3′,4′-Tetrahydroxybutyl)thiazolidine (RibCyst, 2) (Scheme 1). RibCys and RibCyst were synthesized and characterized as described.[16] RibCyst was stored under argon at −15 °C but occasionally displayed evidence of decomposition, such as discoloration and development of a slight odor, which was remedied by recrystallization from methanol.

Thiazolidin-2-one (OxoCyst, 3) (Scheme 2). The synthetic strategy for OxoCyst was based on the methods of d'Ischia et al.[32] 1,1′-Carbonyl diimidazole (30.0 g, 0.185 mol) was dissolved (with heating) in acetonitrile (300 mL), which had been degassed and flushed with argon. To this solution were added 18-crown-6 (0.7 g, 0.025 mol), 2-aminoethanethiol hydrochloride (20.1 g, 0.185 mol), and potassium carbonate (26 g, 0.19 mol). The mixture was heated to reflux and stirred for 18 h. Water (100 mL) was added, and the mixture was refluxed for an additional 4 h. The solution was cooled slightly, and the solvent was removed in vacuo. Ethyl acetate (500 mL) was added to the resulting solid, and the insoluble salts were removed by filtration. The filtrate was dried, and the crude product was purified on silica gel, eluting with 50% ethyl acetate in hexane. Yield: 13 g (68%); mp 48−50 °C; $R_f = 0.22$ (2:1 ethyl acetate:hexane). HRMS (FAB^+): $m/z = 104.01752$ ($M^+ + 1$, theoretical = 104.01701). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.37 (t, 2H, $J = 8$, 6.5 Hz), 3.59 (t, 2H, $J = 8$, 6.5 Hz), 6.75 (br s, 1H) ppm. ^{13}C NMR ($CDCl_3$, 125 MHz): δ 30.0, 43.4, 176.7 ppm. Anal. C, H, N.

3-(*N*-Phthaloyl-3-aminopropyl)thiazolidin-2-one (4) (Scheme 2). To a solution of thiazolidin-2-one (**3**, 6.4 g, 0.062 mol) in acetonitrile (100 mL) was added *N*-(3-bromopropyl)-phthalimide (26.0 g, 0.093 mol), potassium carbonate (17 g, 0.12 mol), and 18-crown-6 (1.6 g, 0.0061 mol). The reaction mixture was stirred at reflux for 16 h, and then the acetonitrile was removed in vacuo. The crude product was redissolved in 0.5 M potassium chloride (60 mL), and the product was extracted with ethyl acetate (3 × 75 mL). The combined organic fractions were washed with a saturated aqueous solution of sodium chloride (50 mL), dried over sodium sulfate, and concentrated in vacuo. The resulting solid was recrystallized from ethyl acetate (150 mL). Yield: 13 g (72%); mp 121−124 °C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 1.68−1.91 (m, 2H), 3.22−3.35 (m, 4H), 3.48−3.65 (m, 4H), 7.75−7.88 (m, 4H) ppm.

3-(3-Aminopropyl)thiazolidin-2-one (5) (Scheme 2). The removal of the phthalimide protecting group was based on the procedure of Osby et al.[33] Sodium borohydride (8.1 g, 0.21 mol) was added to a suspension of 3-(*N*-phthaloyl-3-aminopropyl)-thiazolidine-2-one (**4**, 12 g, 0.043 mol) in a 6:1 solution of 2-propanol:water (425 mL). After being stirred at reflux for 16 h, the mixture was cooled slightly and glacial acetic acid (45 mL) was added. After an additional 4 h at reflux, the solvent was removed in vacuo, and the resulting solid was washed with ether (700 mL). The crude product was purified in three portions on Dowex (50W ×8) cation-exchange resin (18 × 3 cm), washing with water (500 mL) and 1 N HCl (500 mL), and eluting the product, as the hydrochloride salt, with 6 N HCl (500 mL). Yield: 4.8 g (55%); mp 78−82 °C. HRMS (FAB^+): $m/z = 161.07447$ ($M^+ + 1$, theoretical = 161.07486). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 2.49 (t, 2H, $J = 7.5$ Hz), 3.39 (t, 2H, $J = 3$ Hz), 3.95−4.00 (m, 4H), 4.31 (t, 2H, $J = 8$, 6.5 Hz), 8.89 (br s, 3H) ppm. ^{13}C NMR (DMSO-d_6, 125 MHz): δ 25.0, 25.3, 36.4, 41.5, 48.1, 171.1 ppm. Anal. C, H, N.

Biological Evaluation. General Cell Culture Procedures. V79 Chinese hamster lung fibroblasts (V79-4) were obtained from American Type Culture Collection (Manassas, VA). The following supplies were purchased from Sigma Chemical Co. (St. Louis, MO): Dulbecco's modified minimum essential media (D-MEM), Hanks' balanced salt solution, trypsin (1:250), phosphate buffered saline (PBS), antibiotic/antimycotic solution (100X), and OxoCys. Serum was obtained from HyClone Laboratories (Logan, UT). V79 cells were grown in D-MEM containing 3.7 g/L sodium bicarbonate, 10% Fetal Clone I serum, and 0.1% antibiotic/antimycotic solution. Cells were maintained in a humidified 5% CO_2−air atmosphere at 37 °C.

The number of cells was determined using a Coulter Counter, model Z1 (Beckman Coulter, Fullerton, CA) particle counter. Colonies were stained using a solution of 2.5 g of powdered crystal violet in 900 mL of methanol and 100 mL of 10% formalin; colonies consisting of at least 50 cells were counted using the Manostat Colony Counting System (Manostat Corp., New York, NY).

Irradiation of cells was carried out in a Shepherd Mark I irradiator (^{137}Cs source) at a dose rate of 7.5 Gy/min.

Data analysis was conducted using GraphPad Prism (v. 2.0, San Diego, CA).

Clonogenic Survival Assay of Drug Toxicity. Each well of a six-well culture plate was seeded with enough cells in 3 mL of media to yield at least 50 colonies after treatment, and the cells were incubated for 16 h to allow attachment to the plate surface. The media was then replaced with 5 mL of freshly made solutions of each drug in media at various concentrations (pH 7−7.5). The cells were incubated with the treatment solutions for 3 h, and then the media was removed and the cells were washed with Hanks' buffered salt solution for 15 min at room temperature. Fresh media (3 mL) was added, and the plates were incubated 4 d longer to allow colony development. The colonies were stained and counted, and the surviving fraction (SF) was calculated by dividing the number of cells retaining colony forming ability after drug treatment by those receiving media alone. These values were normalized for comparison (controls, SF = 1). The plating efficiency of untreated V79 cells was approximately 60%.

Protection against Radiation-Induced Cell Death. Sufficient cells were plated in each well of a 6-well plate to yield at least 50 colonies after treatment. After overnight incubation, the media was removed and replaced with 5 mL of freshly prepared treatment solutions (pH 7−7.5) at a drug concentration in the relatively nontoxic range (derived from Figure 1). A minimum of three wells per study served as media controls.

Cells were incubated for 3 h, then irradiated at doses of 0, 1, 2, 4, or 8 Gy. After irradiation, treatment solutions were removed, and cells were washed for 10 min at room temperature with Hanks' buffered salt solution. Fresh media was added, and the plates were incubated for 4 d to allow colony development. The colonies were then stained and counted. The ratio of colonies per cells plated in treated versus untreated samples represented the SF. These values were normalized for comparison (controls, SF = 1).

Protection against Radiation-Induced Mutagenesis. Assays of mutagenesis at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus in V79 cells were based on previously reported methods.[28,29] After removing preexisting HGPRT mutants by growth in HAT media (hypoxanthine (0.1 mM), aminopterin (0.4 μM), and thymidine (0.016 mM); Gibco BRL, Rockville, MD) for 1 d, cells were allowed to recover for 6 to 9 d, subculturing every 3 d prior to treatment.

The cells were then trypsinized and counted. Cells (2×10^6) were seeded into each of a series of fresh 25 cm^2 flasks, each in 5 mL of media. For each experiment, sufficient flasks were prepared to investigate both irradiated and nonirradiated samples of each drug studied, as well as of controls, which received no drug treatment. The flasks were incubated for 24 h to allow cell adhesion to the flask surface.

The following day, media was removed from cell cultures and was replaced with fresh media (in control samples) or freshly prepared drug solutions (pH 7). Two flasks were prepared for each drug treatment group. One flask from each drug treatment group was irradiated at 4 Gy. Cells were incubated 3 h longer in the treatment solutions, then washed with Hanks' buffered salt solution. The cells were trypsinized and counted. For each sample, three wells of a six-well plate were seeded with 200−250 cells for plating efficiency determination. Additionally, fresh cultures were initiated with 1.5×10^6 cells each.

After 5 d, colonies in the six-well plates were stained and counted to determine the surviving fraction of each treatment group.

The cultures were grown for a total of 6 d, subculturing at 3 d. After this time, cells were trypsinized and counted. For each sample, each well of five six-well plates was seeded with 8×10^4 cells, each in media containing 6-thioguanine (6TG) at a concentration of 5 μg/mL. In addition, three wells per sample were seeded with 200 cells each, in media without 6TG, for plating efficiency determinations.

All culture plates were incubated for 5 d to allow colony growth, then stained and counted.

Mutation frequencies for each sample were calculated as the number of mutant colonies per number of cells plated. (The latter value was corrected for cell survival rates, based on the plating efficiency determinations.) Mutation frequencies were expressed as mutants per 10^6 surviving cells. The background mutation frequency of untreated cells (6 mutants per 10^6 surviving cells) was subtracted from the value obtained for each treatment sample.

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