In Vitro Antiproliferative Activity of 4-Substituted 2-(2-Hydroxyphenyl)thiazolines on Murine Leukemia Cells

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Two previously synthesized and two structurally novel thiazoline iron chelators are described. N^4-Benzyl-N^1,N^8-bis[[2-(2-hydroxyphenyl)thiazolin-4-yl]carbonyl]homospermidine (**5**) proved to be the most potent antiproliferative and cytocidal compound in the series with in vitro IC_{50} values of 3 and 1 μM on L1210 and P388 murine cell lines. The N^4-acetyl analogue **7** was considerably less active than **5** with IC_{50} and cell viability values that were similar to those of the structurally simple thiazolines **2** and **3**. The antiproliferative activity of **3** and **7** could be substantially reduced or ablated by delivery to cell suspensions as a 1:1 molar mixture with $FeCl_3$, while the activity of **5** was unaffected by Fe(III) chelation. As expected, **3** induced a G_1/S cell cycle block at the 100 μM block consistent with interference with DNA synthesis while 10 μM **5** did not affect L1210 cell cycle distribution. Tritiated thymidine incorporation studies confirmed that **5** was incapable of interfering with DNA synthesis at concentrations below 40 μM. Alkaline elution studies indicate that **5** does not cause DNA strand breaks in vitro at concentrations of 10 μM. The N^4-benzyl group of **5** appears to impart in vitro potency as the N^4-acetyl analogue **7** lacks comparable in vitro antiproliferative and cytocidal activity.

Whether neoplastic cells have higher iron requirements than somatic progenitor cells has yet to be answered. Infiltration of adjacent tissue with iron–dextran is not associated with subsequent concentration of iron by safrole-induced mouse neoplasm or plasmacytoma.[1,2] In contrast, white cells recovered from juvenile acute lymphocytic leukemia patients have higher average iron concentrations than normal juvenile lymphocytes.[3] Transformed white cells have been reported to display 10^3 times the number of transferrin receptors of resting lymphcytes.[4] Thus, some transformed tissues may have higher basal iron utilization than nonmalignant precursor, but malignancies do not necessarily store iron in a depot fashion beyond immediate needs.

A variety of oncolytic compounds including parabactin,[5] omadine,[6] 8-hydroxyquin,[6] tropolone,[6] guanazole,[7] α-(N)-heterocyclic aldehyde thiosemicarbazones,[8] deferoxamine,[9] and substituted α-ketohydroxypyridines[10] have been shown to bind iron. Parabactin, hydroxyurea, and deferoxamine inhibit DNA synthesis by inhibiting the iron-dependent enzyme ribonucleotide reductase.[9-13] We have engaged in the synthesis and in vitro testing of a variety of 4-substituted 2-(2-hydroxyphenyl)thiazoline iron chelators in hope of identifying compounds that display potent in vitro and in vivo oncolytic activity, ease of synthesis, low host toxicity, and potential oral bioavailability. In vitro antiproliferative activity is reported for two chemically novel thiazoline compounds and two previously synthesized compounds.[14,15]

Chemistry

2-(2-Hydroxyphenyl)thiazoline-4-carboxylic acid (**2**; see Chart I) and methyl 2-(2-hydroxyphenyl)thiazoline-4-carboxylate (**3**) were synthesized by similar means. Ethyl 2-hydroxybenzimidate (**1**) was condensed with L-cysteine hydrochloride or L-cysteine hydrochloride methyl ester in dry refluxing methanol to afford the 2-(o-hydroxyphenyl)thiazolines **2** and **3** in yields of 65% and 85%, respectively. Yields proved to be highest when the hydrochloride salt of cysteine rather than the hydrochloride salt of ethyl 2-hydroxybenzimidate was used in the condensation.

Chart I

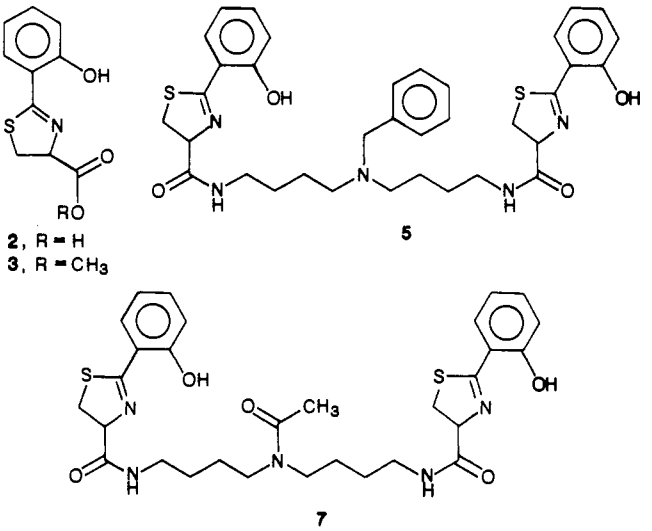

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Table I. In Vitro Antiproliferative and Cytocidal Activity of Various Thiazoline Compounds and Other Reference Iron Chelators

compd no.	chelator	antiproliferative act., IC_{50},[a] μM		viability,[b] %	
		L1210	P388	L1210	P388
	hydroxyurea	85	32	97	96
1	ethyl 2-hydroxybenzimidate	>200	ND[c]	>99	ND
2	2-(2-hydroxyphenyl)thiazoline-4-carboxylic acid	25	17	92	77
3	methyl 2-(2-hydroxyphenyl)thiazoline-4-carboxylate	14	18	87	85
5	N^4-benzyl-N^1,N^8-bis[[2-(2-hydroxyphenyl)thiazolin-4-yl]carbonyl]homospermidine	3	1	6[d]	15[e]
7	N^4-acetyl-N^1,N^8-bis[[2-(2-hydroxyphenyl)thiazolin-4-yl]carbonyl]homospermidine	21	12	95	85

[a] Values listed represent molar ligand concentrations that inhibited by 50% the growth of control cell suspensions from 24 to 48 h after ligand addition. [b] Viability, as determined by trypan blue exclusion 48 h after ligand addition at 100 μM concentrations. [c] ND = not defined. [d] At 35 μM ligand concentration. [e] At 25 μM ligand concentration.

2-(2-Hydroxyphenyl)thiazoline-4-carboxylic acid (**2**) was used as a reagent in the subsequent synthesis of **5** and **7**. In the presence of *N*-hydroxysuccinimide, **2** was condensed with dicyclohexylcarbodiimide in dry DMF to afford the *N*-oxosuccinimide active ester of **2**. After filtration, the active ester of **2**, without further purification, was condensed with either N^4-benzylhomospermidine or N^4-acetylhomospermidine to produce **5** (40% yield) and **7** (35% yield), respectively.

It has been shown that the asymmetric polyamine spermidine and the long-chain analogue homospermidine undergo active transport in L1210 cells and stimulate cell division in the same cell line. Additionally, the synthetic polyamine homospermidine and to a lesser extent nonspermidine effectively compete with spermidine for cellular uptake.[16] In the synthesis of **5** and **7** homospermidine was chosen over the naturally occurring asymmetric polyamine spermidine due to the facility of homospermidine's synthesis. The polyamine reagents were used merely as a means of coupling two thiazoline ligands and were not intended to facilitate cellular uptake or subcellular localization of the thiazolines. In fact, it has previously been shown that derivatization of spermidine or homospermidine in the fashion of **5** and **7** (bis acylation of primary amines) severely reduces active transport of polyamine derivatives in L1210 cells.[17]

Results and Discussion

Table I shows IC_{50} and cell viability data for the ligands tested. All of the ligands showed substantially better antiproliferative potency than hydroxyurea, with **5** being the most potent inhibitor of cell division (IC_{50} = 3 μM) on L1210 cells). The 48-h cytocidal potency of **5**, as determined by trypan blue exclusion, was much greater than that of any other ligand. Clonogenic assays indicated that L1210 cell viabilities were substantially lower, at 100 μM concentrations for all ligands tested, than indicated by trypan blue exclusion methods (Table II). These findings are consistent with the fact that soft agar clonogenic assays are a more sensitive means of assessing cell viability than the trypan blue exclusion technique due to the ability of clonogenic assays to assess cell reproductive capability and delayed cell death. Once again, **5** induced the greatest reduction in cell viability with a complete loss of clonogenic potential at a 50 μM ligand concentration. In contrast, **7** had no effect on L1210 cell clonogenicity at a 50 μM concentration.

Previous work with catecholamide iron chelators has shown that in vitro antineoplastic efficacy of various chelators relates to the lipophilic character of the iron-free ligand.[5,6] Partition ratios were determined for compounds **2**, **3**, **5**, and **7** (Table III). In contrast with the above-mentioned catecholamide chelators, there was found to be little correlation between lipophilicity of the various (*o*-hydroxyphenyl)thiazolines and their in vitro antiproliferative potency. The most potent antiproliferative in this series, **5**, displayed significant aqueous solubility at pH 7.1 in comparison with the less potent **3** and **7**.

As expected, on the basis of past observations with catecholamide iron chelators,[5] exposing stock solutions of **3** and **7** to equimolar $FeCl_3$ solutions, followed by addition to L1210 cell suspensions, was associated with a reduction of antiproliferative effects of the chelators (Table IV).

Table II. L1210 Cell Viability As Determined by Soft Agar Clonogenicity

compd no.	concn, μM	% viability ± SE[a]
2	0	94 ± 1.3
	50	81 ± 9[b-d]
	100	54 ± 8[b-d]
	200	42 ± 7[b,c]
3	0	99 ± 0.8
	50	36 ± 5[b,d]
	100	46 ± 8[b]
	200	42 ± 3[b]
5	0	87 ± 0.9
	12.5	78 ± 5[f]
	25	44 ± 8[b,f]
	50	0 ± 0[b,f]
7	0	99 ± 0.5
	25	92 ± 7
	50	100 ± 10
	100	40 ± 2[b,e]
	200	27 ± 3[b,e]

[a] SE = standard error. [b] Control L1210 cell viabilities differed statistically ($p < 0.05$) from viabilities at indicated chelator concentrations for all compounds. [c] Cell viabilities in the presence of 50 μM vs 100 or 200 μM **2** differed significantly from one another ($p < 0.001$). [d] Cell viabilities in the presence of 50 μM **3** vs 50 μM **2** differed significantly ($p < 0.0005$). [e] Cell viabilities in the presence of 100 μM vs 200 μM **7** differed significantly from one another ($p < 0.006$). [f] Cell viabilities in the presence of 12.5, 25, and 50 μM **5** all differed significantly from each other ($p < 0.006$).

Table III. Partition Coefficients and G° Transfer[a]

compd no.	approximate PBS solubility, M	concn ratio of compd in 1-octanol/PBS	G°_t, kcal M^{-1}
2	7.5×10^{-5}	0.03	0.72
3	5.0×10^{-5}	11.75	−1.47
5	1.5×10^{-5}	0.96	0.02
7	1.0×10^{-5}	>7[b]	<−1.16[b]

[a] Determined in either phosphate-buffered saline (PBS), pH 7.1, or 1-octanol biphasic mixture. [b] Concentration of **7** in PBS was below assay sensitivity as limited by aqueous solubility of **7** in PBS.

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Table IV. Antiproliferative Activity of Iron(III) Chelates versus Iron-Free Ligands

compd(s)	concn, μM	% of control growth ± SE[a]
3	100	4 ± 0.1[b]
3, $FeCl_3$	100 (each)	98 ± 7.0[b]
5	5	22 ± 1.2
5, $FeCl_3$	5 (each)	21 ± 2.7
7	25	29 ± 0.9[b]
7, $FeCl_3$	25 (each)	68 ± 1.6[b]

[a] Values determined 48 h after ligand addition. [b] Antiproliferative activity of 100 μM **3** vs 100 μM (**3**, $FeCl_3$) and 25 μM **7** vs 25 μM (**7**, $FeCl_3$) differed significantly ($p < 0.0001$).

Table V. Flow Cytometric (FCM) Analysis of L1210 Cells[a]

cell cycle phase	control	concn of **5** 10 μM
G_0/G_1	43.2 ± 0.4	43.0 ± 0.4
S	42.7 ± 0.1	44.5 ± 1.5
G_2/M	12.2 ± 0.6	8.8 ± 1.1

cell cycle phase	control	concn of **3** 100 μM	300 μM	500 μM
G_0/G_1	42.9 ± 1.7[b]	46.0 ± 2.8[b,d]	62.6 ± 8.4[b]	61.3 ± 7.4[b]
S	38.8 ± 2.5	36.5 ± 3.3[d]	31.5 ± 9.1	34.3 ± 7.9
G_2/M	18.3 ± 1.4[b]	17.5 ± 2.0	5.9 ± 0.8[b]	4.4 ± 0.9[b]

cell cycle phase	control	concn of hydroxyurea 100 μM	500 μM
G_0/G_1	42.9 ± 1.7[c]	31.9 ± 1.8[c,d]	59.8 ± 7.0[c]
S	38.8 ± 2.5[c]	48.4 ± 3.7[c,d]	53.0 ± 0.6
G_2/M	18.3 ± 1.4[c]	19.7 ± 2.6	0.8 ± 0.4[c]

[a] Percent cells populating each cell cycle phase ± SD. [b] $p < 0.005$ for the Gossett *T*-test comparing control cell cycle phase distribution with distribution in the presence of various concentrations of **3**. [c] $p < 0.005$ for the Gossett *T*-test comparing control cell cycle phase distribution with distribution in the presence of 100 or 500 μM hydroxyurea. [d] $p < 0.001$ for the Gossett *T*-test comparing cell cycle phase distribution for 100 μM concentration of **3** vs 100 μM hydroxyurea.

Conversely, forming the iron(III) chelate of **5** did not reverse antiproliferative efficacy. The iron(III) chelate of **5** versus iron-free ligand showed equal L1210 antiproliferative activity. These findings differ from those reported for catecholamide iron chelators[5] but are supported by examples of other ligands whose antiproliferative activities are not reversed by iron chelation including α-ketohydroxypyridines, omadine, 8-hydroxyquin, tropolone, and α-(N)heterocyclic aldehyde thiosemicarbazones.[6,8]

Flow cytometric (FCM) analysis with **5** indicated that at 10 μM only very small changes in cell population distributions were observed. The changes, which were not statistically significant, consisted of an incomplete block in S phase (Table V). This observation was surprising as a 10 μM concentration of **5** is 3 times the IC_{50} and should have caused a considerable block, if the antiproliferative activity of this compound was due primarily to interference with DNA synthesis. In contrast, 100 μM concentrations of **3** induced a G_1/S cell cycle block that was substantial and significantly ($p < 0.05$) greater than that induced by 100 μM hydroxyurea (G_1 32% vs 46% and S 48% vs 37%) for the two compounds, respectively (Table V).

Tritiated thymidine uptake studies confirmed results seen regarding the effect of **5** on cell cycle kinetics (Table VI). Significant reduction in thymidine uptake was observed only when the concentration of **5** exceeded 50 μM. Even at concentrations greater than 300 μM, thymidine uptake was reduced to only 27% of control values. Delivery to culture media of **5** as an equimolar mixture with $FeCl_3$ inhibited thymidine incorporation to the same extent as iron-free **5**.

Table VI. [^{3}H]Thymidine Uptake Analysis after 120-min Incubation with **5** and Radiolabel

concn of **5**, μM	[^{3}H]TdR incorpn, % of control ± SD	concn of **5**, μM	[^{3}H]TdR incorpn, % of control ± SD
4.8	98 ± 9	76	47 ± 3
19	95 ± 11	200	41 ± 5
38	94 ± 10	300	27 ± 3
50	48 ± 12		

We found no evidence for the production of DNA damage by **5**. Exposure of V79 cells for 24 h at 37 °C to 8 μM **5**, a treatment that reduced cell proliferation by about 2-fold, was associated with no detectable DNA strand breaks as measured by alkaline elution. The elution parameter[18] corresponded to 0 rad of equivalent X-ray DNA damage.

Conclusions

In light of the above findings, the principal oncolytic activity of 10 μM **5** under these experimental conditions is unlikely to be associated with an intracellular removal of iron from critical enzyme systems, such as ribonucleotide reductase. Rather, **5** likely possesses a multifaceted dose-dependent mechanism of oncolytic activity in the cell lines studied. 4-Bis condensation of the 2-(2-hydroxyphenyl)thiazoline chelator residue upon the linear triamine N^4-benzylhomospermidine appears to prevent the cytotoxic inactivation seen with the ferric iron chelate of **3** or **7**. The observation that the N^4-benzyl group of **5** substantially enhances in vitro cytotoxic potency relative to the N^4-acetyl derivative **7** will lead us to synthesize numerous N^4-substituted derivatives in an effort to define compounds with greater in vitro and in vivo antiproliferation efficacy.

Experimental Section

All reagents were obtained from Aldrich Chemical Co., Milwaukee, WI, and used without additional purification unless indicated in the synthesis section. Tissue culture media and penicillin–streptomycin antibiotic solution were purchased from Gibco Labs, Grand Island, NY. Defined equine serum was purchased from Hyclone Labs, Logan, UT. Mopes–Hepes buffer ingredients, hydroxyurea, RNase, and propidium iodide were obtained from Sigma Chemical Co., St. Louis, MO. [^{3}H]TdR and [^{13}C]TdR were obtained from New England Nuclear, Boston, MA.

Melting points were determined on a Thomas-Hoover capillary melting point apparatus. IR spectra were generated on a Beckman Acculab 4 spectrometer using Wilks 25 × 4 mm NaCl plates or Barnes 50 × 25 × 6 mm NaCl rectangles in Beckman window holders. NMR spectra were determined on a Varian EM 360A 60-MHz or a Bruker Instruments WH-270 spectrometer. Mass spectra were generated by electron impact with a direct-exposure probe on a Finnegan Corp. 4023 spectrometer or by fast atom bombardment with a thioglycerol matrix on a Kratos MS-50 spectrometer. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN, and carbon, hydrogen, and nitrogen analyses were within ±0.4% of the theoretical values. Preparative column chromatography was conducted by using EM Reagents silica gel (80–230 mesh, without indicator) or Sigma Co. LH-20 hydrophobic gel resin.

All reagents were dried in vacuo over P_2O_5, and all glassware was acid washed and flame dried prior to use.

Ethyl 2-Hydroxybenzimidate Hydrochloride (1). Compound **1** was prepared by established methods.[19] Upon recrystallization from 95% ethanol and washing with cold diethyl ether, large off-white crystals were obtained (16.77 g, 84 mmol, 40%): mp 150–153 °C, lit. mp 150–151 °C. Ethyl 2-hydroxybenzimidate hydrochloride (**1**) (4.72 g, 23.5 mmol) was converted to the free

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imine **1a** by partitioning between cold ethyl ether (100 mL) and cold aqueous $NaHCO_3$ (2.17 g, 26 mmol, 100 mL) followed by drying of the ether layer over Na_2SO_4. The recovered off-white solid (3.61 g, 85%) had a characteristic highly aromatic "maple sugar" like aroma.

2-(2-Hydroxyphenyl)thiazoline-4-carboxylic Acid (2). L-Cysteine hydrochloride was condensed with **1a** according to methods previously reported by one of these authors.[14,15]

Methyl 2-(2-Hydroxyphenyl)thiazoline-4-carboxylate (3). L-Cysteine methyl ester was condensed with **1a** according to methods previously reported by one of these authors.[14,15]

N^4-Benzylhomospermidine (4). A previously reported[20] method afforded a clear semiviscous oil (8.68 g, 52%) after distillation: bp 170 °C/0.1 Torr (lit.[20] bp 166 °C/0.05 Torr): 60-MHz ^1H NMR ($CDCl_3$) δ 1.2 (s, 4 H), 1.3–1.65 (m, 8 H), 2.15–2.8 (m, 8 H), 3.45 (s, 2 H), 6.95–7.30 (m, 5 H).

N^4-Benzyl-N^1,N^8-bis[[2-(2-hydroxyphenyl)thiazolin-4-yl]carbonyl]homospermidine (5). Compound **2** (0.44 g, 2 mmol) was solubilized in 20 mL of dry, distilled DMF, to which was added *N*-hydroxysuccinimide (0.28 g, 2.4 mmol). The solution was cooled in an ice bath, and dicyclohexylcarbodiimide (0.49 g, 2.4 mmol) in 20 mL of dry, distilled dimethylformamide (DMF) was added by addition funnel dropwise over 3 h. The solution was stirred at room temperature for 18 h. The crude reaction mixture was then filtered to remove dicyclohexylurea byproduct. N^4-Benzylhomospermidine (**4**) (0.23 g, 0.9 mmol) was next added in three aliquots, each aliquot 4 h apart, followed by stirring for 18 h. The crude reaction mixture was filtered, concentrated in vacuo, and chromatographed on silica gel with an 8% methanol in chloroform mobile phase. The semipurified product was next taken up in cold absolute ethanol and acidified with anhydrous HCl gas. The ethanol solution was concentrated in vacuo to dryness and chromatographed on silica gel in 10% methanol in chloroform until all impurities had eluted from the column. At this time the mobile phase was saturated with ammonium hydroxide, which allowed the product of interest to elute from the column. The mobile phase was dried over anhydrous Na_2SO_4 and concentrated in vacuo to afford **5** (0.25 g, 40%) as a white solid: mp 40–42 °C; 270-MHz ^1H NMR [$(CD_3)_2CO$] δ 1.45–1.58 (m, 8 H), 2.34–2.47 (m, 4 H), 3.20–3.30 (m, 4 H), 3.50 (s, 2 H), 3.64–3.82 (m, 4 H), 5.30–5.39 (t, 2 H), 6.89–6.99 (m, 4 H), 7.14–7.34 (m, 5 H), 7.38–7.52 (m, 4 H); IR ($CHCl_3$) 3450 (m), 3020 (w), 2950 (m), 1665 (s), 1620 (w), 1590 (m), 1515 (m), 1455 (w), 1285 (m), 1230 (m), 1155 (w), 940 cm^{-1} (m); mass spectrum *m/e* 661 ($M^{\cdot+}$ + 2), 659 ($M^{\cdot+}$). Anal. ($C_{35}H_{41}O_4N_5S_2 \cdot 1.5H_2O$) C, H, N.

N^4-Acetylhomospermidine (6). A previously described method was used to afford **6** as a clear viscous oil:[17,20] 60-MHz ^1H NMR ($CDCl_3$) δ 1.1–1.7 (m, 8 H), 2.05 (s, 3 H), 2.9–3.5 (m, 8 H).

N^4-Acetyl-N^1,N^8-bis[[2-(2-hydroxyphenyl)thiazolin-4-yl]carbonyl]homospermidine (7). The method described for **5** was used except that N^4-acetylhomospermidine (**6**) was used in the condensation. Compound **1** (0.76 g, 3.4 mmol) was taken up in 20 mL of dry distilled DMF, and *N*-hydroxysuccinimide (0.47 g, 4.1 mmol) was added. Dicyclohexylcarbodiimide (0.84 g, 4.1 mmol) in DMF was added dropwise. The reaction mixture was stirred at room temperature for 18 h and then filtered. N^4-Acetylhomospermidine (**6**) (0.31 g, 1.6 mmol) was added in 5 mL of DMF in three aliquots, 4 h apart. The reaction mixture was stirred at room temperature for 14 h, concentrated in vacuo, and chromatographed in silica gel in 10% methanol in chloroform. Fractions containing semipurified product were concentrated and chromatographed on Sephadex LH-20 in 15% methanol in benzene followed by silica gel chromatography with 5% acetone in benzene until impurities eluted, followed by a mobile phase change to 10% methanol in chloroform, to afford **7** (0.33 g, 35%) as a glassy solid: 60-MHz ^1H NMR [$(CD_3)_2CO$] δ 1.2–1.75 (m, 8 H), 2.0 (s, 3 H), 2.8–3.45 (m, 8 H), 3.45–3.85 (br d, 4 H), 4.95 (distorted t, 2 H), 6.40–6.95 (m, 5 H) 7.0–7.45 (m, 5 H), 11.75–12.05 (br s, 2 H); IR ($CHCl_3$) 3450 (m), 3020 (s), 2950 (s), 1620 (s), 1590 (w), 1500 (m), 1360 (w), 1285 (m), 1235 (m), 1155 (w), 1040 (w), 940 (m), 900 cm^{-1} (w); mass spectrum *m/e* 613 ($M^{\cdot+}$ +2), 612 ($M^{\cdot+}$ +1), 611 ($M^{\cdot+}$); FAB 612 ($M^{\cdot+}$ + 1), 178. Anal. ($C_{30}H_{37}N_5O_5S_2 \cdot 1H_2O$) C, H, N.

Partition Analysis. Four chelators were studied to determine partition equilibrium values between 1-octanol and aqueous phosphate buffer (PBS). 1-Octanol and PBS solvent were saturated with each other overnight prior to use as solvents for standard curves. The compounds, in solution with methanol, were added in the same concentration to four acid-washed test tubes. Nitrogen was then blown into the tubes to evaporate the alcohol and to leave a residue of compound coating the interior surface of the tube. 1-Octanol and PBS (pH 7.1) were then added, each in 5-mL quantities, to generate chelator concentrations that could not theoretically exceed previously determined solubility limits in either solvent. The four tubes were then sealed under nitrogen with Teflon caps and agitated for 3 h at 27 °C. Aliquots from each layer were measured for the different compounds by recording absorbances at 247 nm for each compound. These absorbance values were compared to standard Beer's plots obtained for each compound at 247 nm at concentration ranges of 1.25–10 μM for **5** and **7** and 3.25–50 μM for **2** and **3**.

Cell Culture. American Type Culture Collection, Rockville, MD, provided murine L1210 leukemia, P388 murine lymphoid neoplasm, and CHO cell lines. L1210 and P388 cells were passed and tested in RPMI 1640, with L-glutamine and 10% defined equine serum, 2% Mopes–Hepes buffer (pH 7.40), 50 units/mL penicillin, and 50 μg/mL streptomycin. Chinese hamster V79 lung fibroblast cells (GM 0215, Human Genetic Mutant Cell Repository, Camden, NJ) were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air, in a 1:1 mixture of Dulbecco's modified Eagle's medium plus Hams F12 medium (DME/F12) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and antibiotics. Cells were subcultured according to standard techniques. Cells were periodically tested for *Mycoplasma* contamination and found to be negative.

Chelator concentrations that inhibit 50% of the growth of control flasks were calculated. Cells were tested in logarithmic growth (0.5×10^5–2×10^6 cells/mL). Growth inhibition assays were run in duplicate as previously described.[15] Compounds were added to culture medium as 10-μL aliquots of sterile DMSO stock solutions, except for methyl 2-(2-hydroxyphenyl)thiazoline-4-carboxylate, which was delivered as above in an ethanol stock solution, and hydroxyurea, which was delivered as an aqueous solution. Control flasks were spiked with 10 μL of the corresponding chelator-free solvent. Cells were counted 24 and 48 h after chelators were added by electronic particle counting (Coulter Counter, Model ZF, Hialea, FL). Cell survival at 24 and 48 h was determined by the trypan blue exclusion technique. Soft agar clonogenic assays were conducted as previously described.[15]

Iron reversal experiments were carried out at chelator concentrations of at least twice the IC_{50} for each compound by using 10 μL of a sterile aqueous stock solution of $FeCl_3$ added in a 1:1 molar ratio to 10-μL aliquots of chelator stock solution. Upon the development of an intense purple color, 20 μL of the iron chelate solution was added to cell-containing media and compared for cell proliferation at 24 and 48 h against flasks containing chelator alone, ferric chloride alone, or 10 μL of DMSO alone.

Cell Cycle Analysis. Cultured L1210 cells were exposed for 24 h to various chelators at concentrations of approximately twice the IC_{50} value. Cells were then centrifuged at 8000 rpm for 2 min, the medium was decanted, and the cells were resuspended in a propidium iodide and RNase solution to a final cell density of approximately 5×10^5 cells/mL.[21] DNA content was measured by fluorescent multichannel analysis with a FACS II flow cytometer FCM (Becton Dickinson FACS Systems, Sunnydale, CA). Previously reported data analysis programs were used to display data.[22] Gossett T tests were used to carry out channel by channel overlay and statistical evaluation at a significance threshold of $p < 0.05$.[22]

DNA Synthesis. DNA synthetic activity was estimated by measuring the incorporation of [^3H]TdR into acid-insoluble DNA

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macromolecules. Attached V79 cells were cultured in 60-mm plastic culture dishes, to a density of about 1×10^5 cells/cm^2 at which time the cells were undergoing a logarithmic rate of growth. Growth medium was then replaced with fresh medium containing [^{3}H]TdR ([*methyl*,1′,2′-^{3}H]thymidine: 95 Ci/mmol, 2 μCi/mL, New England Nuclear, Boston, MA), and incubation at 37 °C was resumed for 10 min. DNA synthesis inhibitors were then added from freshly prepared stock solutions: hydroxyurea was dissolved in balanced salt solution, and NBT or its iron chelate was dissolved and diluted in dimethyl sulfoxide (DMSO). Control samples included untreated cells and cells treated with appropriate concentrations of the vehicle alone.

After incubation in the presence of chelators for 0–120 min, the dishes were removed from the incubator and placed on ice, and ice-cold 70% percholoric acid (PCA) was added to a final concentration of 0.5 N in the previously incubated balanced salt solution. After 60 min, the precipitated cellular macromolecules were transferred to a Gelman type A/E glass fiber filter, which was washed twice with 5 mL of cold PCA. The radioactivity on the filters was then counted and converted to ^{3}H dpm using a Beckman LS-9800 liquid scintillation spectrometer.

Alkaline Elution of DNA. DNA damage was measured by the alkaline elution technique,[23] as modified by Moss et al. and described elsewhere.[18,24,25] Briefly, control V79 lung fibroblast cells labeled with [^{3}H]TdR and experimental cells labeled with [^{14}C]TdR (New England Nuclear, Boston, MA) each were added to a lysing solution in a filter assembly containing cellulose triacetate filter membranes. The two DNA populations were then coeluted with tetrapropylammonium hydroxide. The filter eluate was collected with 1-mL fractions, and the radioactivity per fraction was measured and expressed as ^{3}H and ^{14}C dpm. Under these conditions, about 85% of the DNA from untreated cells eluted from the filters; thus, there was no need to use irradiated cells as a source of reference DNA for coelution.

DNA damage was measured as the mass of DNA from treated cells (proportional to the ^{14}C dpm) eluting prior to the DNA from untreated control cells (proportional to the ^{3}H dpm). The damage is expressed as the "elution parameter", which is directly proportional to the number of DNA lesions expressed as strand breaks in alkali, i.e., DNA strand scissions plus alkali-labile sites.[24]

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Electrophilic Derivatives of Purines as Irreversible Inhibitors of A_1 Adenosine Receptors

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Functionalized congeners derived from 1,3-dipropyl-8-phenylxanthine and from N^6-phenyladenosine were derivatized to contain electrophilic groups (isothiocyanate, *N*-hydroxysuccinimide ester, maleimide, sulfonyl chloride, or α-haloacyl group) capable of reaction with nucleophiles on biopolymers. The goal was to inhibit chemically the A_1 adenosine receptor by using reactive agonist and antagonist ligands. Some of the electrophilic derivatives were synthesized through acylation of amine-functionalized congeners using hetero- or homobifunctional reagents available for protein cross-linking. The affinity for A_1 adenosine receptors was evaluated in competitive binding assays by using rat and bovine brain membranes. Several xanthine and adenosine thiourea derivatives prepared from 1,3- and 1,4-phenylene diisothiocyanate (DITC) were potent irreversible inhibitors of adenosine receptors. Derivatives of *m*-DITC, at concentrations between 10 and 500 nM, irreversibly eliminated binding at 90% of the A_1-receptor sites. Receptor affinity of both xanthine and adenosine derivatives containing distal phenylthiourea substituents was diminished by electron-donating groups on the ring.

Adenosine modulates a variety of physiological functions.[1] It acts as an inhibitor of neuronal firing and the release of neurotransmitters,[1b] an inhibitor of platelet aggregation,[1c] a cardiac depressant and a vasodilator,[1d] a vasoconstrictor[1e] (e.g., in the renal afferent arterioles and in the skin), and an immunosuppressant.[1f] Most of the physiological effects of adenosine result from binding to discrete membrane-bound adenosine receptors of the A_1 or A_2 subtypes.[1g] The xanthine drugs caffeine and theophylline, and many synthetic analogues,[2] act as competitive antagonists at adenosine receptors.

Alkylating or acylating ligands that form a stable covalent bond with a receptor have been synthesized for a number of receptors, including opiate,[3,4] phencyclidine,[5]

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