Cancer Chemother Pharmacol (1988) 21: 233–236

Cancer Chemotherapy and Pharmacology

In vitro antiproliferative activity of 2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid and its methyl ester on L1210 and P388 murine neoplasms

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Summary. The activity of three iron chelators, methyl [2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylate] (MTL); 2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid (TFAL); and 2-hydroxyphenyl-imido-ethyl-ether (Imidate), regarding antiproliferative, cytocidal, and cell-cycle effects are reported and compared with hydroxyurea (HU). In vitro, against L1210 and P388 murine neoplasms, MTL and TFAL displayed substantially greater antiproliferative activity than HU, although Imidate displayed no appreciable activity. MTL also induced a statistically more complete G_1/S cell-boundary block than did HU at equimolar concentrations (100 μM). The IC_{50} values produced by MTL and TFAL were low enough (≤ 20 μM) to warrant further testing of these chelators as potential antineoplastic agents.

Introduction

It is well recognized that neoplastic disease in humans [3, 11] is associated with iron overload. A 30% incidence of the normally rare, primary hepatic carcinoma is associated with ideopathic hemochromatosis [8] and siderosis secondary to high dietary iron [2, 14]. Iron dextran-induced sarcoma at injection sites has also been well documented [21]. The "sham anemia" associated with malignancy has been hypothesized by some to represent an iron-withholding defense by which the host attempts to attenuate neoplastic proliferation [22, 23]. It is uncertain whether malignant tissues have a higher iron requirement than nonmalignant precursors. Safrol-induced mouse neoplasm and plasmacytoma do not appear to concentrate iron when adjacent tissues are infiltrated with iron dextran [10, 16]. On the other hand, white cells harvested from juvenile ALL patients do have higher iron contents (5.5 ± 0.2 $\mu g/10^{10}$ cells) than nonmalignant juvenile lymphocytes (2.5 ± 0.2 $\mu/10^{10}$ cells) [6]. In comparison with resting lymphocytes, transformed white cells have 1,000 times as many transferrin surface receptors [18]. One might conclude that, although some neoplasms have a higher basal iron requirement than nontransformed precursors, it is unlikely that malignancies concentrate iron in a repository fashion.

A number of compounds with the ability to bind iron have been shown to exert antineoplastic activity in vitro. Hydroxyurea [17, 19], deferoxamine [8], guanazole [4], 1-formylisoquinoline thiosemicarbazone [15], parabactin [1, 7], and compound II [1, 7], among others, have been shown to inhibit replication of tumor cells in vitro by interference with ribonucleotide reductase and hence DNA synthesis [13]. In search for novel iron chelators that might enjoy ease of synthesis, low host toxicity, and oral bioavailability, our laboratory turned its attention to the in vitro cytotoxicity testing of 2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid and its methyl ester analog.

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Materials and methods

Chemicals. All reagents were purchased from Aldrich Chemical Company (Milwaukee, Wis, USA) and used without further purification. Tissue culture media and penicillin-streptomycin antibiotic solution were obtained from Gibco Laboratories (Grand Island, NY, USA). Equine serum was purchased from Hyclone Laboratories (Logan, Utah, USA). Mopes-Hepes buffer ingredients and hydroxyurea were obtained from Sigma Chemical Company (St. Louis, Mo, USA).

Synthesis

2'-(2-Hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid was prepared by the condensation of 2-(hydroxyphenyl)-imido-ethyl ether [12] (2.0 g, 1.2×10^{-2} *M*) with *L*-cysteine · HCl (1.7 g, 1.1×10^{-2} *M*) in refluxing dry methanol for 22 h. The crude reaction mixture, after concentration in vacuo, was purified by eluting from Sephadex LH-20 in 15% methanol/benzene to afford 2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid (1.54 g, 63% yield) as a crystalline, yellow solid. Purity was confirmed by thin-layer silica gel chromatography.

[^{1}H] NMR ($CDCl_3$) 3.45–3.90 (m, 2H) 5.30 (t, 1H) 6.55–7.0 (m, 2H) 7.0–7.5 (m, 3H), IR (neat) 3000(m), 1750(s), 1610(s), 1510(m), 1310(m), 1240(s), 1070(m), 950(m), 750(m)

Anal. calculated for C_{10} H_9 N_1 O_3 S_1: C, 53.07; H_1 5.35; N_1 6.19; S_1 14.16. Found: C, 53.05; H, 5.33; N, 6.17; S, 14.18

Methyl-[2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylate] was made by the condensation of 2-(hydroxyphenyl)-imido-ethylether (0.7 g, 4.24×10^{-3} *M*) with L-cysteine · HCl (0.73 g, $4.24 \times 10^{-3} M$) in refluxing dry methanol for 18 h. The crude reaction mixture was concentrated in vacuo and chromatographed on silica gel 60A in 3% ac-

etone/benzene to afford the final product (0.83 g, 85% yield) as a clear yellow oil. Purity was confirmed by thin-layer silica gel chromatography.

[^{1}H] NMR ($CDCl_3$ 3.35 (d, 1H), 3.65 (d, 1H), 3.75 (s, 3H), 5.30 (t, 1H), 6.90 (m, 2H) 7.35 (m, 2H) 12.30 (s, 1H)
IR (neat) 3000(m), 2950(m), 1740(s), 1620(s), 1580(s), 1490(m), 1220(s), 760(s)
Anal. calculated for C_{11} H_{11} O_3 S_1: C, 55.68; H, 4.67; N, 5.90; S, 13.51. Found: C, 55.57; H, 4.72; N, 5.88; S, 13.44

Cell culture. Murine L1210 leukemia and P388 murine lymphoid neoplastic cells were obtained from American Type Culture Collection (Rockville, Md, USA). Cells were maintained and tested in RPMI 1640 with L-glutamine containing 10% defined equine serum, 2% Mopes-Hepes buffer (pH 7.40), 50 IU/ml penicillin, and 50 μg/ml streptomycin. The concentration of chelators inhibiting 50% of the growth of untreated cells (IC_{50}) was determined on cells in logarithmic growth (1×10^5 cells/ml to 2×10^6 cells/ml). Drug concentrations in the range of 5 to 200 μ*M* for first runs and 50–5 μ*M* for refined runs were used. Drugs were delivered as stock solutions in 10 μl of sterile DMSO for free acid thiazoline ligand (TFAL); 100% ethanol for methyl ester thiazoline ligand (MTL) and imidate; and H_2O for hydroxyurea. Control flasks were spiked with 10 μl corresponding drug-free solvent. All IC_{50} determinations were run in duplicate. Cells were quantitated at 24 and 48 h after drug administration using electronic particle counting (Coulter Counter Model ZF, Hialea, Fla, USA). Cell viability was measured by trypan blue exclusion and soft agar clonogenic assays. Soft agar clonogenic techniques represent a more reliable method of assessing the ability of various chelators to affect cell viability than does trypan blue exclusion. L1210 cells were incubated at 5×10^4 cell/ml in 25-cm^2 culture flasks in the presence of MTL or TFAL at 25, 50, 100, and 200 μM concentrations for 24 h, quantitated by Coulter counting and serially diluted in RPMI media as fortified above to a theoretical cell concentration of 30 cells/100 μl. Double-strength, serum-fortified RPMI-1640 Mopes-Hepes buffered media was mixed with an equal volume of sterile 0.9% Bacto agar aqueous solution and pipetted at 42° C in 2-ml aliquots into six-well multiwell plates. Thirty cells were delivered in 100 μl culture media to each well containing 2 ml soft agar media, and were swirled and allowed to cool and solidify. Wells were incubated at 37° C, 5% CO_2, 95% relative humidity for 10 days, at which time colonies were visually counted. Each drug concentration was run in duplicate flasks, and 30 cells from each flask were spiked into triplicate wells. All experiments were run in duplicate and the results combined. Significant differences in cell viability were assessed using a two-tailed *t*-test for independent samples at a significant threshold of $P < 0.05$. Iron reversal experiments were conducted by mixing 100 μl ethanolic MTL and aqueous $FeCl_3$ stock solutions in a 1:1 molar equivalent ratio to produce a deeply purple-colored stock solution. This Fe-MTL stock was next spiked in 20-μl volumes to media-containing culture flasks, generating 50 μ*M* Fe-MTL chelate concentrations. L1210 cell growth at 24 and 48 h was compared with cells in media containing either 50 μ*M* $FeCl_3$ or 50 *M* MTL.

Flow cytometric analysis. Cultured L1210 cells were treated with methyl-[2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylate] or hydroxyurea at varying concentrations for 24 h. At this time cells were diluted to a concentration of 5×10^5 cells/ml and treated with propidium iodide and RNAase [20]. Fluorescence was next analyzed with a FACS II flow cytometer (Becton Dickinson FACS systems, Sunnyvale, Calif). Phase-fraction populations were determined by previously reported data analysis programs [5]. Channel-by-channel overlay and statistical evaluation by Gossett *t*-test were carried out, with a significance threshold set at $P < 0.05$ as previously described [5].

Results

Cytostatic and cytocidal analysis

Methyl-[2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylate], or methyl thiazoline ligand (MTL), 2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid, or thiazoline free acid ligand (TFAL), 2-(hydroxyphenyl)-imido-ethyl ether, or imidate, and hydroxyurea were all tested on L1210 and/or P388 cells in vitro, generating growth curves (Fig. 1) and 48 h IC_{50} values (Table 1). As expected, the most lipophilic compound displayed the most potent antiproliferative activity. This observation is consistent with those previously reported by this author and colleagues regarding catecholamide iron chelators [1]. Both MTL and TFAL were considerably more active at preventing cell division than hydroxyurea, which is currently the only iron-chelating ribonucleotide reductase inhibitor approved for clinical use in the United States. The antiproliferative activity of MTL was reversed when a molar equivalent of $FeCl_3$ was added to ligand stock solutions prior to media inoculation. Compared to control cells grown in the presence of 50 μ*M* $FeCl_3$, MTL (50 μ*M*) suppressed cell growth by 88% ±1.6%, whereas MTL (50 μ*M*) plus $FeCl_3$ (50 μ*M*) suppressed cell growth by only 6% ±6%. Interestingly, imidate, which was used as a reagent in the synthesis of the two thiazoline ligands, also binds iron but was found to be devoid of significant antiproliferative activity.

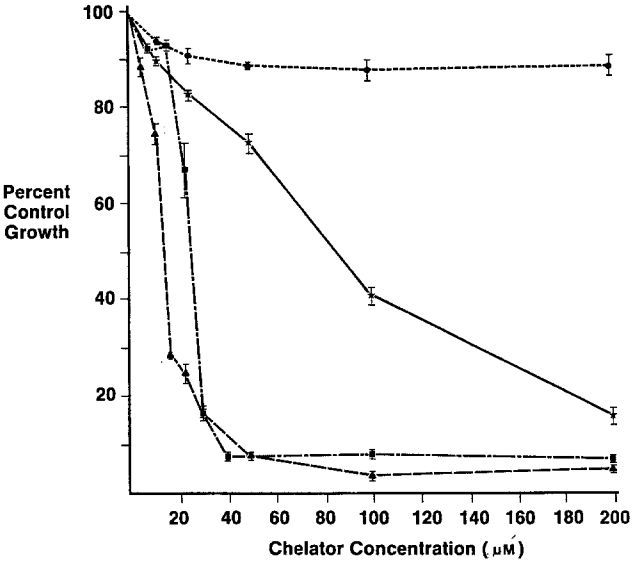

Fig. 1. Percentage of relative growth compared with control values of L1210 cells in RPMI 1640 media plus 10% equine serum, Mops-Hepes buffer, in the presence of (●) Imidate, (*) hydroxyurea, (■) TFAL, or (▲) MTL. Average values and experimental ranges are indicated

Table 1. Cytostatic and cytocidal activity of various iron chelators in L1210 and P388 murine neoplasms grown in RPMI 1640 media plus 10% defined equine serum

Iron chelator	Antiproliferative activity		Cell viability[a]	
	IC_{50}	48 h	48 h	
	L1210	P388	L1210	P388
Methyl-[2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylate] "Methyl thiazoline ligand"	14 *M*	18 *M*	87%	85%
2'-(2-Hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid "Thiazoline free acid ligand"	25 μ*M*	19 μ*M*	92%	75%[b]
2-Hydroxyphenyl-imido-ethyl ether "Imidiate"	>200 μ*M*	ND[c]	>99%	ND[c]
Hydroxyurea	85 μ*M*	ND[c]	97%	ND[c]

[a] At 100 μ*M* chelator concentrations, determined by trypan blue exclusion
[b] At 60 μ*M* chelator concentration, determined by trypan blue exclusion
[c] ND, Not determined

Table 2. Flow cytometric analysis of L1210 cells

Cell cycle phase	Control	Antiproliferative compound: Methyl thiazoline ligand		
		100 *M*	300 *M*	500 *M*
G_0/G_1	42.9±1.7[a]	46.0±2.8[a,b]	62.6±8.4[a]	61.3±7.4[a]
S	38.8±2.5	36.5±3.3[b]	31.5±9.1	34.3±7.9
G_2/M	18.3±1.4[a]	17.5±2.0	5.9±0.8[a]	4.4±0.9[a]
	Control	Hydroxyurea		
		100 *M*	500 *M*	
G_0/G_1	42.9±1.7[a]	31.9±1.8[a,b]	59.8±7.0[a]	
S	38.8±2.5[a]	48.4±3.7[a,b]	53.0±0.6	
G_2/M	18.3±1.4[a]	19.7±2.6	0.8±0.4[a]	

[a] $P < 0.005$ for the Gossett *t*-test comparing control cell-cycle population distribution with distribution in the presence of ligand
[b] $P < 0.001$ for the Gossett *t*-test comparing cell-cycle population distribution for 100 μ*M* methyl thiazoline ligand vs 100 μ*M* hydroxyurea

Cytocidal activity of the above compounds are defined in Table 1 by the trypan blue exclusion method as percentage of viable cells remaining 48 h after drug administration. None of the compounds tested caused a great deal of trypan blue-documented cell death; however MTL appears to be the most cytocidal agent on L1210 cells, whereas TFAL was most cytocidal on P388 cells.

Soft agar clonogenic analysis indicated that at MTL or TFAL concentrations of 50 μ*M* or greater there was a statistically significant reduction in L1210 cell viability (Table 3). Both chelators induce a 58% reduction in cell clonogenicity at 200 μ*M*, although MTL appears to reduce viability to a statistically greater extent at 50 μ*M* than does TFAL. Neither compound affected cell clonogenicity at 25 μ*M* concentrations. At 100 μ*M* MTL and TFAL chelator concentrations, soft agar analysis revealed lower L1210 cell viability (about 50% viable) than predicted by trypan blue exclusion methods (about 90% viable). This finding is consistent with the enhanced sensitivity of soft agar clonogenic methods for analyzing cell viability.

Table 3. L1210 cell viability as determined by soft agar clonogenicity

Chelator	Concentration (μM)	Percent control viability ± SE
Methyl thiazoline ligand (MTL)	50	36±5[a,c]
	100	46±8[a]
	200	42±3[a]
Thiazoline free acid ligand (TFAL)	50	81±9[a,b,c]
	100	54±8[a,b,c]
	200	42±7[a,b]

SE, Standard error
[a] Control L1210 cell viabilities differed statistically ($P < 0.05$) from viabilities at 50, 100, and 200 μ*M* chelator concentrations for both compounds
[b] Cell viabilities in the presence of 50 μ*M* vs 100 or 200 μ*M* TFAL differed significantly from one another ($P < 0.001$)
[c] Cell viability in the presence of 50 μ*M* MTL vs 50 μ*M* TFAL differed significantly ($P < 0.0005$)

Flow cytometric analysis

Methyl thiazoline ligand induced statistically significant changes in L1210 cell-cycle distribution (G_1/S boundary block), possibly indicative of interference with DNA synthesis (Table 2). A significant increase in the fraction of cells populating G_0/G_1 was seen after 24-h incubation of cells in 100 μ*M* MTL. A significant drop in cells populating G_2/M was observed when MTL concentration was increased to 300 μ*M*, and this change was associated with a further increase in cells populating G_0/G_1 phase. At a 100 μ*M* concentration, HU did not induce a G_1/S boundary block in the fashion of MTL but appeared to produce an S-phase block associated with a reduction in cells populating G_0/G_1; however, at a 500 μ*M* concentration, HU appeared to significantly increase cell distribution in G_0/G_1 in addition to S phase, while profoundly reducing cell population in G_2/M.

Discussion

Methyl-[2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylate and 2'-(2-hydroxyphenyl)-2'-thiazoline-4'-carboxylic acid define a new chemical class of ferric iron-binding antiproliferative compounds. The IC_{50} values generated in vitro indicate the compounds to be potent enough to meet the informal NCI criteria warranting further in vitro and in vivo study[1]. Both compounds appear more potent than hydroxyurea in antiproliferative and cytocidal activity in vitro. Antiproliferative effects of MTL could be reversed by delivering the ligand to cell suspensions as the ferric iron chelate, supporting the idea that the drugs antiproliferative activity is associated with iron chelation either in the inter-

[1] personal communication: Betty Abbott, Biological Screening Division, National Cancer Institute. Potentially active compounds should have an IC_{50} of roughly 20 μ*M* or lower on L1210 and P388 in tissue culture.

nal or external environment of the tumor cell. Presently we are synthesizing a number of derivatives of these compounds in hopes of enhancing iron-formation constants and antiproliferative activity. We have also begun studies aimed at documentation of the mechanism by which cell division is blocked, specifically, interference with DNA synthesis via ribonucleotide reductase inhibition.

Should these compounds prove to have low, acute host toxicity yet effective in vivo antineoplastic efficacy, there are a number of advantages they might possess over other antiproliferative iron chelators. The simplicity of their synthesis should lower production costs. Methyl thiazoline ligand has the potential of good oral bioavailability due to its lipophilic nature, provided that the thiazoline ring shows adequate acid stability in the stomach.

Acknowledgements. This work was supported in part through grants provided by the Arkansas Cancer Research Center, University of Arkansas for Medical Sciences Foundation, and the University of Arkansas for Medical Sciences College of Pharmacy.

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Received November 13, 1986/Accepted October 12, 1987