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DEVELOPMENT OF INHIBITORS OF ALKALINE PHOSPHATASE—AN ENZYME INVOLVED IN RESISTANCE OF SARCOMA 180/TG TO 6-THIOPURINES*

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Abstract—A number of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones with metal chelating activity were tested as inhibitors of alkaline phosphatase purified about 20 times from a murine ascitic cell line of Sarcoma 180 resistant to the antileukemic agents, 6-mercaptopurine and 6-thioguanine. Derivatives of this class of compounds having a morpholino group in the side chain were found to be the most potent and exhibited considerably greater inhibition of alkaline phosphatase than did some of the more conventional metal-binding agents. The inhibition caused by these agents was readily prevented and reversed by addition of equimolar concentrations of zinc and cobalt, and to a lesser extent by other transition metals. Spectral studies indicated that one of these agents, 5-hydroxy-l-formylisoquinoline 4′-diethyleneoxythiosemicarbazone (A-851), was capable of forming a complex with zinc, cobalt and other transition metals. Optimal interaction between A-851 and zinc occurred when the ratio of drug to zinc was 2:1. Structural modifications that interfered with the metal coordinating potential of these compounds decreased enzyme inhibitory activity. The results support the view that metal binding is involved in the inhibition of alkaline phosphatase by compounds of this class.

THE ANTINEOPLASTIC purinethiols, 6-mercaptopurine (6-MP) and 6-thioguanine (TG) are known to require conversion to their respective nucleotide derivatives by the enzyme guanine-hypoxanthine phosphoribosyltransferase (IMP-pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) for tumor inhibitory activity. The biochemical mechanism of acquired resistance to these thiopurines most prevalent in both transplanted animal tumors and microorganisms is that of loss or marked decrease in the activity of guanine-hypoxanthine phosphoribosyltransferase.[1,2] However, in human leukemic cells, loss of sensitivity to these antineoplastic agents was not attributable to a decrease in the activity of guanine-hypoxanthine phosphoribosyltransferase,[3] nor was there a consistent correlation between drug responsiveness and inhibition of purine nucleotide biosynthesis by these agents.[3,4]

To investigate potential mechanisms by which leukemic cells of man might achieve resistance to these antineoplastic agents, a subline of the murine ascitic cancer, Sarcoma 180, resistant to purinethiols (Sarcoma 180/TG) was developed as a model system. This subline is resistant not only to 6-MP and 6-TG, but also to their respective nucleosides.[5] Initial studies with this variant indicated that insensitivity to

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thiopurines was not the result of impaired uptake or increased catabolism of the base, nor of a decreased capacity to synthesize the inhibitory nucleotide form.[6] It was found, however, that the rate of loss of newly formed thiopurine nucleotide was greater in resistant cells than in the parent neoplasm,[6] and that this phenomenon correlated with the presence of eight times more particulate-bound alkaline phosphatase activity in the resistant variant,[7] suggesting an increased rate of catabolism of active inhibitory nucleotide forms. The findings infer that enhanced breakdown of the nucleotide form of the thiopurines by alkaline phosphatase is at least partially responsible for the insensitivity of the resistant subline to 6-MP and 6-TG. A similar mechanism of resistance to 6-methylmercaptopurine has been proposed by Henderson *et al.*[8] The development of a potent inhibitor of alkaline phosphatase would appear to result in a potentially useful therapeutic agent when employed in combination with either 6-MP or 6-TG in those neoplasms attaining insensitivity by an increase in alkaline phosphatase activity.

Alkaline phosphatases from both microbial and mammalian sources have been found to contain zinc,[9-13] and in several of these biocatalysts zinc was shown to be an active component.[12,14] One approach to the development of inhibitors of this enzyme with potential therapeutic utility is to employ metal-binding agents. For this purpose, a number of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones and related compounds, which were shown to be potent inhibitors of the growth of a variety of transplanted rodent neoplasms[15-21] and which possessed the potential to form coordination compounds with certain transition metals,[15,16,22,23] have been tested as potential inhibitors of alkaline phosphatase from Sarcoma 180/TG ascites cells which was purified about 20 times over cell sonic extracts. Structure–inhibitor relationships and initial studies on the possible mechanism of enzyme inhibition by these compounds are presented in this report.

MATERIALS AND METHODS

Materials. Tris, *p*-nitrophenylphosphate (PNPP), 1,10-phenanthroline, 8-hydroxyquinoline and EDTA were purchased from Sigma Chemical Co. All compounds tested as inhibitors of alkaline phosphatase were synthesized in this laboratory[24,25] and were chemically analyzed for purity. Other chemicals were of analytical grade.

Enzyme source and assay. Alkaline phosphatase from murine ascites cells of Sarcoma 180/TG was purified by butanol extraction of the particulate fraction obtained by sedimenting the sonicated cell suspension at 105,000 *g* for 1 hr. The method of preparing the 105,000 *g* particulate fraction from transplanted cells was described previously,[7] except that centrifugation at 12,000 *g* for 20 min was omitted. Butanol extraction was then carried out by adding one-half vol. of butanol to the particulate fraction in Erlenmeyer flasks and extraction was carried out at 37° for 15 min with shaking at a speed of 60 rev/min. The material was then centrifuged at 12,000 *g* for 10 min at 4°. The upper butanol layer was discarded and the aqueous layer was collected. The cell debris was again suspended in the original volume of 0·05 M Tris–HCl (pH 7·6), homogenized and the butanol extraction was repeated. The aqueous layers were combined. The enzyme thus prepared was approximately 20 times pure over sonicated cell suspensions. Enzyme was assayed at 25° according to the method of Simpson *et al.*[26] Activity was measured by the initial rate of hydrolysis of PNPP using the change in absorbancy at 410 nm with a Gilford thermostated

spectrophotometer. The reaction mixture contained 3·0 ml of 0·8 M Tris–HCl (pH 9·2) and 10^{-3} M substrate; the reaction was initiated by addition of an appropriate amount of enzyme. For assay in the presence of inhibitors, stock solutions of inhibitors at 10^{-3} M were dissolved in 20–50% dimethylsulfoxide, unless otherwise specified, and varying amounts of inhibitor were added to the reaction mixture. With the largest concentrations of drug, dimethylsulfoxide was found to be slightly inhibitory; however, in all cases, the concentration of dimethylsulfoxide was less than 5% in the assay mixture and an appropriate control containing dimethylsulfoxide was included. One unit of activity is expressed as hydrolysis of 1 nmole substrate/min under the conditions employed, assuming a molar absorbancy of *p*-nitrophenol in 0·8 M Tris–HCl (pH 9·2) of $1{\cdot}7 \times 10^4$/mole/l./cm. Specific activity refers to nanomoles Pi/minute/milligram of protein. Protein was measured by the method of Lowry *et al.*[27]

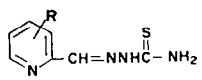
Determination of inorganic phosphate. Since several of the compounds tested formed complexes with metals which absorbed strongly in the region of 400 nm and therefore interfered with the spectrophotometric assay, activity was alternately determined by measuring the inorganic phosphate liberated according to the method of Dryer *et al.*[28] The incubation procedure was a modification of the method used by Paterson and Hori,[29] and the reaction mixture contained 100 mM carbonate–bicarbonate buffer (pH 9·2), 2 mM PNPP and 0·1 ml enzyme (0·28 mg protein) in a total volume of 1·0 ml. Incubations were carried out at 37° for 30 min. Reactions were terminated by the addition of 1·0 ml of 20% trichloroacetic acid. Precipitated protein was removed by centrifugation and 1·0-ml aliquots of the supernatant were assayed for inorganic phosphate. The appropriate blanks containing no enzyme or substrate were subtracted from the values obtained.

Absorption analyses. Absorption spectra were taken at room temperature using a Cary 15 spectrophotometer.

RESULTS AND DISCUSSION

Previous work in this laboratory has demonstrated that the tumor-inhibitory agents, 2-formylpyridine thiosemicarbazone (PT) and 1-formylisoquinoline thiosemicarbazone (IQ-I) and their related compounds, interfered with DNA synthesis by virtue of inhibition of ribonucleoside diphosphate reductase,[21,23,30] which is known to be an iron-containing enzyme. The mechanism of inhibition of ribonucleoside diphosphate reductase, as well as inhibition of tumor growth by these agents, has been postulated to be due to the chelation of the active metal component.[15,16,22,23] The hypothesis was that this class of compounds was acting as tridentate ligands (N*-N*-S*) with a predilection for forming coordination compounds with divalent transition metals.[15,16] Since alkaline phosphatase in general appears to be a zinc-containing enzyme, these agents were tested as possible inhibitors of alkaline phosphatase. Table 1 shows the approximate concentration of PT, the parent compound of this series, as well as the levels of related derivatives, required to produce 50 per cent inhibition of enzyme activity. PT required 0·28 mM to depress phosphatase activity by 50 per cent. Substitution of PT in the 3, 4, 5 or 6 positions with either a methyl function or, in some cases, with a hydroxyl group did not markedly alter the inhibitory potency of this agent, indicating that the enzyme had reasonable tolerance for substituents in the pyridine nucleus.

TABLE 1. CONCENTRATION OF 2-FORMYLPYRIDINE THIOSEMICARBAZONE (PT) AND RELATED DERIVATIVES REQUIRED FOR 50 PER CENT INHIBITION OF ALKALINE PHOSPHATASE ACTIVITY*

Compound	ID_{50} (mM)
	
R=	
H (PT)	0·28
3-OH (3-HP)	0·25
5-OH (5-HP)	0·25
3-CH_3	0·12
4-CH_3	0·53
5-CH_3	0·35
6-CH_3	0·71

* The enzyme (0·7 mg) was incubated at 25° for 15 min in 0·8 M Tris–HCl (pH 9·2) with various concentrations of inhibitors; substrate was then added to initiate the reaction. Activity of the enzyme in the absence of inhibitor, determined spectrophotometrically as described in Methods, was 64 nmoles Pi/min/mg.

The effects of removal of the sulfur moiety of the side chain, an atom essential for metal binding, are shown in Table 2. Replacement of sulfur (5-HP) by selenium (A-611) did not markedly alter inhibitory potency, but substitution of an =NH group (A-601) or an oxygen atom (A-591) resulted in inactive compounds. These later derivatives are much weaker chelating agents than are the thio- and seleno-semicarbazones.[22,23] The results, therefore, support the concept that the chelating potential of these agents for transition metals correlates with biological activity.

Substitution of the protons of the thioamide group markedly enhanced the inhibitory potency of these agents (Table 2). Replacement of both terminal protons by a diethyleneoxy group (e.g. A-191) resulted in compounds with considerably more inhibitory activity than did substitution of only one hydrogen atom (e.g. A-181), which in turn had equal or slightly more activity than an unsubstituted thiosemicarbazone (e.g. IQ-1). An increase in inhibitory potency was also observed when the pyridine ring of the thiosemicarbazone was replaced by an isoquinoline moiety [see PT (Table 1) and IQ-1 (Table 2)]. Furthermore, substitution of a hydroxyl group on either heterocyclic ring was without significant effect (see for example, PT vs 5-HP and A-851 vs A-191).

Substitution of a methyl group on the aldehyde carbon of the side chain (A-291) or on the 2′-nitrogen of the side chain (A-281) resulted in compounds that did not inhibit the activity of alkaline phosphatase at concentrations up to 10^{-2} M (Table 2). Also, removal of the nitrogen atom from the isoquinoline ring (A-871) resulted in an agent with no inhibitory activity. The ring nitrogen atom in the 2-position is essential for chelation, and removal of this nitrogen as well as other changes in the side chain which interfered with metal binding markedly reduced or eliminated inhibitory activity. The data in Table 2 also demonstrate that the diethyleneoxythiosemicarbazide portion of the molecule (A-182), thiosemicarbazide (TC) itself, and 5-hydroxy-1-methylisoquinoline (A-102) did not possess inhibitory activity at concentrations up

TABLE 2. EFFECT OF MODIFICATIONS IN THE HETEROCYCLIC RING AND SIDE CHAIN ON THE ACTIVITY OF ALKALINE PHOSPHATASE*

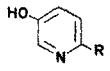
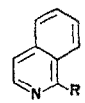
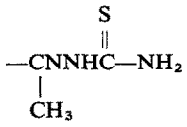
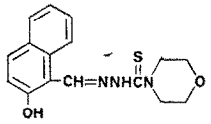
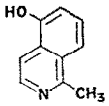
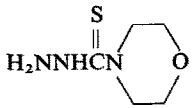
Code	Compound	ID_{50} (mM)
		
	R=	
5-HP	—CHNNHC(=S)—NH_2	0·25
A-611	—CHNNHC(=Se)—NH_2	0·39
A-601	—CHNNHC(=NH)—NH_2	Inactive
A-591	—CHNNHC(=O)—NH_2	Inactive
A-861	—CHNNHC(=S)—N(CH$_2$CH$_2$)$_2$O	0·013
		
	R=	
IQ-1†	—CHNNHC(=S)—NH_2	0·058
A-181†	—CHNNHC(=S)—$NHCH_2CH(CH_3)_2$	0·032
A-201	—CHNNHC(=S)—$NHCH_2CH_2OH$	0·05
A-191	—CHNNHC(=S)—N(CH$_2$CH$_2$)$_2$O	0·009
A-851	—CHNNHC(=S)—N(CH$_2$CH$_2$)$_2$O, 5-hydroxy	0·008
A-281	—CHNNC(=S)(CH_3)—NH_2	Inactive

TABLE 2.—*cont.*

Code	Compound	ID_{50} (mM)
A-291		Inactive
A-871		Inactive
A-102		Inactive
A-182		Inactive
TC		Inactive

* The conditions of assay were the same as those described in Table 1.

† Stock solutions of these compounds at 10^{-2} M were dissolved in 100% dimethylsulfoxide and their appropriate controls, containing the same amount of dimethylsulfoxide, were included.

to 10^{-2} M; however, when the two portions form the derivative, 5-hydroxy-1-formyl-isoquinoline 4′-diethyleneoxythiosemicarbazone (A-851), marked inhibition of enzyme activity resulted, suggesting that the sulfur and nitrogen atoms of both portions of the molecules are required to cause inhibition of the enzyme.

When alkaline phosphatase was assayed in the presence of 5×10^{-5} M A-851, about 90 per cent of the activity was lost. Simultaneous addition of a molar equivalent quantity of zinc, cobalt or manganese, and to a lesser extent nickel and iron, prevented inhibition by this agent (Table 3). Furthermore, zinc and cobalt were able to effectively reverse the enzyme inhibition by A-851 when the enzyme was preincubated with inhibitor for up to 20 min, supporting the concept that A-851 inhibited alkaline phosphatase by either binding to a zinc metallo-protein or by removing zinc from the enzyme. Magnesium neither prevented nor reversed inhibition of alkaline phosphatase activity by A-851.

The degree of inhibition of the enzyme was not influenced by changes in the concentration of PNPP over a 10-fold range from 10^{-3} M to 10^{-2} M in the assay mixture, indicating that A-851 did not bind to the enzyme at the site occupied by the substrate. The degree of inhibition in the presence of 5×10^{-5} M A-851 at various substrate concentrations within this range was approximately 90 per cent.

TABLE 3. EFFECT OF METAL IONS ON THE ACTIVITY OF ALKALINE PHOSPHATASE*

	No inhibitor†	With inhibitor	
		No preincubation‡	Preincubation§ (20 min, 25°)
E	1·00	1·00	1·00
E + Zn^{2+}	0·77		
E + Co^{2+}	1·06		
E + Ni^{2+}	0·98		
E + Mn^{2+}	1·16		
E + Fe^{3+}	1·00		
E + Mg^{2+}	1·07		
E + I		0·07	0·02
E + I + Zn^{2+}		0·71	0·68
E + I + Co^{2+}		0·86	0·66
E + I + Ni^{2+}		0·46	0·07
E + I + Mn^{2+}		0·74	0·23
E + I + Fe^{3+}		0·51	0·31
E + I + Mg^{2+}		0·07	0·03

* The activity of the enzyme obtained in the absence of inhibitors was 50 nmoles Pi/min/mg. This was equated to 1·00 and all data were expressed relative to this value. Abbreviations: E, enzyme; I, inhibitor.

† Enzyme (0·28 mg) was assayed in 1·0 ml of 0·1 M bicarbonate buffer (pH 9·2) in the presence and absence of $5·0 \times 10^{-5}$ M metal chloride by the method of Dryer *et al.*[28]

‡ Enzyme (0·28 mg) was added to 1·0 ml of 0·1 M bicarbonate buffer (pH 9·2) containing $5·0 \times 10^{-5}$ M A-851 and 2 mM PNPP. Immediately after mixing, molar equivalent amounts of metal chlorides were added and activities were measured as described in † above.

§ Enzyme (0·28 mg) was preincubated with $5·0 \times 10^{-5}$ M A-851 in 1·0 ml of 0·1 M bicarbonate buffer (pH 9·2) for 20 min at 25°. Immediately following the addition of substrate, molar equivalent quantities of metal chlorides were added and activities were determined as described in † above.

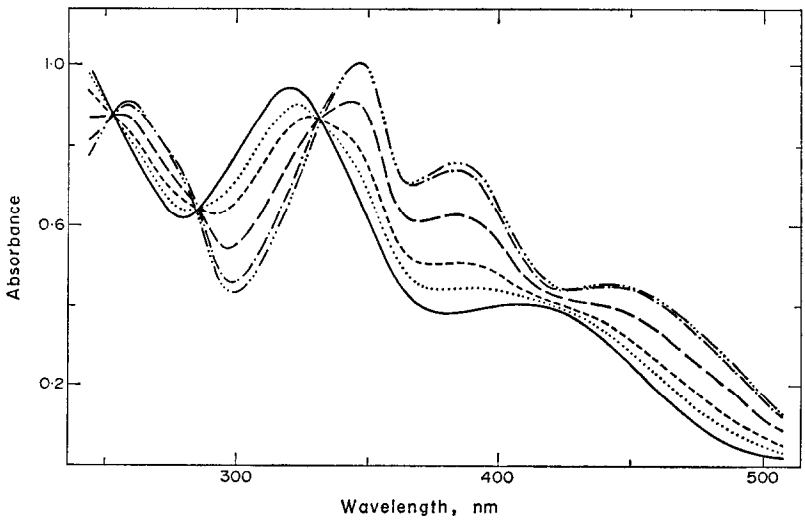


FIG. 1. Spectrum of compound A-851 and its zinc complexes. The concentration of A-851 was $5·33 \times 10^{-5}$ M in 0·05 M carbonate–bicarbonate buffer (pH 9·2). Zinc chloride was added in increments and the spectrum of the formed complexes was measured. ——, A-851; ·····, A-851 + $0·33 \times 10^{-5}$ M Zn; – – –, A-851 + $0·66 \times 10^{-5}$ M Zn; — —, A-851 + $1·33 \times 10^{-5}$ M Zn; — · —, A-851 + $1·99 \times 10^{-5}$ M Zn; ——·——, A-851 + $2·66 \times 10^{-5}$ M Zn.

Spectral evidence to demonstrate that the diethyleneoxy derivative, A-851, was capable of forming a coordination complex with zinc is shown in Fig. 1. In 0·05 M bicarbonate buffer (pH 9·2), A-851 ($5{\cdot}33 \times 10^{-5}$ M) exhibits maximal absorption at wavelengths of 320 and 410 nm with a negligible shoulder at 340 nm. Addition of zinc in increasing increments caused a gradual shift toward longer wavelengths and

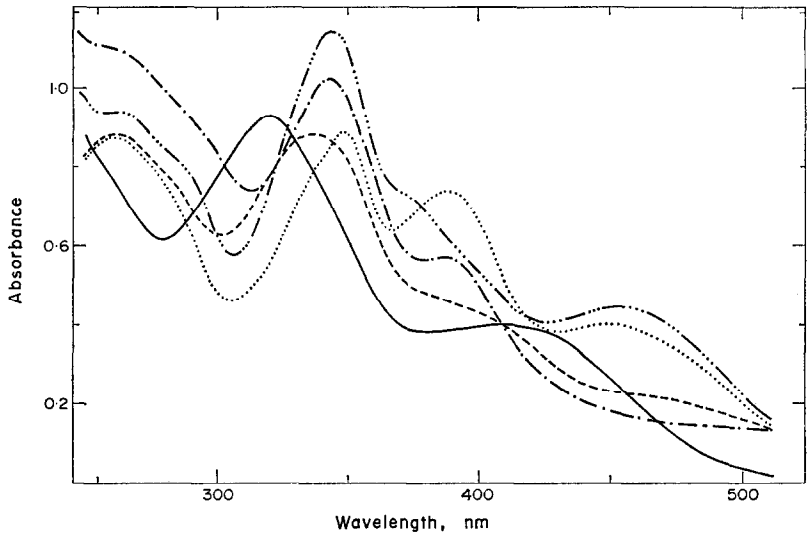


FIG. 2. Spectrum of compound A-851 and its metal complexes. The concentration of A-851 was $5{\cdot}33 \times 10^{-5}$ M in 0·05 M carbonate–bicarbonate buffer (pH 9·2). The ratio of each metal chloride to A-851 in the buffer was 1:2. ——, A-851; ·····, A-851 + Mn; – – –, A-851 + Cu; — · —, A-851 + Co; —···—, A-851 + Ni.

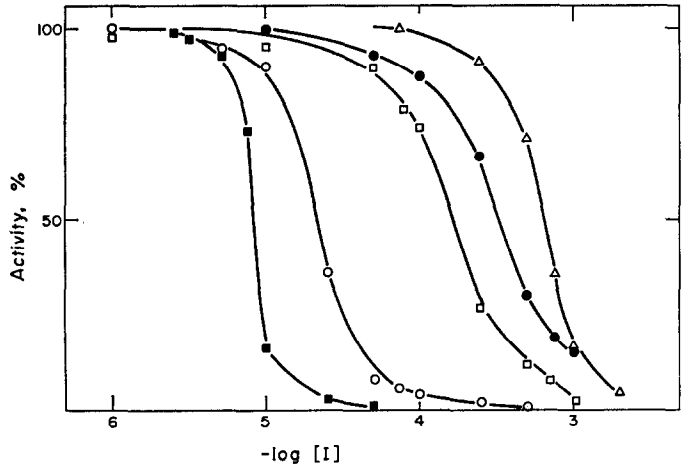


FIG. 3. Inhibition of alkaline phosphatase from Sarcoma 180/TG by EDTA (○——○), 1,10-phenanthroline (△——△), 8-hydroxyquinoline (□——□), PT (●——●) and A-851 (■——■). The enzyme was incubated in 0·8 M Tris–HCl (pH 9·2) with inhibitors for 15 min at 25° prior to addition of substrate to start the reaction. Activity was measured spectrophotometrically at 410 nm by the initial rate of hydrolysis of *p*-nitrophenylphosphate.

created a new absorption band, until the ratio of zinc to A-851 was 1:2. The extinction coefficients at 257, 346, 384 and 445 nm for the A-851–zinc complex were $1{\cdot}7 \times 10^4$, $1{\cdot}9 \times 10^4$, $1{\cdot}4 \times 10^4$ and $0{\cdot}9 \times 10^4$ mole/l./cm, respectively. Further addition of zinc did not cause any change in the spectrum of A-851, suggesting that optimal interaction of zinc and A-851 occurred at a ratio of 1:2. Similar data were generated to show that A-851 formed a complex with cobalt, nickel, copper and manganese; however, no spectral change was induced by the addition of magnesium (Fig. 2).

A comparison of the inhibitory potency of the morpholino derivative A-851 and PT, to the known metal-binding agents, EDTA, 8-hydroxyquinoline, and 1,10-phenanthroline, is shown in Fig. 3. The results demonstrate that A-851 was considerably more potent than PT, EDTA, 8-hydroxyquinoline or 1,10-phenanthroline as an inhibitor of alkaline phosphatase. The relatively great potency of A-851 suggests that this agent may be a useful biochemical tool to manipulate this enzymic activity.

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