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INHIBITORS OF ALKALINE PHOSPHATASE OF SARCOMA 180/TG*

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Abstract—The inhibitory potential of a variety of agents of different chemical classes has been examined using a particulate-bound alkaline phosphatase partially purified from the 6-thioguanine-resistant variant, Sarcoma 180/TG ascites cells, in an effort to discover inhibitory potential which might be useful in blocking this enzyme activity *in situ*. The most potent inhibitors tested were derivatives of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones, tetramisole, permanganate, and beryllium. Kinetic studies of the mode of inhibition of alkaline phosphatase by some of these inhibitors indicated that derivatives of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones and those of tetramisole and beryllium inhibited the enzyme by different mechanisms. The kinetics of inhibition by beryllium and derivatives of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones were mixed, while inhibition by a derivative of tetramisole was uncompetitive.

6-Mercaptopurine (6-MP) and 6-thioguanine (6-TG) are effective agents, primarily in combination with other drugs, in the therapy of acute leukemia of man. One of the factors which limits the continued use of these drugs in the treatment of these neoplasms is the acquisition of resistance. Although the biochemical mechanism(s) by which human leukemic cells achieve insensitivity to these agents has not yet been fully delineated, evidence has been attained, both in an experimental animal system [1] and in leukemic cells of man [2], to suggest that a particulate-bound alkaline phosphatase present in neoplastic cells is in some instances at least partially responsible for: (1) an increased rate of degradation of the nucleotides of the 6-thiopurines, which are the active tumor-inhibitory forms of these agents or their direct precursors; and (2) the consequent insensitivity of these neoplastic cells to these tumor-inhibitory thiopurines.

The availability of a potent inhibitor of alkaline phosphatase would appear to have potential as a therapeutic agent when employed in combination with either 6-MP or 6-TG in those neoplasms attaining insensitivity by an increase in particulate alkaline phosphatase activity. In this communication, the inhibitory properties of a variety of inhibitors of alkaline phosphatase have been examined using a particulate alkaline phosphatase partially purified from the 6-thiopurine-resistant variant, Sarcoma 180/TG ascites cells, in an attempt to ascertain the relative potential of a variety of inhibitor classes for further drug development.

MATERIALS AND METHODS

Tris; *p*-nitrophenylphosphate; 1,10-phenanthroline; 8-hydroxyquinoline; and EDTA were purchased from Sigma Chemical Co. Periodic acid was obtained from G. Frederick Smith Chemical Co. and potassium permanganate from Fisher Scientific Co. Derivatives of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones were synthesized in this laboratory [3, 4]. Analogs of tetramisole were kindly donated by Dr. H. Van Belle of Janssen Pharmaceutical Research Laboratories, Belgium, and Dr. R. K. Johnson of the Division of Cancer Treatment, National Cancer Institute (U.S.A.).

The butanol-extractable particulate alkaline phosphatase (enzyme B) of the murine ascitic neoplasm Sarcoma 180/TG was partially purified according to the procedure previously described [5], except that an additional DEAE-cellulose column chromatography step was inserted prior to gel filtration on Sephadex G-200. Enzyme activity was measured at 25° by determining the initial rate of hydrolysis of *p*-nitrophenylphosphate using the change in absorbance at 410 nm with a Gilford thermostated recording spectrophotometer. The reaction mixture contained 1·0 M Tris–HCl (pH 9·4), 10^{-3} M substrate, and 50 μl enzyme (0·246 mg/ml; 89 units of activity/ml) in a final volume of 3·0 ml, except when otherwise specified. The enzyme was incubated with an appropriate amount of inhibitor in the reaction buffer at room temperature for 15 min, and the reaction was then initiated by the addition of the substrate. For water-insoluble inhibitors, stock solutions (10^{-3} to 10^{-2} M) were dissolved in 50–100% dimethylsulfoxide, and varying amounts of inhibitor were added to the reaction buffer. An appropriate control containing dimethylsulfoxide was included in all determinations of inhibitory activity. One unit of activity is expressed as hydrolysis of 1 nmole substrate per min under the conditions employed, based on a molar absorbancy of *p*-nitrophenol in the buffer used of $1{\cdot}7 \times 10^4$/moles/l./cm. Specific activity refers to nmoles Pi formed/min/mg of protein. Protein was determined by the method of Lowry *et al.* [6].

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RESULTS AND DISCUSSION

The design of an effective inhibitor of alkaline phosphatase would appear to be a particularly difficult task, since the enzyme is relatively nonspecific and hydrolyzes phosphorylated substrates; possible inhibitory phosphorylated substrate derivatives would not appear to be useful chemotherapeutic agents, since they would not be expected to traverse cellular membranes readily. In search of useful leads to assist in the design and synthesis of potent inhibitors of this catalyst, a number of different classes of potentially inhibitory materials were tested against a highly purified particulate alkaline phosphatase deemed to be involved in the acquired resistance of Sarcoma 180/TG to 6-thiopurines; these included substituted derivatives of the previously reported active inhibitory class of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones [4, 7] and other classical metal coordinating agents, tetramisole and its analogs [8], amino acids and related agents, metals and anions. The concentrations of these various materials required to produce 50 per cent inhibition of alkaline phosphatase from Sarcoma 180/TG ascites cells under the conditions employed are shown in Tables 1 and 2.

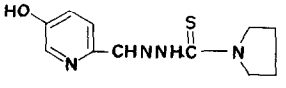
Evidence is available to show that alkaline phosphatase enzymes from a number of sources require Zn^{2+} as a cofactor [9, 10]; thus, metal-binding agents have known utility as phosphatase inhibitors. The classical chelating agents, 1,10-phenanthroline, EDTA, and 8-hydroxyquinoline, were all reported to be inhibitory to a 20-fold purified particulate-bound alkaline phosphatase from Sarcoma 180/TG [7]; however, since the fraction employed in these previous studies consisted of a mixture of two similar catalysts [5], it was deemed important to retest these agents against the enzyme which appeared to prefer 5′- nucleotides as substrates (i.e. alkaline phosphatase B) [5]. Slight differences existed in the inhibitory potencies of these agents for the highly purified enzyme (alkaline phosphatase B) and the less purified mixed enzymes from the butanol extraction described previously [7]. The differences between these findings were attributed to the presence of alkaline phosphatase A in the latter preparation. In both instances, however, EDTA was the most potent of these classical chelating agents.

Previous studies from this and other laboratories have demonstrated that: (1) α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones with a potential to form coordination compounds with certain transition metals exhibit tumor-inhibitory activity [11–13], and (2) inhibition of alkaline phosphatase by these agents is readily prevented and reversed by the addition of molar equivalent concentrations of zinc and cobalt [7]. The derivatives of α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazones employed (i.e. A171 and A1121, see Table 1), which were substituted in the side chain, were more potent inhibitors of alkaline phosphatase than conventional metal-binding agents. Compound A1121 appeared to be less active on this purified preparation of alkaline phosphatase B than reported earlier [4]. Introduction of a benzo group at the 3,4-positions of the pyridine ring in A1121 produced an isoquinoline derivative, A171, which was

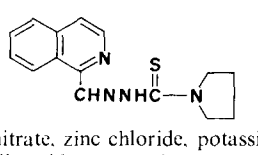
Table 1. 50 per cent inhibitory concentrations of some inhibitors of alkaline phosphatase of Sarcoma 180/TG

Inhibitor class	Compound	ID_{50} (M)
Classical chelating agents	1,10-Phenanthroline	7.5×10^{-4}
	EDTA	2.5×10^{-5}
	8-Hydroxyquinoline	4.3×10^{-5}
α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone derivatives	A1121*	1.4×10^{-5}
	A171†	5×10^{-7}
Amino acids and related agents	Imidazole	5×10^{-3}
	L-Histidine	1.7×10^{-3}
	Cysteine	1.4×10^{-4}
	L-Phenylalanine	$>1.0 \times 10^{-2}$
Metals	Be^{2+}‡	5×10^{-7}
	Zn^{2+}	1.0×10^{-5}
Anions§	IO_4^-	1.4×10^{-4}
	MnO_4^-	3.5×10^{-7}

* A1121: 5-Hydroxy-2-formylpyridine 4′-tetramethylenethiosemicarbazone.



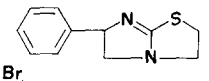
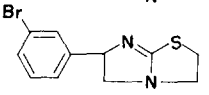
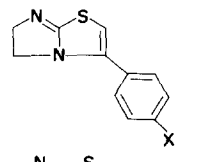
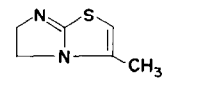
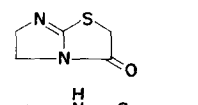
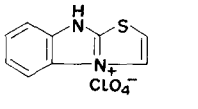
† A171: 1-Formylisoquinoline 4′-tetramethylenethiosemicarbazone.



‡ Beryllium nitrate, zinc chloride, potassium permanganate and periodic acid were used.

§ Assayed in 0·01 M sodium barbital buffer (pH 9·4). The other assay conditions were the same as described in the Methods section.

Table 2. Structure inhibitory activity relationships of derivatives of tetramisole

Formula		ID (M)
	DL-Form (tetramisole)*	5.8×10^{-5}
	L-Form (levamisole)†	1.3×10^{-5}
	R8231‡	2.0×10^{-4}
	X = H	$>1.0 \times 10^{-3}$
	Cl	7.8×10^{-4}
	OH	$>1.0 \times 10^{-3}$
	NO_2	$>1.0 \times 10^{-3}$
		1.0×10^{-3}
		1.3×10^{-3}
		1.3×10^{-3}

* Tetramisole: (±)-2,3,5,6-tetrahydro-6-phenylimidazo-(2,1-b)thiazole hydrochloride.

† Levamisole: *levo*-isomer of tetramisole.

‡ R8231: (±)-6(*m*-bromophenyl)-5,6-dihydroimidazo(2,1-b)thiazole oxalate.

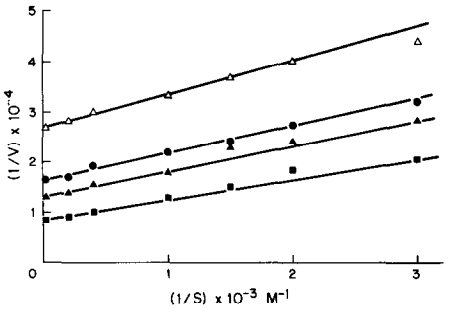


Fig. 1. Lineweaver–Burk plots of inhibition of alkaline phosphatase B of Sarcoma 180/TG ascites cells by compound R8231. The enzyme was incubated in 1·0 M Tris–HCl, pH 9·4, with the inhibitor at room temperature for 15 min prior to addition of various concentrations of the substrate, *p*-nitrophenylphosphate, as described in the Methods section. The initial velocity, as measured by the rate of hydrolysis of *p*-nitrophenylphosphate, was expressed as nmoles Pi formed/min. The inhibitor concentrations were: none, ■——■; 1×10^{-6} M, ▲——▲; 2×10^{-6} M, ●——●; 4×10^{-6} M, △——△.

3-fold more active as an inhibitor than A1121, requiring a concentration of only 5×10^{-6} M for 50 per cent inhibition of enzyme activity. Whether this increase in activity is due to a hydrophobic interaction between the benzenoid portion of the molecule and the enzyme is yet to be ascertained.

Amino acids (i.e. L-phenylalanine, L-histidine and cysteine), which have inhibitory potency toward alkaline phosphatase enzymes, in some instances demonstrating tissue specificity [14], were, in general, relatively weak inhibitors of the activity of the enzyme from Sarcoma 180/TG. Imidazole, which was reported by Brunel and Cathala [15] to be relatively potent against alkaline phosphatase of brain, kidney and bone, required 5×10^{-3} M for 50 per cent inhibition of the phosphatase of Sarcoma 180/TG.

Beryllium and relatively high concentrations of zinc have been shown to be inhibitory to alkaline phosphatase enzymes from a number of sources [16–19]. Beryllium proved to be one of the most potent inhibitors tested of the enzyme from Sarcoma 180/TG, producing 50 per cent inhibition at a concentration of 5×10^{-7} M; $1{\cdot}0 \times 10^{-3}$ M zinc was required to produce a similar degree of depression of enzymatic activity.

Ohlsson and Wilson [20] have reported that *Escherichia coli* alkaline phosphatase is strongly inhibited by periodate and permanganate. The mechanism of inhibition by these two anions was described [20]. Periodate was a reversible competitive inhibitor, whereas permanganate was an irreversible inhibitor. The enzyme from Sarcoma 180/TG was strongly inhibited by permanganate in barbital buffer (Table 1); however, periodate was less potent under the same conditions. Considerably less inhibition of alkaline phosphatase was produced by these anions in Tris buffer, as reported by Ohlsson and Wilson [20].

Certain derivatives of tetramisole have been reported by Van Belle [8] to be relatively potent stereospecific uncompetitive inhibitors of canine alkaline phosphatase. These derivatives are also strong inhibitors of alkaline phosphatase B of Sarcoma 180/TG (Table 2). Structure–inhibitory activity relationships were carried out with a number of analogous compounds; these investigations revealed that: (1) the L-form (levamisole) is probably the active material, since more than twice the quantity of the DL-form (tetramisole) was required to produce a similar degree of inhibition of enzymatic activity; (2) substitution of the phenyl ring of teteramisole in the *meta*-position by bromine (R8231) increased inhibitory potency by about 6-fold; and (3) movement of the phenyl group from the 6-position to the 3-position of the imidazo (2,1-b)thiazole ring system reduced inhibitory activity markedly. Other derivatives with substituents on the 3-position of the imidazo (2,1-b)thiazole ring were also relatively inactive. The mechanism of inhibition by this class is not due to chelation of the active metal component of alkaline phosphatase, since Van Belle [8] with the canine enzyme, and this investigation with the catalyst from Sarcoma 180/TG (data not shown), have found that the inhibition caused

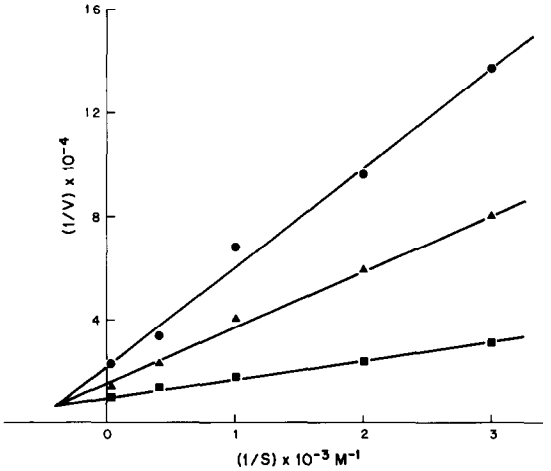


Fig. 2. Lineweaver–Burk plots of inhibition of alkaline phosphatase B of Sarcoma 180/TG ascites cells by compound A1121. The enzymatic assay and conditions were the same as described in Fig. 1, except that the inhibitor was A1121. The A1121 concentrations were: none, ■——■; $1{\cdot}5 \times 10^{-5}$ M, ▲——▲; $2{\cdot}5 \times 10^{-5}$ M, ●——●.

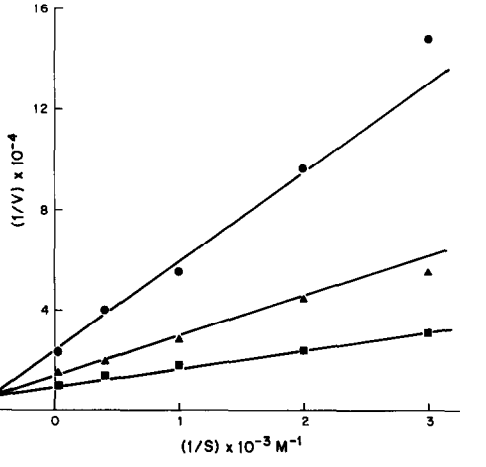


Fig. 3. Lineweaver–Burk plots of inhibition of alkaline phosphatase B of Sarcoma 180/TG ascites cells by $Be(NO_3)_2$. The assay conditions were the same as described in Fig. 1, except that the inhibitor was $Be(NO_3)_2$. The inhibitor concentrations were: none, ■——■; $2{\cdot}5 \times 10^{-7}$ M, ▲——▲; $1{\cdot}0 \times 10^{-6}$ M, ●——●.

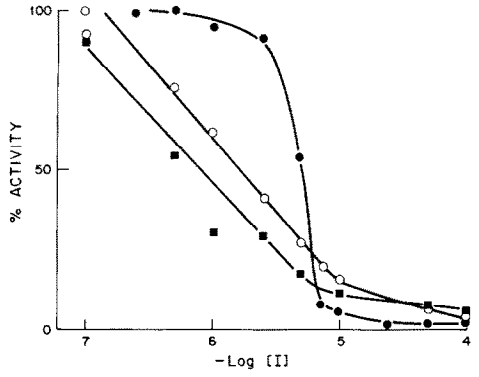


Fig. 4. Plots of inhibition of alkaline phosphatase B of Sarcoma 180/TG ascites cells versus log of effective inhibitor concentrations. The assay conditions were the same as described in the Methods section. Inhibition by A171, ●——●; R8231, ○——○; $Be(NO_3)_2$ ■——■.

by levamisole is not reversed by zinc or other transition metals.

The kinetic mechanism of inhibition of alkaline phosphatase of Sarcoma 180/TG by three of the most potent inhibitors, A1121, R8231 and Be^{2+}, was determined in double reciprocal plots shown in Figs. 1–3. The K_i values for A171, R8231 and $Be(NO_3)_2$, determined from the intercepts of plots of the reciprocal of the velocity versus inhibitor concentration, were $1{\cdot}5 \times 10^{-6}$, $1{\cdot}0 \times 10^{-6}$ and $5{\cdot}8 \times 10^{-7}$ M respectively. The K_m value for the enzyme in the absence of inhibitor in 1·0 M Tris–HCl (pH 9·4) with *p*-nitrophenylphosphate as the substrate was $8{\cdot}3 \times 10^{-4}$ M. The inhibition kinetics for A171 (data not shown), A1121 and Be^{2+} were of a mixed type, indicating a degree of competitive interaction between inhibitor and substrate [21], whereas inhibition by R8231 was uncompetitive, implying interaction between inhibitor and the enzyme–substrate complex [21]. A plot of the observed inhibition of this enzyme as a function of the log of inhibitor concentration is shown in Fig. 4. Under the conditions employed, the chelating agent A171 produced a sigmoidal shaped inhibition curve whereas differences existed in the slope of the inhibition curve produced by R8231 and Be^{2+}.

The results encourage the further modification of compounds in both the tetramisole and α-(*N*)-heterocyclic carboxaldehyde thiosemicarbazone series in an attempt to further enhance inhibitory activity towards alkaline phosphatase.

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