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HUMAN LEUCOCYTE ALKALINE PHOSPHATASE INHIBITION BY ALLERGIC MEDIATOR RELEASE INHIBITORS

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Abstract— Allergic mediator release inhibitors such as cromolyn sodium have been reported to act by blockade of antigen-induced uptake of extracellular calcium. They also promote the formation of a phosphoprotein during inhibition of mediator release. Since alkaline phosphatase is associated with calcium uptake mechanisms in certain tissues as well as with phosphoprotein phosphatase action, inhibition studies with a series of mediator release inhibitors were done. The mediator release inhibitors, cromolyn sodium, doxantrazole, AA-344, M & B-22948, ICI-74917, and W-13560, were shown to uncompetitively inhibit human leucocyte alkaline phosphatase. The most potent inhibitor, W13560, had a K_i of 7.9×10^{-6} M. Cromolyn sodium was the least effective inhibitor of the series while ICI-74917 and W-13560 were the most potent. Doxantrazole, AA-344, and M & B-22948 were intermediate in their inhibitory activities against alkaline phosphatase. Since uncompetitive inhibitors are known to inhibit, activate, or have no effect on an enzyme, depending upon conditions, the unpredictable clinical activity observed with this class of agent may be explained by their incompetitive nature of inhibition.

Antigen-induced release of mediators of the allergic reaction, such as histamine and slow reacting substance of anaphylaxis (SRS-A), is dependent upon calcium uptake into mast cells of the rat [1] and guinea pig [2]. Allergic mediator release inhibitors such as cromolyn sodium and doxantrazole [3] inhibit antigen-induced ^{45}Ca uptake at concentrations similar to those that inhibit histamine secretion [1]. Bufrolin (ICI-74917) also has been reported to block antigen-induced uptake of calcium [4].

No clearly defined mechanism has emerged to explain the activity of mediator release inhibitors. Inhibition of cyclic 3′,5′-nucleotide phosphodiesterase has been offered as a mode of action [5], but it is not consistent with all agents since nucleotide specificity varies widely among a number of release inhibitors [6]. Then, too, direct bronchodilator effects, generally observed with phosphodiesterase inhibitors such as methylxanthines, are not observed at drug levels which inhibit mediator release [7].

Recently, evidence was presented [8] showing that cromolyn sodium may inhibit histamine release from mast cells by regulating phosphorylation of a specific mast cell protein. A certain 78,000-dalton protein band is phosphorylated in the presence of cromolyn sodium. It was suggested that a possible association exists between phosphorylation of this protein band and cellular mechanisms for physiological termination and pharmacological block of the secretory mechanism.

Alternatively, an enzyme or enzyme complex directly involved with calcium uptake may be modulated by mediator release inhibitors. A close correlation between alkaline phosphatase, Ca^{2+}-ATPase, and calcium transport has been noted in several tissues including the intestinal brush border of chicks, [9, 10] and rats [11] and canine vascular smooth muscle [12]. The authors concluded that Ca^{2+}-ATPase and alkaline phosphatase may be the same enzyme or two enzymes closely associated in the calcium transport–enzyme complex. Hattori *et al.* [13] recently reported that certain immunoglobulin G fractions complexed with alkaline phosphatase with species and isoenzyme specificity of binding. Two known inhibitors of human leucocyte alkaline phosphatase, Zn^{2+} and levamisole [14, 15], block histamine release from sensitized rat mast cells [16, 17].

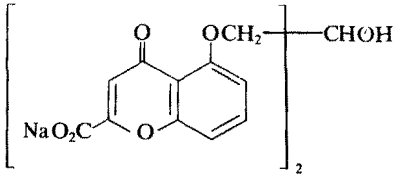
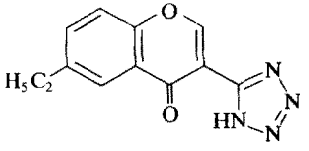
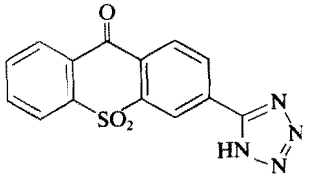
A series of structurally diverse mediator release inhibitors were therefore studied to determine their possible affects upon human leucocyte alkaline phosphatase (EC 3.1.3.1). This enzyme was selected as a suitable model since it is associated not only with calcium uptake, but also with the membrane fraction of neutrophils [15], a cell known to release allergic mediators [18].

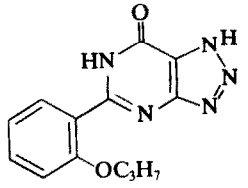
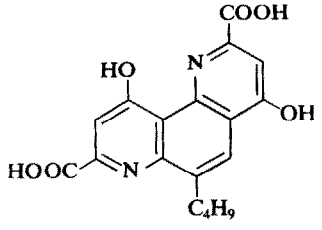
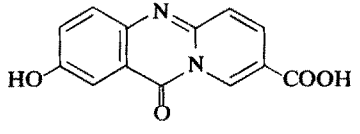
MATERIALS AND METHODS

M & B-22948 was provided by May & Baker, Ltd. (U.K.). Tris–hydrochloride and *p*-nitrophenyl phosphate were purchased from Calibochem-Behring Corp., La Jolla, CA. Analytical samples of cromolyn sodium, AA-344, doxantrazole, ICI-74917, and W-13560 were prepared by the chemical research group at the Warner-Lambert Co., Ann Arbor, MI. All other chemicals used were reagent grade materials.

Human leucocyte alkaline phosphatase was obtained from 1 pint of heparinized whole normal human blood as described previously [14]. The leucocytes were separated from plasma by centrifugation at 2000 *g*, resuspended in 15 ml of 1 mM Tris–

Table 1. Comparison of allergic mediator release inhibitor activity against leucocyte alkaline phosphatase and allergic models*

Inhibitor	Structure	I_{50} human leucocyte alkaline phosphatase† [S] = 0.2 mM	I_{50} human leucocyte alkaline phosphatase† [S] = 10 mM	Inhibitor potency x cromolyn sodium Alkaline phosphatase‡	Inhibitor potency x cromolyn sodium Rat PCA§	Inhibitory concentrations chopped human lung‖ (M)
Cromolyn sodium·		>10¶	9.68 ± 0.52**	1.0	1	$6–20 \times 10^{-5}$
AA-344		2.77 ± 0.43**	2.88 ± 0.67	3.4	4	
Doxantrazole		2.51 ± 0.50	2.00 ± 0.43	4.8	1	$0.1–3 \times 10^{-6}$

M & B-22948		3.44 ± 0.45	1.75 ± 0.75	5.5	35–50	4–11 × 10^{-6}
ICI-74917		1.96 ± 0.16	0.97 ± 0.03	10	100–300	10^{-5}–10^{-9}
W-13560		0.42 ± 0.01	0.34 ± 0.02	28	30††	

* The enzymic reaction was followed measuring *p*-nitrophenolate production at 405 nm. Each assay was run at 24° and the total volume of 1 ml contained 0.2 or 10 mM *p*-nitrophenyl phosphate, 2.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 0.2 M Tris–hydrochloride, pH 9.2, and sufficient enzyme to give an easily measurable initial reaction rate. The specific activity of the prepared enzyme used for the experiments was 1.1 μmoles of *p*-nitrophenol formed per hr mg protein.

† The inhibitor concentration (mM) required for 50 per cent inhibition of leucocyte alkaline phosphatase (*in vitro*) at a substrate concentration, [*S*], of 0.2 or 10 mM was determined graphically by plotting percent inhibition vs inhibitor concentration.

‡ Inhibition compared at [*S*] = 10 mM.

§ Comparison of the intravenous doses required for 50 per cent inhibition of the rat passive cutaneous anaphylaxis test as reported in Ref. 7.

‖ Comparison of the *in vitro* concentrations required to inhibit mediator release from chopped human lung as cited in Ref. 7.

¶ Inhibition was less than 50 per cent at a 0.2 mM substrate concentration.

** Mean ± S.E.M.

†† Cited in Ref. 22.

hydrochloride in 0.155 M sodium chloride, pH 7.5, sonicated, and extracted with 5 ml of 1-butanol. The butanol phase was washed with 5 ml of the Tris/NaCl mixture. The combined aqueous extracts were dialyzed from 18 hr at 0° against 2 l. of 0.05 M Tris–hydrochloride, pH 7.5. The dialysate was then stored at 0° until used in the enzymatic assay. Protein determination, according to Lowry *et al.* [19], indicated a 3-fold purification of the enzyme as reported previously [14]. The specific activity of the enzyme preparation was 1.1 μmoles of *p*-nitrophenol produced per hr per mg of protein. During the course of these studies, blood samples from several subjects were used. Similar results in enzyme activity and response to inhibitors were obtained for all samples.

The enzymatic reaction was followed by measuring *p*-nitrophenolate production at 405 nm, using a Gilford model 250 spectrophotometer. Each assay was run at 24°, and the total volume of 1 ml contained 0.2 or 10 mM *p*-nitrophenyl phosphate, 2.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 0.2 M Tris–hydrochloride, pH 9.2, and sufficient enzyme amounts to give an easily measurable initial rate of reaction. Solutions of the substrate were prepared daily in water. The concentration of inhibitor (I) required for 50 per cent inhibition (I_{50}) was determined graphically by plotting percent inhibition vs I; each assay was run in duplicate. All reported values were determined at least twice using several inhibitor concentrations.

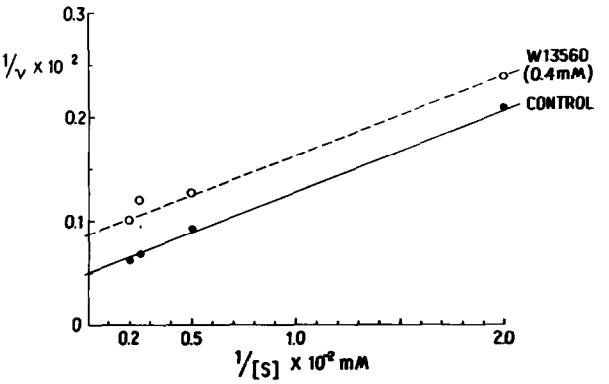


Fig. 1. Plot of $1/v$ vs $1/[S]$ showing uncompetitive inhibition of W-13560 of human leucocyte alkaline phosphatase. The enzyme assay was conducted as described in the legend of Table 1 and in Materials and Methods. The amount of substrate contained in each assay, however, was varied in the absence and presence of inhibitor at a concentration approximately equal to I_{50} for the inhibitor. The specific activity of the enzyme used was 1.1 μmoles of *p*-nitrophenol produced per hr per mg protein. Values are the means of at least two experiments. A representative experiment is shown for the inhibitor.

RESULTS

Using *p*-nitrophenyl phosphate as substrate, the apparent K_m of 1.51 ($\pm$ 0.14) $\times$ 10^{-4} M (mean $\pm$ S.E.M.) was determined from reciprocal plots [20]. A K_m of 1.1×10^{-4} M has been reported [14].

Cationic effects were studied with the enzyme. The enzyme was activated 28 $\pm$ 5 per cent by 2 mM $CaCl_2$, 66 $\pm$ 20 per cent by 2 mM $MgCl_2$, and 72 $\pm$ 28 per cent by 2 mM $CaCl_2$ + 2 mM $MgCl_2$ compared with the assay not containing added cations. This activation is minimal when one considers the 14- to 20-fold activations of several isoenzymes due to magnesium. The low-level activation of the leucocyte enzyme resembles the intestinal and placental isoenzymes [21].

Several known mediator release inhibitors were evaluated for their effects upon human leucocyte alkaline phosphatase activity. It was found that all mediator release inhibitors tested inhibited the enzymatic reaction. Several of the compounds tested appear in Table 1. A comparison of the relative potency order for enzyme inhibition, inhibition of the allergy-mediated rat passive cutaneous anaphylaxis assay (PCA) [7, 22], and inhibition of histamine release from chopped human lung [7] showed similarities. In the three assays compared, cromolyn sodium was the least potent inhibitor. The most active agents were ICI-74917 and W-13560 [22], while doxantrazole, AA-344, and M & B-22948 were intermediate in activity. The high potency of bufrolin

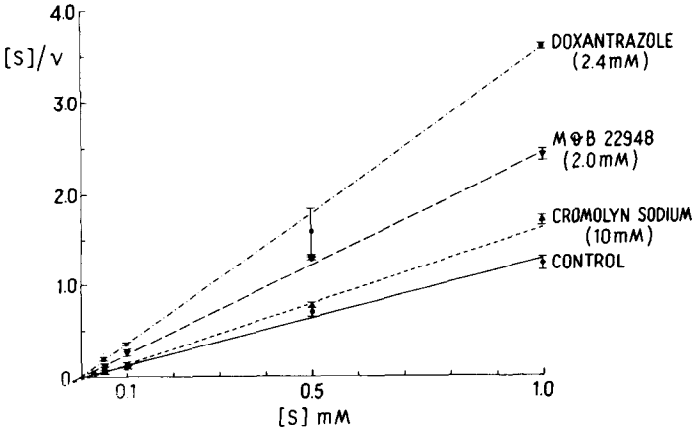


Fig. 2. Plot of $[S]/v$ vs $[S]$, showing uncompetitive inhibiton by cromolyn sodium, doxantrazole, and M & B-22948 of human leucocyte alkaline phosphatase. See the legend of Fig. 1 for details. Values are the means of at least two experiments. A representative experiment is shown for each inhibitor.

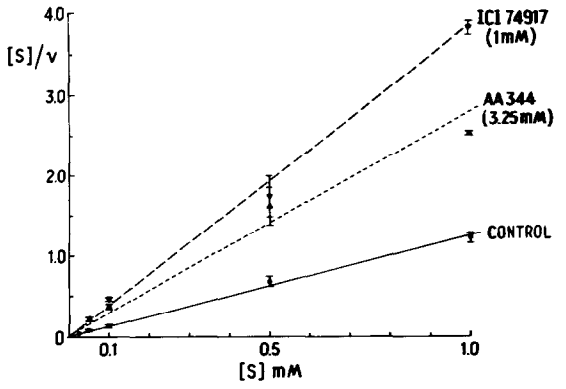


Fig. 3. Plot of $[S]/v$ vs $[S]$, showing uncompetitive inhibition by AA-344 and ICI-74917 of human leucocyte alkaline phosphatase. See the legend of Fig. 1 for details. Values are the means of at least two experiments. A representative experiment is shown for each inhibitor.

(ICI-74917) in the rat PCA was not apparent in the enzymic inhibition. Kinetic analysis of the inhibition type observed for this series of compounds indicated inhibition which was not competitive with substrate. A double reciprocol plot $1/v$ vs $1/S$ for each compound reported in the table yielded parallel lines characteristic of uncompetitive inhibition. A plot of S/v vs S for each compound was also characteristic for uncompetitive inhibition [23] (Figs. 1–3).

K_i values for each of the inhibitors were estimated graphically from double-reciprocal plots. The most potent inhibitor, W-13560, had a K_i of 7.89 ($\pm$1.64) $\times 10^{-6}$ M (mean $\pm$ S.E.M.). The remaining compounds studied had K_i values in a narrow range of 1–5 $\times 10^{-5}$ M. The K_i values, reported as the mean of two determinations, are cromolyn sodium, 2.5×10^{-5} M; AA-344, 5.5×10^{-5} M; doxantrazole, 1.5×10^{-5} M; M & B-22948, 3.0×10^{-5} M; and ICI-74917, 1.0×10^{-5} M.

Each inhibitor was studied at two concentrations of substrate (0.2 and 10 mM). The inhibitor potency was not reduced by the increased substrate concentration. This is also indicative of the uncompetitive nature where the inhibitor does not interact at the substrate binding site [20].

DISCUSSION

The mechanism of monophosphate ester cleavage by alkaline phosphatase has been studied [23]. At least two steps are involved in the sequence: (1) cleavage of the monoester by the enzyme, forming a phosphorylated enzyme intermediate, and (2) dephosphorylation of the enzyme to regenerate the free enzyme. At nonsaturating substrate concentrations and physiological pH, the formation of the phosphorylated enzyme is the rate-limiting step. Higher concentrations of substrate, however, make the dephosphorylation step rate-limiting. Van Belle [23] concluded that levamisole is an uncompetitive inhibitor of alkaline phosphatase and that it acts at the dephosphorylation step since it is a better inhibitor of the enzyme at high substrate concentrations. Similarly, mediator release inhibitors, such as those in Table 1, are uncompetitive inhibitors and probably are acting at the dephosphorylation step of the hydrolysis sequence. Several of the compounds tested appear to be better inhibitors at high substrate concentrations.

The fact that ICI-74917 and M & B-22948 are not as highly potent inhibitors of alkaline phosphatase as reported for allergic models may not be contradictory to their involvement with alkaline phosphatase. Generally, the high potency of inhibition in allergy test models such as rat PCA or chopped human lung has not been predictive of clinical potency or efficacy for agents like cromolyn sodium. Pharmacologically, the kinetics of binding and dose–response of cromolyn sodium are complex. The bell-shaped dose–response observed may be crucial in understanding the unpredictable clinical activity observed with current mediator release inhibitors such as cromolyn sodium [7].

The observation that cromolyn sodium promotes the formation of a phosphoprotein during inhibition of mediator secretion [8] could be explained by inhibition of alkaline phosphatase. Alkaline phosphatase is reported to possess phosphoprotein phosphatase activity [24]. Also, uncompetitive inhibition of the enzyme alkaline phosphatase prevents the dephosphorylation of a phosphorylated enzyme intermediate [23]. Therefore, prevention of mediator release through alkaline phosphatase inhibition by cromolyn sodium could be expected to promote the retention or formation of certain phosphorylated protein intermediates involved in calcium uptake or mediator secretion mechanisms.

The chemical structures of mediator release inhibitors are quite diverse and possess only certain common features such as carbonyl and acidic functional groups. It may be significant that all of the compounds reported in Table 1 are uncompetitive inhibitors of leucocyte alkaline phosphatase. Inhibition of alkaline phosphatase may be an important mode of action, possibly related to the calcium uptake inhibition mechanism proposed for certain allergic mediator release inhibitors. The uncompetitive nature of their inhibition may be reflected in their complex binding kinetics and dose–response pharmacologically. Depending upon substrate concentrations, an uncompetitive inhibitor may activate, inhibit, or exert no effect upon an enzyme reaction [20].

Other uncompetitive inhibitors of alkaline phosphatase are known. L-Phenylalanine inhibits intestinal, placental, and leucocyte enzymes [14], while levamisole inhibits enzymes from bone, kidney, leucocyte, liver, and spleen. El-Aaser *et al.* [25], however, has reported that leucocyte alkaline phosphatase isoenzyme may be different than placental or intestinal isoenzymes. The determination of possible organ specificity for mediator release inhibitor modulation of alkaline phosphatase is currently under investigation.

In summary, antigen–antibody-induced calcium uptake into cells could involve alkaline phosphatase in the biochemical mechanism leading to the release of allergic mediators. The inhibiton of alkaline phosphatase by certain allergic mediator release inhibitors, such as cromolyn sodium, suggests the involve-

ment of alkaline phosphatase in antigen–antibody-induced allergic responses.

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