L-PAM. These results are in agreement with the observations of Parsons *et al.* [7] using human melanoma cells. However, our results are not in agreement with a report [8] which indicated that L-PAM sensitive cells transport more L-PAM than do resistant cells. However, differences in the mechanism of resistance to L-PAM may exist in different cell types. Our results do indicate that L-PAM resistant cells convert to 2 to 2.5 times more L-PAM to its non-cytotoxic derivative 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine than do L-PAM sensitive cells and that this dechlorination is inhibited by the sulfhydryl reagent *N*-ethylmaleimide. This detoxification of L-PAM positively correlates with the intracellular concentration of glutathione, the principal non-protein thiol of the cell.

Several studies have demonstrated that cells sensitive to certain alkylating agents generally have a lower cellular content of protein-free thiols [9–13] or a lower ratio of protein-free to protein-bound thiol [14] than do cells which have acquired resistance to these antineoplastic drugs. The results described here, and previously [1], clearly implicate glutathione as being the critical determinant in L-PAM cytotoxicity. The precise mechanism by which glutathione reduces the cytotoxic potency of L-PAM is not clearly understood at the present time. However, it is possible that direct interaction of L-PAM with glutathione and glutathione-*S*-transferase, an enzyme which participates in the dehalogenation of many electrophilic substrates [15], results in dechlorination of L-PAM and a loss of cytotoxicity. Alternatively, the higher concentration of glutathione present in L-PAM resistant cells may either result in protection from L-PAM of a cellular site critical for the expression of L-PAM cytotoxicity or glutathione may aid in displacing L-PAM which is loosely bound to cellular macromolecules during the period immediately following drug exposure. Finally, the possibility exists that glutathione somehow reduces L-PAM cytotoxicity by altering the nature of interstrand, intrastrand or DNA-protein cross-links induced by the drug [16–18]. Our observation that L-PAM resistant cells can be completely sensitized to the drug by lowering the intracellular concentration of glutathione [1] provides an important means to investigate and determine the critical cellular site(s) with which L-PAM must interact for its cytotoxicity to be expressed.

* Address correspondence to: David T. Vistica, Ph.D., Laboratory of Medicinal Chemistry and Biology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Building 37, Room 6D28, Bethesda, MD 20205, U.S.A.

Laboratory of Medicinal Chemistry and Biology
Developmental Therapeutics Program
Division of Cancer Treatment
National Cancer Institute
Bethesda, MD 20205, U.S.A.

KAYOKO SUZUKAKE
BARBARA P. VISTICA
DAVID T. VISTICA*

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Design and testing of potential activators for hydrolytic enzymes

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Inborn errors of metabolism can arise either from a total lack of an enzyme or from a reduced enzymatic activity that results from the synthesis of an impaired enzyme or a failure to synthesize sufficient enzyme [1]. There are only a few approaches to the treatment of such enzyme insufficiency diseases, and their therapy with drugs has been a particularly intractable problem. In favorable cases, such as galactosemia [1], control of the diet may be effective but in many other cases, such as the lysosomal storage diseases [2], the accumulated metabolite is synthesized internally and cannot be controlled by regulating the diet. In such cases, there is still [3] no effective treatment

although very promising results have been obtained with various kinds of enzyme replacement therapy [4, 5].

Another therapeutic strategy is to stimulate the deficient enzyme and thereby increase its biochemical effectiveness [6–10]. Several metabolic pacemaker enzymes are known which can be markedly activated [7], but the activators bind to special allosteric sites. Presumably most target enzymes do not have such sites. Not all activators, however, act allosterically. Many of the lysosomal enzymes of interest have the unique property that product dissociation is the rate-determining step [11–13], and this makes it possible to design drugs as potential enzyme activators.

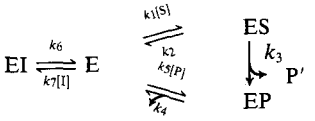
Theory

Irreversible enzymatic hydrolytic reactions can be formulated

$$S \longrightarrow P' + P$$

where P is the last of the two products to dissociate from the enzyme. The conventional formulation (Scheme I) for such a reaction is unsatisfactory [13] for the following four reasons: (a) the enzyme complex dissociations are not written as balanced chemical equations, (b) X-ray crystallography has shown that the active site is occupied by a surrogate anion when anionic substrates are not bound [14], (c) enzymes cannot be eluted from affinity columns containing immobilized substrates (S), products (P) or inhibitors (I) by water alone as is implied by Scheme I, and (d) with certain competitive inhibitors the ratio of the apparent K_m values for the substrate (K_m') and the product (K_p') can be shown to vary with concentration.

Scheme I



where

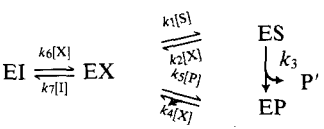
$$K_m' = \frac{k_2 + k_3}{k_1}(1 + k_7[I]/k_6)$$

and

$$K_p' = \frac{k_4}{k_5}(1 + k_7[I]/k_6).$$

These defects are corrected by writing the complex dissociations as ligand substitution reactions involving a surrogate ligand (X) as in Scheme II.

Scheme II



where

$$K_m' = \frac{k_2[X] + k_3}{k_1}(1 + k_7[I]/(k_6[X]))$$

$$K_p' = \frac{k_4[X]}{k_5}(1 + k_7[I]/k_6[X]))$$

and the ratio K_m'/K_p' varies with the concentration of the surrogate ligand (X).

The unique feature of Scheme II is that, in the absence of any inhibitor (I), it predicts that the dissociation constant for the product (K_p') is strictly proportional to the ligand concentration. Furthermore, if the dissociation of the second product (P) is the rate-determining step, then the surrogate ligand (X) can be both an activator and an inhibitor. Activation by the surrogate ligand (X) can also occur if the enzyme is inactivated by another ligand (I) [6, 9, 10] whose complex with the enzyme (EI), unlike the surrogate complex (EX), will not react directly with the substrate.

Because product dissociation is the rate-determining step for most hydrolytic enzymes, activation by nucleophiles (N) that react with the (EP) complex to form substrate analogues (NP) also occurs. This type of activation, however, is probably of only limited use as a strategy for the therapeutic activation of deficient enzymes since the substrate analogues (NP) produced are usually potent competitive inhibitors of the natural substrates. For example, alkaline phosphatase is activated by Tris buffer due to the formation of the Tris phosphate ester but Tris–phosphate is a potent competitive substrate analogue.

For the therapy of enzyme insufficiencies, what one requires is a drug (X) which does not compete with the substrate ($k_2 \ll k_1$ in Scheme II) but does compete very effectively with either the inhibitory product ($k_4 \gg k_5$ in Scheme II) or another endogenous inhibitor (I) ($k_6 \gg k_7$ in Scheme II). Since the surrogate ligand (X) is presumed to act by binding to the active site, such drugs are substrate and product analogues. It is easy to determine which substrate and product analogues are potentially useful because they, characteristically, increase the rate of the enzymatic reaction in the presence of inhibitory concentrations of the reaction product (P) that competes with the substrate.

In this paper we will illustrate this screening procedure with bovine intestinal alkaline phosphatase, a hydrolytic enzyme comparable to the lysosomal hydrolases.

Materials and methods

Intestinal alkaline phosphatase and *p*-nitrophenylphosphate were obtained from the Sigma Chemical Co. (St. Louis, MO). The enzymatic reactions were followed for 1 min at 405 nm in a Cary, model 15, recording spectrophotometer, thermostatted at 30°.

Results

Since the product of the alkaline phosphatase reaction (P) is the dianion HPO_4^{2-}, the surrogate ligand (X) must also be anionic. We chose inorganic anions as the simplest product analogues to test. Reaction mixtures were buffered with 0.05 M aminoethylpropanediol–nitrate and contained 45 μM substrate (about twice the K_m). To test the effects of anions on the phosphate inhibition, sufficient phosphate was included to give about 80% inhibition. Table 1 shows that all the anions activated the reaction in the presence of the product phosphate but only sulfate and sulfite inhibited in the absence of product.

The fact that chloride was acting as a surrogate ligand was confirmed by measuring the K_m and V_m together with the product K_i by conventional Dixon plots [15]. Figure 1 shows that chloride did not affect the apparent Michaelis constants but that the K_i for phosphate was strictly proportional to the chloride concentration, as predicted. At lower pH values, both the V_m and K_m decrease with lower chloride concentrations [16, 17]. This is predicted also by Scheme II, for when product dissociation becomes the rate-determining step ($k_4[X] \ll k_3$) the following relationship holds:

$$(K_m)_{\text{observed}} \times \frac{V_m}{(V_m)_{\text{observed}}} = \frac{k_2[X] + k_3}{k_1}$$

where V_m is the maximum value of the Michaelis V_m constant which is observed at high chloride concentrations where chloride acts only as a classical competitive inhibitor.

Because the product is a divalent anion, a single chloride anion is insufficient to write a balanced chemical equation for K_i. However, both K_i and the rate of product dissociation are proportional to the hydroxide anion concentration

Table 1. Effects of 0.1 M potassium salts on the hydrolysis of *p*-nitrophenylphosphate by bovine intestinal alkaline phosphatase in 0.05 M aminoethylpropanediol–nitrate buffer, pH 9.0, 30°*

		F	Cl	Br	I	SO_4	SO_3	NO_3	NO_2
45 μM Substrate	(%)†	128 ± 2	105 ± 3	94 ± 2	96 ± 3	74 ± 3	33 ± 2	94 ± 2	96 ± 3
180 μM Substrate 500 μM Phosphate	(%)‡	162 ± 6	129 ± 5	129 ± 5	129 ± 4	167 ± 4	167 ± 3	132 ± 4	131 ± 5

* Errors are based on S.E.M. (N = 4) with 100% in the absence of the potassium salt.
† 0.2 μg Enzyme, 100% = 0.145 ± 0.002 Abs·min^{-1}.
‡ 1.0 μg Enzyme, 100% = 0.157 ± 0.003 Abs·min^{-1}.

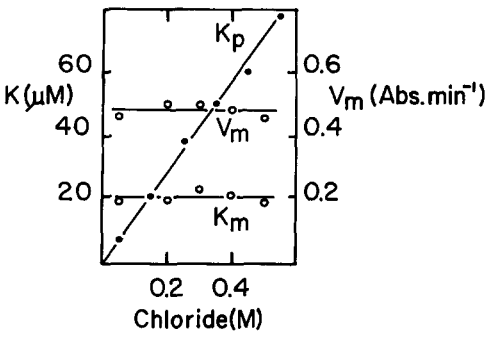


Fig. 1. Effect of chloride on the K_m, V_m and K_p (K_i) values for alkaline phosphatase at 30° in 0.05 M aminoethylpropanediol buffer, pH 9.0, containing variable concentrations of KCl.

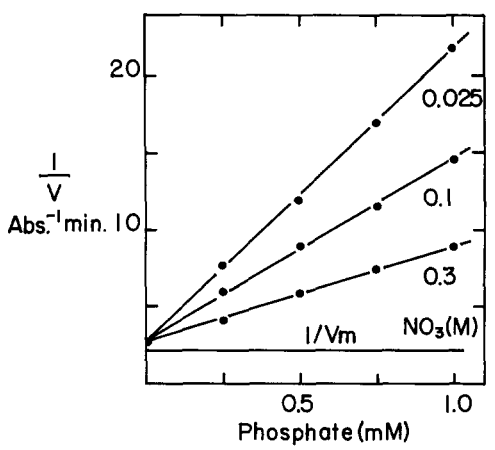


Fig. 2. Activation of alkaline phosphatase by nitrate anions in the presence of phosphate. Reaction mixtures contained 0.05 M aminomethylpropanediol–nitrate buffer, pH 8.8, and 160 μM *p*-nitrophenylphosphate. Data are illustrated in the form of a Dixon plot [15]. The maximum velocity, determined separately, is indicated by the horizontal line.

[16, 17] so one can write the following hypothetical balanced chemical equation for the product dissociation.

$$Cl^- + OH^- + E{\cdot}P_i \rightleftharpoons (HO\text{-}E\text{-}Cl) + HPO_4^{2-}$$

A more thorough investigation of the activation by nitrate in the presence of inhibitory product concentrations is illustrated in Fig. 2. This graph is effectively a Dixon plot [15] but a high substrate concentration (about 8 × K_m) was chosen to yield almost the maximum velocity in the absence of phosphate. It can be seen that nitrate gave a marked stimulation in the presence of phosphate but had no effect in the absence of phosphate.

Discussion

Although the surrogate ligand (X) has many of the properties ascribed to classical competitive inhibitors (I), the kinetics observed do not follow the expected equation [18]:

$$\frac{V_m}{V} = 1 + \frac{K_m(1 + [I]/K_i + [P]/K_p)}{[S]}.$$

This equation predicts a family of parallel lines in a Dixon plot such as Fig. 2.

A further characteristic property of surrogate ligands [13] is that they may both activate and inhibit at low substrate concentrations. The activation is due to rate-determining product displacement ($k_4[X] \ll k_3$) and is reflected in an increase in the Michaelis V_m up to a limiting value ($k_3 \cdot E_t$). The inhibition at higher surrogate ligand (X) concentrations is due to competition with the substrate which increases the Michaelis K_m. This biphasic activation and then inhibition means that drugs designed to be competitive inhibitors may actually activate at low concentrations and consequently give unexpected pharmacological effects. For example, Radin *et al.* [8] unexpectedly found that a ceramide analogue (*N*-decanoyl aminopropanediol) activated the cerebrosidase that forms ceramides.

In summary, four points should be noted: (1) hydrolytic enzymes may be activated by either nucleophiles or certain competitive inhibitors which facilitate dissociation of the reaction product from the enzyme; (2) there is a probability that some substrate analogues will compete with the reaction product much more effectively than with the reaction substrate; (3) substrate and product analogues (surrogate ligands) which are potential activators may be readily identified by the fact that they activate in the presence of the product that is a competitive inhibitor of the substrate; and (4) sulfate and sulfite were shown to compete with both the substrate and product of alkaline phosphatase but many other anions were found to compete with only the product, phosphate dianion.

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Department of Chemistry
Indiana University
Bloomington, IN 47405, U.S.A.

W. Terry Jenkins*
Rahman A. Mohd Yunus

* Author to whom correspondence should be sent.

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Inhibition of soybean lipoxygenase by sulfasalazine and 5-aminosalicylic acid: a possible mode of action in ulcerative colitis

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For over 30 years, sulfasalazine [1] has been the drug of choice in the treatment of ulcerative colitis. The exact mode of action remains unclear, but it has been hypothesized that the action of sulfasalazine and its metabolite, 5-aminosalicylic acid (5-ASA), is through inhibition of colonic mucosal prostaglandin synthesis [2, 3], inhibition of mucosal prostaglandin metabolism [4–6], inhibition of absorption of folates [7] or inhibition of DNA synthesis [8]. The apparent contradictions about the mode of action of sulfasalazine and 5-ASA suggest that a totally different mode of action might be involved.

Ulcerative colitis is characterized by an acute mucosal inflammation dominated by polymorphonuclear leukocyte accumulation by random migration [9]. Sulfasalazine and 5-ASA inhibit such random migrations, phagocytosis and oxidative metabolism [10].

Leukotrienes, hydroperoxyeicosatetraenoic acids (HPETE), and hydroxyeicostetraenoic acids (HETE) are products of arachidonic acid metabolism via the lipoxygenase pathway. They have been shown to be highly potent and stereospecific factors stimulating polymorphonuclear leukocyte (PMNL) migration [11–14] and enhancing vascular permeability [15]. Since sulfasalazine and 5-ASA inhibit the migration of PMNL, it seems possible that sulfasalazine and 5-ASA might be working in part through inhibition of the lipoxygenase pathway. To test the above hypothesis, we evaluated sulfasalazine and 5-ASA for possible lipoxygenase inhibitor activity.

Materials and methods

Sulfasalazine and 5-ASA were tested against the lipoxygenase of arachidonic acid using soybean lipoxygenase (EC 1.13.11.12) and linoleic acid as substrate.

Sulfasalazine was a gift from Salsbury Laboratories, Charles City, IA, and 5-ASA was obtained from the Aldrich Chemical Co., Milwaukee, WI. Soybean lipoxygenase, linoleic acid and Tris were purchased from the Sigma Chemical Co., St. Louis, MO.

Determination of lipoxygenase activity. The conversion of linoleic acid to hydroperoxylinoleic acid was followed spectrophotometrically by the appearance of a conjugated diene at 234 nm. The enzymatic reaction was monitored using a Gilford model 250 spectrophotometer at 24°. Each assay had a total volume of 1 ml and contained sodium linoleate, 100 μM; 0.1 M Tris hydrochloride, pH 9.0; 2% ethanol; 4.2% propylene glycol; and sufficient enzyme to give an easily measurable initial rate of reaction [16, 17]. Substrate solutions were prepared fresh prior to assays with 20% ethanol in Tris buffer, and inhibitors were dissolved in a 5.25% propylene glycol–Tris buffer solution in such a manner that an aliquot of each yielded a final concentration of 4.2% propylene glycol and 2% ethanol in each assay. The effects of inhibitors on the enzymatic reaction were compared against controls under identical conditions. The substrate concentration used for all assays was 100 μM. Under the conditions of this assay, an IC_{50} value of 6.1 μM was obtained for the known lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA). The reported IC_{50} values for NDGA are 2.4 to 10 μM [16, 18].

Results and discussion

The inhibitory activity of sulfasalazine and 5-ASA was measured against soybean lipoxygenase, an enzyme source shown to be predictive of human lipoxygenase [18]. In the presence of sulfasalazine, there was a dose-related inhibition of lipoxygenase enzyme. Based upon three separate determinations, the IC_{50} for sulfasalazine was found to be 66.2 μM, while 5-ASA, the metabolite of sulfasalazine, inhibited the enzyme with an IC_{50} of 170 μM. A representative IC_{50} determination is shown in Fig. 1.

Sulfasalazine inhibits prostaglandin biosynthesis with an IC_{50} of about 1500 μM [5], whereas 5-ASA is weaker than sulfasalazine [5]. While the IC_{50} concentrations are very high, local or topical application of these agents such as with enemas [19–21] might have therapeutic effect.

Sulfasalazine has been shown to inhibit the breakdown