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SHORT COMMUNICATIONS

Specific inhibitors of aminopeptidase M—Relationship to anti-inflammatory activity

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A great variety of *N*-terminal exopeptidases are found in nature, but only a few have been well characterized, and very little is known about their precise functions [1]. Since intracellular proteolytic enzymes have become of interest in the study of the possible treatment of degenerative conditions [2], the study of the biological roles of the aminopeptidases has received increased impetus. In any study of the biological role of an enzyme, a specific inhibitor is of great importance. Thus far, only one specific inhibitor has been described for this type of peptidase. Jost *et al.* [3, 4] have described a threonine tertiary butyl peptide,

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H—Thr—Phe—Pro—OH, which is a specific competitive inhibitor of leucine aminopeptidase. It has no effect on aminopeptidase M. This report describes the discovery of aminopeptidase inhibitors with the opposite specificity. These compounds are good inhibitors of aminopeptidase M, but have no effect on leucine aminopeptidase, nor on the proteolytic enzymes renin, pepsin and cathepsin D.

Colorimetric assays for animopeptidase M (APM) and for leucine aminopeptidase (LAP) were developed using alanine-4-methoxy-β-naphthylamide and leucine-4-methoxy-β-naphthylamide as the substrates. The substrates were synthesized by Dr. E. L. Smithwick and Mr. R. Shuman of our laboratories, who also prepared the 4-methoxy-β-naphthylamine tosylate used as the colorimetric standard. Leucine aminopeptidase was obtained from Worthington Biochemicals, Freehold, NJ, and aminopeptidase M from Henley & Co., New York City, NY. The MES and HEPES buffers were obtained from Sigma Chemical Co., St. Louis, MO, and the Fast Blue dye (3,3'-dimethoxy-4,4'-biphenyl ether bis-diazonium zinc tetrachloride) was from General Aniline Corp, New York. The *o*-(phenylthio)-phenylacetic acid, its sulfinyl derivative, and their benzoic and propionic acid analogs were obtained from Dr. Lamar Field of Vanderbilt University.

Aminopeptidase M assay. The MES buffer used is 0.05 M 2-(*N*-Morpholino)-ethane sulfonic acid adjusted to pH 5.6 with 10% KOH. The substrate is 1 mM alanine-4-methoxy-β-naphthylamide, or the leucine analog, in the MES buffer. The enzyme is kept as a stock solution at 20,000 mU/ml in 0.9% NaCl containing 1:10,000 merthiolate at 4°. For the assay, it is diluted to the appropriate concentration with MES buffer. The dye used for the color reaction is Fast Blue (3,3'-dimethoxy-4,4'-biphenyl ether bis-diazonium zinc tetrachloride from General Aniline) freshly prepared in distilled water at 1 mg/ml.

To each tube (13 × 100 mm disposable glass) is added 2 ml of substrate solution, plus the appropriate volumes of enzyme solution, inhibitor sample, and MES buffer to make a final volume of 3 ml. For inhibition studies, a final concentration of 4 mU/ml of enzyme in the incubation mixture is used. After a 20-min incubation at 37°, 0.1 ml of 4N HCl is added, the tube vortexed, 1.0 ml of the dye solution added, and the tube vortexed again. After 35 min at room temperature to develop maximum color, the optical density of the solution is read at 520 nm. A substrate control without enzyme is used as the instrument blank.

Leucine aminopeptidase assay. The HEPES buffer used is 0.05 M *N*-2-hydroxyethylpiperazine-N^1-2-ethane sulfonic acid adjusted to pH 7.0 with 10% KOH. The substrate is 1 mM leucine-4-methoxy-β-naphthylamide in the HEPES buffer. The enzyme (Worthington LAPC 53E335) is received as a suspension and is kept at 4°. It is activated by incubating a mixture of 0.05 M Tris–HCl buffer, pH 8.5, 5 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.5 mg of the enzyme in a total volume of 2.0 ml for 3 hr at 37°.

For the assay, 2 ml of substrate solution is added to each tube (13 × 100 mm disposable glass), plus the appropriate volumes of activated enzyme solution, inhibitor sample, and HEPES buffer to make a final volume of 3 ml. For inhibition studies, a final concentration of 17 μg/ml of enzyme in the incubation mixture is used. After incubating for 30 min at 37°, the mixture is acidified and the color developed as for the APM assay. A substrate control without enzyme is used as the instrument blank.

A four point standard curve is constructed for both assays using 4-methoxy-β-naphthylamine tosylate at 5, 10, 25 and 50 nmoles/ml in the appropriate buffer. The standard samples are acidified and color is developed in the same manner as for the assay samples. The standard curves are linear over this range of concentrations, as can be seen in Fig. 1.

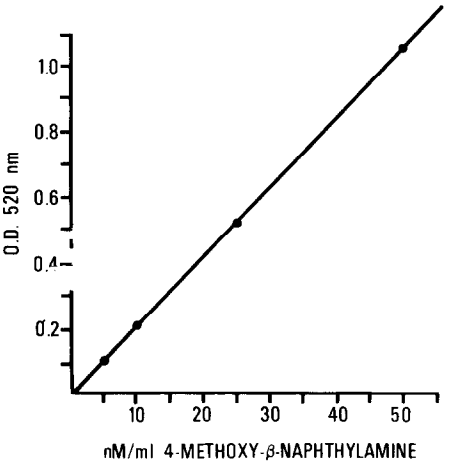

Fig. 1. Standard curve for aminopeptidase assay.

While testing synthetic organic compounds for inhibitory activity against peptidases and proteases, it was found that *o*-(phenylthio)-phenylacetic acid was a potent inhibitor of aminopeptidase M. The sulfinyl derivative was equally active, and was more soluble in the buffer systems used. Because of this, all subsequent work was done with it.

The inhibition curve obtained with *o*-(phenylsulfinyl)-phenylacetic acid is shown in Fig. 2. The concentration giving a 50 per cent inhibition of aminopetidase M was 6.15×10^{-6} M. It failed to inhibit leucine aminopeptidase at up to thirty times that concentration. It also failed to shown any inhibition when tested against the proteases pepsin, renin and cathepsin D. Hence, it appears to be a relatively specific inhibitor of aminopeptidase M.

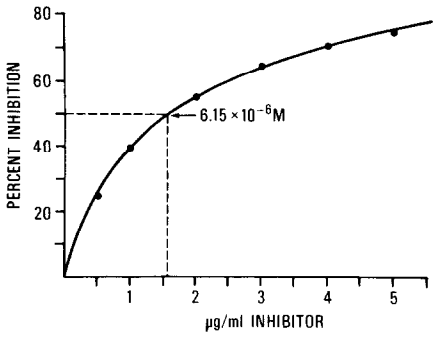

Fig. 2. Inhibition of aminopeptidase M by *o*-(phenylsulfinyl)-phenylacetic acid. Enzyme concentration was 2 mU/ml. Substrate was 0.67 mM alanine-4-methoxy-β-naphthylamide. Incubated at 37° for 20 min.

The influence of the substrate concentration on the velocity of the reaction of aminopeptidase M with the leucine-4-methoxy-β-naphthylamide substrate was studied with various concentrations of inhibitor present. Lineweaver–Burk plots of the data indicated that the inhibition was of the mixed type (Fig. 3). The same plots gave a K_m value of 0.24×10^{-3} M for this substrate, and a V_{max} value of 4.35 nmoles/ml/min for aminopeptidase M in this system.

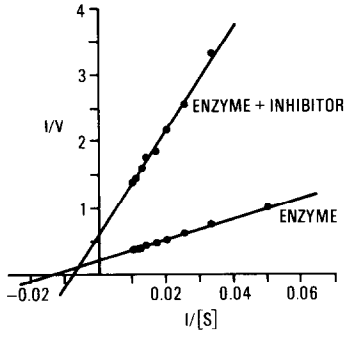

Fig. 3. Lineweaver–Burk plot of the influence of substrate concentration on the velocity of the reaction of aminopeptidase M with the leucine-4-methoxy-β-naphthylamide substrate in the presence and absence of the inhibitor *o*-(phenylsulfinyl)-phenylacetic acid.

A limited number of structural analogs of *o*-(phenylthio)-phenylacetic acid were tested in an attempt to establish some structure–activity relationships. The results obtained are shown in Table 1.

The benzoic acid analogs proved to be only marginally active. However, compounds V and VI in Table 1, with the acetyl and propionyl groups in the para position, were almost as active as the original compounds. On the other hand, the weak activity of compounds VII and VIII in Table 1, indicates a strong reduction in activity when the propionyl group is in the ortho position.

Table 1. Inhibition of aminopeptidase M

Compound	Structure	Per cent inhibition 4 μg/ml	20 μg/ml
I	2-(C_6H_5S)—$C_6H_4CH_2$-COOH	67	93
II	2-[$C_6H_5S(O)$]—$C_6H_4CH_2$-COOH	67	93
III	2-(C_6H_5S)—C_6H_4COOH	4	11
IV	2-[$C_6H_5S(O)$]—C_6H_4-COOH	3	12
V	4-(C_6H_5S)—$C_6H_4CH_2$-COOH	50	84
VI	4-(C_6H_5S)—C_6H_4CH-(CH_3)COOH	58	88
VII	2-(C_6H_5S)—C_6H_4CH-(CH_3)COOH	14	41
VIII	2-[$C_6H_5S(O)$]—C_6H_4CH-(CH_3)COOH	3	9

Since *o*-(phenylthio)-phenylacetic acid and some of its analogs had been synthesized originally as potential non-steroidal anti-inflammatory agents [5], other anti-inflammatory compounds were tested as inhibitors of aminopeptidase M. The results obtained are shown in Table 2. The data in Table 2 afford some additional structure–activity relationships, but there is little correlation of APM inhibition with the anti-inflammatory activity of known non-steroidal anti-inflammatory agents. Those anti-inflammatory agents which are structurally related to the arylacetic and the arylpropionic acid derivatives also inhibit APM; however, there is no direct correlation of the two activities. In fact, the best inhibitors of APM, *o*-(phenylthio)-phenylacetic acid and its sulfinyl analog, are relatively weak anti-inflammatory agents [5]. Also, replacing the acetyl group with a propionyl group in the ortho position in the APM inhibitor series results in a strong reduction in activity, whereas in the arylacetic and arylpropionic acid anti-inflammatory series, the effect is just the reverse. In that series, a major increase in activity is the result of such a substitution. Among the active non-steroidal anti-inflammatory agents, only indomethacin is active in the same range of concentration as *o*-(phenylthio)-phenylacetic acid.

Acid proteases and, by implication and association, aminopeptidases have been implicated in a number of degenerative conditions including muscular dystrophy, denervation atrophy, inflammatory and allergic conditions, experimental allergic encephalomyelitis, and multiple sclerosis [2]. Since pepstatin has been shown to be a highly potent inhibitor of acid proteases [6, 7] and there are now inhibitors

Table 2. Inhibition of aminopeptidase M

Compound	Per cent inhibition 4 μg/ml	20 μg/ml
Indomethacin	60	84
Fenclozic acid	43	77
Ibuprofen	41	76
Fenoprofen	28	64
Naproxen	27	65
Tolectin	13	30
Ketoprofen	11	31
Chloroquin	Inactive	
Phenylbutazone	Inactive	
Sudoxicam	Inactive	
Penicillamine	Inactive	
Aspirin	Inactive	
Flufenamic acid	Inactive	

available which are specific for aminopeptidase M and for leucine-aminopeptidase [3], it appears that the tools are becoming available with which to investigate the possible roles of these enzymes in various pathological conditions. The (phenylthio)-phenylacetic and propionic acid compounds might also prove useful in studying the catabolism of certain biologically active peptides, such as the polypeptide hormones, and the more recently discovered hormone releasing factors.

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Binding of nonsteroidal anti-inflammatory agents to proteins—I. Ibuprofen–serum albumin interaction

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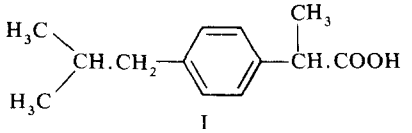
A generally accepted explanation of the mechanism of action of nonsteroidal anti-inflammatory drugs (NSAID) is the inhibition of prostaglandin synthesis [1–5]. Other explanations which have been proposed include displacement of corticosteroids from serum proteins [6], displacement of endogenous anti-inflammatory polypeptides [7] and stabilization of protein structure [8].

Whether nonsteroidal anti-inflammatory drugs act by a process of plasma protein stabilization, or of displacement, or by inhibition of prostaglandin synthetase, an evaluation of their protein binding characteristics is directly relevant to all mechanisms. Binding to the active site of prostaglandin synthetase might be expected to parallel albumin binding although the latter may require a much smaller degree of ligand structural specificity than the enzyme. Furthermore, binding of drugs to serum and tissue proteins is known to be an important determinant of their disposition kinetics and pharmacodynamics [9–13].

Ibuprofen (I), reported in 1967 [14], is widely used in the treatment of rheumatoid arthritis and osteoarthroses. Mills *et al.* [15] have reported that ibuprofen at a concentration of 20 μg ml^{-1} was 99 per cent bound in whole human plasma. It was reported that interaction occurred with a single primary albumin binding site with an association constant of 10^5 molar^{-1}; however, it is not clear how this was established using whole plasma.



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Ibuprofen, 2-(4-isobutylphenyl)-[3-^{14}C] propionic acid with a specific activity of 13.11 ± 0.19 μCi mg^{-1}, was a gift from The Boots Pure Drug Co. Ltd., Nottingham, U.K. The pK_a was determined, by the solubility technique of Albert and Serjeant [16], to be 4.13 ± 0.05. Binding of ibuprofen to bovine serum albumin (BSA) (fraction V, Sigma Chemical Co.) and human serum albumin (HSA) (fraction V, Behringewerke A.G.) was determined by equilibrium dialysis using 10-ml sterilized glass cells. The "Visking" cellophane membrane was boiled in several changes of distilled water before use.

Each cell was equilibrated for 36 hr at the required temperature (5–45° ± 0.1). Binding of ibuprofen was characterized alone and in the presence of bilirubin, uric acid, cholesterol and palmitate. The molecular weight of albumin was taken to be 69,000. Apparent contributions to binding by osmotic pressure and Donnan membrane effects were negligible under the experimental conditions. Where necessary albumin was defatted by the charcoal treatment described by Chen [17]. Palmitate was added by the technique of Avigan [18] and assayed [19]. Solutions were analyzed by liquid scintillation counting of [^{14}C]ibuprofen in Brays solution, to <3.5 per cent standard deviation in a Hewlett Packard scintillation spectrometer (model 3375). Correction for quenching was made using an automatic external standard technique.

Data were fitted to the generalized Scatchard equation [20] using FUNFIT, an interactive program for nonlinear regression [21]. Binding parameters were compared by a two-tailed t-test ($P \leq 0.05$). Each test parameter was assumed to be normally distributed.

Ibuprofen binding to HSA (Fig. 1) and BSA was determined in 0.033 M phosphate buffer at 37°. Correction for possible electrostatic interactions between binding sites, resulting from ligand binding and subsequent protein valency changes, were performed according to the Debye–Huckel–Born model [20]. However, the Scatchard plots remained curved, suggesting multiple class binding.

The quality of fit of the generalized Scatchard model with and without electrostatic correction was distinguished by comparison of residual variances. No improvement in fit was achieved by electrostatic correction or using numbers of classes of sites greater than 2($P \leq 0.05$). The data were thus best described by binding to two classes of sites. Binding parameters obtained using this model are summarized in Table 1 together with the coefficient of variation as an indication of the precision of each estimate. Ibuprofen binds strongly to a single primary site on HSA with an association constant of 2.73 × 10^6 molar^{-1}, and six to seven secondary sites with an association constant of 1.95 × 10^4 molar^{-1}.

Comparison of the binding parameters estimated at 1% and 0.4% HSA reveals an apparent protein concentration dependence.* Lowering the albumin concentration results in an apparent increase in the binding capacity (n_1 and n_2) and a decrease in the strength of binding (k_1 and k_2). These

* One of the reviewers directed our attention to a previous report [J. Cassel, J. Gallagher, J. A. Reynolds and J. Steinhardt, *Biochemistry* **8**, 1706 (1969)] which established that apparent protein concentration-dependent binding of dodecylsulfate and other compounds to BSA was due instead to the very slow approach to equilibrium of this and other ligands particularly when the ionic ligand was dialyzed into concentrated protein solutions. In all our experiments, the drug was dialyzed from the protein compartment and a preliminary study using 1% HSA established that thermodynamic equilibrium was established well within the 36-hr equilibration period.