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Pharmacology

Chemical, biochemical, pharmacokinetic, and biological properties of L-680,833: A potent, orally active monocyclic β-lactam inhibitor of human polymorphonuclear leukocyte elastase

(synthetic elastase inhibitors/lung injury/fibrinogen cleavage)

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ABSTRACT A series of potent and highly selective time-dependent monocyclic β-lactam inhibitors of human polymorphonuclear leukocyte elastase (PMNE, EC 3.4.21.37) is described. The intrinsic potency of these compounds, as exemplified by L-680,833 ($k_{inactivation}/K_i$ of 622,000 $M^{-1}s^{-1}$), is reflected at the cellular level where it inhibits generation of the specific N-terminal cleavage product Aα-(1–21) from the Aα chain of fibrinogen by enzyme released from isolated polymorphonuclear leukocytes stimulated with fMet-Leu-Phe with an IC$_{50}$ of 0.06 μM. The inhibitory activity of L-680,833 is also apparent in whole blood stimulated with A23187, where it inhibits formation of Aα-(1–21) and PMNE–α$_1$-proteinase inhibitor complex formation with IC$_{50}$ values of 9 μM. Pharmacokinetic studies indicate that after oral dosing L-680,833 is bioavailable in rats and rhesus monkeys. This oral bioavailability is reflected by the inhibition (*i*) of tissue damage elicited in hamster lungs by intratracheal instillation of human PMNE and (*ii*) enzyme released from human PMN stimulated after their transfer into the pleural cavity of mice. The properties of L-680,833 allow it to effectively supplement the activity of natural inhibitors of PMNE *in vivo*, suggesting that this type of low-molecular-weight synthetic inhibitor could have therapeutic value in diseases where PMNE damages tissue.

The extracellular activity of polymorphonuclear leukocyte (PMN) elastase (PMNE, EC 3.4.21.37) is tightly regulated by potent natural inhibitors, notably α$_1$-proteinase inhibitor (α$_1$PI) (1) and α$_2$-macroglobulin, in blood and by antileukoprotease (ALP) (2), also known as secretory leukocyte protease inhibitor (3), in the environment of mucosal surfaces. There are indications, however, that elastase released from PMN can express extracellular activity leading to tissue damage that is not effectively controlled by natural inhibitors of the enzyme. Moreover, genetically based deficiencies or, more rarely, absence of circulating α$_1$PI is associated with a more frequent onset of emphysema at an early age (4).

We recently disclosed that the appendages of the cephalosporin nucleus can be modified to alter the selectivity of inhibition from that of bacterial enzymes to human PMNE (5–10). Compounds of this class show excellent selectivity

and potency for PMNE but suffer from the drawbacks of poor oral bioavailability and modest stability in plasma, minimizing their potential value as systemically administered therapeutic agents. Given the constraints imposed by the structure of the cephem nucleus on the substitution pattern of the β-lactam ring, it was considered that the monocyclic β-lactam nucleus could be optimized for each substituent attached to it, leading to the optimum balance between intrinsic chemical stability, potency toward PMNE, and specificity against other serine proteases. We now report on the properties of a monocyclic β-lactam, 4-{[1-({[1-(R)-(4-methylphenyl)-butyl]amino}carbonyl)-3,3-diethyl-4-oxo-2-(S)-azetidinyl]oxy}-benzeneacetic acid L-680,833, (M_r = 466) (1, Fig. 1). This compound shows potent, time-dependent inhibition of human PMNE, possesses a high degree of selectivity against other serine proteases, and inhibits the activity of PMNE released from stimulated PMN. It exhibits good oral bioavailability in rats and rhesus monkeys. This activity is reflected functionally by inhibition of human PMNE-mediated lung injury in the hamster and the inhibition of this enzyme released from human PMN transferred into the pleural cavity of mice.

METHODS

Synthesis of Compound 1 and Other Monocyclic β-Lactam PMNE Inhibitors. The synthesis of 1 has been described in European Patent 0 337,549 (11), and additional details are given by Shah *et al.* (12). [³H]-labeled 1 was prepared with the label in the ethyl group to a specific activity of 172.3 mCi/mg (1 Ci = 37 GBq) and with a radiochemical purity of 98.5%.

Assay of PMNE. The activity of PMNE (7–30 nM, Elastin Products, St. Louis) was determined spectrophotometrically in 450 mM NaCl/10% dimethyl sulfoxide/45 mM Tes (1× Tes is 20 mM Tris/1 mM EDTA/250 mM sucrose) at pH 7.5 (buffer A) with 1 mM MeO-Succ-Ala-Ala-Pro-Val-pNA (Calbiochem, where Succ is succinyl and pNA is p-nitroanilide)

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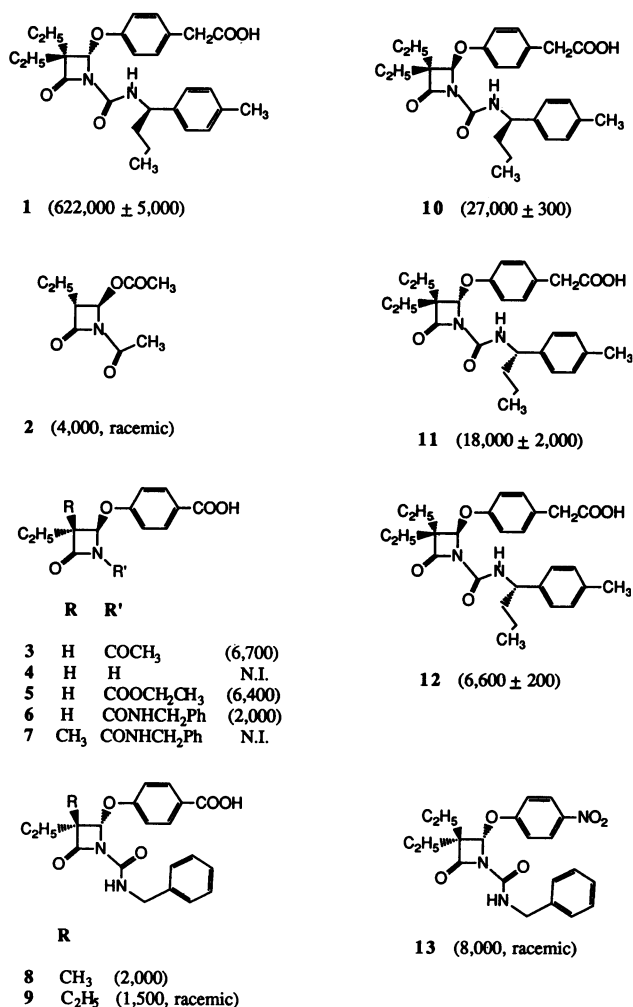
Abbreviations: PMN, polymorphonuclear leukocyte; PMNE, PMN elastase; E–I, enzyme–inhibitor; Aα-(1–21), N-terminal peptide generated by PMNE from the Aα chain of fibrinogen; α$_1$PI, α$_1$-proteinase inhibitor; ALP, antileukoprotease; Succ, succinyl; pNA, p-nitroanilide.
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FIG. 1. The structure of compound **1** and related analogs. The second-order rate constants for inhibition of PMNE in $M^{-1} \cdot s^{-1}$ are in parentheses. N.I., no inhibition at 2 μM.

or 0.2 mM Succ-Ala-Ala-Pro-Ala-pNA (Chemical Dynamics, South Plainfield, NJ) as substrate according to Green *et al.* (13). The presence of these β-lactam inhibitors in the activity assay resulted in a pseudo-first-order loss of PMNE activity with time. The second-order rate constants ($k_{inactivation}/K_i$) for the inactivation of PMNE by inhibitors were determined from these progress curves (13, 14).

Generation of Aα-(1-21) from Fibrinogen by Isolated PMN. The formation of the N-terminal peptide generated by PMNE from the Aα chain of fibrinogen (15) [Aα-(1-21)] after the degranulation of human PMN adhered onto a matrix of fibrinogen was assayed as described (16, 17). Human PMN (1×10^6) isolated by 3% dextran sedimentation followed by Ficoll centrifugation were plated in Hanks' balanced salt solution/20 mM Hepes/0.1% bovine serum albumin (HHBSA) in the presence of 1.0 nM recombinant human tumor necrosis factor α (16) on Costar 24-well cluster tissue-culture plates. Cells were incubated for 60 min at 37°C. The plates were coated with 0.25 ml of a solution of human fibrinogen at 2.4 mg/ml (Sigma) in 1.5% sodium bicarbonate for at least 3 hr at 37°C and washed with phosphate-buffered saline before PMN addition. After the 60 min cell attachment period, nonadherent PMN were removed by washing with HHBSA. Fresh HHBSA was added containing inhibitor and incubated for 10 min at 37°C. Cytochalasin B (2.5 μg/ml) was then added 10 min before the addition of fMet-Leu-Phe (0.1 μM). Cells were incubated for 60 min at 37°C, at which time medium was removed. Aα-(1-21)

was assayed by direct RIA using a polyclonal antibody raised in rabbits against Aα-(1-21), as described by Mumford *et al.* (15).

Generation of Aα-(1-21) from Fibrinogen in Blood Stimulated with A23187. Human heparinized blood (1-ml aliquots) was preincubated with test compounds for 10 min before addition of A23187 (100 μM) and incubation for 30 min at 37°C, at which time plasma was prepared. Inhibitors were prepared as solutions in dimethyl sulfoxide, which was diluted to a final concentration of 0.1% in blood. Plasma proteins were removed by addition of acetone (60%, final concentration), and the amount of Aα-(1-21) in the aqueous/acetone extract was determined by RIA (15). Aliquots of plasma were assayed for PMNE–α_1PI complex using a sandwich/trapping ELISA on Immunolon 96-well microtiter plates (18).

Human PMNE-Induced Lung Hemorrhage in Hamster. Intratracheal administration of PMNE causes an acute hemorrhagic response in the terminal airways of the hamster. Methods for inducing this hemorrhage and measuring the effects of inhibitors were those described by Fletcher *et al.* (19). Drug was administered orally as a suspension of the isobutanolamine salt in Methocel (Dow) 5 hr before challenge with enzyme. Animals were sacrificed 3 hr later, and the extent of hemorrhage and its inhibition by compound **1** was determined by lavage of erythrocytes and spectrophotometric analysis of their hemoglobin content.

Effects of Synthetic Inhibitors on Elastase Activity Released from Human PMN Transferred into the Mouse Pleural Cavity. Human PMN were isolated from heparinized blood, and 3-5 $\times$ 10^6 cells in a 0.3-ml vol were transferred into the pleural cavity of C57BL/6J female mice. Pharmacological agents were dosed orally 15 min after cell transfer. At various times subsequently an intrapleural injection of A23187 was given to induce degranulation of PMN. Pleural cavity fluid was removed 40 min after A23187 injection, cells were removed by centrifugation, and the supernatant was stored at -20°C until assay of enzyme activity. PMNE activity was assayed by hydrolysis of MeO-Succ-Ala-Ala-Pro-Val-pNA.

Pharmacokinetics. Pharmacokinetic studies were done in rats and rhesus monkeys. Compound was administered as [^{3}H]1, diluted with unlabeled compound, dissolved in saline (rhesus monkeys) or 5% dextrose (rats) and 0.5% methyl cellulose for i.v. and oral dosing, respectively. Rats received doses of 2 mg/kg, and rhesus monkeys received 10 mg/kg. A number of other species received unlabeled **1** to determine blood levels and tissue distribution of drug.

RIA of Compound 1. A polyclonal antibody to **1** was produced by immunization of rabbits with a conjugate of the compound with bovine thyroglobulin emulsified in complete Freund's adjuvant. The concentration of **1** in rat and monkey plasma was measured by a direct RIA with [^{3}H]1 (specific activity, 172.3 μCi/mg) as a radiolabeled probe.

HPLC Analysis of Compound 1. Blood was mixed with 10 vol of acetonitrile. After vigorous mixing samples were centrifuged at 2000 $\times$ g for 5 min, and supernatants were dried under nitrogen. Samples were reconstituted to one-half original volume with HPLC mobile phase, sonicated in a water bath, centrifuged at 10,000 $\times$ g for 5 min, and 50 μl aliquots of the supernatants were analyzed on a Waters HPLC system by using a Zorbax phenyl column (2.5 $\times$ 15 cm) with an isocratic mixture of 65% acetonitrile/35% H$_2$O (all in 0.2% trifluoroacetic acid) at 1 ml/min.

RESULTS AND DISCUSSION

Chemistry. The starting point for the development of an orally bioavailable PMNE inhibitor was the azetidinone **2** (Fig. 1), which had previously been shown by Firestone *et al.* (20) in these laboratories to be a time-dependent inhibitor of

the enzyme. Studies on the chemical stability of 2 showed that the compound had a half-life of 2.4 hr in 0.4 M Mops buffer, pH 8, at 25°C, whereas it decomposed virtually instantaneously in human blood at 25°C. Systematic examination of the appendages of 2 revealed several key points. The C-4 acetoxy group was considered likely to be unstable to nucleophiles and/or esterases, requiring replacement with a more robust functionality. Substituted phenols were thought to be reasonable alternatives, giving compounds such as 3, which retained enzyme inhibitory activity and had improved aqueous solubility. Examination of the stability of 3 under the conditions described above showed that the compound had a half-life of 8.6 hr in 0.4 M Mops buffer at pH 8.0. Furthermore, the analysis of its decomposition products showed the production of two compounds, *p*-hydroxybenzoic acid and the deacetylated compound 4 in a ratio of 3/2. Compound 4 was found to hydrolyze slowly ($t_{1/2} \geq 28$ hr) under these conditions, and thus the appearance of *p*-hydroxybenzoic acid was assumed to result principally from hydrolysis of the β-lactam ring in 3.

Lability of the *N*-acetyl group of 3 led us to search for substituents more stable to nucleophilic attack. Alkoxycarbonyl substituents, such as the carboethoxy group in 5, successfully prevented the formation of 4 at pH 8, but the half-life of hydrolysis of the β-lactam ring in 5 did not differ substantially from 3 ($\approx$30 hr). However, *N*-carbamoyl substituents such as found in 6 imparted at least an additional order of magnitude in stability to the series ($t_{1/2} >> 80$ hr), presumably because the carbamoyl moiety is intrinsically less electron-withdrawing than an acyl or a carboxyalkyl residue. It is also noteworthy that 6 showed only a 3-fold decrease in inactivation rate of PMNE relative to 5 or 3.

Incubation of 6 in mouse blood at 37°C resulted in a 50% loss of intact compound after 30 min. In marmoset blood, the stability of 6 was greater: 67% of the compound remained after incubation for 2.5 hr at 37°C. Moreover, an oral dose of 6 at 100 mg/kg to marmosets resulted in peak blood levels of 20–35 μg/ml at 30–90 min after dosing.

It was felt that the stability of the β-lactam nucleus in a compound, such as 6, could be further bolstered by introducing a second alkyl group on the β face at the 3 position, which should hinder attack by nucleophiles on the β-lactam carbonyl from that side of the ring. This addition was anticipated not to adversely affect inhibition of PMNE because crystallographic data on the related enzyme porcine pancreatic elastase suggested that the serine hydroxyl should attack the β-lactam carbonyl from the α face of a molecule such as 6 (M. A. Navia, personal communication). Preparation of the optical isomers of 6 showed that essentially all inhibitory activity resided in the enantiomer depicted in Fig. 1 ($k_{observed}/[I] = 4000$ M^{-1}s^{-1} versus 400 M^{-1}s^{-1} for its antipode, where I is inhibitor concentration). Under the conditions of these experiments, values for $k_{observed}/[I]$ did not differ from those for $k_{inactivation}/K_i$ (13, 14). Compound 7 was significantly more stable than 6 ($\approx$20-fold) but was found to be very weakly active against PMNE. However, isomer 8 *with the stereochemistry inverted at C-4* showed good time-dependent inhibition of PMNE as well as the improved stability of 7. The 3,3-diethyl analog 9 showed PMNE inhibitory activity comparable to 8 and was approximately two orders of magnitude more stable than 6 to nonspecific nucleophiles, such as ε-aminocaproic acid or *N*-acetylcysteine. Further, there was no degradation of 9 when it was incubated in human or rat blood for 12 hr at 37°C.

Subsequent efforts at optimization of the *in vitro* potency showed, *inter alia*, that modification of the *N*-carbamoyl residue on the phenyl ring and in the benzylic position (11, 12) could increase activity. These studies ultimately led to the finding that the *N*-{1-[1-(4-methyl)phenyl]butyl} substituent imparted a 100-fold improvement in activity over the corre-

sponding *N*-benzyl analog (9). It was suggested by crystallographic studies (K. Hoogsteen, personal communication) and confirmed here that the isomer with the absolute stereochemistry depicted for 1 has a second-order rate constant for inhibition of PMNE that is 1–2 orders of magnitude greater than those of other possible isomers (Fig. 1, compare 1 with 10, 11, and 12).

In addition to its potency, the specificity of 1 is noteworthy, not only in relation to other serine, thiol, metallo, and aspartyl proteases (14), but also to its relatively weak activity against the elastases of various mammalian species, especially porcine pancreatic elastase, which has been well-characterized by crystallography (21) and which has served as a model for inhibitor binding to human PMNE in our and other laboratories (22, 23). Furthermore, the potency of 1 against MeO-Succ-Ala-Ala-Pro-Val-pNA-hydrolyzing activity in rat and dog PMN is but a small fraction of that seen against human PMNE, and it is only in the primate that activity approaching that obtained with the human enzyme is seen (14). No significant effect was observed on the blood clotting or complement cascades at concentrations to 200 μM.

Studies on the mechanism of inhibition of PMNE by 1 and analogues (14) suggest that acylation of the active-site serine by the β-lactam carbonyl occurs with subsequent or concerted loss of the C-4 substituent. In those studies an initial intermediate enzyme–inhibitor (E–I) complex partitioned between inhibitor turnover and inactivation. A final, stable E–I complex was produced that required 1.3 equivalents of 1 to completely inactivate PMNE. This very limited turnover was also confirmed by the production of only 1.3 equivalents of *p*-hydroxyphenylacetic acid. In another example, inactivation of PMNE by 13 liberated *p*-nitrophenol, implying once again that the C-4 substituent is not present in the stable E–I complex. These results represented a significant improvement over the partitioning of E–I complexes produced from the cephalosporins (13, 24) and simple β-lactams (25). Because the C-4 substituent is not present, the stability of the final E–I complexes depended only on the identity of the urea moiety. Thus 1 and 10 produce E–I complexes that reactivated with half-lives of 11–15 hr at 37°C, whereas 11 and 12 produce complexes that reactivated with half-lives of 2–3 hr (14).

Oral Bioavailability and Pharmacokinetics. Studies on the pharmacokinetics of 1 in several species showed that improvements in intrinsic stability were reflected in oral bioavailability and pharmacokinetic profiles of this compound. Studies with ^{3}H-labeled compound showed that orally administered 1 was completely bioavailable in Sprague–Dawley rats. In rhesus monkeys, the oral bioavailability of 1 was determined to be $\approx$23%. The terminal elimination half-lives of 1 were 2.3 hr and 5.8 hr in the rat and rhesus monkey, respectively, after a single i.v. dose.

Oral dosing of unlabeled 1 at 10 mg/kg gave high plasma levels with a prolonged half-life in several species. In marmosets, peak blood levels of 3–7 μg/ml were achieved 45 min after dosing, whereas the African Green monkey achieved peak blood levels of 6 μg of compound 1 per ml at 60 to 120 min after dosing. In the dog, a peak blood level of 34 μg/ml was achieved 60 min after dosing; a level of 13 μg/ml was maintained for 12 hr after dosing, indicative of a long half-life in this species. The drug distributes well into tissues. In hamsters receiving a single i.v. dose of 3 mg of compound 1 per kg, a peak level of 3 μg/g in lung, comparable to that seen in blood, was measured 15 min after administration of drug. Drug persisted in lung tissue and blood for up to 20 hr when levels of 0.4 μg/g and 0.4 μg/ml, respectively, were detected. The drug also penetrates into the inflamed synovial cavity of the dog. When an oral dose of 1 at 10 mg/kg was administered to beagle dogs at the same time as an intraarticular injection

of urate crystals, blood levels of 12.4 ± 6.7 μg/ml and synovial fluid levels of 4.1 ± 1.1 μg/ml of compound 1 were measured 2 hr later.

Cell Biology of Compound 1. *Comparison of the potency of 1 with natural inhibitors to inhibit PMNE released from human PMN in vitro.* In vitro studies have shown that low-molecular-weight PMNE inhibitors modulate the activity of the enzyme as it is released from cells onto matrices containing substrates such as fibronectin (26), fibrinogen (27), artificial basement membrane (28), or elastin (29). The potency of 1 was compared with that of α_1PI and ALP by assay of its inhibition of the formation of $A\alpha$-(1-21). Fig. 2 shows that the compound inhibits the generation of $A\alpha$-(1-21) from fibrinogen when PMN are stimulated to degranulate onto a matrix of this substrate with an IC$_{50}$ of 0.06 μM. This potency compares favorably with the two major natural inhibitors of the enzyme, α_1PI and ALP, which gave IC$_{50}$ values of 0.12 μM and 0.25 μM, respectively, in this system. α_1PI was from Athens Research & Technology (Athens, GA) and ALP was from Hans Kramps, University of Leiden, The Netherlands.

Compound 1 is also effective against elastase released from PMN into blood plasma, which contains 30–50 μM of α_1PI (Fig. 3). The stimulation of PMN in blood with the A23187 results in the extracellular release of azurophilic-granule contents with the appearance in blood of marker enzymes, including elastase complexed with α_1PI. After incubation with A23187 (100 μM) for 30 min at 37°C PMNE is detected in plasma complexed with α_1PI (PMNE–α_1PI) in amounts from 0.1 to 0.4 μM, as compared with unstimulated control levels of ≈0.01 μM. Under these conditions, $A\alpha$-(1-21) released (15) is also detected. This signal of enzyme activity is probably generated during the brief time when PMNE is released in high concentrations from azurophilic granules of the stimulated PMN (30). Both the activity of the enzyme, as measured by formation of $A\alpha$-(1-21), and the formation of its complex with α_1PI are inhibited by 1 with IC$_{50}$ values of 9 μM (Fig. 3). This result is surprising in view of the high concentration of α_1PI in plasma and its greater activity against the enzyme than that of 1 (14). This result suggests that 1 will be active against PMNE under conditions where the natural inhibitors are quantitatively deficient or cannot gain access to the enzyme at the time of its release from the PMN.

Inhibition of PMNE in vivo by orally dosed 1. The observations described above that 1 is orally bioavailable encouraged us to evaluate its potential to inhibit human PMNE *in vivo*, under conditions where it causes tissue damage that is not effectively controlled by endogenous natural inhibitors of the enzyme. Intratracheal administration of the human enzyme to hamsters causes dose-dependent hemorrhage into the alveolar space (19). Fig. 4 illustrates that 1 inhibits this hemorrhage with an ED$_{50}$ of 1.5 mg/kg when the compound
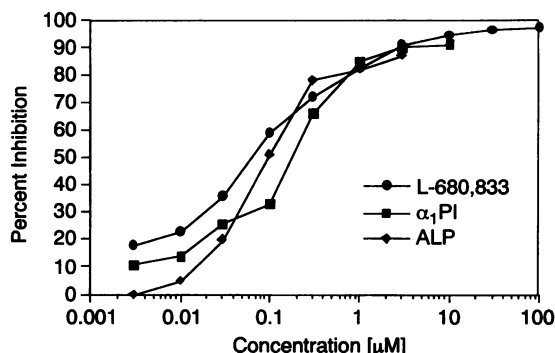

FIG. 3.   Compound 1 inhibits the formation of $A\alpha$-(1-21) fibrinopeptide and PMNE–α_1PI complex formation in human blood incubated with A23187. Data are the mean of duplicate incubations ± the range. ■, $A\alpha$-(1-21); □, PMNE–α_1PI complexes.

is administered orally 5 hr before the enzyme. The compound has a prolonged duration of action because at an oral dose of 3 mg/kg it maintains >50% inhibition of hemorrhage for between 13 and 14 hr, an activity commensurate with detection of the compound in the plasma of hamsters as long as 20 hr after a single oral dose of 3 mg/kg.

The activity of 1 against endogenous PMNE in experimental animals is difficult to evaluate because the compound is much less active against PMNE of the several lower species examined (14) as compared with humans. The capacity of 1

FIG. 2.   ALP, α_1PI, and 1 inhibit the production of $A\alpha$-(1-21) caused by elastase released from stimulated PMN adhered to a fibrinogen matrix.

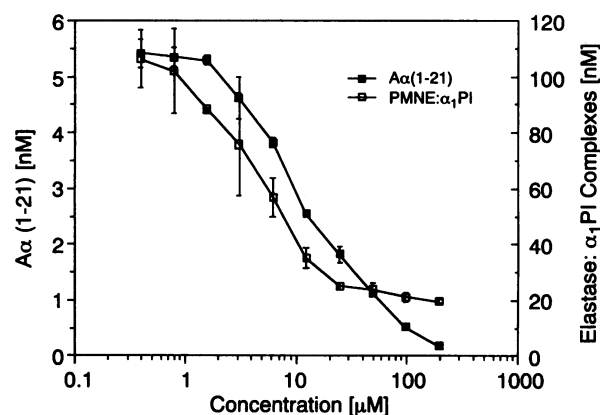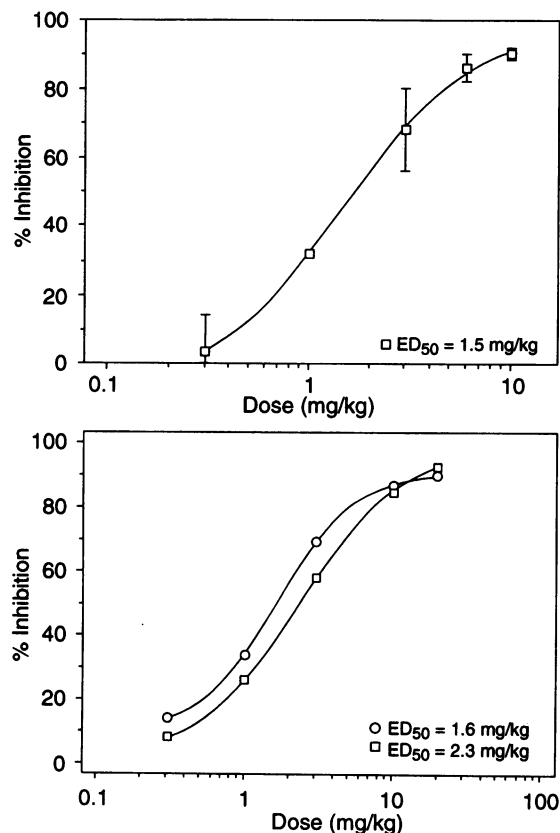FIG. 4.   (*Upper*) Inhibition of human PMNE *in vivo* after oral administration of 1 inhibits PMNE-induced lung hemorrhage in the hamster in a dose-dependent manner. Each point represents the mean ± SEM of three experiments. (*Lower*) Compound 1 inhibits the activity of elastase released from human PMN transferred into mouse pleural cavity. ○ and □, Data from two separate representative experiments.

to inhibit elastase released from human PMN *in vivo* was therefore evaluated in a model where isolated human peripheral blood PMN were transferred into the pleural cavity of mice and stimulated to release their elastase *in situ* by A23187. Under these conditions enzyme activity can be detected in a lavage of the pleural cavity. Oral dosing of the mice with 1 allowed potent and long-lived inhibition of the released enzyme (Fig. 4 *Lower*), giving an ED$_{50}$ of ≈2 mg/kg for up to 6 hr after drug administration.

The great destructive potential of PMNE has been long recognized (31), and it has been widely hypothesized that it may contribute to tissue damage in inflammatory diseases where the accumulation of PMN and the extracellular release of elastase are demonstrable. The studies described here indicate that time-dependent low molecular weight inhibitors such as 1 have the specificity and biochemical potency as well as the pharmacokinetic properties to determine whether oral administration of PMNE inhibitors will be useful in human disease, as recently recommended by a workshop considering new approaches to the therapy of emphysema (32).

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