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# INHIBITION OF $^{125}$I-CHEMOTACTIC PEPTIDE UPTAKE BY PROTEASE INHIBITORS

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Summary: A chymotrypsin inhibitor, 1-tosylamido-2-phenylethyl chloromethyl ketone and two chymotrypsin substrates, L-tyrosine-benzyl ester-p-tosylate and L-phenylalanine-benzyl ester-p-tosylate, irreversibly inhibited the uptake of formyl-Nle-Leu-Phe-Nle-Tyr($^{125}$I)-Lys by human neutrophils. The carboxylproteinase inhibitor pepstatin A reversibly inhibited the same process and induced chemotaxis of human neutrophils. For pepstatin A, a close correlation was found between inhibition of formyl peptide uptake and chemotaxis demonstrating that the biological activity of pepstatin A was mediated through the formyl peptide chemotactic receptor. The chymotrypsin inhibitors and substrates were one to two logs more potent as inhibitors of chemotaxis than as inhibitors of formyl peptide uptake demonstrating that the mechanism of inhibition of chemotaxis was independent of inhibition of uptake.

Extensive data suggest an essential role for a membrane-bound serine esterase in the chemotactic response of leukocytes to various chemotactic factors (1-4). This hypothesis is based on parallel inhibition of serine esterase activity and chemotaxis by small synthetic and natural macromolecular esterase inhibitors. Recently, pepstatin A, an inhibitor of carboxyproteinases secreted by species of actinomycetes, has been shown to induce chemotaxis of human peripheral leukocytes (5). The authors speculated that the chemotactic activity of this compound may have been related to its proteinase inhibitory activity.

However, interpretation of studies with protease inhibitors should be made

Abbreviations: 1-tosylamido-2-phenylethyl chloromethyl ketone, TPCK; L-phenylalanine-benzyl ester-p-tosylate, PBET; L-tyrosine-benzyl ester-p-tosylate, TBET; N-tosyl-L-lysine-chloromethyl ketone, TLCK; p-tosyl-L-arginine methyl ester, TAME; phenyl methylsulfonylfluoride, PMSF; soybean trypsin inhibitor, SBTI; ε-amino caproic acid, EACA; alpha-1-antitrypsin, $\alpha_1$-AT, N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys, formyl peptide; N-formyl-Nle-Leu-Phe-Nle-Tyr($^{125}$I)-Lys, $^{125}$I-formyl peptide.

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with caution as many of these inhibitors interact with proteins other than esterases or proteases. For example, N-tosyl-L-lysine-chloromethyl ketone, a covalent trypsin inhibitor, will covalently inhibit protein kinase (6). Phenylmethylsulfonylfluoride will covalently label many E. coli proteins in addition to serine esterases (7) and several esterase inhibitors will irreversibly inhibit binding to the cortisol receptor (8).

This latter observation suggested that the effect of the protease inhibitors on chemotaxis may also be at the level of the receptors for the various chemotactic factors. One such chemotactic factor, N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys, has been shown to bind to specific receptors on the plasma membrane of human neutrophils and be rapidly internalized in a receptor-mediated process (9,10). Here we report that serine esterase inhibitors with chymotrypsin specificity, irreversibly inhibit the uptake of this formyl peptide by human peripheral neutrophils, whereas pepstatin A reversibly inhibits the same process. There is a close correlation between the pepstatin A concentrations which inhibit formyl peptide uptake and those which induce chemotaxis, demonstrating that pepstatin A is simply acting as a low affinity analog of the formyl peptides. However, the concentrations of the serine esterase inhibitors which will inhibit chemotaxis are one to two logs lower than the concentrations required to achieve a comparable degree of uptake inhibition. Additionally, phenylmethylsulfonylfluoride inhibits chemotaxis without affecting $^{125}$I-formyl peptide uptake. This suggests that inhibition of chemotaxis by the serine esterase inhibitors is independent of their effect on formyl peptide uptake.

Materials and Methods: Radioiodine- and tetramethylrhodamine-labeled N-formyl-Nle-Leu-Phe-Nle-Tyr-Lys were prepared as previously described (9,10). TPCK, TLCK, PBET, TBET, PMSF, EACA, TAME, type III ovomucoid, type IV ovoinhibitor, leupeptin and pepstatin A were from Sigma. Alpha-1-antitrypsin and soybean trypsin inhibito were from Worthington.

Human peripheral neutrophils were obtained from healthy paid volunteers and isolated by the method of Boyum (11). Chemotaxis was estimated by the Boyden chamber technique (12). The $^{125}$I-formyl peptide uptake assay and the rhodamine-formyl peptide fluorescent assay were done as described previously (9,10).

Treatment of purified neutrophils with the protease inhibitors was performed as follows: 2 x $10^6$ cells/mL in 15 mM sodium phosphate, 123 mM NaCl, 1 mM $CaCl_2$, pH 6.75 were treated with the indicated concentration of inhibitor for 30 min at 37°C. The cells were pelleted and resuspended in the same buffer containing the inhibitor and 0.1% bovine serum albumin for the binding assays or

in Gey's balanced salt solution with the inhibitor and 2% bovine serum albumin for the chemotaxis assays. An aliquot of the treated cells was washed once and suspended in the same buffer without inhibitor to determine reversibility of the inhibition.

Results and Discussion: Several protease inhibitors and substrates with chymotrypsin specificity, including 1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), L-phenylalanine benzyl ester-p-tosylate (PBET) and L-tyrosine benzyl ester-p-tosylate (TBET), inhibited uptake of the $^{125}$I-formyl peptide by human neutrophils (table I). Analogous inhibitors and substrates with trypsin specificity, tosyl-L-lysine-chloromethyl ketone (TLCK) and p-tosyl-L-arginine methyl

Table 1. Effect of protease inhibitors on the uptake of $^{125}$I-formyl peptide by human neutrophils. Treatment and washing as described in Methods.

| | | Treated Uptake % of Control | After Washing Uptake % of Control |
|---|---|---|---|
| TPCK | 10 μM | 94 | 97 |
| | 25 μM | 62 | 60 |
| | 100 μM | 40 | 44 |
| PBET | 50 μM | 110 | 106 |
| | 250 μM | 66 | 71 |
| | 1 mM | 14 | 23 |
| TBET | 200 μM | 16 | 8 |
| | 1 mM | 21 | 12 |
| TLCK | 10 μM | 100 | |
| | 200 μM | 96 | |
| | 1 mM | 98 | |
| TAME | 500 μM | 109 | |
| | 2 mM | 112 | |
| | 10 mM | 61 | |
| Pepstatin A | 0.25 μM | 83 | 97 |
| | 1 μM | 55 | 88 |
| | 5 μM | 18 | 62 |
| PMSF | 50 μM | 100 | |
| | 200 μM | 98 | |
| | 500 μM | 103 | |
| SBTI | 1 μg/mL | 107 | |
| | 5 μg/mL | 107 | |
| | 20 μg/mL | 98 | |
| EACA | 1 mM | 108 | |
| | 20 mM | 103 | |
| | 80 mM | 104 | |
| $\alpha_1$-AT | 0.2 μg/mL | 100 | |
| | 1.0 μg/mL | 89 | |
| | 5.0 μg/mL | 92 | |
| Ovomucoid | 0.82 μg/mL | 96 | |
| | 8.2 μg/mL | 97 | |
| | 82 μg/mL | 100 | |
| Ovoinhibitor | 1 μg/mL | 98 | |
| | 5 μg/mL | 90 | |
| | 50 μg/mL | 93 | |
| Leupeptin | 24 mM | 97 | |
| | 48 mM | 103 | |
| | 96 mM | 96 | |

ester (TAME), did not affect uptake, except at much higher concentrations. Phenylmethylsulfonylfluoride (PMSF), a structurally dissimilar chymotrypsin inhibitor, also did not inhibit uptake.

As expected because of the reactivity of the chloromethyl ketone group, TPCK inhibition was irreversible, suggesting that a nucleophilic group, perhaps a sulfhydryl or imidazole nitrogen, was involved in the uptake process. However, inhibition produced by the non-reactive compounds, PBET and TBET, was also irreversible. This demonstrated that the inhibition was not due to direct covalent modification of the receptor, but to some other process, perhaps receptor endocytosis or a conformational change induced by binding of each of the analogs. TPCK, PBET and TBET did not affect cell viability as assayed by trypan blue exclusion.

Pepstatin A was also a potent inhibitor of uptake, although in this case the inhibition was readily reversible with washing. This may indicate that following binding, pepstatin A does not induce endocytosis or other processes producing irreversible inhibition. While this manuscript was in preparation, Nelson et al., demonstrated that pepstatin A would inhibit binding of N-formyl-Met-Leu-[$^{3}$H]Phe to human neutrophils (13).

Although the protease specificities of pepstatin A and the chymotrypsin inhibitors are quite different, they share several structural features. All are peptide analogs composed of hydrophobic residues with a blocked amino-terminus. These are the same features previously defined for biological activity of the formyl peptides (14). It is likely that the ability of these compounds to inhibit $^{125}$I-formyl peptide uptake is due to their structural similarity to the formyl peptides rather than to their activity as protease inhibitors.

When the potencies of these compounds as inhibitors of chemotaxis was compared with their potencies as inhibitors of formyl peptide uptake, a direct correlation was seen only for pepstatin A (Fig. 1). An $IC_{50}$ = 1.4 μM was determined for the inhibition of uptake of 1.5 nM $^{125}$I-formyl peptide and an $IC_{50}$ = 1.0 μM for inhibition of chemotaxis induced by the same concentration of non-

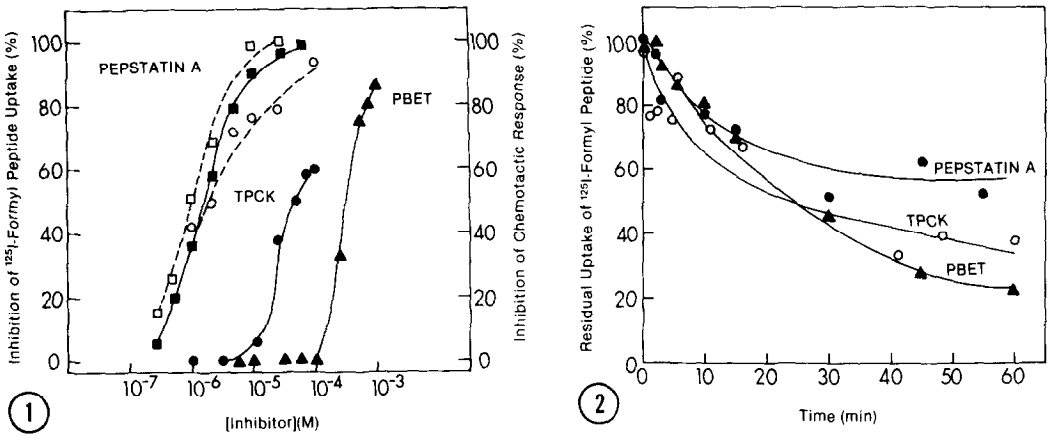


Figure 1. Correlation of inhibition of uptake of the $^{125}$I-formyl peptide (solid lines) with inhibition of chemotaxis (dashed lines) as a function of increasing protease inhibitor concentration. Treatment and assays were as described in Methods. TPCK (●—● and O---O), pepstatin A (■—■ and □---□) and PBET (▲—▲).

Figure 2. Inhibition of $^{125}$I-formyl peptide uptake as a function of time of preincubation with the various protease inhibitors. 2 μM pepstatin A (●—●), 25 μM TPCK (O—O) and 250 μM PBET (▲—▲). Following preincubation for the indicated times in the presence of the inhibitor at 37°C, the standard uptake assay was performed at 22°C for 30 min.

radioactive formyl peptide. Pepstatin A was itself chemotactic, 1.0 μM induced maximal chemotaxis (data not shown). This close correlation between inhibition of formyl peptide uptake and chemotaxis indicates that the chemoattractant activity of pepstatin A is due to its ability to interact with the formyl peptide receptor rather than to its anti-protease activity. The fact that inhibition of binding by pepstatin A was readily reversible, may suggest that chemotaxin endocytosis and chemotaxis can be dissociated for pepstatin A.

No similar correlation was seen for the serine esterase inhibitors. TPCK was 25-fold more potent as an inhibitor of chemotaxis than as an inhibitor of formyl peptide uptake. An $IC_{50}$ = 2 μM for chemotaxis and an $IC_{50}$ = 50 μM for uptake were determined (Fig. 1). PMSF, which did not inhibit uptake at concentrations up to 500 μM, abolished the chemotactic response at 200 μM. PBET and TBET caused cell aggregation in Gey's buffer so that chemotactic response could not be determined. TPCK, TBET, PBET and PMSF did not induce a chemotactic response by themselves.

The time dependence of the inhibition was similar for all three compounds and suggests that a process in addition to receptor binding was required for

inhibition (Fig. 2). This may be related to receptor aggregation or endocytosis, to a conformational change in the receptor or to masking by secretory or hydrolytic products as suggested by Gallin et. al., (15). However, the process was obviously not identical for all the compounds because the pepstatin A inhibition was reversible at all time points whereas the TPCK and PBET inhibition was not.

All three compounds would inhibit binding of $^{125}$I-formyl peptide to purified neutrophil membranes with an apparent $K_i$ of 1.5 μM for pepstatin A, $K_i$ of 50 μM for TPCK and $K_i$ of 250 μM for PBET. Even in membranes the inhibition was time dependent (data not shown). Additionally, 25 μM TPCK, which inhibited chemotaxis approximately 80%, did not inhibit uptake of the rhodamine-labeled formyl peptide as visualized using video intensification microscopy (10).

These data extend the observation of Aswanikumar et. al., (16) that natural bacterial products, gramicidin S, tyrocidin, bacitracin and now pepstatin A are chemotaxins by virtue of their ability to bind to the formyl peptide receptor. This raises the possibility that other, as yet undefined, natural mammalian products involved in the inflammatory response may also interact with this receptor. Recently, Tanswell and Lynn (17) have described a superoxide stimulating factor associated with the membrane of freshly isolated human neutrophils, which can be displaced by N-formyl-Met-Phe. This may be a natural mammalian product which interacts with the formyl peptide receptor.

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