*Biochemical Pharmacology*, Vol. 44, No. 9, pp. 1725–1730, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00


# INHIBITION OF AMINOPEPTIDASES N, A AND W

## A RE-EVALUATION OF THE ACTIONS OF BESTATIN AND INHIBITORS OF ANGIOTENSIN CONVERTING ENZYME

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(*Received* 29 *June* 1992; *accepted* 4 *August* 1992)

**Abstract**—The effects of a range of metallopeptidase inhibitors on the activities of the porcine kidney cell surface zinc aminopeptidases, aminopeptidase A (AP-A; EC 3.4.11.2), aminopeptidase N (AP-N; EC 3.4.11.7) and aminopeptidase W (AP-W; EC 3.4.11.16), have been directly compared. Amastatin and probestin were effective against all three aminopeptidases, with the concentration of inhibitor required to cause 50% inhibition ($I_{50}$) in the low micromolar range ($I_{50}$ = 1.5–20 $\mu$M), except for probestin with AP-N which displayed an $I_{50}$ of 50 nM. Actinonin failed to inhibit significantly either AP-A or AP-W, and thus can be considered a relatively selective inhibitor ($I_{50}$ = 2.0 $\mu$M) of AP-N. In contrast, bestatin was a relatively poor inhibitor of AP-N ($I_{50}$ = 89 $\mu$M) and failed to inhibit AP-A, but was more potent towards AP-W ($I_{50}$ = 7.9 $\mu$M). Thus, some of the observed chemotherapeutic actions of bestatin may be due to inhibition of cell-surface AP-W. A number of other metallopeptidase inhibitors, including inhibitors of endopeptidase-24.11 (EC 3.4.24.11) and membrane dipeptidase (EC 3.4.13.11), and the carboxylalkyl and phosphoryl inhibitors of angiotensin converting enzyme (EC 3.4.15.1) failed to inhibit significantly AP-A, AP-N or AP-W. However, AP-W was inhibited with $I_{50}$ values in the micromolar range by the sulphydryl converting enzyme inhibitors rentiapril ($I_{50}$ = 1.6 $\mu$M), zofenoprilat ($I_{50}$ = 7.0 $\mu$M) and YS 980 ($I_{50}$ = 17.7 $\mu$M). Neither AP-A nor AP-N were affected by these sulphydryl compounds. Inhibition of AP-W may account for some of the side effects noted with the clinical use of the sulphydryl converting enzyme inhibitors. The availability of compounds which are totally selective for AP-W over any of the other mammalian cell surface zinc aminopeptidases may aid in identifying endogenous substrates, and thus physiological or pathophysiological role(s) of AP-W.

Mammalian cell surface peptidases are involved in the metabolism of a range of biologically active peptides, including peptide hormones, neuropeptides and dietary peptides [1]. Some of them are proving to be therapeutic targets in a variety of disease states, including heart disease, inflammation and metastasis [2, 3]. Several cell surface peptidases have recently been identified as cluster differentiation (CD†) antigens. For example, endopeptidase-24.11 (EC 3.4.24.11) has been shown to be identical to the common acute lymphocytic leukaemia antigen (CALLA; CD10) [4], aminopeptidase N (AP-N; EC 3.4.11.2) to CD13 [5], dipeptidyl peptidase IV (EC 3.4.14.5) to CD26 [6] and aminopeptidase A (AP-A; EC 3.4.11.7) to the murine $\beta$-lymphocyte differentiation antigen BP1/6C3 [7]. The role(s) of these peptidases in metastasis is unclear, although the termination or generation of peptide signals affecting the proliferation of transformed and normal cells is a possibility. In addition, AP-N has been identified recently as a receptor in both pigs and humans for the coronaviruses TGEV and 229E, respectively [8, 9].

The zinc aminopeptidases constitute a relatively large subdivision of the mammalian cell surface peptidase family. To date four distinct enzymes have been characterized: AP-N, AP-A, aminopeptidase P (AP-P; EC 3.4.11.9) and aminopeptidase W (AP-W; EC 3.4.11.16). AP-P is somewhat different from the other three in being anchored in the plasma membrane by a glycosyl-phosphatidylinositol moiety [10] and in having a strict requirement for Pro in the penultimate position of susceptible substrates [11]. AP-N, the best characterized of this class, displays a broad substrate specificity [12] and has been identified as the major activity releasing the N-terminal Tyr from the enkephalins [13]. The enzyme may also play a role in the metabolism of cholecystokinin-8 [14] and neurokinin A [15]. AP-A hydrolyses acidic residues from the N-terminus of susceptible substrates and may be involved in the *in vivo* conversion of angiotensin II to angiotensin III [16]. AP-W preferentially hydrolyses short peptides and exhibits maximal rates towards dipeptides in which the $P_1'$ residue is aromatic [17, 18]. So far, AP-W has not been implicated in the metabolism of any biologically active peptide, due in part to the lack of a selective inhibitor. The specificities of these latter three aminopeptidases overlap to a certain extent often leading to difficulties in identifying unequivocally the enzyme responsible for the metabolism of a particular peptide.

Although several inhibitors of the aminopeptidases are available, most of these are effective on more than one enzyme. We observed recently that certain compounds designed as supposedly selective inhibitors of angiotensin converting enzyme (EC

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† Abbreviations: AP-A, aminopeptidase A; AP-N, aminopeptidase N; AP-W, aminopeptidase W; AP-P, aminopeptidase P; CD, cluster differentiation antigen; $I_{50}$, concentration of inhibitor required to cause 50% inhibition.

3.4.15.1) also inhibited AP-P with $I_{50}$ values in the micromolar range [19]. In the present study we have extended this work by directly comparing the effect of a range of converting enzyme inhibitors on the activities of AP-A, -N and -W in an attempt to identify compounds which could be used to discriminate between these activities and thus be useful tools for delineating the role of a particular peptidase in the metabolism of a biologically active peptide. In addition, we have reassessed the inhibitory profile of a number of compounds which have been characterized as inhibitors of the zinc aminopeptidases.

## MATERIALS AND METHODS

*Materials.* AP-N and AP-W were purified from pig kidney cortex as described previously [17, 20]. Both enzyme preparations were apparently homogenous as assessed by SDS–PAGE. AP-N had a specific activity of 41.2 μmol 4-methyl-7-coumarylamide/min/mg of protein, and AP-W a specific activity of 15.03 μmol $PheNH_2$/min/mg of protein. Microvillar membranes were prepared from pig kidney cortex as described by Booth and Kenny [21], except that the 15,000 *g* centrifugation steps were each extended from 12 to 15 min. Enalaprilat (MK 422), lisinopril (MK 521), L155,212, L155,502 and L155,524 were gifts from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ, U.S.A.). Spiraprilat (SCH 33861) was a gift from Schering-Plough Research (Bloomfield, NJ, U.S.A.). Pentoprilat (CGS 13934) was a gift from Ciba-Geigy Corporation (Summit, NJ, U.S.A.). Quinaprilat (PD 109548-1) and indolaprilat (PD 110021-0) were gifts from Parke-Davis Pharmaceutical Research Division (Ann Arbor, MI, U.S.A.). Cilazaprilat (Ro 31-3113/000) was a gift from Roche Products Ltd (Welwyn Garden City, U.K.). Rentiapril (SA 446) and YS 980 were gifts from Santen Pharmaceutical Co. Ltd (Osaka, Japan). Ramiprilat was a gift from Hoechst Pharmaceutical Research Laboratories (Milton Keynes, U.K.). Zofenoprilat (SQ 26,703), fosinoprilat (SQ 27,519), ceranopril (SQ 29,852) and captopril (SQ 14,225) were gifts from Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ, U.S.A.). Probestin was a gift from Dr T. Aoyagi (Tokyo, Japan). Arphamenines A and B and Asp-$PheNH_2$ were obtained from the Sigma Chemical Co. (Poole, U.K.). All other materials were from sources noted previously.

*Enzyme assays.* AP-A was assayed in pig kidney microvillar membranes (50 μg protein) with α-Glu-4-methyl-7-coumarylamide (0.2 mM) as substrate in 0.1 M Tris–HCl, 1 mM $CaCl_2$, pH 7.4 at 37°. The product, 4-methyl-7-coumarylamide, was detected fluorimetrically at an excitation wavelength of 370 nm and an emission wavelength of 442 nm [22]. AP-N (100 ng) was assayed with L-Ala-4-methyl-7-coumarylamide (0.2 mM) as substrate in 0.1 M Tris–HCl, pH 7.4 at 37° [22]. AP-W (100 ng) was assayed with Asp-$PheNH_2$ (1 mM) as substrate in 0.1 M Tris–HCl, pH 7.4 at 37°. The product, $PheNH_2$, was separated from the substrate and was quantified by reverse phase HPLC as described for Gly-D-Phe [23]. Incubations were performed in duplicate with each concentration of inhibitor. Enzyme and inhibitors were preincubated for 15 min at 4°.

## RESULTS

The effect on the activity of AP-A, -N and -W of specific metallopeptidase inhibitors (at a final concentration of 0.1 mM) was examined (Table 1). AP-A was inhibited significantly (>69%) only by amastatin and probestin. Actinonin and arphamenine B caused partial inhibition of AP-A. AP-N was inhibited significantly (>91%) by actinonin, amastatin and probestin, but less so (52%) by bestatin. AP-W was inhibited significantly (>89%) by amastatin, bestatin and probestin. Arphamenine A also caused partial inhibition of AP-W. The inhibitors of endopeptidase-24.11, phosphoramidon, thiorphan and LI55,524, and the inhibitor of membrane dipeptidase, cilastatin, failed to inhibit

Table 1. Effect of metallopeptidase inhibitors on the activities of AP-A, -N and -W

| Class of peptidase | Inhibitor | Relative activity (%) at an inhibitor concentration of 0.1 mM AP-A | AP-N | AP-W |
|---|---|---|---|---|
| | None | 100.0 | 100.0 | 100.0 |
| Aminopeptidase | Actinonin | 75.0 ± 2.4 | 9.0 ± 1.0 | 104.0 ± 1.1 |
| | Amastatin | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | Arphamenine A | 100.0 ± 3.0 | 74.0 ± 4.7 | 50.0 ± 3.0 |
| | Arphamenine B | 68.8 ± 2.0 | 105.0 ± 8.2 | 93.0 ± 6.2 |
| | Bestatin | 100.0 ± 2.5 | 48.0 ± 11.0 | 11.0 ± 1.0 |
| | Probestin | 31.0 ± 6.8 | 0.0 ± 0.0 | 6.8 ± 2.0 |
| Endopeptidase | Phosphoramidon | 100.0 ± 0.0 | 95.0 ± 0.0 | 97.3 ± 3.0 |
| | Thiorphan | 105.0 ± 2.0 | 84.0 ± 3.2 | 70.0 ± 14.0 |
| | LI55,524 | 100.0 ± 0.0 | 93.0 ± 0.0 | 86.0 ± 2.0 |
| Dipeptidase | Cilastatin | 103.0 ± 2.6 | 103.0 ± 4.0 | 83.0 ± 4.4 |

The aminopeptidases were assayed with the appropriate substrate as described in Materials and Methods.

The results are the means ± SEM of three independent determinations with each inhibitor.

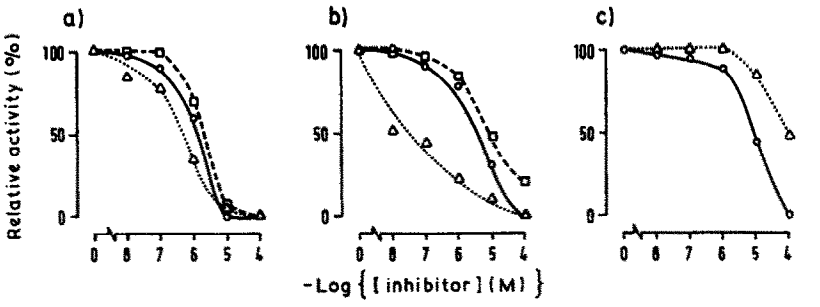


Fig. 1. Inhibition of AP-A, -N and -W by amastatin, bestatin and probestin. The aminopeptidases were assayed with the appropriate substrate as described in Materials and Methods. Each point is the mean of triplicate determinations. (a) Amastatin; (b) probestin; (c) bestatin. (□) AP-A; (△) AP-N; (○) AP-W.

Table 2. Effect of angiotensin converting enzyme inhibitors on the activities of AP-A, -N and -W

| Class of inhibitor | Inhibitor | Relative activity (%) at an inhibitor concentration of 0.1 mM AP-A | AP-N | AP-W |
|---|---|---|---|---|
| | None | 100.0 | 100.0 | 100.0 |
| Sulphydryl | Captopril | 88.2 ± 0.0 | 94.2 ± 2.0 | 43.6 ± 3.0 |
| | Rentiapril | 87.2 ± 1.7 | 90.0 ± 0.4 | 0.0 ± 0.0 |
| | YS 980 | 94.0 ± 3.4 | 92.0 ± 1.0 | 20.0 ± 2.0 |
| | Zofenoprilat | 94.0 ± 0.0 | 95.0 ± 0.0 | 17.6 ± 6.6 |
| Carboxylalkyl | Benazeprilat | 98.0 ± 6.1 | 100.0 ± 0.0 | 92.0 ± 2.6 |
| | Cilazaprilat | 110.0 ± 5.0 | 88.0 ± 2.0 | 73.0 ± 4.6 |
| | Enalaprilat | 120.0 ± 0.0 | 86.0 ± 3.4 | 83.9 ± 3.5 |
| | Indolaprilat | 104.0 ± 3.0 | 70.0 ± 0.5 | 80.5 ± 2.0 |
| | Lisinopril | 103.0 ± 2.6 | 100.0 ± 0.0 | 72.3 ± 6.0 |
| | L155,212 | 96.0 ± 4.0 | 98.0 ± 1.6 | 86.5 ± 0.2 |
| | L155,502 | 100.0 ± 11.0 | 100.0 ± 0.0 | 90.0 ± 2.0 |
| | Pentoprilat | 100.0 ± 5.0 | 76.0 ± 0.0 | 73.0 ± 0.5 |
| | Quinaprilat | 104.0 ± 11.0 | 85.0 ± 2.2 | 90.5 ± 2.5 |
| | Ramiprilat | 105.0 ± 10.0 | 76.0 ± 0.6 | 107.0 ± 5.0 |
| | Spiraprilat | 95.2 ± 5.0 | 77.0 ± 0.8 | 72.0 ± 0.7 |
| Phosphoryl | Ceranopril | 100.0 ± 0.0 | 100.0 ± 0.0 | 87.0 ± 6.0 |
| | Fosinoprilat | 100.0 ± 0.0 | 98.0 ± 0.6 | 94.8 ± 2.5 |

The aminopeptidases were assayed with the appropriate substrate as described in Materials and Methods.

The results are the means ± SEM of three independent determinations with each inhibitor.

any of the aminopeptidases significantly (maximally 17%), except for thiorphan which caused 30% inhibition of AP-W. The inhibition of the three aminopeptidases by amastatin, bestatin and probestin was examined in more detail (Fig. 1). Amastatin was equipotent with all three aminopeptidases ($I_{50}$ = 1.5–3.2 μM). However, probestin was some 100–400-fold more potent an inhibitor of AP-N ($I_{50}$ = 50 nM) than of either AP-A or AP-W ($I_{50}$ = 19.9 and 5.0 μM, respectively). In contrast, bestatin was an 11-fold more potent inhibitor of AP-W ($I_{50}$ = 7.9 μM) than AP-N ($I_{50}$ = 89.1 μM).

The effect on the activity of AP-A, -N and -W of specific inhibitors of angiotensin converting enzyme (at a final concentration of 0.1 mM) was also examined (Table 2). AP-A was not significantly inhibited (maximally 13%) by any of the inhibitors tested. Only indolaprilat, pentoprilat, ramiprilat and spiraprilat caused partial inhibition (maximally 30%) of AP-N. None of the carboxylalkyl or phosphoryl inhibitors caused >28% inhibition of AP-W. However, the sulphydryl compounds, captopril, rentiapril, YS 980 and zofenoprilat, all caused significant (>54%) inhibition of AP-W. The effect of these sulphydryl compounds on the activity of AP-W was examined in more detail (Fig. 2). These results indicate that rentiapril ($I_{50}$ = 1.6 μM) was the most potent at inhibiting AP-W, with zofenoprilat ($I_{50}$ = 7.0 μM), YS 980 ($I_{50}$ = 17.7 μM) and captopril ($I_{50}$ = 199 μM) being increasingly less potent.

## DISCUSSION

In the present study we have directly compared

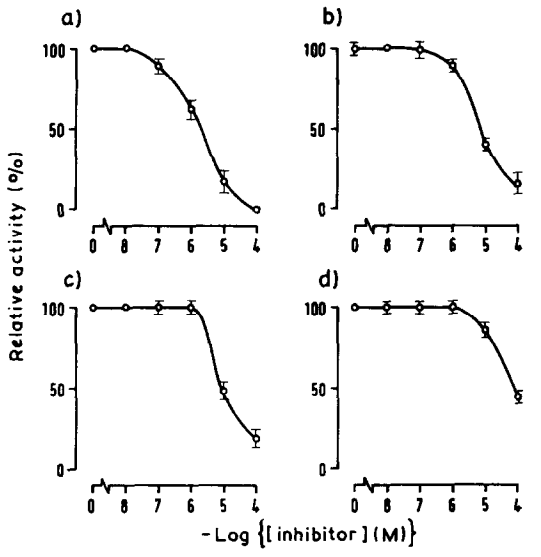


Fig. 2. Inhibition of AP-W by converting enzyme inhibitors. Pig kidney AP-W was assayed with 1 mM Asp-PheNH$_2$ as described in Materials and Methods. Each point is the mean of triplicate determinations. (a) Rentiapril; (b) zofenoprilat; (c) YS 980; (d) captopril.

Table 3. Comparison of the potency of inhibitors on the activities of AP-A, -N and -W

| Inhibitor | $I_{50}$ ($\mu$M) AP-A | AP-N | AP-W |
|---|---|---|---|
| Actinonin | >100 | 1.99 | NI |
| Amastatin | 2.00 | 3.16 | 1.58 |
| Bestatin | NI | 89.1 | 7.9 |
| Probestin | 19.9 | 0.05 | 5.0 |
| Captopril | NI | NI | 199 |
| Rentiapril | NI | NI | 1.6 |
| YS 980 | NI | NI | 17.7 |
| Zofenoprilat | NI | NI | 7.0 |

The $I_{50}$ value is the concentration of inhibitor giving 50% inhibition of enzyme activity under the assay conditions described in Materials and Methods.

NI, negligible inhibition observed at an inhibitor concentration of 0.1 mM.

the effect of several metallopeptidase inhibitors on the activities of the cell surface zinc aminopeptidases A, N and W in an attempt to identify compounds which could be used in metabolic studies to discriminate between these activities. During this work we also reassessed the inhibitory action of a number of previously characterized aminopeptidase inhibitors. Bestatin (Ubenimex) was originally identified as an inhibitor of the soluble aminopeptidase B and the cytosolic leucine aminopeptidase [24, 25]. Subsequently, it was shown to inhibit purified AP-N [26] and AP-W [18]. The results of the present study indicate that bestatin is a more potent inhibitor of AP-W than AP-N ($I_{50}$ values of 8 and 89 $\mu$M, respectively) (see Table 3). These results are somewhat surprising as bestatin has long been considered a potent inhibitor of AP-N and has been used extensively to inhibit this activity in metabolic studies [27, 28]. It was originally noted, however, that bestatin inhibited monkey and canine kidney cell surface leucine aminopeptidase activity (probably AP-N) with an $I_{50}$ value in the range 32–162 $\mu$M [29]. Bestatin has been shown to display immunomodulating properties [29–31] and to act as an antitumour agent [32–34]. These chemotherapeutic actions are believed to be due, in part, to its inhibition of cell surface AP-N. However, in the light of the present results, these actions may either/also be due to inhibition of cell surface AP-W.

Probestin was isolated recently from *Streptomyces azureus* and shown to inhibit AP-N [35]. In the present study, we show that probestin also inhibits AP-A and AP-W with $I_{50}$ values in the micromolar range (see Table 3). Thus, probestin appears to be a relatively broad acting aminopeptidase inhibitor, although it is somewhat more potent against AP-N. Amastatin was originally identified as an inhibitor of AP-A [36], and has since been shown to inhibit also AP-N [26] and AP-W [18]. In this study, we have directly compared the inhibitory potency of amastatin with AP-A, -N and -W and obtained results in agreement with these earlier reports, indicating that amastatin is essentially equipotent against these three enzymes (see Table 3). Actinonin was originally identified as an inhibitor of AP-N which lacked activity towards AP-A [37], and it has also been shown not to inhibit AP-W [18]. These results have been confirmed in the present study (see Table 3). Thus, within the cell surface aminopeptidase family, actinonin can be considered as a selective inhibitor of AP-N.

Due to the key role played by angiotensin converting enzyme in the renin–angiotensin system, numerous inhibitors of this zinc peptidyl dipeptidase have been synthesized and some have been used successfully in the treatment of hypertension and congestive heart failure [38, 39]. These compounds can be divided into three classes depending on the group that coordinates to the active site zinc atom: (i) sulphydryl, (ii) carboxylalkyl and (iii) phosphoryl. Recently, we observed that AP-P was sensitive to inhibition by some of these converting enzyme inhibitors [19]. In the present study we have extended these observations by assessing the effect of inhibitors of angiotensin converting enzyme on AP-A, -N and -W. None of the compounds examined caused significant inhibition of AP-A or AP-N. However, the sulphydryl compounds rentiapril, zofenoprilat and YS 980 all inhibited AP-W with $I_{50}$ values in the micromolar range (1.6–17.7 $\mu$M). These sulphydryl compounds are all analogues of captopril [38]. YS 980 is structurally identical to captopril apart from having a sulphur atom at position 4 of the prolyl ring. Zofenoprilat differs from captopril in possessing an aromatic ring attached to position 4 of the prolyl ring by a sulphur bridge, while rentiapril differs from YS 980 in having a hydroxylated aromatic ring attached to position 5 of the prolyl ring. The increased potencies towards AP-W of rentiapril and

zofenoprilat may be due to their large aromatic moieties mimicking the aromatic $P_1'$ residue of the substrate. The order of potency of these sulphydryl compounds is essentially the reverse of that seen with AP-P [19] where the order was YS 980 ($I_{50}$ = 19.5 $\mu$M), captopril ($I_{50}$ = 110 $\mu$M), zofenoprilat ($I_{50}$ = 440 $\mu$M) and rentiapril caused only 35% inhibition at a concentration of 1 mM. The slight inhibition of AP-W observed with thiorphan (Table 1) is probably also due to the zinc coordinating sulphydryl group in this compound.

Even though the sulphydryl converting enzyme inhibitors are 1000-fold less potent at inhibiting AP-W than angiotensin converting enzyme [19], an *in vitro* $I_{50}$ value in the micromolar range may be significant in causing inhibition of AP-W *in vivo*. For example, teprotide, the first effective converting enzyme inhibitor *in vivo*, displays an $I_{50}$ toward the enzyme of 1.0 $\mu$M [40]. In addition, the inhibitor of membrane dipeptidase (EC 3.4.13.11), cilastatin, which is coadministered with the $\beta$-lactam antibiotic imipenem to prevent its metabolism by dipeptidase in the kidney, displays an $I_{50}$ value of 0.1 $\mu$M *in vitro* [41]. In clinical use, numerous side effects have been noted with captopril, including coughing, skin rashes and taste disturbance [39, 42]. The cause of these adverse effects remains unclear, although similar side effects have been noted with penicillamine leading to speculation that the sulphydryl group may be responsible. The results of the present study suggest that these effects may be due to additional inhibition of AP-W by the sulphydryl converting enzyme inhibitors.

In conclusion, we have shown that both amastatin and probestin have broad inhibitory profiles acting on the three cell surface zinc aminopeptidases A, N and W. We also confirm that actinonin is a relatively selective inhibitor of AP-N, but that bestatin is a relatively poor inhibitor of this peptidase and is much more potent towards AP-W. This raises the question as to whether the chemotherapeutic properties of bestatin may involve inhibition of AP-W. The observation that the sulphydryl converting enzyme inhibitors, rentiapril and zofenoprilat, are relatively potent and totally specific for AP-W over any of the other cell surface zinc aminopeptidases should enable the role of this enzyme in the metabolism of bioactive peptides under normal and disease situations to be assessed, and may explain some of the side effects noted with the clinical use of these compounds. However, no selective inhibitor of AP-A has been identified.

*Acknowledgements*—We thank Mrs J. Ingram for the purified samples of aminopeptidases N and W. S.T. is in receipt of a British Council Studentship.

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