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CHARACTERIZATION OF THE DEAMIDASE ENZYME RESPONSIBLE FOR THE METABOLISM OF THE ANTI-CANCER PEPTIDE: H–Arg–D-Trp–N^{me}Phe–D-Trp–Leu–Met–NH_2

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Abstract—H–Arg–D-Trp–N^{me}Phe–D-Trp–Leu–Met–NH_2, a broad spectrum neuropeptide growth factor antagonist (antagonist G), is soon to enter a phase I clinical trial for the treatment of small-cell lung cancer (SCLC). The pre-clinical pharmacology of this peptide has revealed that its metabolism proceeds from the C-terminus via deamidation. In this study the class of enzyme responsible for the degradation of antagonist G has been characterized. Tissue distribution studies on the enzyme have shown it to be very widespread with high specific activity being detected in the spleen, kidney, H69 SCLC xenograft and liver (12.64, 9.58, 8.00 and 6.94 nmols G/mg protein/hr, respectively). HPLC gel filtration indicated that the G-deamidase enzyme had an apparent molecular mass of 81 kDa. The sub-cellular distribution of the enzyme using differential centrifugation indicates that it is largely soluble with > 85% of the activity located in the cytosolic fraction. The distribution of activity towards antagonist G closely resembles that of esterase and acid carboxypeptidase activity, two activities, along with deamidase activity, known to be possessed by serine carboxypeptidases. Studies using a range of protease inhibitors showed clear inhibition of metabolism by phenylmethylsulphonylfluoride and benzyloxycarbonyl-phenylalanine chloromethylketone, indicating that the enzyme is a chymotrypsin-like serine carboxypeptidase. This knowledge of the enzyme will be invaluable in the further development of antagonist G and similar compounds. Moreover, the widespread distribution of this enzyme together with its broad specificity for C-terminal group suggests that it should be given serious consideration when designing C-terminally modified peptide drugs.

Key words: peptide drug; small-cell lung cancer; metabolism; peptidase; deamidation; inhibition

Antagonist G† will be the first of a new class of anti-cancer agents, broad spectrum neuropeptide growth factor antagonists, to enter into a phase I clinical trial for the treatment of SCLC. The biological activity of this type of drug is thought to reside in its ability to competitively inhibit the binding of several neuropeptide growth factors, including bombesin/GRP, bradykinin and vasopressin, to their specific cell surface receptors [1]. In SCLC a variety of these neuropeptides have been shown to stimulate cell proliferation as part of a complex paracrine/autocrine network [2–4]. Interruption of this growth stimulatory network is likely to prove an effective means of therapy for this disease, as shown in the clinical trial of a monoclonal antibody that blocked the bombesin/GRP response [5]. Antagonist G has previously been shown to inhibit the growth of subcutaneous SCLC xenografts (H69, WX322) *in vivo* following administration to *nu/nu* mice [6].

As with any peptide drug, metabolism plays a critical role in its effectiveness *in vivo*, peptidase action could degrade and consequently inactivate the drug prior to it exerting its biological effect [7]. Metabolic stability is of greater importance to this type of anti-cancer agent than to conventional cytotoxic agents since its activity relies upon it being present in sufficient concentrations to exhibit competitive inhibition of the neuropeptide growth factors involved. As part of the preclinical evaluation of antagonist G, its metabolism was thoroughly characterized and insights were gained into the fate of this compound *in vivo*. The measures taken in the design of antagonist G to increase its peptidase resistance, namely the introduction of N^{α}-methylphenylalanine and D-tryptophan, were shown to be effective but metabolism did still occur [8]. The major metabolites of antagonist G were isolated and their structures elucidated to reveal a pathway of metabolism that was initiated by C-terminal deamidation followed by carboxypeptidase removal of the terminal methionine residue (see Fig. 1) [9]. The type of enzyme(s) involved were demonstrated to be serine protease-like in nature, a result not unexpected since serine carboxypeptidases are known to possess the ability to hydrolyse peptide

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† Abbreviations: antagonist G, H–Arg–D-Trp–N^{me}Phe–D-Trp–Leu–Met–NH_2; BTEE, benzoyl-tyrosine ethyl ester; EDTA, ethylenediaminetetraacetic acid; FAPP, *N*-(3(2-furyl)-acryloyl)-phenylalanine; GRP, gastrin-releasing peptide; IAA, iodoacetamide; PMSF, phenylmethylsulphonylfluoride; RP-HPLC, reverse-phase high-performance liquid chromatography; SCLC, small-cell lung cancer; SKP buffer, 0.1 M potassium phosphate buffer, pH 7.0, 0.25 M sucrose; TFA, trifluoroacetic acid; TLCK, *p*-tosyl-lysine-chloromethylketone; ZFCK, benzyloxy-carbonyl-phenylalanine-chloromethylketone.

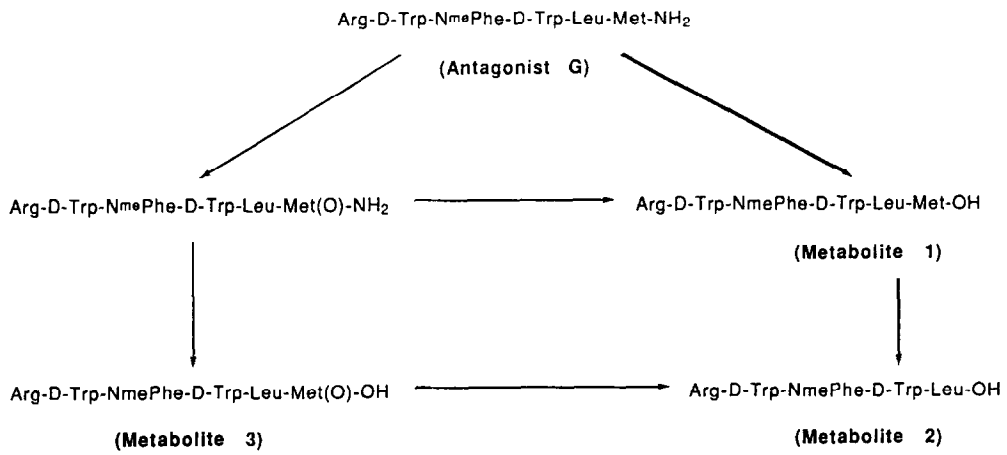

Fig. 1. Pathway of metabolism of H–Arg–D-Trp–N^{me}Phe–D-Trp–Leu–Met–NH_2 (antagonist G).

amides as well as peptide free acids and peptide esters [10]. In this paper we report further characterization of the enzymes involved in the degradation of antagonist G, including tissue and sub-cellular distribution, gel-filtration properties and the effect of more selective protease inhibitors than previously used. Also reported is the sub-cellular distribution of the esterase and acid carboxypeptidase activity, this could be expected to mirror the distribution of the enzymes responsible for antagonist G degradation if, as believed, it is serine carboxypeptidases that are the active enzyme group.

MATERIALS AND METHODS

Materials. Antagonist G was supplied by Peptech (Europe) (Copenhagen, Denmark). Acetic acid, sucrose, potassium dihydrogen phosphate and di-potassium hydrogen phosphate were from BDH Chemicals (Poole, Dorset, U.K.). Acetonitrile was HPLC grade and purchased from Rathburn Chemicals (Walkerburn, Scotland, U.K.). TFA, sodium chloride and all protease inhibitors were from Sigma Chemical Co. (Poole, Dorset, U.K.). All chemicals were of the highest grade commercially available and were used without further purification. Water was de-ionized and bi-distilled in a quartz glass still.

Animals. Female nude (*nu/nu*) mice were obtained from OLAC (Oxford, U.K.) and maintained in negative pressure isolators (La Calhene, Cambridge, U.K.). NCI-H69 SCLC xenografts were derived by implantation of 10^7 cells of NCI-H69 cell line into the flanks of the female mice described above. Xenografts were grown as subcutaneous tumours until used.

RP-HPLC. The HPLC system consisted of two 510 pumps, a 712 WISP autosampler, a TCM column heater, a Model 441 UV absorbance detector (all Waters, Northwich, U.K.), a Primesphere 5u ODS-MC analytical column fitted with a 3 cm pre-column (Phenomenex, Macclesfield, U.K.) and a 1046A fluorescence detector (Hewlett Packard, Walborn, Germany). Waters MAXIMA 820 computer software was used with a Waters system interface module to control the chromatographic system and to collect and integrate data. Peptides were separated by isocratic elution with 45% (v/v) acetonitrile in 0.1% (v/v) aqueous TFA detection being by fluorescence (Ex 233 nm; Em 395 nm).

Tissue distribution studies. Mouse organs and tumour xenografts were removed and stored at −20° until used. The tissues were thawed and homogenized in phosphate buffered saline (PBS; 10% (w/v); pH 7.4) to give a final homogenate consisting of 10% (w/v) tissue. The protein concentration in each homogenate was estimated by the Biuret method using BSA as a standard [11]. The degradation of antagonist G by each tissue was studied by introducing 0.1 mg of the peptide into 1.0 mL of incubation mixture containing 0.2 mL of 10% tissue homogenate (w/v) and 0.8 mL PBS. The mixture was incubated at 37° for 60 min before the reaction was terminated by the addition of 0.1 mL of glacial acetic acid to each sample followed by vigorous vortexing. A 0.2 mL sample of the acidified mixture was then taken into 0.8 mL of 0.1 M acetic acid and this solution was centrifuged at 14 000 rpm in an Eppendorf bench-top centrifuge for 5 min. The degree of metabolism was ascertained by analysing 0.2 mL of the resultant supernatant on RP-HPLC as described above.

Sub-cellular fractionation. The sub-cellular distribution of the enzyme activity towards antagonist G (G-deamidase), BTEE (esterase) and FAPP (acid carboxypeptidase) was studied in mouse liver and NCI-H69 xenograft. First the mouse liver (or tumour xenograft) was washed with PBS and then transferred to cold 0.25 M sucrose, 0.1 M potassium phosphate buffer pH 7.0 (SKP buffer) in which an homogenate (10%, w/v) was produced. This homogenate was then used to prepare nuclear, mitochondrial, microsomal and cytosolic sub-cellular fractions by differential centrifugation. The nuclear fraction was produced by resuspension of the pellet formed by a low-speed spin (800 *g* for 10 min at 4°). The pellet

was resuspended in 6 mL of SKP buffer and the low-speed spin repeated. The washed pellet was resuspended in 10 mL of SKP buffer and this constituted the nuclear fraction. The mitochondrial fraction was prepared from the post-nuclear supernatant by centrifugation at 10 000 *g* for 25 min at 4°. The pellet was resuspended in 6 mL of SKP buffer and the centrifugation repeated. The pellet was then resuspended in 10 mL of SKP buffer and this constituted the mitochondrial fraction. The post-mitochondrial supernatant was then subjected to a high-speed spin (100 000 *g* for 70 min at 4°). The pellet produced was resuspended in 6 mL of SKP buffer and the high speed spin repeated. The washed pellet was resuspended in 10 mL of SKP buffer and this constituted the microsomal fraction. The post-microsomal supernatant was subjected to another high-speed spin as above and the resultant supernatant was made up to a total volume of 10 mL with SKP buffer. This constituted the cytosolic fraction. The protein content of each fraction was estimated by the Biuret method using BSA as a standard [11], typical yields were as follows: nuclear fraction 1.11 mg protein/mL; mitochondrial fraction 6.14 mg protein/mL; microsomal fraction 7.82 mg protein/mL and cytosol 15.38 mg protein/mL.

Sub-cellular distribution of enzyme activity. Degradation of antagonist G (G-deamidase activity) was measured by mixing 0.2 mL of the respective fraction with 0.6 mL of 0.1 M potassium phosphate buffer (pH 7.4) and 0.1 mL of 1.0 M sodium chloride. The incubation mixture was brought to 37° before the addition of 0.1 mg of antagonist G. The incubation was maintained at 37° for 60 min before the reaction was terminated by the addition of 0.1 mL of glacial acetic acid. The degree of degradation was quantified in the same manner as described above for the tissue distribution studies.

Esterase activity was measured by the degradation of BTEE [12]. Typically, 0.02 mL of sub-cellular fraction was placed in 1.0 mL of 0.1 M potassium phosphate buffer (pH 7.4 37°) containing 0.1 M sodium chloride and 0.1 mM substrate. The exception to this was the cytosolic fraction where 0.3 mM substrate was used. The mixture was incubated at 37° for 60 min then 0.1 mL was removed and mixed with 0.1 mL of 1.0 M acetic acid. The metabolism was analysed on the above described RP-HPLC system using an isocratic elution with acetonitrile (40%, v/v) in aqueous TFA (0.1%, v/v). The degree of decomposition of the substrate was determined by the decrease in size of the substrate peak detected at 254 nm.

Acid carboxypeptidase activity was measured by the degradation of FAPP [10] when 0.02 mL of sub-cellular fraction was placed in 1.0 mL of PBS, which had been adjusted to pH 5.5 with 1.0 M acetic acid, containing 0.1 mM substrate. Incubations were performed at 37° for 60 min before 0.1 mL of the mixture was removed, mixed with 0.1 mL of 1.0 M acetic acid and analysed on RP-HPLC using an isocratic elution in acetonitrile (40%, v/v) in aqueous TFA (0.1%, v/v). The degree of decomposition was determined by the decrease in the size of the substrate peak detected at 340 nm.

HPLC gel filtration chromatography. HPLC gel filtration chromatography was performed on the system described above using a BIOSEP™-SEC-S3000 (300 mm × 7.8 mm) column (Phenomenex, Macclesfield, U.K.) fitted with a BIOSEP™-EXPRESS (35 mm × 7.8 mm) pre-column. The column was equilibrated with 0.5 M potassium phosphate buffer pH 6.8 at ambient room temperature. Calibration of the column was achieved using the Bio-Rad protein standard (Bio-Rad, Hemel Hempstead, U.K.). Peaks eluting from the column were monitored at 280 nm. Separation of the components in the cytosolic fraction of mouse liver or NCI-H69 xenograft was achieved by loading 0.02 mL of the respective fraction onto the column and collection of the eluted peaks. This procedure was repeated several times for each cytosol and the identical peaks from each elution were combined and stored on ice until assayed for enzyme activity. The peaks were assayed for G-deamidase activity, esterase activity and acid carboxypeptidase activity by mixing 0.1 mL of the peak with 0.9 mL of PBS (pH 7.4 for G-deamidase and esterase assay and pH 5.5 for acid carboxypeptidase assay) containing the appropriate substrate (0.05 mM antagonist G, 0.1 mM BTEE or 0.1 mM FAPP) and incubating at 37° for 60 min prior to analysis on RP-HPLC as described earlier for the sub-cellular studies. The protein content of each peak was estimated by its absorbance at 280 nm and based on the formula 1.0 mg/mL = 1.48 OD units [13].

Effect of protease inhibitors on the degradation of antagonist G. The degradation of antagonist G (0.1 mM) in 1.0 mL of mouse liver homogenate (2%, w/v) when incubated at 37° for 60 min was studied in the presence or absence of protease inhibitors ranging in concentration from 0 to 2.5 mM. The inhibitors studied were PMSF, IAA, EDTA, TLCK and ZFCK. Where inhibitors were present they were pre-incubated with the homogenate for 15 min at 23° prior to substrate addition. After 60 min the reaction was arrested by the addition of 0.1 mL of glacial acetic acid and the mixture analysed by RP-HPLC as previously described.

RESULTS AND DISCUSSION

Tissue distribution of G-deamidase activity

The enzyme, or isoforms thereof, capable of degrading antagonist G was shown to be widely distributed (see Table 1). The tissue with greatest G-deamidase activity was the spleen, its homogenate having a specific activity of 12.64 nmols G/mg protein/hr. Activity was also high in H69 xenograft, liver and kidney. Plasma was devoid of G-deamidase activity, a result that reflects earlier results reported with human plasma [8]. These results indicate that as soon as antagonist G is absorbed from the plasma into any tissue, following systemic administration, it is likely to be metabolized. The same pattern of metabolism appeared to be proceeding in all tissues studied suggesting that similar if not identical enzymes were active in each case. There were other minor metabolites detected after incubation with kidney homogenate and this may reflect a slightly different enzyme profile in this tissue as compared to all the others.

Table 1. Tissue distribution of G-deamidase activity

Tissue homogenate	Protein concentration (mg/mL)	G-deamidase activity (SD*) (nmolsG/mg protein/hr)
Brain	26.97	3.34 (0.95)
Heart	26.44	3.14 (0.61)
Kidney	27.08	9.58 (0.18)
Liver	173.2	6.94†
Lung	36.67	3.30 (0.35)
Plasma	4.70	ND‡
Spleen	20.15	12.64 (0.59)
H69 xenograft	18.98	8.00 (1.24)

* SD, standard deviation from three to five values.
† Mean of two experiments.
‡ ND, no activity detected.

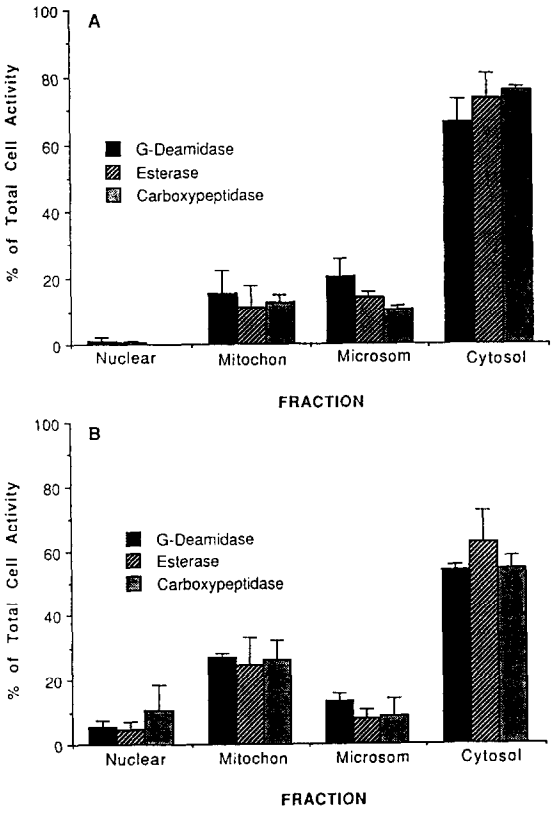

Fig. 2. Sub-cellular distribution of G-deamidase (0.05 mM antagonist G), esterase (0.1 mM BTEE) and acid carboxypeptidase (0.1 mM FAPP) activity in (A) mouse liver homogenate, and (B) human H69 xenograft homogenate. Error bars represent the standard deviation from three to five values.

Sub-cellular distribution of enzyme activity

The determination of the sub-cellular distribution of G-deamidase, esterase and acid carboxypeptidase activity in mouse liver homogenate revealed that these three activities were distributed in almost identical locations within the cell (see Fig. 2A). The majority of the activity, greater than 75% of the total cell activity in all cases, was detected in the cytosolic fraction and would suggest that the enzyme is soluble. There was a high specific activity recorded in the mitochondrial fraction and it is possible that the activity detected here was due to enzyme that was lysosomal in origin [14]. Virtually no activity was detected in the nuclear fraction with respect to any of the three substrates and this supports the theory that the enzyme is soluble and not membrane bound. Studies on the sub-cellular fractions of the human H69 xenograft demonstrated that the pattern of distribution here was almost identical to that seen in the mouse liver (see Fig. 2B), suggesting a great deal of similarity between the enzyme profile in both tissues. It could be speculated that the enzymes involved are in fact very similar in nature in both species and that the metabolism results obtained

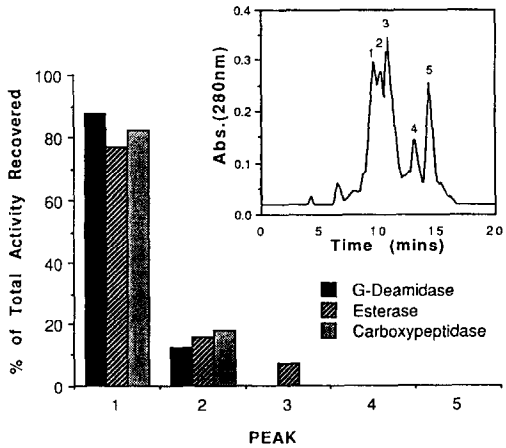

Fig. 3. The G-deamidase (0.05 mM antagonist G), esterase (0.1 mM BTEE) and acid carboxypeptidase (0.1 mM FAPP) activity in the HPLC gel filtration peaks of mouse liver cytosol. Inset: the HPLC gel filtration profile of a 20 μL injection of mouse liver cytosol.

Table 2. Properties of peaks isolated by HPLC gel-filtration of liver cytosol

Peak	Protein (mg/mL)	Fraction volume (mL)	% of Total G-deamidase activity recovered	Apparent molecular mass (kDa)
1	0.150	7.60	87.47	81
2	0.169	2.35	12.53	50
3	0.153	5.70	0	25
4	0.056	2.50	0	2.4
5	0.098	7.35	0	0.55

from pre-clinical studies in *nu/nu* mice are a very good indication of what one may expect to see when antagonist G is administered to humans in the planned phase I clinical trial.

HPLC gel-filtration

Separation on the basis of molecular size allowed the separation of five major peaks (see the inset of Fig. 3). The peaks were collected and assayed for G-deamidase, esterase and acid carboxypeptidase activity (see Fig. 3). Activity towards all three substrates followed a similar pattern with the highest activity being detected in peak 1, with a smaller amount in peak 2. There was a very small amount of esterase activity detected in peak 3, but this was most likely due to contamination from peak 2 since the two peaks were not well resolved on HPLC. Accounting for the respective activities and the total volume of eluent in which each peak was collected it was possible to determine what proportion of the total activity recovered was in each peak (see Table 2). These results have demonstrated that the vast majority of the G-deamidase activity (87.47%) is possessed by an enzyme with an apparent molecular mass of 81 kDa. The serine-carboxypeptidase reported to deamidate biologically active peptides such as substance P and endothelin-1 had an apparent molecular mass of 94 kDa by gel-filtration [9]. The same pattern of enzyme distribution was seen when the H69 tumour cytosol was subjected to HPLC gel-filtration (data not shown).

Effect of protease inhibitors

Further confirmation of the type of enzyme involved in the deamidation of antagonist G was obtained when the effect of a range of protease inhibitors was monitored (see Fig. 4). No effect was seen when EDTA was used and the small amount of inhibition seen with iodoacetamide is more likely due to the modification of a structurally important cysteine rather than an active-site cysteine which one would expect to abolish all activity. Considerable inhibition was achieved with PMSF (63% at 0.5 mM), indicating the enzyme was indeed a serine protease. TLCK, an inhibitor of trypsin-like enzymes, exhibited only slight inhibition which at these high concentrations suggests that this was non-specific inhibition. On the other hand, ZFCK, an inhibitor of chymotrypsin-like enzymes, was a more potent inhibitor than PMSF giving 90% inhibition at 0.5 mM.

In conclusion, the characterization of the enzyme

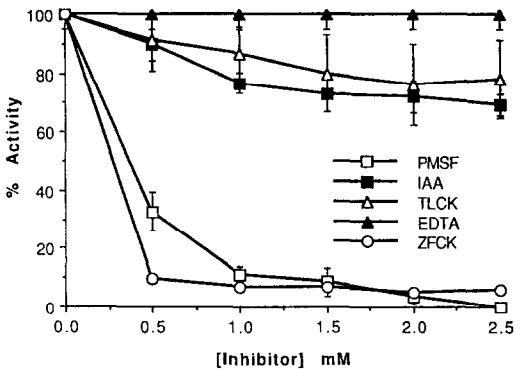

Fig. 4. The effect of protease inhibitors on the degradation of antagonist G by 2% (w/v) mouse liver homogenate at 37° for 60 min. Inhibitors were pre-incubated with the homogenate for 15 min at 23°, prior to antagonist G (0.1 mM) addition and activity is expressed relative to a control with no inhibitor added. Error bars represent the standard deviation from three experiments.

responsible for the degradation of this broad spectrum neuropeptide growth factor antagonist has shown that the activity is very widespread, the enzyme has an apparent molecular weight of 81 kDa by HPLC gel-filtration and is a chymotrypsin-like enzyme. The sub-cellular distribution of the enzyme suggests that it is soluble and not located in the cell membrane like many of the enzymes that degrade similar peptides *in vivo*, such as neutral endopeptidase 24.11 [15] and angiotensin-converting enzyme [16]. The localization of the enzyme in the cytosol with the site of action of the peptide antagonist being extracellular does raise some questions with respect to the actual site of degradation of antagonist G *in vivo* and there are two possible explanations. The peptide antagonist could be internalized into the cell prior to degradation. This process may be receptor-mediated in similar fashion to natural G-protein linked receptor ligands [17] or, due to its hydrophobic nature, it is possible that antagonist G may cross the cell membrane unaided [18]. Alternatively, the enzyme may be secreted into the extracellular domain following an appropriate stimulus at the cell surface. This process has been observed previously where platelets treated with epinephrine were shown to secrete a soluble serine-protease like enzyme that can also act as a deamidase

[10]. Direct evidence is not yet available that can distinguish between these possible mechanisms of degradation *in vivo*. The broad specificity of this widespread enzyme that enables it to degrade peptide esters, peptide amides and peptide acids indicates that it may be one of the major factors that should be considered when designing any peptide drug possessing a C-terminal modification.

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