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Metabolism of the Anticancer Peptide: H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH$_2$

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JONES, D. A., J. CUMMINGS, S. P. LANGDON, A. J. MACLELLAN, T. HIGGINS, E. ROZENGURT AND J. F. SMYTH. *Metabolism of the anticancer peptide: H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH$_2$.* PEPTIDES **16**(5) 777–783, 1995.—H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH$_2$ (Antagonist G) will be the first broad-spectrum neuropeptide antagonist to enter a phase I clinical trial. Its in vitro and in vivo metabolism has been extensively characterized. The major metabolites were identified and their structures elucidated by mass spectroscopy and amino acid analysis. Metabolism occurred almost exclusively at the *C*-terminus and was arrested by phenylmethylsulfonylfluoride, a known serine-protease inhibitor. Biological characterization of the metabolites demonstrated that the degradation of Antagonist G produces metabolites that retain neuropeptide antagonist properties.

Broad-spectrum neuropeptide antagonist Anticancer Metabolism Small cell lung cancer

H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH$_2$ (Antagonist G) is one of a new class of anticancer agent, broad-spectrum neuropeptide growth factor antagonists being developed for the treatment of small cell lung cancer (SCLC). Lung cancer is the most common fatal malignancy in the developed world with SCLC accounting for 25% of the total (14). The incidence of SCLC is still increasing, particularly amongst women, and the prognosis in patients with SCLC remains poor with an expected 2-year survival rate a little over 10% (18,20). The disease follows an aggressive clinical course and is characterized by its ability to secrete a wide range of hormonal, Ca^{2+}-mobilizing neuropeptides including bombesin/gastrin-releasing peptide (GRP), vasopressin, cholecystokinin, and neurotensin. This secretion of peptides and the concomitant expression of their respective cell surface receptors leads to a complex autocrine/paracrine growth-stimulating network (2,7). Antagonism of this neuropeptide growth-stimulation network has been shown in a clinical trial to be a possible strategy for the treatment of SCLC (13).

A family of broad-spectrum neuropeptide growth factor antagonists has now been described. In particular, two analogues based around the structure of substance P, H-D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH$_2$ and H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH$_2$ (Antagonists D and G, respectively) have been shown to inhibit the binding of GRP, vasopressin, bradykinin, cholecystokinin, galanin, and neurotensin to their specific cell surface receptors in vitro (23). It is thought that, via this competitive inhibition, these analogues block the mitogenic signaling induced by these neuropeptides (16). Furthermore, Antagonists D and G have been shown to inhibit the growth of human SCLC xenografts (WX322, H69) in vivo (14). Consequently, these two broad-spectrum antagonists have undergone preclinical evaluation. Antagonist G has progressed through toxicology studies with a phase I clinical trial planned for the near future in Edinburgh.

A common limiting factor in the effectiveness of peptide drugs is their instability when administered systemically. They can be rapidly hydrolyzed by a host of widely distributed proteases/peptidases, and their catabolism and consequent inactivation remains a constant problem in peptide drug development (19). Peptides can be rendered more metabolically stable by the incorporation of amino acid analogues into the peptide chain. This approach has seen the development of stable analogues of several biologically active peptides, including substance P (17). A common means of conferring metabolic stability is the introduction of D-amino acids and/or selective methylation of specific peptide bonds that are known to be most vulnerable to peptidase action (25). Antagonist G contains modified amino acid residues that have already been shown to protect this peptide against peptidase activity (5).

In the research reported here, the in vitro and in vivo metabolism of antagonist G has been investigated and the metabolites fully characterized. An insight into the pathway of catabolism has been gained to aid in the rational design of a second generation of related peptide drugs with greater stability and hence increased therapeutic potential.

METHOD

Materials

Antagonist G was supplied by Peptech (Europe) (Copenhagen, Denmark). Acetic acid and ammonium acetate (Aristar

[1] Requests for reprints should be addressed to Dr. D. A. Jones.

grade) were from BDH Chemicals (Poole, Dorset, UK). All methanol and acetonitrile was HPLC grade from Rathburns Chemicals (Walkerburn, Scotland, UK). Trifluoroacetic acid (TFA) and hydrogen peroxide (30% solution) were from Sigma Chemical Co. (Poole, Dorset, UK). All other chemicals were of the highest grade commercially available and were used without further purification. Water was deionized and bidistilled in a quartz glass still.

Animals

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

High Performance Liquid Chromatography

The HPLC system used consisted of two 510 HPLC pumps, a 712 WISP autosampler, a TCM column heater (all Waters, Northwich, UK), and a Hewlett–Packard 1046A fluorescence detector (Hewlett–Packard, Walborn, Germany). A Waters MAXIMA 820 computer package was used with a Waters system interface module to control the system operation and collect and integrate data.

In Vitro Production of Metabolites

Mouse liver (or NCI-H69 xenograft) was homogenized in phosphate-buffered saline (PBS; pH 7.4; 10% solution w/v) to a final concentration of 2% w/v tissue. To 10 ml of the homogenate was added 1 mg of Antagonist G and the mixture incubated at 37°C for 4 h. After this 1.0 ml of glacial acetic acid was added followed by vigorous vortexing. The sample was then centrifuged at 800 × *g* for 10 min and the metabolites were isolated from the supernatant via solid-phase extraction (SPE). The SPE procedure employed was a modified protocol to that previously reported (6) using a 3-cc (200 mg sorbent) C2 Bond-Elut column (Varian Sample Preparation Products, Harbour City, CA). Columns were eluted under negative pressure using an SS24 sample preparation station (Varian) connected to a vacuum pump (Waters).

The 3-cc Bond-Elut column was activated with methanol (7 ml) then washed with water (7 ml). The sample was applied in 1.0-ml aliquots. When all the sample had been applied, the column was washed with water (10 ml) and allowed to air dry for 60 min. Finally, the metabolites were eluted with methanol:1 M ammonium acetate (90:10 v/v; 7 ml). After elution the collected sample was concentrated to 1.0 ml in a UNIVAP (Uniscience, London, UK) at 30°C. The metabolites were purified by reverse-phase HPLC on a C18 μBondapak column (300 × 3.8 mm; Waters). Separation was achieved using an isocratic elution buffer consisting of 39% (v/v) acetonitrile in 0.1% aqueous TFA at 45°C. The purity of the isolated metabolites was evaluated by isocratic analytical RP-HPLC using the chromatographic system described for the purification. To confirm metabolite purity, the purified samples were analyzed by gradient elution whereby separation was achieved on a linear gradient of acetonitrile in 0.1% aqueous TFA (20–60% v/v over 25 min; 45°C) on a C18 μ-Bondapak column (300 × 3.8 mm).

Amino Acid Analysis of the Metabolites

The amino acid composition of the isolated metabolites was determined by employing the Waters AccQ.Tag Chemistry Package (Waters, Northwich, UK). The reagents and procedure used are described in detail in the literature (4). Typically, 2 μg of peptide was hydrolyzed under vacuum at 150°C for 2 h in the presence of 6 N hydrochloric acid and phenol. Hydrolyzed samples were reconstituted in 20 μl 50% (v/v) acetonitrile in 0.1% (v/v) aqueous TFA. The precolumn derivatization step was performed by adding 60 μl borate buffer (pH 8.8) followed by 20 μl of Waters AccQ.Fluor™ reagent. The samples were gently heated at 55°C for 10 min prior to analysis of the derivatized sample on reverse-phase HPLC using a Waters AccQ.Tag™ column (3.9 × 150 mm) at a running temperature of 37°C and component detection was by fluorescence (Ex =250 nm and Em = 395 nm). The identity of the liberated amino acids was determined by external calibration of the system with a known amino acid mixture and quantification of the amino acids was by comparison with the quantity obtained after hydrolyzing a known amount of standard Antagonist G.

Positive Ion Fast Atom Bombardment (FAB) Mass Spectrum Analysis of the Metabolites

Purified metabolites were mass analyzed by Mr. A. Taylor (Department of Chemistry, University of Edinburgh, UK) on a Kratos MS50 TC mass spectrometer. The samples were dissolved in a thioglycerol matrix and subjected to static FAB using argon gas (99.99% purity).

Isolation of In Vivo Metabolites

Six female mice (*nu/nu*) were injected IP with 45 mg/kg Antagonist G as an aqueous solution such that each animal received 0.1 ml/20 g body weight. One hour post-peptide administration the mice were sacrificed and the livers removed and placed immediately in liquid nitrogen. To isolate the metabolites each liver was thawed, homogenized in 1 *M* acetic acid (10 ml) and centrifuged at 800 × *g* for 10 min. The metabolites were isolated from the supernatant by solid-phase extraction, concentration, and RP-HPLC purification in the manner described for the purification of the in vitro metabolites. The metabolites obtained from all six livers were combined and submitted to a final purification step using gradient elution RP-HPLC. Separation was achieved by running a gradient of acetonitrile in 0.1% aqueous TFA (20–60% v/v over 25 min; 45°C) on a C18 μBondapak column (300 × 3.8 mm) at a flow rate of 1.0 ml/min.

In Vitro Biological Activity

Confluent, quiescent cultures of Swiss 3T3 cells in 33-mm^3 nunc plates were washed twice with DMEM and incubated in a humidified atmosphere of 10% CO_2 at 37°C with DMEM/Waymouth's medium (1:1, v/v) containing [^{3}H]thymidine (0.25 μCi/ml, 1 μ*M*), insulin (1 μg/ml), vasopressin (1 n*M*), and various concentrations of Antagonist G or its metabolites as described. After 40 h, the cultures were washed twice with PBS and incubated in 5% trichloroacetic acid (TCA) at 40°C for 30 min to remove acid-soluble radioactivity. Cultures were washed with industrial methylated spirits, solubilized in 1.0 ml of 2% sodium hydrogen carbonate, 0.1 *M* sodium hydroxide, 1% sodium dodecyl sulphate, and the radioactivity in the acid-soluble fraction was determined by scintillation counting in 6.0 ml Ultima Gold (Packard).

Metabolite Flux in Mouse Liver and NCI-H69 Xenograft Homogenates

The rate of disappearance/appearance of the parent peptide and its metabolites was monitored under the following condi-

tions. Tissue homogenate (1 ml; 2% w/v) was incubated at 37°C and 0.1 mg of antagonist G was added. At appropriate time points 0.1 ml of glacial acetic acid was added to the incubation mixture followed by vigorous vortexing. The sample was then centrifuged and the metabolites extracted from the supernatant using solid-phase extraction according to the method described previously (6). Once extracted, the metabolites were analyzed by RP-HPLC on a C18 μBondapak column (300 × 3.8 mm; Waters). Separation was achieved using a linear gradient of acetonitrile in 0.1% TFA (20–60% over 25 min). Quantitation of the metabolites was based on the integration of the response given on the fluorescence detector (Ex = 250 nm, Em = 395nm).

Effects of Protease Inhibitors on the Degradation of Antagonist G

The degradation of Antagonist G (100 μg) in 1.0 ml of mouse liver homogenate (2% w/v), when incubated at 37°C for 2.5 h, was studied in the presence of protease inhibitors ranging in concentration from 0 to 2.5 m*M*. The inhibitors studied were phenylmethylsulfonylfluoride (PMSF), iodoacetamide, and ethylenediaminetetraacetic acid (EDTA). Where inhibitors were to be present they were preincubated with the liver homogenate for 15 min at 23°C prior to the addition of Antagonist G. After 2.5 h at 37°C the reaction was stopped by the addition of 100 μl glacial acetic acid and the reaction mixture subjected to solid-phase extraction and analysis on RP-HPLC as described earlier.

RESULTS

Detection of the Metabolites of Antagonist G

Under optimal chromatographic conditions it has proved possible to separate Antagonist G from at least three major metabolites. The rate of metabolism of Antagonist G was saturable, suggesting it is an enzyme-catalyzed reaction. The similar chromatographic profiles obtained when separating the metabolites isolated in vitro from mouse liver and human NCI-H69 SCLC xenograft homogenates [Fig. 1(A,B)] and in vivo [Fig. 1(C)] suggests that the same metabolic products are formed. The three major metabolites were ascribed names on the basis of their chromatographic similarity to the parent peptide. The pure metabolites isolated in this manner were metabolite 1 (retention time = 11.02 min), metabolite 2 (retention time = 8.31 min), and metabolite 3 (retention time = 5.79 min). Treating the metabolite mixture with 0.5% v/v hydrogen peroxide changed metabolite 1 into a compound that coeluted with metabolite 3 whereas metabolite 2 was unaffected. This result indicates that the methionine residue is present in metabolite 1, but not in metabolite 2 (5). Metabolites 1, 2, and 3 were purified to a single peak on RP-HPLC (Fig. 2) by means of a two-step procedure involving a solid-phase extraction on C2-bonded silica and preparative HPLC. The purified metabolites were subjected to chemical and biochemical analysis to determine their chemical identity and their biological properties.

Amino Acid Analysis

The purified metabolites were hydrolyzed for 2 h before precolumn derivatization and separation on RP-HPLC (described in the Method section). The basis of detecting the residues present was fluorescence, which does have its limitations. Tryptophan is particularly difficult to detect in amino acid analysis for two reasons. First, the indole side chain of tryptophan is unstable during the acid hydrolysis at elevated temperatures. Second, the derivatized tryptophan that is obtained cannot be detected by fluorescence because the side chain quenches the fluorescence of the

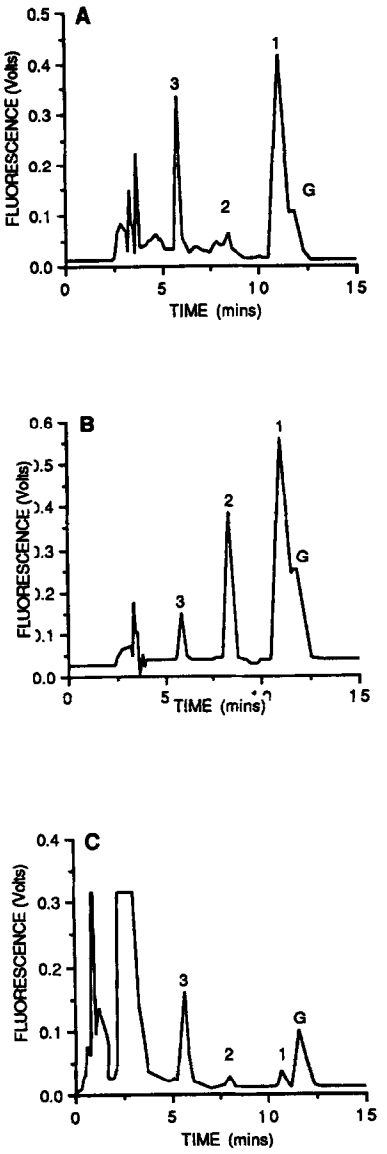

FIG. 1. RP-HPLC of extracted mixtures of Antagonist G and its metabolites. The isocratic separation of Antagonist G and its metabolites isolated by C2 solid-phase extraction after incubating Antagonist G under the following conditions. (A) In vitro using 2% (w/v) mouse (*nu/nu*) liver homogenate in PBS at 37°C for 2 h. (B) In vitro using 2% (w/v) NCI-H69 xenograft homogenate in PBS at 37°C for 2 h. (C) Metabolites isolated from mouse (*nu/nu*) liver 1 h post-IP administration of Antagonist G at a dose of 45 mg/kg.

amino label. N^{α}-Methylphenylalanine is also problematic when subjected to this system of amino acid analysis. The problems encountered here are different than those seen with tryptophan in that the residue is stable during the acid hydrolysis step, but the yield of the reaction between the methylated N^{α}-amino function and the fluorescent label is very poor. Presumably this is a direct result of steric hindrance around the amino moiety. Consequently, when performing the amino acid analysis of Antagonist G and its metabolites the residues that one could expect to detect are limited to the arginine, leucine, and methionine, a result that is confirmed when hydrolyzing a known standard of Antagonist G.

Table 1 summarizes the results of the amino acid analyses. As can be seen from the results, metabolite 1 and metabolite 3 both contained the same residues as the parent peptide. The fact that none of the residues appeared to have been removed suggested that hydrolysis of a peptide bond had not occurred in metabolites 1 and 3, but some other modification was responsible for the change in their chromatography compared to that of the parent peptide. Methionine-containing peptides are known to be susceptible to oxidation and it has previously been demonstrated that Antagonist G is no exception (5). Treatment of Antagonist G or metabolite 1 with hydrogen peroxide resulted in a product that coeluted on RP-HPLC with metabolite 3. On the other hand, no such change in character was observed when metabolite 3 was treated in the same way. This effect of oxidation meant that the identity of metabolite 3 was probably an oxidized form of metabolite 1 or Antagonist G because these two compounds were not resolved on HPLC. A clear difference was observed between these metabolites and metabolite 2. Methionine was not detected in metabolite 2 and treatment with hydrogen peroxide had no effect, which indicated that the identity of this metabolite was H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-OH. The metabolites isolated in vitro from mouse liver and NCI-H69 xenograft homogenates and those produced in vivo gave the same results under amino acid analysis, again indicating that the metabolic pathway followed in all three situations was the same.

TABLE 1

AMINO ACID ANALYSIS

Metabolite	Arginine	Methionine	Leucine
Liver 1*	1.23	1.06	0.94
Liver 2*	1.20	ND	0.88
Liver 3*	1.00	0.95	1.00
Tumor 1†	0.98	1.00	1.03
Tumor 2†	0.99	ND	0.85
Tumor 3†	1.16	1.04	1.04
Liver 1‡	1.05	0.96	1.02
Liver 2‡	0.64	ND	0.73
Liver 3‡	0.82	0.63	1.06
Standard antagonist G	1.00	1.00	1.00

ND = not detected.

* Metabolites purified from mouse liver homogenate.

† Metabolites purified from NCI-H69 xenograft homogenate.

‡ Metabolites purified from mouse liver 1 h post-IP administration of antagonist G.

Positive Ion FAB Mass Spectral Analysis

Mass analysis of the three metabolites and standard Antagonist G was performed and the molecular ions detected (M + H) supported the conclusions made from the amino acid analysis. A molecular ion of 821 was obtained for metabolite 2, which corresponds to the expected *m/z* for H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-OH. The *m/z* value seen for metabolite 1 was 952, one mass unit higher than Antagonist G, a difference that can be explained by conversion from a peptide amide to a peptide acid. This change in structure is in agreement with the change in retention time seen between metabolite 1 and the parent peptide. The conclusion is thus drawn that the identity of metabolite 1 is H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-OH. The mass analysis of metabolite 3 allowed the identity of this metabolite to be deduced. The

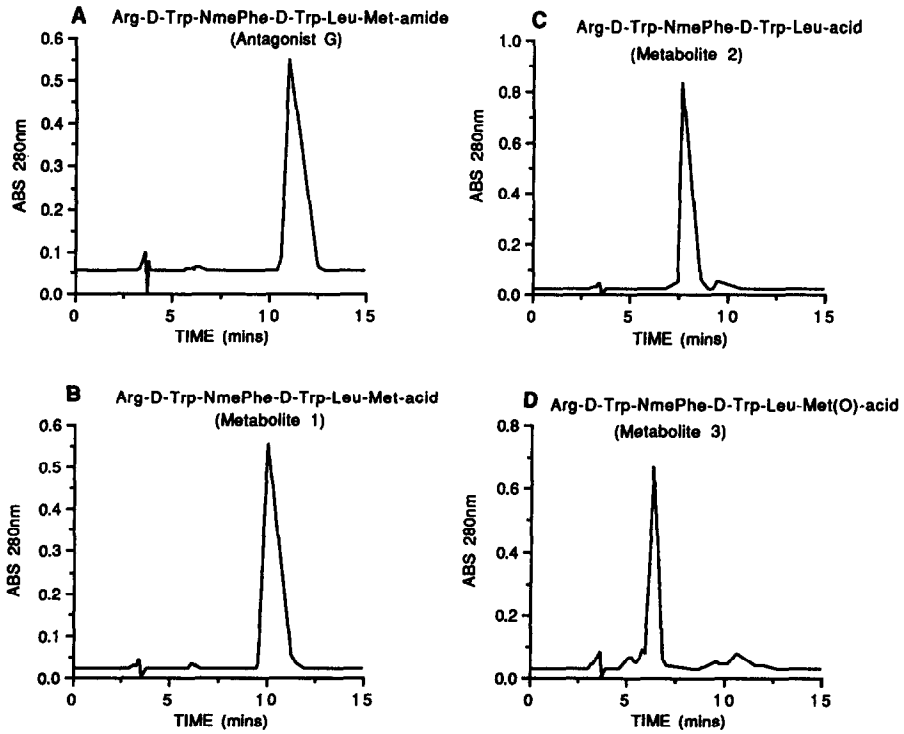

FIG. 2. RP-HPLC analysis of Antagonist G and its purified metabolites. Isocratic elution of standard Antagonist G (A), metabolite 1 (B), metabolite 2 (C), and metabolite 3 (D). Purification was by C2 solid-phase extraction followed by preparative RP-HPLC. Purity of the peptides was confirmed by gradient elution and all peptides were adjudged > 95% pure.

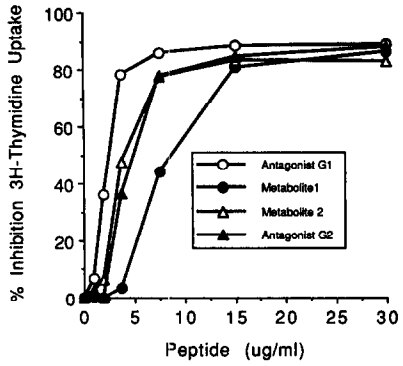

FIG. 3. In vitro biological characteristics. Expressed as percent inhibition of [^{3}H]thymidine uptake by Swiss 3T3 cells when incubated at 37°C in DMEM/Waymouth's medium (1:1, v/v) in the presence of increasing concentrations of Antagonist G or its metabolites. Results shown are the mean of duplicate samples where variation between samples did not exceed ±5%.

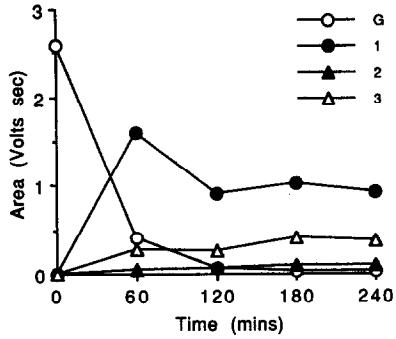

FIG. 4. Metabolite flux. The in vitro degradation/formation of Antagonist G and its three major metabolites in 2% mouse (*nu/nu*) liver homogenate in PBS at 37°C over a 4 h period. Analysis was performed by isocratic RP-HPLC following C2 solid-phase extraction of the incubation mixture.

molecular weight of an oxidized peptide would be expected to be 16 mass units higher than its reduced counterpart. The *m/z* obtained for metabolite 3 was 968, which corresponds to the mass expected for the oxidized form of metabolite 1. The chemical structure of metabolite 3 is therefore H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met(O)-OH.

In Vitro Biological Activity of the Purified Metabolites

To ascertain whether the major metabolites retained neuropeptide antagonism properties, the purified metabolites were tested in vitro for their ability to inhibit the uptake of [^{3}H]thymidine by Swiss 3T3 cells treated with vasopressin. The metabolites studied appeared to retain the properties of the parent peptide in this respect. Dose–response results (Fig. 3) show that a half-maximal inhibition of vasopressin-stimulated growth was seen with 8.9 and 5.0 μM metabolites 1 and 2, respectively, compared with 2.3 μM observed when treating cells with an external standard Antagonist G (Antagonist G1, Fig. 3). An internal standard Antagonist G (Antagonist G2, Fig. 3), which is the parent peptide repurified from the in vitro incubations and hence had been subjected to the same purification, etc., as the metabolites, was also assayed against vasopressin. Half-maximal inhibition with this internal standard was observed at 5.1 μM. The difference between the internal and external standards is unlikely to be due to any modification or contamination, but it demonstrates the problems encountered when quantifying such small amounts of recovered peptide. Taking this into consideration, to state that metabolites 1 and 2 have IC_{50} values that are comparable to Antagonist G is as accurate a conclusion that can realistically be drawn at this stage.

Metabolite Flux

The discovery that the major metabolites of Antagonist G possess antagonistic activity places more importance on the flux of the metabolites. As can be seen (Fig. 4), the degradation of Antagonist G proceeded under our in vitro conditions such that 90% of the parent peptide had been degraded by 90 min. On the other hand, maximum levels of metabolite 1 were detected after 60 min with the degradation of metabolite 1 proceeding much more slowly than that of Antagonist G, with substantial levels detected after 4 h. The levels of metabolite 2 detected increased more slowly than metabolite 1, reaching a plateau after around 240 min. The peak concentration of metabolite 2 is considerably lower than that of metabolite 1, and this is likely to be due to further degradation occurring at an almost equivalent rate to its formation. It was interesting to note that there was a more pronounced accumulation of metabolite 2 in the in vitro incubations with homogenized NCI-H69 xenograft than in liver homogenate [Fig. 1(A,B)]. There was no difference in the structure of the metabolites produced in either system, and it could be speculated that the difference observed in metabolite flux is most likely due to a variation of respective enzyme activity and/or enzyme abundance, which affords the tumor homogenate a more efficient metabolism of metabolite 1.

Effect of Protease Inhibitors

Elucidation of the metabolic pathway of Antagonist G has shown its catabolism to occur almost exclusively at the *C*-terminus via deamidation. Few enzymes active in the deamidation of peptides have been reported and in vitro studies employing protease inhibitors were expected to provide a valuable insight into the type of enzyme involved. PMSF caused 90% inhibition at a concentration of 1.0 mM, whereas the effects of EDTA or iodoacetamide were less dramatic (Fig. 5). Slight inhibition was observed in the presence of iodoacetamide, but even at high con-

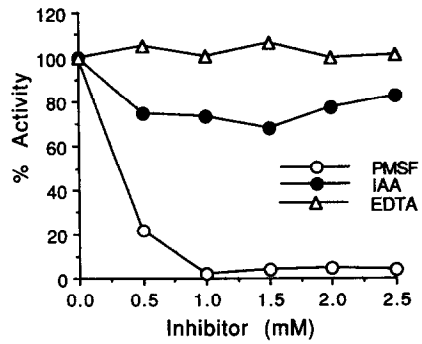

FIG. 5. Effect of protease inhibitors on the metabolism of Antagonist G in vitro. Antagonist G (100 μM) was incubated at 37°C with 2% mouse liver homogenate in PBS in the presence of varying concentrations of the three protease inhibitors: phenylmethylsulfonylfluoride (PMSF), iodoacetamide (IAA), and ethylenediaminetetraacetic acid (EDTA). These results are the mean of between two and five experiments where variation in inhibition observed was never greater than 10% in any duplicate sample.

centrations of this inhibitor (2.5 m*M*) only 30% inhibition occurred. These results suggest that the deamidating enzyme catalyzing the first step in the degradation of Antagonist G is a serine protease-like enzyme.

DISCUSSION

The successful purification and characterization of the metabolites of the hexapeptide, H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2 (Antagonist G) has allowed the deduction of the metabolic pathway followed by this broad-spectrum neuropeptide growth factor antagonist both in vitro and in vivo. The pathway of metabolism is summarized in Fig. 6. The major route of degradation is an enzyme-catalyzed process occurring at the *C*-terminus of the peptide. First, a deamidation step occurs producing H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-OH (metabolite 1). Second, a carboxypeptidase action removes the methionine residue to yield H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-OH (metabolite 2). A small amount of oxidation of the methionine residue is also observed, producing H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met(O)-OH (metabolite 3), although it is unclear at this stage whether the oxidation occurs in vivo or is a sample handling artefact. The peptide is significantly protected from the action of endopeptidases such as CD10/neutral endopeptidase, which has been shown to degrade bombesin-like peptides that regulate the growth of SCLC cells (18). When administered exogenously, unmodified peptides are metabolized very rapidly. For instance, substance P has been reported to have a half-life of 1.8–2.1 min (12) whereas pharmacokinetic studies on Antagonist G have shown its half-life to be 28.9 min (Cummings et al., submitted). However, the modifications are positioned towards the *N*-terminal portion of the peptide, leaving the *C*-terminal portion less protected. The *C*-terminal amide function and the leucine-methionine peptide bond are therefore the most susceptible sites for peptidase action. The elucidation of the metabolic pathway (Fig. 6) has shown these two sites to be attacked most readily and with the furthest removed from any modification being the most labile. Cleavage of the less hindered bonds could be expected to produce metabolites that are more stable with respect to peptidases, but still possessing the hydrophobic core important for receptor binding (9). Although comparison of metabolite half-lives with the half-life of Antagonist G calculated on the basis of the metabolite flux study would not be reliable due to the absence of an appropriate time zero where all concentrations are equal, the metabolites produced are detected long after the parent peptide has been metabolized (Fig. 4), suggesting that they are more metabolically stable.

A knowledge of the route of degradation of a peptide gives some strong indications as to which peptidases are responsible. Relatively few mammalian enzymes have been reported to be active in the deamidation of peptides as seen with Antagonist G. Some examples that have been noted are calpain (10), butyrylcholinesterase (3), an unidentified rat liver microsomal enzyme (22), and a deamidase enzyme (lysosomal protective protein) that also possesses carboxypeptidase activity (11). Studies in vitro, adding inhibitors to the crude liver homogenate, have demonstrated that the enzyme active in deamidating Antagonist G is serine protease-like in nature. Metabolism is virtually halted by the addition of PMSF, a known serine-protease inhibitor, to the incubation mixture (Fig. 5). The degradation of Antagonist G is not arrested by the addition of iodoacetamide, an inhibitor of cysteine proteases or by the addition of ethylenediaminetetraacetic acid, a metalloprotease inhibitor. The partial inhibition caused by iodoacetamide is more indicative that a structurally important cysteine residue was modified rather than an active site cysteine. Furthermore, under acidic conditions (pH 5.5) the deamidase activity is markedly decreased whereas the carboxypeptidase attack is increased, producing metabolite 2 at a much faster rate than metabolite 1 (unpublished observations). A recently proposed mechanism for the serine carboxypeptidase deamidation of peptides explains this effect on the basis of the ionization state of a catalytically important glutamate residue of the enzyme (15).

It is interesting that the smaller, metabolically more stable metabolites are approximately equipotent to Antagonist G in vitro. It may be speculated that the metabolites would be longer lived than Antagonist G and their smaller size may be beneficial with regards to absorption into the tumor. The IC_{50} for Antagonist G against SCLC cell lines (H209 and H510A) in vitro is around 15 μM, although these values were observed when the cells were treated with the peptide antagonist for 12–13 days (23). Tissue distribution studies performed after a single IP administration of 45 mg/kg in mice (*nu/nu*) revealed a maximum concentration of Antagonist G targeted to the NCI-H69 xenograft of 2.0 μM (Cummings et al., submitted). Due to the shallow dose–response curves observed in vitro (23), significant growth inhibition (approximately 20%) occurred at levels as low as 4 μM in the H510A cell line. Given that the metabolites studied possess comparable growth factor antagonist properties to the parent peptide and that they have been seen to accumulate to significant levels in the NCI-H69 xenograft (metabolite 1 = 1.0 μM and metabolite 2 = 1.1 μM), the overall antagonist concentration achieved in the xenograft is capable of causing an antitumor effect.

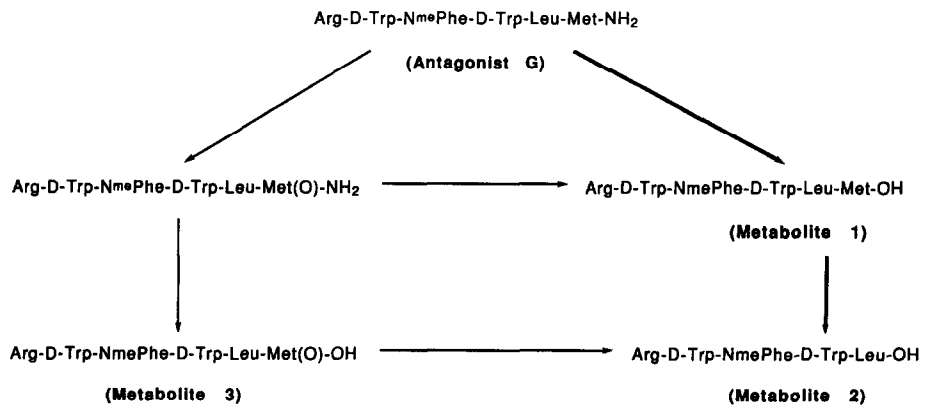

FIG. 6. Pathways of metabolism of Antagonist G.

In conclusion, H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2 is metabolized in vitro and in vivo into metabolites exhibiting greater metabolic stability. Catabolism is almost exclusively due to the action of a serine carboxypeptidase causing deamidation followed by carboxypeptidase removal of the terminal methionine. The metabolites have been purified, chromatographically and chemically characterized, and they appear to retain the growth factor antagonist properties of the parent peptide. Antagonist G has previously been shown to possess antitumor activity in vivo (14); with this knowledge of its metabolism it may now prove possible to rationally design more stable but equally potent analogues for use as anticancer agents. The identification of the type of enzyme involved in the degradation of Antagonist G may allow more effective use of this antitumor agent itself by the coadministration of suitable protease inhibitors, an approach that has previously been employed with other peptide drugs (1,8).

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