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Short Communication

PROCESSING OF THE NEUROPEPTIDE GROWTH FACTOR ANTAGONIST [ARG^6, D-TRP^{7,9}, NmePHE^8]-SUBSTANCE P (6-11) BY A SMALL CELL LUNG CANCER CELL LINE (H69)

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Abstract—[Arg^6, D-Trp^{7,9}, NmePhe^8]-substance P (6-11) (antagonist G) is a broad spectrum neuropeptide growth factor antagonist about to enter clinical trials as an anticancer drug. Its fate has been studied after incubation with two densities (5×10^4 cells/mL and 1×10^6 cells/mL) of the H69 small cell lung cancer cell line for up to 7 days at a concentration of 20 μM, corresponding to the IC_{50} for growth inhibition. HPLC analyses were conducted on cell pellets and media and in controls consisting of cell free media and water. Over 7 days in media containing cells a 70.4% reduction in parent peptide concentration occurred at the high density and a 44.1% reduction at low density. Despite this, there was a steady elevation in peptide associated with cells reaching a 189% increase by day 7. Oxidation of G at the C-terminal methionine residue occurred in all media studied indicative of a chemical process. The two major active metabolites of antagonist G (deamidated G and G minus Met^{11}) were detected only in media in the presence of cells. These accumulated with time in media and cells together with oxidized products. These results reveal complex cellular pharmacology for antagonist G where H69 cells are increasingly exposed to 4 different peptide products rather than 1.

Key words: metabolism; neuropeptides; growth factors; antagonists; small cell lung cancer; cell lines

Antagonist G§ represents one of the first neuropeptides to be advanced forward for clinical evaluation as a potential new treatment for SCLC [1]. Its proposed mechanism of action is based on receptor antagonism and subsequent inhibition of cellular signalling induced by a large number of neuropeptides including vasopressin, bradykinin, cholecystokinin, galanin, neurotensin and GRP/bombesin which are believed to drive the growth of SCLC via autocrine and paracrine growth loops [2, 3]. Antagonist G therefore belongs to a new class of anticancer drugs: broad spectrum neuropeptide growth factor antagonists.

A major part of the design philosophy behind antagonist G (and related substance P analogues [4]) was that they should be stable against the action of peptidases. This is especially important in the case of a non-cytotoxic anticancer drug like antagonist G, whose growth inhibitory effect is reversible and is only likely to be manifested when it is associated with plasma membrane receptors at critical concentrations [2]. Thus, antagonist G has incorporated into its structure D-amino acids and N-methylated amino acids, both of which have been shown to protect substance P derivatives from plasma and tissue peptidases [5, 6].

In vitro metabolism of antagonist G by mouse liver homogenates and H69 SCLC xenograft homogenates has recently been fully characterized by our laboratory [7]. The parent peptide is converted into two major products: metabolite 1 (deamidated antagonist G) and metabolite 2 (antagonist G minus C-terminal methionine). Both of these products retain equal potency to the parent peptide in antagonizing vasopressin and insulin-stimulated growth in Swiss 3T3 cells [7]. In addition, recent results have revealed that the two metabolites contribute significantly to the *in vivo* antitumour activity of antagonist G against the H69 SCLC xenograft [8].

In tissue culture experiments with SCLC cell lines such as WX322, H69, H510A and H209 antagonist G is incubated over several days, which can be extended to between 12 and 21 days, although significant effects on growth are observed after 5 days [2, 9]. Throughout this period it is unknown if the peptide is stable or if it is metabolized into stable products (such as metabolites 1 and 2) which could in their own right exert significant biological effects. In this report the fate of antagonist G incubated with the H69 cell line at its IC_{50} concentration of 20 μM has been studied for up to 7 days under conditions identical (and at a higher cell density to amplify possible effects) to those used in the above growth inhibition studies.

Materials and Methods

Standards of antagonist G and its major metabolites. Antagonist G was supplied as a kind gift from Peptech (Europe) (Copenhagen, Denmark). Standards of

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§ Abbreviations: antagonist G, [Arg^6, D-Trp^{7,9}, NmePhe^8]-Substance P (6-11); metabolite 1, H-Arg-DTrp-NmePhe-DTrp-Leu-Met-OH; metabolite 2, H-Arg-DTrp-NmePhe-DTrp-Leu-OH; metabolite 3a, H-Arg-DTrp-NmePhe-DTrp-Leu-Met(O)-NH_2; metabolite 3b, H-Arg-DTrp-NmePhe-DTrp-Leu-Met(O)-OH; SCLC, small cell lung cancer; GRP, gastrin releasing peptide; HPLC, high-performance liquid chromatography; HITES, (10 nM hydrocortisone; 5 μg/mL insulin; 10 μg/mL transferrin; 10 nM 17 β-estradiol and 30 nM sodium selenite).

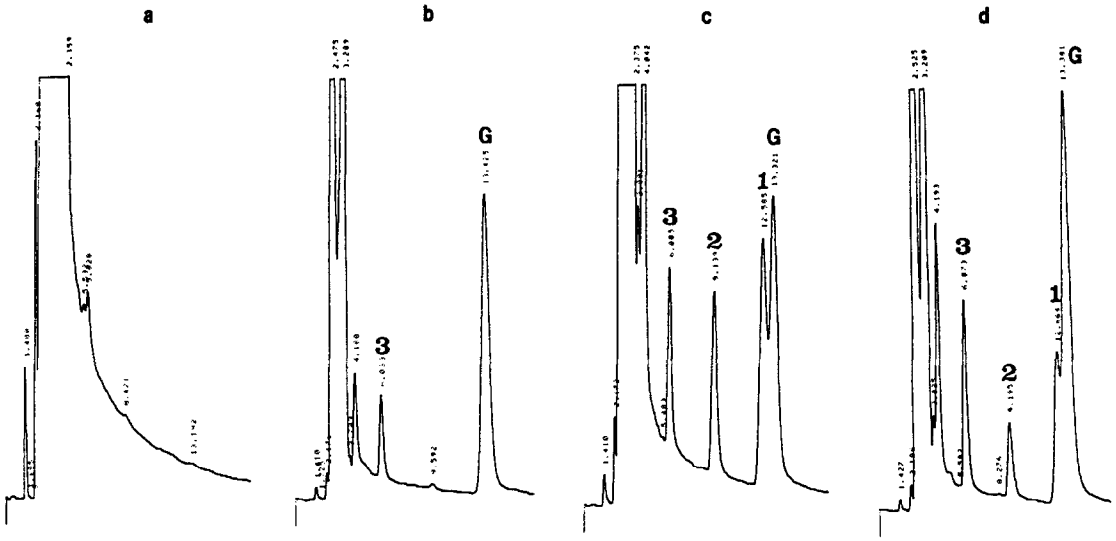

Fig. 1. HPLC analysis of [Arg^6, D-Trp^{7,9}, NmePhe^8]-Substance P (6-11) (antagonist G) incubated with H69 small cell lung cancer cells (10^6 cells/mL) for 7 days at its IC_{50} concentration of 20 μM (19 μg/mL). (a) Media containing cells prior to addition of peptide showing that no peaks are present which interfere with the identification of antagonist G and its metabolites. (b) Cell-free media: the concentration of antagonist G is 15.9 μg/mL and oxidized metabolite 3 is 3.2 μg/mL. (c) Media containing cells: the concentration of antagonist G is 5.6 μg/mL; metabolite 1, 4.7 μg/mL; metabolite 2, 2.6 μg/mL and metabolite 3, 2.5 μg/mL. (d) H69 cells (10^6 cells/mL): the concentration of antagonist G is 4.4 μg/mL; metabolite 1, 1.6 μg/mL; metabolite 2, 0.59 μg/mL and metabolite 3, 1.5 μg/mL.

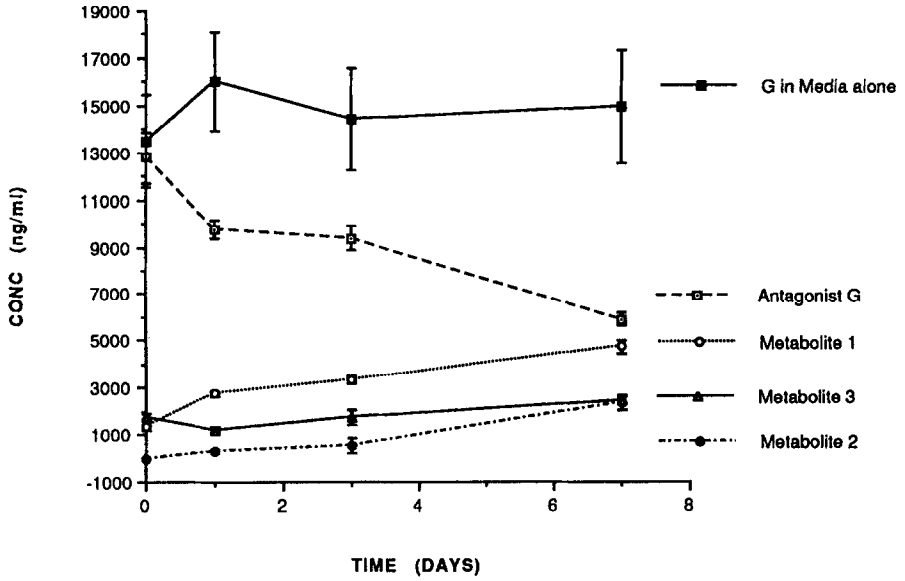

Fig. 2. Concentration–time profiles of [Arg^6, D-Trp^{7,9}, NmePhe^8]-Substance P (6-11) (antagonist G) and its metabolites in tissue culture media containing H69 small cell lung cancer cells (10^6/mL) after incubation with the peptide at its IC_{50} concentration of 20 μM (19 μg/mL). Each point represents the mean value ± SD from three replicate experiments.

metabolite 1 (deamidated antagonist G); metabolite 2 (antagonist G minus C-terminal methionine); and metabolite 3b (oxidized deamidated antagonist G) were prepared in-house by *in vitro* incubations of antagonist G with mouse liver homogenates [7]. The resultant metabolites were isolated and purified using scaled-up versions of the analytical methodologies presented below. Metabolite 3a (oxidized antagonist G) was synthesized chemically by treatment of antagonist G with hydrogen peroxide as previously described [10]. The structures of all these metabolites were confirmed by mass spectroscopy and by analysis of amino acid composition [7]. The purified metabolite standards ran as a single chromatographic peak when determined by isocratic HPLC as detailed below.

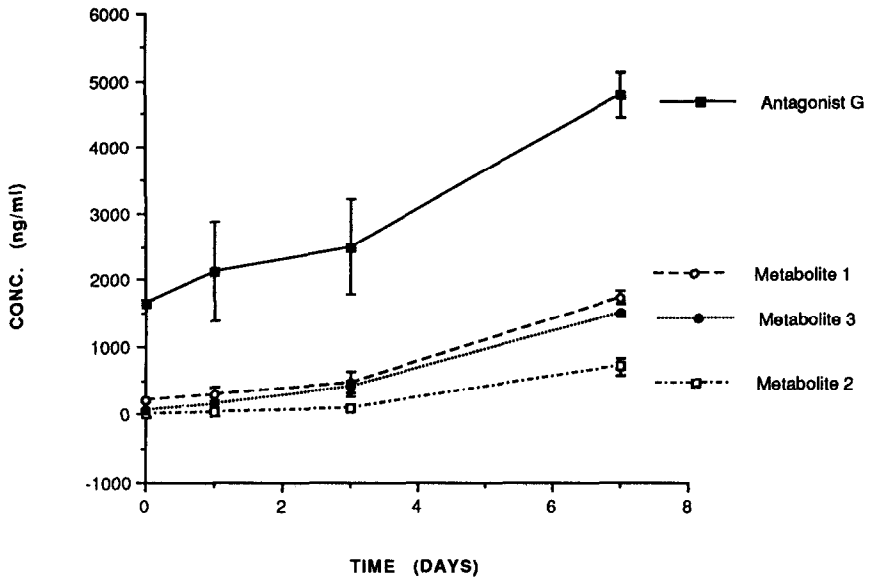

Fig. 3. Concentration–time profiles of [Arg^6, D-Trp^{7,9}, NmePhe^8]-Substance P (6-11) (antagonist G) and its metabolites associated with H69 cells after incubation with the peptide at its IC_{50} concentration of 20 μM (19 μg/mL). Each point represents the mean value ± SD from three replicate experiments.

However, metabolites 3a and b were not routinely resolved by this method and normally co-eluted as a single peak termed metabolite 3.

Drug analysis techniques. Antagonist G and its metabolites were determined by HPLC with coulometric electrochemical detection as described previously [11]. Antagonist G and metabolites were extracted from culture medium, cell pellets and water by solid phase extraction [11]. For culture medium and water, 200 μL was applied directly on to the extraction columns; cell pellets were reconstituted in 1 mL 1 M acetic acid, homogenized and then centrifuged prior to loading the complete sample on to the extraction columns.

Tissue culture incubations. The H69 SCLC cell line was maintained in suspension culture in RPMI 1640 supplemented with HITES (10 nM hydrocortisone; 5 μg/mL insulin; 10 μg/mL transferrin; 10 nM 17β-oestradiol and 30 nM sodium selenite) and 0.25% BSA [2]. Incubations were performed at two different cell densities (5×10^4 cells/mL and 1×10^6 cells/mL) in a total volume of 10 mL of tissue culture medium in 25 cm^3 tissue culture flasks which were maintained at 37° in a humidified atmosphere of 10% CO_2/90% air. In control experiments, antagonist G was added to 25 cm^3 flasks containing 10 mL of tissue culture medium without cells, or water, and incubated as above. The final concentration of antagonist G was 20 μM and all experiments were performed in three replicate flasks. At various times (pre-addition of peptide, time zero after addition of peptide and, day 1, day 3 and day 7) 1 mL was withdrawn from each flask for the determination of antagonist G and its major metabolites by HPLC as described above. Samples from flasks containing cells were immediately centrifuged; supernatants and cell pellets were separated and stored separately. All samples were stored at −80° prior to HPLC analysis.

Results and Discussion

In situ *metabolism of antagonist G by H69 SCLC cells.* Typical HPLC chromatograms illustrating the profiles of metabolites/degradation products detected under the various incubation conditions studied are contained in Fig. 1. Oxidized metabolites of antagonist G (Met^{11}(O), metabolite 3) were present in all media studied including water controls and cell-free tissue culture medium, implicating a non-enzymatic mechanism of formation and confirming previous results [10]. Changes in the concentration of metabolite 3 with time showed no strong pattern suggesting that its appearance may be explained as an artefact of sample preparation. Only in the presence of cells were the two major metabolites of antagonist G detected: H-Arg-DTrp-NmePhe-DTrp-Leu-Met-OH (deamidated antagonist G, metabolite 1) and H-Arg-DTrp-NmePhe-DTrp-Leu-OH (metabolite 2). Thus, the same pattern of metabolism that has been observed *in vitro* with mouse liver homogenates and H69 xenograft homogenates [7, 9], and *in vivo* after administration of the peptide to mice [8, 11] has now been shown to occur in human SCLC cells growing in tissue culture.

Extensive characterization of the enzyme(s) responsible for catalysing this pathway of metabolism has been completed in our laboratory [12]. These studies have revealed that the enzyme, which is predominately localized in the cytoplasm, belongs to the serine carboxypeptidase class of peptidases, which also act as peptide amide hydrolases at neutral pH and are implicated in the turnover of a number of biologically active peptide amides including substance P and bradykinin [13, 14]. The appearance of metabolites 1 and 2 in the incubation with H69 cells suggests that this class of enzyme is also present in SCLC. However, other candidate enzymes such as neutral endopeptidase-24.11 (NEP, enkephalinase, EC 3.4.24.11, [15]) and lysosomal peptidases [16], both of which have been shown to be active in SCLC cell lines against peptide growth factors and their antagonists, cannot be completely ruled out at this stage.

Concentration–time profiles of antagonist G and its metabolites in tissue culture media. The concentration of the parent peptide decreased by 70.4% over the 7 day incubation with a calculated half-life of 6.8 days at the high cell density (see Fig. 2) and decreased by 44.1% over 7 days at low cell density (data not shown). The decreases in the concentration of antagonist G could be completely accounted for by the appearance of metabolites and oxidized products and by association with H69 cells. In all analyses performed, the total yield of recovered material was 92.8 ± 13.1% (SD). No significant reduction in the

concentration of parent peptide occurred in cell free media (Fig. 2) or in water (not shown). Concentrations of metabolites 1 and 2 steadily increased over the seven day incubation period indicating possible metabolic stability of these products in the H69 cell line (Fig. 2). These metabolites were present at approximately four-fold lower levels at the lower cell density.

While one would ideally wish to observe no reduction in antagonist G concentration in tissue culture experiments running over several days, a half-life of 6.8 days has to be compared against the stability of other related peptide antagonists used in SCLC. Half-lives of 3.3–23.1 hr have been reported for bombesin and a series of chemically modified neuropeptide antagonists when incubated at 50 μM with 0.4×10^6 cells of the NCI-H345 SCLC cell line [15]. A half-life of considerably less than 24 hr has been shown for GRP in H69 cells [17].

Concentration-time profiles of antagonist G and its metabolites associated with H69 cells growing in tissue culture. Concentration–time profiles for antagonist G, its two major metabolites (metabolite 1 and 2), and oxidized metabolites (metabolite 3, which includes a mixture of oxidized parent peptide and oxidized deamidated antagonist G) are shown in Fig. 3. For technical reasons data were only derived for the higher cell density. Despite the fact that the concentration of antagonist G was continually falling in tissue culture media, the level of antagonist G associated with cells steadily increased by up to 2.9-fold from a high level at time 0. The concentrations of metabolites 1–3 associated with cells also increased with time but from a concentration of zero at time 0.

Binding of neuropeptides such as bombesin/GRP and the substance P antagonist spantide ([D-Arg^1, D-Trp^{7,9}, Leu^{11}] substance P) to cell surface receptors occurs rapidly sometimes reaching a peak level after only 10 min, with receptor internalization being a much slower process [18, 19]. The high level of antagonist G associated with H69 cells at time zero, probably, includes a component bound to receptors. However, antagonist G is a hydrophobic peptide that can cross lipid bilayers making it likely that it enters directly into cells by passive diffusion (Secl *et al.*, unpublished observations). In these preliminary studies no attempt has been made to distinguish between receptor bound and intracellular fractions of peptide.

In summary, the results presented in this report reveal a complex cellular pharmacology for antagonist G in the H69 SCLC cell line. First, although levels in media fall significantly, levels associated with cells increase significantly, indicating that estimations of antagonist G exposure to cells in culture by analysing only the peptide concentration in the medium are likely to be misleading. Second, antagonist G is metabolized to two stable, active products by the H69 cell line and, together with oxidized products, these increasingly associate with cells.

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