Advances in Brief

[D-Arg^1,D-Trp^5,7,9,Leu^11]Substance P: A Novel Potent Inhibitor of Signal Transduction and Growth in Vitro and in Vivo in Small Cell Lung Cancer Cells

Michael J. Seckl, Theresa Higgins, Fred Widmer, and Enrique Rozengurt

Imperial Cancer Research Fund, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom [M. J. S., T. H., E. R.]; Department of Medical Oncology, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, United Kingdom [M. J. S.]; and Peptech, 4–10 Inman Road, P.O. Box 444, Dee Why, New South Wales 2099, Australia [F. W.]

Abstract

[D-Arg^1,D-Trp^5,7,9,Leu^11]Substance P (SP) was identified out of a panel of novel SP analogues as the most potent inhibitor of small cell lung cancer (SCLC) cell growth. This analogue inhibited proliferation of H-510 and H-69 SCLC cells in liquid culture and in semisolid media (IC_50 5 μM). Colony formation stimulated by multiple neuropeptides, including vasopressin and bradykinin, was also blocked by [D-Arg^1,D-Trp^5,7,9,Leu^11]SP. Furthermore, this new SP analogue inhibited vasopressin- or bradykinin-induced Ca^{2+} mobilization and mitogen-activated protein kinase activation. Administration of [D-Arg^1,D-Trp^5,7,9,Leu^11]SP blocks the growth of an H-69 xenograft in nude mice. Our results support the hypothesis that SP analogue broad-spectrum neuropeptide antagonists could be of therapeutic value in SCLC.

Introduction

SCLC constitutes 25% of all pulmonary cancers and has a sub-5-year survival rate of less than 5% despite initial chemotherapeutic and radiosensitivity (1). Thus, novel therapeutic strategies are urgently required, and these will most likely arise from a better understanding of the factors and signaling pathways that stimulate the proliferation of SCLC. A variety of neuropeptides including bombesin/gastrin-releasing peptide, bradykinin, vasopressin, galanin, and gastrin promote clonal proliferation of SCLC cell lines and have been proposed to act as autocrine/paracrine growth factors for these cells (2–5). Consequently, broad-spectrum neuropeptide antagonists could provide a novel therapeutic approach for SCLC. Indeed, the synthetic SP analogues, [D-Arg^1,D-Phe^3,D-Trp^5,7,9,Leu^11]SP and [Arg^6,D-Trp^5,7,9,MePhe^8]SP (6–11), which block the biological effects of a broad range of neuropeptides (6–11), also inhibit SCLC cell proliferation in vitro and in vivo (12–14). As a result, [Arg^6,D-Trp^5,7,9,MePhe^8]SP (6–11) is in Phase I clinical trials. It is now important to develop more potent analogues and to understand their mechanism of action. In this report, we have examined a panel of known and novel SP analogues and demonstrated that [D-Arg^1,D-Trp^5,7,9,Leu^11]SP is the most potent broad-spectrum neuropeptide inhibitor for SCLC cells so far identified. We show that [D-Arg^1,D-Trp^5,7,9,Leu^11]SP blocks SCLC growth in vitro, reversibly inhibits neuropeptide-induced Ca^{2+} mobilization and MAPK activation, and blocks the growth of a SCLC xenograft in nude mice.

Received 10/15/96; accepted 11/15/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom requests for reprints should be addressed. Phone: 44-171-269-3455; Fax: 44-171-269-3417. The abbreviations used are: SCLC, small cell lung cancer; [Ca^{2+}], intracellular calcium concentration; HITESA, RPMI 1640 supplemented with 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 μg/ml estradiol, 30 μM selenium, and 0.25% BSA; MAPK, mitogen-activated protein kinase; SP, substance P; AME, tetra-acetoxymethyl ester.

Materials and Methods

Cell Culture. SCLC cell lines H-510 and H-69 were donated generously by Dr. A. Gazdar (Bethesda, MD) and purchased from the American Type Culture Collection. Stocks were maintained in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (heat inactivated at 57°C for 1 h) in a humidified atmosphere of 10% CO_{2}/90% air at 37°C. They were passaged every 7 days. For experimental purposes, the cells were grown in HITESA.

Liquid Culture Assay. SCLC cells, 3–5 days postpassage, were washed and resuspended in HITESA. Cells were then aliquoted in 24-well Falcon plates at a density of 5 X 10^6 cells in 1 ml HITESA in the presence or absence of SP analogues. At various times, the cell number was determined from a minimum of three wells per condition using a Coulter counter, after cell clumps were disaggregated by passing the cell suspension five times through a 19- and subsequently a 21-gauge needle.

Clonogenic Assay. SCLC cells, 3–5 days postpassage, were washed and resuspended in HITESA. Cells were then disaggregated into a single-cell suspension by two passages through a 19-gauge needle and then through a 20 μm nylon gauze. The cell number was determined using a Coulter counter, and 10^6 viable cells were mixed with HITESA containing 0.3% agarose and agamin with or without SP analogue at the concentrations indicated and layered over a solid base of 0.5% agarose in HITESA with or without SP analogue at the same concentration in 33-mm plastic dishes. The cultures were incubated in humidified 10% CO_{2}/90% air at 37°C for 21 days and then stained with the vital stain nitroblue tetrazolium. Colonies of >120 μm diameter (16 cells) were counted using a microscope.

Xenografts. The H-69 SCLC xenograft was derived by implantation of 10^7 cells of the H-69 SCLC cell line into the flanks of the female nude (nude) mice. The xenograft was maintained as a subcutaneous tumor in the flanks of these animals. Histological analysis confirmed the pathology of the xenografts, and this was checked with every passage.

Animals. Female nude/nu mice were bred and maintained in negative pressure isolators by the animal-breeding facility of the Imperial Cancer Research Fund (London, United Kingdom).

Antitumor Testing. The in vivo-propagated cell lines were excised from donor animals, cut into small pieces, and implanted into the flanks of recipient animals. After approximately 1 month, when the tumors were measurable, animals were randomized into control and test groups and given ear tags to allow individual identification. Groups contained 8–10 mice. Treatment was started when tumors reached a mean diameter of 3.5 mm, and the 1st day of treatment was designated day 0. Tumor growth was assessed by caliper measurement, and tumor volume (V) was estimated as

\[ V = (\pi/6) \times l \times w^2 \]

where l is the longest diameter and w is the perpendicular to this.

For injection into animals, [D-Arg^1,D-Trp^5,7,9,Leu^11]SP was dissolved in sterile water and administered either percutaneously or i.p.

Measurement of Intracellular Calcium. [Ca^{2+}], was measured with the fluorescent Ca^{2+} indicator fura-2 using a modification of the procedure described previously (15). SCLC cells in HITESA were loaded with fura-2 solution, and transferred to a quartz cuvette as described previously (13). The
Table 1 Comparison of structure/inhibitory activity of multiple SP analogues on the growth of the H-69 SCLC cell line in liquid culture

<table>
<thead>
<tr>
<th>SP analogue structure</th>
<th>Percentage of growth inhibition at 25 μM^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg-o-Trp-MePhe-o-Trp-Leu-o-Met-NH₂</td>
<td>30</td>
</tr>
<tr>
<td>Arg-o-Trp-MePhe-o-Trp-Leu-o-Met-NH₂</td>
<td>32</td>
</tr>
<tr>
<td>Arg-o-Trp-MePhe-o-Trp-Leu-o-Met-NH₂</td>
<td>37</td>
</tr>
<tr>
<td>Arg-o-Trp-MePhe-o-Trp-Leu-o-Met-NH₂</td>
<td>27</td>
</tr>
<tr>
<td>Arg-o-Trp-MePhe-o-Trp-Leu-o-Met-NH₂</td>
<td>19</td>
</tr>
<tr>
<td>H-Arg-o-Trp-MePhe-o-Trp-Leu-o-Met-OH</td>
<td>16</td>
</tr>
<tr>
<td>H-Arg-o-Trp-MePhe-o-Trp-Leu-o-Met-OH</td>
<td>13</td>
</tr>
<tr>
<td>d-Arg-Pro-Lys-Pro-o-Phe-GLn-o-Trp-Phe-o-Trp-Leu-o-Met-NH₂</td>
<td>61</td>
</tr>
<tr>
<td>d-Arg-Pro-Lys-Pro-o-Phe-GLn-o-Trp-Phe-o-Trp-Leu-o-Met-NH₂</td>
<td>92</td>
</tr>
<tr>
<td>d-Arg-Pro-Lys-Pro-o-Phe-GLn-o-Trp-Phe-o-Trp-Leu-o-Met-NH₂</td>
<td>36</td>
</tr>
<tr>
<td>d-Arg-Pro-Lys-Pro-o-Phe-GLn-o-Trp-Phe-o-Trp-Leu-o-Met-NH₂</td>
<td>20</td>
</tr>
<tr>
<td>d-Arg-Pro-Lys-Pro-o-Phe-GLn-o-Trp-Phe-o-Trp-Leu-o-Met-NH₂</td>
<td>16</td>
</tr>
<tr>
<td>Ac-Lys-Pro-o-Phe-GLn-o-Trp-Phe-o-Trp-Leu-o-Met-NH₂</td>
<td>0</td>
</tr>
<tr>
<td>Ac-Lys-Pro-o-Phe-GLn-o-Trp-Phe-o-Trp-Leu-o-Met-NH₂</td>
<td>39</td>
</tr>
</tbody>
</table>

^a H-69 SCLC cells were incubated in HITESA in the absence or presence of 25 μM [d-Arg^1,o-Trp^3,7,9,Leu^1]SP and counted after 12 days as described in “Materials and Methods.” In all cases, each value represents the mean of three determinations and is expressed as a percentage of growth inhibition calculated as 100 — (cell number + SP analogue/cell number in the absence of SP analogue) X 100.

^b Denotes the SP analogue that was most effective at inhibiting SCLC growth.

Suspension was stirred continuously and maintained at 37°C. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrophotometer with an excitation wavelength of 336 nm and emission wavelength of 510 nm. Various additions were made as indicated in the figure legend (Fig. 2) after a 1-min stabilization period. [Ca^{2+}]_i was calculated using the formula [Ca^{2+}] = K(F - F_{min})/(F_{max} - F), where F is the fluorescence at the unknown [Ca^{2+}], F_{max} is the fluorescence after addition of 0.02% Triton X-100, and F_{min} is the fluorescence after the Ca^{2+} in the solution is chelated with 10 mM ethylenebis(oxyethylenenitrilo) tetra-acetic acid. The value of K was 220 nm for fura-2 (15).

Immune Complex Kinase Assay for p42^MAPK Activation. SCLC cells in HITESA for 3–5 days were washed twice and resuspended in RPMI 1640, and...
Fig. 2. A, effect of [D-Arg',D-Trp5,7,9,Leu1]SST on either vasopressin or bradykinin dose-response curves of Ca2+ mobilization in the H-510 SCLC cell line. Cells loaded with fura-2/AM were resuspended in 2 ml of solution stimulated and measured increasing concentrations of either vasopressin or bradykinin in the presence (□) or absence (○) of [D-Arg',D-Trp5,7,9,Leu1]SST at 10 or 15 µM, respectively. In all cases, a representative of three independent experiments is shown, where each point is the mean of two determinations. B, effect of [D-Arg',D-Trp5,7,9,Leu1]SST reversibly inhibits MAPK activation induced by either vasopressin (left) or bradykinin (right) in the H-510 SCLC cell line. H-510 SCLC cells were incubated in the absence or presence of [D-Arg',D-Trp5,7,9,Leu1]SST at 10 µM (left) or 15 µM (right) for 10 min. The incubation was continued for an additional 3 min without (CON) or with addition of vasopressin (VP) or bradykinin (BK) at 30 nm or 3 µM prior to performing p42MAPK immuno complex assay as described in “Materials and Methods.” Results are the mean of at least four independent experiments performed in duplicate ± SE and are expressed as a percentage of the maximum vasopressin (left)- or bradykinin (right)-stimulated P42MAPK activation (1800—2400 cpm/3 X 106 cells at 5 min).

Results and Discussion

To identify SP analogues with increased growth-inhibitory activity, new peptides were synthesized based on the structures of either [Arg6,D-Trp7,9,MePhe8]SP (6—11) or [D-Arg1,D-Phe5,D-Trp7,9,Leu1]SST and are shown in Table 1. These SP analogues were screened at 25 µM for their inhibitory effect on liquid culture growth of the H-69 SCLC cell line. [D-Arg1,D-Trp5,7,9,Leu1]SST was identified as the most active (Table 1). This was verified in additional experiments comparing the dose responses of [D-Arg1,D-Phe5,D-Trp7,9,Leu1]SST with [D-Arg1,D-Trp5,7,9,Leu1]SST on growth inhibition of the H-510 and H-69 SCLC cell lines (data not shown). In view of these results, [D-Arg1,D-Trp5,7,9,Leu1]SST was selected for further investigation. Fig. 1A shows that the proliferation of both H-510 and H-69 SCLC cell lines in liquid culture was inhibited profoundly in the presence of 20 µM of this new SP analogue. In both cell lines, [D-Arg1,D-Trp5,7,9,Leu1]SST caused a similar dose-dependent inhibition of cell growth (Fig. 1A, insets).

Tumor and transformed cells are able to form colonies in agarose medium. There is a positive correlation between cloning efficiency of the cells and the histological involvement and invasiveness of the tumour in SCLC specimens taken from patients with the disease (16). Consequently, we determined the effect of [D-Arg1,D-Trp5,7,9,Leu1]SST on colony formation in SCLC cells. Fig. 1B (left) demonstrates that [D-Arg1,D-Trp5,7,9,Leu1]SST inhibited colony formation in a dose-dependent fashion (IC50 = 5 µM). Addition of the neuropeptides galanin, gastrin, vasopressin, and bradykinin increased colony formation in the H-510 SCLC cells. As shown in Fig. 1B (right), addition of 10 µM of [D-Arg1,D-Trp5,7,9,Leu1]SST markedly inhibited both basal and neuropeptide-stimulated colony formation.
Novel therapeutic strategies are urgently required for patients with SCLC. In this report, we have screened a panel of novel SP analogues and identified \([\text{o-Arg'}\text{,o-Trp}^7\text{,Leu}^1\text{]}\text{SP}\) as the most potent broad-spectrum neuropeptide inhibitor for SCLC cell growth in vivo. Moreover, this new SP analogue potently inhibited signal transduction pathways in vitro and significantly delayed the growth of an SCLC xenograft in vivo. Our findings with \([\text{o-Arg'}\text{,o-Trp}^7\text{,Leu}^1\text{]}\text{SP}\) support the hypothesis that broad-spectrum neuropeptide antagonists could be useful antiproliferative agents against SCLC. Furthermore, the results provide structural information for future improvements in the potency of these compounds.

Acknowledgments

We thank Dr. Merilyn Sleigh (Peptech, New South Wales, Australia) for her help with the development of the new SP analogues described here and Sandra Peak (Imperial Cancer Research Fund, London, United Kingdom) for her help with the animal experiments.

References

[d-Arg¹,d-Trp⁵,⁷,⁹,Leu¹¹]Substance P: A Novel Potent Inhibitor of Signal Transduction and Growth in Vitro and in Vivo in Small Cell Lung Cancer Cells

Michael J. Seckl, Theresa Higgins, Fred Widmer, et al.


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/57/1/51

E-mail alerts Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.