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# [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

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## 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. 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 inhibited 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[2] constitutes 25% of all pulmonary cancers and has a usb5-year survival rate of less than 5% despite initial chemo- 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[5],D-Trp[7,9],Leu[11]]SP and [Arg[6],D-Trp[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[7,9],MePhe[8]]SP (6–11) is in a Phase I clinical trial. 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 demonstrate 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.

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[2] The abbreviations used are: SCLC, small cell lung cancer; $[Ca^{2+}]_i$, intracellular calcium concentration; HITESA, RPMI 1640 supplemented with 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM estradiol, 30 nM 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 $5 \times 10^4$ 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 passes through a 19-gauge needle and then through a 20 μm nylon gauze. The cell number was determined using a Coulter counter, and $10^4$ viable cells were mixed with HITESA containing 0.3% agarose and agonist with or without SP analogue at the concentrations indicated and layered over a solid base of 0.5% agarose in HITESA with agonist 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 nu/nu (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 nu/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 peritumorally or i.p.

**Measurement of Intracellular Calcium.** $[Ca^{2+}]_i$ 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 tetra-acetoxymethyl ester, centrifuged, resuspended in 2 ml of electrolyte 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*

| SP analogue structure | Percentage of growth inhibition at 25 μM[a] |
|---|---|
| Arg-D-Trp-MePhe-D-Trp-Leu-D-Met-$NH_2$ | 30 |
| Arg-D-Trp-MePhe-D-Trp-Leu-D-Met-$NH_2$ | 32 |
| Arg-D-Trp-MePhe-D-Trp-D-Leu-Met-$NH_2$ | 37 |
| Arg-D-Trp-MePhe-D-Trp-D-Leu-Val-$NH_2$ | 27 |
| Arg-D-Trp-MePhe-D-Trp-D-Leu-Gly-$NH_2$ | 19 |
| H-Arg-D-Trp-MePhe-D-Trp-Leu-Met-OH | 16 |
| H-Arg-D-Trp-MePhe-D-Trp-Leu-OH | 13 |
| D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-$NH_2$ | 61 |
| D-Arg-Pro-Lys-Pro-D-Trp-Gln-D-Trp-Phe-D-Trp-Leu-Leu-$NH_2$[b] | 92 |
| D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Val-$NH_2$ | 36 |
| D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Gly-$NH_2$ | 20 |
| D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-$NH_2$ | 21 |
| Ac-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-$NH_2$ | 16 |
| Ac-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Gly-$NH_2$ | 0 |
| Arg-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-$NH_2$ | 39 |
| Arg-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Gly-$NH_2$ | 42 |

[a] H-69 SCLC cells were incubated in HITESA in the absence or presence of 25 μM [D-Arg$^1$, D-Trp$^{5,7,9}$, Leu$^{11}$]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 × 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 (see Fig. 2) after a 1-min stabilization period. $[Ca^{2+}]_i$ was calculated using the formula $[Ca^{2+}]_i$ (nM) = $K(F - F_{min}/(F_{max} - F)$, where $F$ is the fluorescence at the unknown $[Ca^{2+}]_i$, $F_{max}$ 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

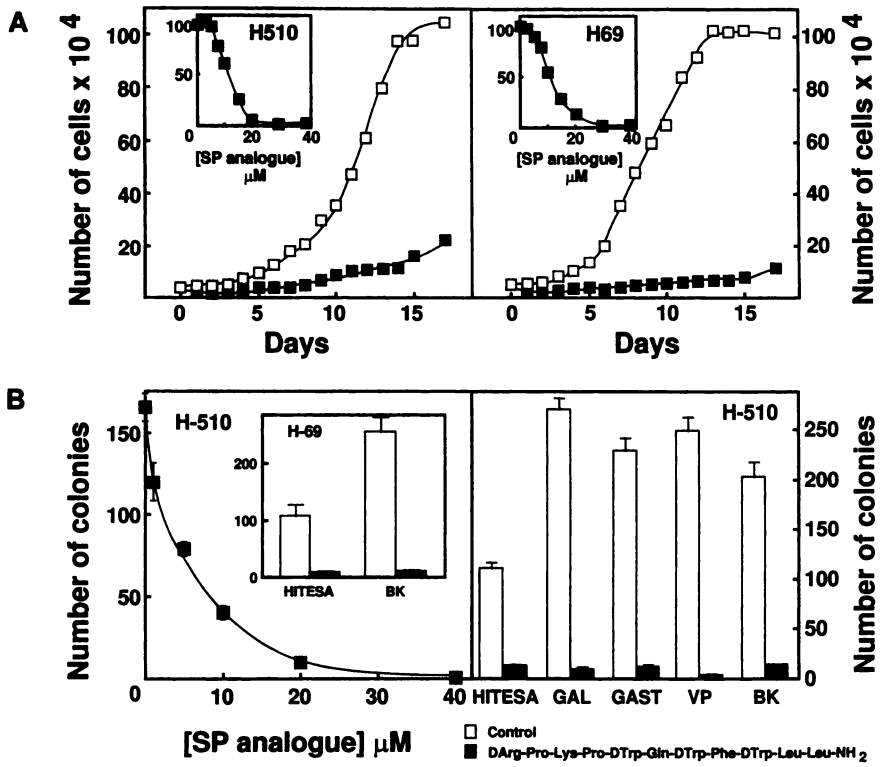


Fig. 1. *A*, inhibitory effect of [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP on the liquid culture growth of H510 and H-69 SCLC cell lines. SCLC cells were incubated in HITESA in the absence (□) or presence (■) of 20 μM [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP and counted at the indicated times as described in "Materials and Methods." *Insets*, SCLC cells were incubated in HITESA containing increasing concentrations of [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP and grown for 12–14 days before being counted. In all cases, each point represents the mean of three determinations. A representative of at least three independent experiments is shown. *SE bars* lie within the symbols. *B*, effect of [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP on the colony growth of H-510 and H-69 SCLC cell lines. *Left*, dose response of [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP on H-510 SCLC colony growth. H-510 cells were plated in agarose medium containing HITESA at a density of $1 \times 10^4$ cells/dish in the absence or presence of increasing concentrations of [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP and incubated for 3 weeks as described in "Materials and Methods." *Left inset*, inhibitory effect of [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP on basal or bradykinin-induced colony formation in H-69 cells. H-69 cells were plated in agarose medium containing HITESA with or without 10 nM bradykinin (*BK*) in the absence (□) or presence (■) of 10 μM [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP and incubated for 3 weeks. *Right*, inhibitory effect of [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP on basal or various neuropeptide-induced colony formations in H-510 cells. H-510 cells were plated in agarose medium containing HITESA with or without either 25 nM galanin (*GAL*), 25 nM gastrin (*GAST*), 25 nM vasopressin (*VP*), or 10 nM bradykinin (*BK*) in the absence (□) or presence (■) of 10 μM [D-Arg$^1$,D-Trp$^{5,7,9}$,Leu$^{11}$]SP and incubated for 3 weeks. In all cases, each point represents the mean number of colonies formed on five separate dishes ± SE. A representative of three independent experiments is shown. *Bars*, SE. Where no error bar is visible, it lies within the symbol.

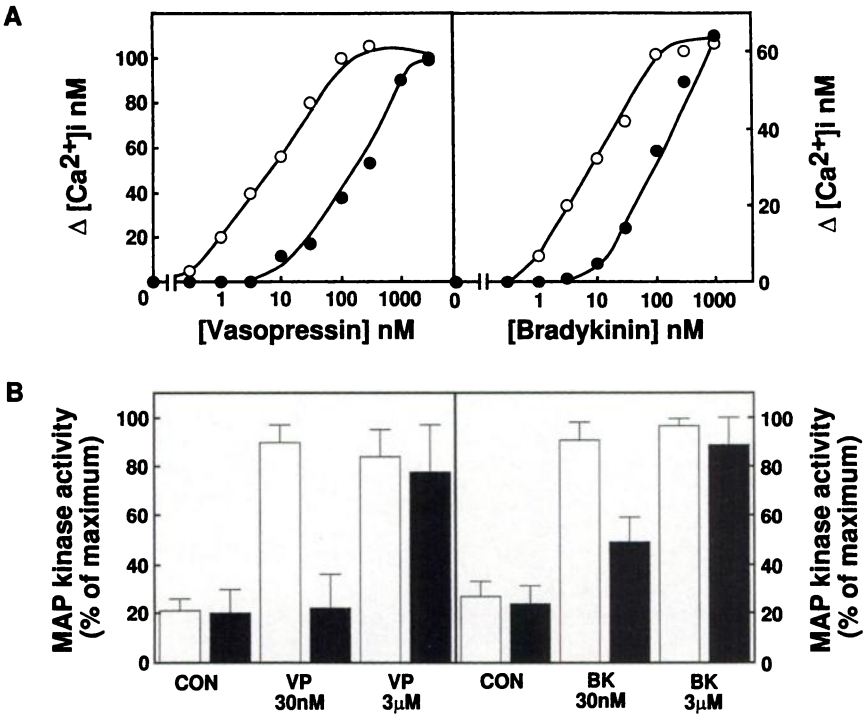


Fig. 2. *A*, effect of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP on either vasopressin or bradykinin dose-response curves of $Ca^{2+}$ mobilization in the H-510 SCLC cell line. Cells loaded with fura-2/AME were resuspended in 2 ml of electrolyte solution and stimulated with increasing concentrations of either vasopressin or bradykinin in the absence (○) or presence (●) of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP 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. For clarity, the SE bars have been omitted. The $\Delta[Ca^{2+}]_i$ was determined as described in "Materials and Methods." *B*, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP 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-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP at 10 μM (*left*) or 15 μM (*right*) for 10 min. The incubation was continued for an additional 5 min without (*CON*) or with addition of vasopressin (*VP*) or bradykinin (*BK*) at 30 nM or 3 μM prior to performing $p42^{MAPK}$ immune complex assays 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 $p42^{MAPK}$ activation (1800–2400 cpm/3 × $10^6$ cells at 5 min).

3 × $10^6$ cell aliquots were incubated for 30 min at 37°C. The cells were then treated with factors as described in the figure legend (see Fig. 2), lysed at 4°C in 1 ml of a solution containing 10 mM Tris-HCL (pH 7.6), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 2 mM $Na_3VO_4$, 1% Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (lysis buffer) and clarified by centrifugation at 15,000 × *g* for 10 min at 4°C. Immunoprecipitation was performed for 2 h using a polyclonal anti-$p42^{MAPK}$ antibody. Protein A-agarose beads (40 μl, 1:1 slurry) were added for the 2nd h. Immune complexes were centrifuged and washed twice in lysis buffer and twice in kinase buffer [15 mM Tris/HCl (pH 7.4), 15 mM $MgCl_2$). The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay mixture containing kinase buffer, 100 μM ATP, 100 μCi/ml [γ-$^{32}$P]ATP, 200 μM microcystin LR (leucine arginine analogue), and 1 mg/ml myelin basic protein peptide (APRTPGGRR). Incubations were at 30°C for 10 min (linear assay conditions) and were terminated by spotting 20 μl of the supernatant onto p81 chromatography paper (Whatman, Inc.). Filters were washed four times for 5 min in 0.5% orthophosphoric acid, immersed in acetone, and dried before Cerenkov counting. The average radioactivity of the two blank samples containing no immune complex was subtracted from the result of each sample. The specific activity of the [γ-$^{32}$P]ATP used was 900–1200 cpm/pmol.

**Materials.** Vasopressin, galanin, gastrin, and bradykinin were obtained from Sigma Chemical Co. (St. Louis, MO). The polyclonal anti-MAPK (anti-ERK-2) antibody raised against a COOH-terminal peptide (EETARFQPGYRS) was a generous gift from Dr. J. Van Lint (Katholieke Universiteit Leuven, Louvain, Belgium). Protein A agarose was from Boehringer Mannheim. [γ-$^{32}$P]ATP was from Amersham Corp. (Little Chalfont, United Kingdom). Fetal bovine serum was purchased from Life Technologies, Inc. and diluted with HITESA to the amounts indicated. Fura-2-tetracetoxymethyl ester was from Calbiochem Corp. (La Jolla, CA), and agarose was from SeaKem (Rockland, ME). [D-Arg¹,D-Phe⁵,D-Trp⁷,⁹,Leu¹¹]SP was from BACHEM, Inc., Torrance, CA. All other SP analogues were provided kindly by Peptech, New South Wales, Australia. All other reagents were of the highest grade available.

## 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,Leu¹¹]SP 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,Leu¹¹]SP 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,Leu¹¹]SP with [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP 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,Leu¹¹]SP was selected for further investigation. Fig. 1*A* 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,Leu¹¹]SP caused a similar dose-dependent inhibition of cell growth (Fig. 1*A*, *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,Leu¹¹]SP on colony formation in SCLC cells. Fig. 1*B* (*left*) demonstrates that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP inhibited colony formation in a dose-dependent fashion ($IC_{50}$ = 5 μM). Addition of the neuropeptides galanin, gastrin, vasopressin, and bradykinin increased colony formation in the H-510 SCLC cells. As shown in Fig. 1*B* (*right*), addition of 10 μM of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP markedly inhibited both basal and neuropeptide-stimulated colony formation.

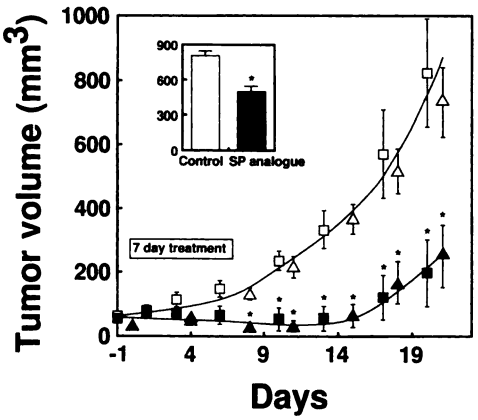


Fig. 3. *In vivo* effect of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP on an H-69 xenograft. Animals bearing single-flank tumors were randomized to receive once-daily peritumoral injections of water (□, △) or 35 μg/g/day of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP (■, ▲) for 7 days as described in "Materials and Methods." Two independent experiments shown as *squares* or *triangles* have been superimposed. Each point represents the mean ± SE (*bars*). *Inset*, i.p. administration of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP. Animals bearing single-flank tumors were randomized to receive twice-daily i.p. injections of water (□) or 35 μg/g twice daily of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP (■) for 7 days as described in "Materials and Methods." The mean effect ± SE at day 23 after the start of injections is shown. In all cases, * indicates $P < 0.05$ significantly different from control (Student's *t* test).

Similar results were obtained with the H-69 SCLC cell line (Fig. 1*B*, *left inset* and data not shown).

One of the earliest events induced by neuropeptides including vasopressin and bradykinin is a rapid phospholipase C-β-mediated hydrolysis of phosphatidyl inositol-4,5-bisphosphate to produce the second messenger inositol-1,4,5-trisphosphate, which promotes mobilization of $Ca^{2+}$ from intracellular stores (17, 18). [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP blocked the transient increase in $[Ca^{2+}]_i$ induced by vasopressin, bradykinin, gastrin, and galanin in H-510 SCLC cells (data not shown). As shown in Fig. 2*A*, the dose-response curves of vasopressin- or bradykinin-induced $Ca^{2+}$ mobilization were shifted to the right (14- and 11-fold, respectively) in the presence of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP.

Another consequence of phospholipase C-β-mediated hydrolysis of phosphatidyl inositol-4,5-bisphosphate is the generation of diacylglycerol, which activates protein kinase C. Neuropeptides such as vasopressin and bradykinin, which stimulate protein kinase C (5, 19–21) can activate the MAPK cascade in SCLC cell lines (22). [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP had no effect on the basal level of immunoprecipitable $p42^{MAPK}$ activity in H-510 SCLC cells but blocked the activation of this kinase by either 30 nM vasopressin or 30 nM bradykinin (Fig. 2*B*). This inhibitory effect was reversed when the vasopressin or bradykinin concentration was increased to 3 μM. Thus, [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP is a potent inhibitor of proliferation and signal transduction in SCLC *in vitro*.

We next examined whether [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP could inhibit SCLC growth *in vivo* using an H-69 xenograft in nude mice. Fig. 3 demonstrates that [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP given peritumorally at 35 μg/g/day for 7 days produced a marked inhibition of the growth of the H-69 xenograft, which was maintained beyond the duration of the antagonist treatment. Indeed, in 4 of 19 animals, the tumors disappeared completely, and 3 months later when those animals were sacrificed, there was no histological evidence of tumor.

To determine whether systemic (rather than peritumoral) administration of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP was effective, this analogue was given i.p. to nude mice bearing flank H-69 tumors. Although the inhibitory effect of [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP administered via this route was smaller, the reduction in tumor volume was statistically significant (Fig. 3, *inset*). No weight loss was seen in the animals receiving peritumoral or intraperitoneal SP analogue.

Novel therapeutic strategies are urgently required for patients with SCLC. In this report, we have screened a panel of novel SP analogues and identified [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]SP as the most potent broad-spectrum neuropeptide inhibitor for SCLC cell growth *in vitro*. 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 [D-Arg¹,D-Trp⁵,⁷,⁹,Leu¹¹]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.

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