[CANCER RESEARCH 52, 4554–4557, August 15, 1992]

Advances in Brief

Broad Spectrum Neuropeptide Antagonists Inhibit the Growth of Small Cell Lung Cancer *in Vivo*

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Abstract

The proliferation of small cell lung cancer (SCLC) cells appears sustained by multiple autocrine and paracrine circuits involving Ca^{2+} mobilizing neuropeptides. Consequently, broad spectrum neuropeptide antagonists which inhibit SCLC growth *in vitro* have been suggested as potential anticancer agents. Here we evaluated this hypothesis using xenografts of WX322 cells, a SCLC cell line that responds to multiple Ca^{2+} mobilizing neuropeptides. The broad spectrum neuropeptide antagonists [Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11) and [D-Arg^1,D-Phe^5,Trp^{7,9}Leu^{11}[substance P were shown to inhibit the growth of WX322 xenografts in nude mice. Similar results were obtained with xenografts of the SCLC cell line H69. The results indicate that broad spectrum neuropeptide antagonists can inhibit the growth of SCLC *in vivo* and suggest that these antagonists could be useful in the treatment of SCLC.

Introduction

Lung cancer is the most common malignancy in the developed world. SCLC[2] constitutes 25% of all pulmonary cancers and follows an aggressive clinical course. In spite of initial sensitivity to radio and chemotherapy, the 2-year survival of patients with SCLC remains very low (1). Thus, novel therapeutic strategies are needed, and most likely they will arise from a better understanding of the factors and signaling pathways that stimulate the proliferation of SCLC.

SCLC is characterized by the ability to secrete a variety of hormonal neuropeptides including GRP, vasopressin, cholecystokinin, and neurotensin (2–7). Among these, GRP has been shown to act as an autocrine growth factor for certain SCLC cell lines (8, 9). Furthermore, a variety of neuropeptides including those secreted by SCLC induce rapid mobilization of Ca^{2+} from internal stores of SCLC cell lines (10–12) and promote clonal growth of these cells in semisolid medium (12). Consequently, the emerging view is that SCLC growth appears to be regulated by multiple autocrine and paracrine circuits involving Ca^{2+} mobilizing neuropeptides. Thus antagonists capable of blocking the biological effects of multiple neuropeptides (*i.e.*, broad spectrum neuropeptide antagonists) could provide an effective approach in the treatment of SCLC.

The compounds [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P and [Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11) have been shown to inhibit signal transduction and DNA synthesis stimulated by bombesin, GRP, bradykinin, and vasopressin by preventing agonist receptor binding in a reversible fashion (13, 14). In SCLC cell lines, these neuropeptide antagonists also blocked Ca^{2+} mobilization by multiple neuropeptides, inhibited cell proliferation in liquid culture, and markedly reduced colony formation in semisolid medium either in the absence or in the presence of exogenously added stimulating neuropeptides (13, 15–17). Thus, broad spectrum neuropeptide antagonists can block multiple autocrine and paracrine growth loops in SCLC. In order to test whether these neuropeptide antagonists could be useful anticancer agents in SCLC, we have evaluated the effects of [Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11) and [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P on the growth of xenografts of the SCLC cell lines WX322 and H69 in nude mice.

Materials and Methods

Cell Culture. SCLC cell line WX322 was grown in HITESA. The cells were passaged every 7 days.

Determination of Intracellular Ca^{2+} Concentration. Aliquots of 4–5 $\times$ 10^6 SCLC cells cultured in HITESA for 3–5 days were washed, and incubated for 2 h at 37°C in 10 ml fresh HITESA medium. Then, 1 μM fura-2-tetracetoxy methyl ester from a stock of 1 mM in dimethyl sulfoxide was added and the cells were incubated for a further 5 min. The cell suspension was centrifuged at 2000 rpm for 15 s, and the cells were resuspended in 2 ml of electrolyte solution (140 mM NaCl, 5 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM CaCl, 25 mM glucose, 16 mM hepes, 16 mMTris, and a mixture of amino acids at pH 7.2), transferred to a quartz cuvet, and stirred continuously at 37°C. Fluorescence was recorded continuously in a Perkin-Elmer LS5 luminescence spectrometer with an excitation wavelength of 336 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calculated as

$$[Ca^{2+}]_i \text{ nM} = \frac{K(F - F_{min})}{(F_{max} - F)}$$

where F is the fluorescence at the unknown $[Ca^{2+}]_i$, F_{max} is the fluorescence after the trapped fluorescence is released by the addition of 0.2% Triton X-100, and F_{min} is the fluorescence remaining after the Ca^{2+} in the solution is chelated with 10 mM [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid. The value of K was 220 for fura-2 (16).

Growth Assay. SCLC cells, 3–5 days postpassage, were washed and resuspended in HITESA. Cells were resuspended at a density of 5 $\times$ 10^4 cells in 1 ml HITESA in the presence or absence of antagonists in triplicate. At various times, cell number was determined using a Coulter Counter, after cell clumps were disaggregated by passing the cell suspension through 19- and 21-gauge needles.

Xenografts. The WX322 SCLC xenograft was derived from a s.c. metastasis of an untreated SCLC tumor (18). The NCI-H69 xenograft was derived by implantation of 10^7 cells of the NCI-H69 SCLC cell line into the flanks of female *nu/nu* (nude) mice. Both xenografts were maintained as s.c. tumors in the flanks of these animals. Histological analysis confirmed the pathology of the xenografts and this was checked every passage.

Received 5/15/92; accepted 7/2/92.

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[2] The abbreviations used are: SCLC, small cell lung carcinoma; GRP, gastrin-releasing peptide; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}; 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% bovine serum albumin.

Animals. Female *nu/nu* mice were obtained from OLAC, Ltd. (Oxford, United Kingdom) and maintained in negative pressure isolators (La Calhene, Cambridge, United Kingdom).

Antitumor Testing. The *in vivo* propagated cell lines were excised from donor animals, cut into small pieces, and implanted as 2–3-mm cubed fragments into the flanks of recipient animals. After approximately 1 month, animals were randomized into control and test groups and given ear tags to allow individual identification. Groups contained 6–8 mice. Treatment was started when tumors reached a mean diameter of 4 mm and the first day of treatment was designated Day 0. The only exception to this was the experiment when [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P was given as early treatment when treatment was started 3 h after tumor implantation. Tumor growth was assessed by caliper measurement and tumor volumes (V) were estimated as

$$V = \frac{\pi}{6} \times l \times w^2$$

where l is the longest diameter and w is the perpendicular to this. The relative tumor volume, V_t/V_0 (where V_0 is the tumor volume at the start of the treatment and V_t is the tumor volume at any given point) was calculated for each individual tumor at every time point.

For injection into animals, antagonists were dissolved in sterile water. To imitate continuous infusion, Alzet osmotic minipumps were used (model 1007D; pumping rate, 0.5 μl/h for 7 days; Charles Rivers UK, Ltd.). The antagonist was diluted in sterile water and 90 μl solution were placed into the pump. The pump was then implanted s.c. in the flank opposite to the tumor of the anesthetized animal. Pumps were removed after 7 days, again while animals were anesthetized.

Materials. Antagonists were obtained from Peninsula Laboratories Belmont, CA. Agonists were purchased from Sigma Chemical Co., St. Louis, MO; fura-2-tetracetoxy methyl ester from Calbiochem Corporation, La Jolla, CA; and agarose from SeaKem, Rockland, ME. All the other reagents were of the highest grade commercially available.

Results and Discussion

Initially we determined whether WX322, a cell line that readily forms tumors in nude mice (18), expresses receptors for multiple neuropeptides. As shown in Fig. 1*A*, sequential addition of substance P, vasopressin, neurotensin, and bradykinin caused rapid and transient increases in $[Ca^{2+}]_i$ in WX322 cells loaded with the fluorescent Ca^{2+} indicator fura-2. Table 1 shows that WX322 cells respond in this assay to a surprisingly large number of neuropeptides suggesting that the growth of these cells could be regulated by multiple autocrine and paracrine circuits.

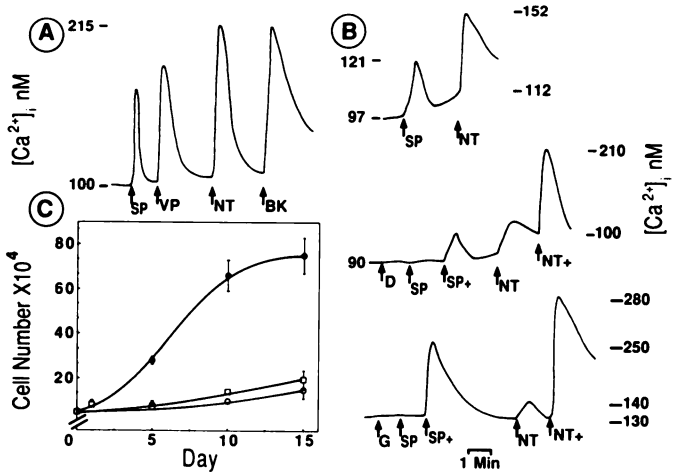

Fig. 1. Effect of agonists and broad spectrum neuropeptide antagonists on $[Ca^{2+}]_i$ and growth in the SCLC cell line WX322. $[Ca^{2+}]_i$ values were determined as described in "Materials and Methods." *A*: SP, substance P; *VP*, vasopressin; *NT*, neurotensin; *BK*, bradykinin. All peptides were added at a final concentration of 100 nM. *B*: SP, substance P (25 nM); *SP+*, substance P (100 nM); *NT*, neurotensin (5 nM); *NT+*, neurotensin (100 nM); *D*, [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]-substance P (20 μM); *G*, [Arg[6],D-Trp[7,9],MePhe[8]]substance P(6–11) (20 μM). *C*: effect of broad spectrum antagonists on growth of WX322 SCLC cells. Cells were incubated at a density of 5×10^4 cells in 1 ml HITESA substance P(6–11) (□) or [D-Arg[1],D-Phe[5],D-Trp[7,9]Leu[11]]substance P (○), each at 20 μM. Each *point* represents mean ± SD (*bars*) of 3 determinations.

Table 1 *Effect of multiple peptide hormones and neuropeptides on $[Ca^{2+}]_i$ mobilization in the SCLC cell line WX322*

$[Ca^{2+}]_i$ was measured as described in "Materials and Methods." All peptides were tested at a final concentration of 100 nM. $[Ca^{2+}]_i$ mobilization: +, increase in $[Ca^{2+}]_i$ of 20–30 nM; ++, increase $[Ca^{2+}]_i$ of 60–100 nM.

Effective	Increase of $[Ca^{2+}]_i$	Noneffective
Angiotensin I	++	ACTH[a]
Bradykinin	++	Atrial natriuretic peptide
Cholecystokinin	+	Calcitonin
Dynorphin	+	Chorionic gonadotropin
Endothelin	+	α-Endorphin
GHRH	+	Epinephrine
Bombesin/GRP	+	Galanin
Neurotensin	++	GIP
Neuromedin B	+	Glucagon
Oxytocin	++	5-Hydroxytryptamine
Substance P	++	Leu-enkephalin
Vasopressin	++	Neuropeptide Y
		Parathyroid hormone
		Substance K
		TRH

[a] ACTH, adrenocorticotrophic hormone; GHRH, growth hormone releasing hormone; GIP, gastric inhibitory peptide; TRH, thyrotropin releasing hormone.

Next, we examined whether broad spectrum antagonists could prevent neuropeptide signal transduction in WX322 cells. Addition of either [D-Arg[1],D-Phe[5],D-Trp[7,9],Leu[11]]substance P or [Arg[6],D-Trp[7,9],MePhe[8]]substance P(6–11) prevented the increase in $[Ca^{2+}]_i$ induced either by substance P or by neurotensin, which acts through a distinct receptor (Fig. 1*B*). The antagonists (20 μM) also blocked the increase in $[Ca^{2+}]_i$ induced by bradykinin, vasopressin, cholecystokinin, and bombesin in this cell line (results not shown) although the relative affinities of these antagonists for the neuropeptide receptors are different (14). Furthermore, both antagonists added at 20 μM caused a profound inhibition of the proliferation of WX322 cells (Fig. 1*C*).

To test whether broad spectrum neuropeptide antagonists can inhibit SCLC growth *in vivo*, we examined the effect of [Arg[6],D-Trp[7,9],MePhe[8]]substance P(6–11) on the growth of WX322 xenograft. Fragments of the xenograft were implanted s.c. in the flanks of nude mice and allowed to grow to a measurable size. Then, a group of animals were treated with the antagonist given peritumorally (45 μg/g) once a day for 1 week. In other experiments, we found that this was the maximum tolerated dose that could be administered i.p. to non-tumor bearing nude mice for 14 days without lethality. Fig. 2 shows that the antagonist profoundly inhibited the growth of the tumor, as compared with the control group. The inhibitory effect was clearly maintained beyond the duration of administration.

The SCLC cell line H69 is also known to express multiple neuropeptide receptors (10–12). Addition of bradykinin, galanin, or neurotensin induced a marked increase in $[Ca^{2+}]_i$ in H69 cells whereas GRP caused only a slight effect (10–12). Signal transduction and colony formation of this cell line in response to neuropeptides are markedly inhibited by broad spectrum neuropeptide antagonists (15, 16).[3] Fig. 2 (*right*) shows that [Arg[6],D-Trp[7,9],MePhe[8]]substance P(6–11) given peritumorally

[3] Unpublished results.

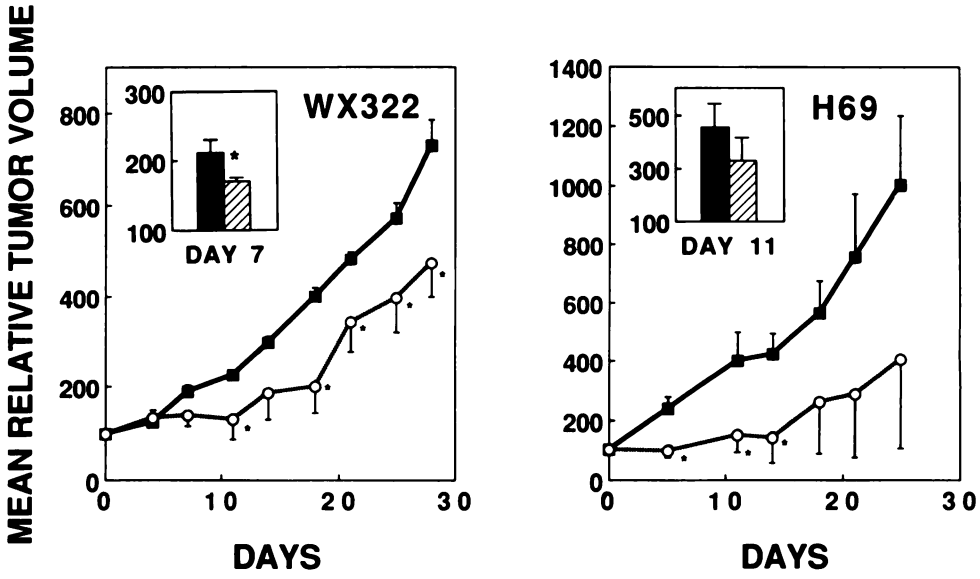

Fig. 2. Peritumoral administration of antagonists to SCLC xenografts. *Points*, mean ± SE (*bars*). ■, untreated control; ○, [Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11), 45 μg/g/day for 7 days. *Inset*, i.p. administration of antagonists to SCLC xenografts. *Points*, mean ± SE (*bars*). (■), untreated controls; ▨, [Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11), 45 μg/g/day. The relative percentage difference in mean body weights between the control and treated groups at any point of the treatment was never more than 3% (accounted for at least in part by smaller tumors in the treatment group) for any of these experiments. *, $P < 0.05$ significantly different from control, Student's *t* test.

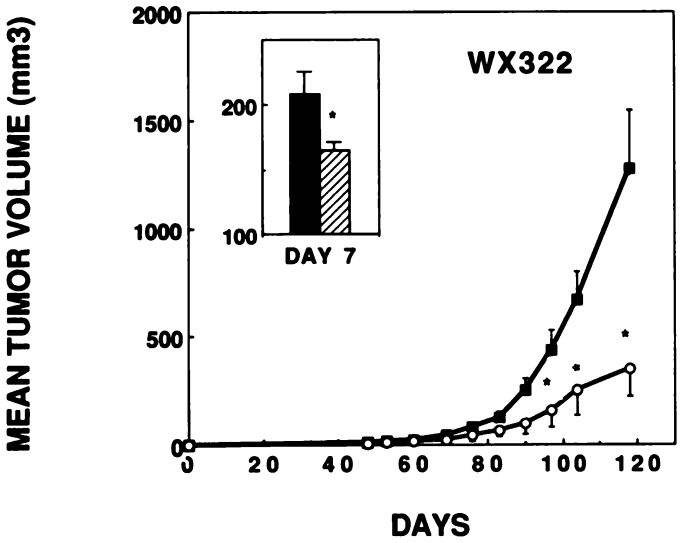

Fig. 3. Alzet pump administration of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P from the time of tumor implantation. *Points*, mean ± SE (*bars*) ■, pumps containing water alone; ○, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P. A single animal in each of those groups was considered as a no-take if no tumor had appeared by Day 118. These were excluded from the analysis. *, $P < 0.05$ significantly different from control, Student's *t* test. *Inset*, effect of continuous infusion of [D-Arg^1,D-Phe^5, D-Trp^{7,9},Leu^{11}]substance P for 7 days on previously implanted WX322 xenografts. [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P was infused at a rate of 100 μg/g/day for 7 days. There was no difference in mean body weights between the treated and control group at any point of treatment for either of these experiments.

at 45 μg/g/day for 7 days also produced a marked inhibition of the growth of the H69 xenograft which was maintained beyond the duration of antagonist treatment.

To determine whether systemic (rather than peritumoral) administration of [Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11) was also effective, we tested the effects of i.p. injection of this antagonist on the growth of WX322 and H69 xenografts. Although the inhibitory effect of the antagonist administered through this route was smaller, the effect was statistically significant against WX322 xenografts (Fig. 2, *insets*).

Next we determined whether the broad spectrum antagonist [D-Arg^1,D-Phe^5,Trp^{7,9},Leu^{11}]substance P(13) could also inhibit SCLC growth *in vivo*. An initial experiment demonstrated that this antagonist could be administered for 7 days by continuous infusion using Alzet osmotic minipumps at doses equivalent to 100 μg/g/day without any indication of toxicity while a dose of 20 μg/g/day given as i.p. injections produced lethalities in non-tumor bearing nude mice. Fig. 3 shows that administration of this antagonist at the time of tumor implantation produced a pronounced inhibition of the growth of the WX322 xenograft. Furthermore, continuous infusion of [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]substance P for 7 days also inhibited the growth of previously implanted WX322 xenografts (Fig. 3, *inset*). In other experiments, administration of [D-Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11) by Alzet osmotic minipumps also inhibited the growth of previously implanted H69 xenografts (results not shown). In all of the above described antitumor experiments, there was no evidence of toxicity as indicated by lethalities or body weight loss after injection of these antagonists.

There is currently great interest in developing new treatment strategies for SCLC. It has been proposed that the broad spectrum neuropeptide antagonists [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]-substance P and [Arg^6,D-Trp^{7,9},MePhe^8]substance P(6–11), provide a novel approach to the treatment of these complex tumors, in which multiple neuropeptides interact to stimulate growth. Here we evaluated this hypothesis by testing the effect of these antagonists on the growth of SCLC *in vivo*. We used the WX322 cell line because we identified that it expresses receptors for multiple neuropeptides. We found that the broad spectrum neuropeptide antagonists inhibited the growth of the WX322 and H69 SCLC xenografts in nude mice. The results support the hypothesis that these compounds could constitute useful antiproliferative agents against SCLC.

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