Effects of Neuropeptide Analogues on Calcium Flux and Proliferation in Lung Cancer Cell Lines

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ABSTRACT

Small cell lung cancers (SCLC) and some non-small cell lung cancers (NSCLC) have neuroendocrine features which include production of a variety of neuropeptides, cell surface expression of the receptors for these peptides, and autocrine stimulation by the peptides. Previous studies showed that some peptide antagonists and anti-peptide antibodies inhibited the growth of SCLC cell lines which expressed receptors for the specific peptide. We and others showed that the heterogeneity of peptide receptor expression and responsiveness was a major potential obstacle for developing therapeutic uses of peptide antagonists. In this manuscript we evaluate the effects of 11 peptide antagonists (3 bombesin-specific, 2 cholecystokinin-specific, 1 arginine vasopressin (AVP)-specific, and 5 substance P derivatives with broad specificity) on peptide-induced calcium mobilization and growth of SCLC and NSCLC cell lines. For each antagonist, we determined the dose-response effects, specificity of peptide antagonism, and biological stability in serum using Indo-1AM-based flow cytometric assays. We found that the three bombesin antagonists, S30, SC196, and L336,175, varied in potency from 10 nm to 10 μM, varied in serum stability from 6 h to more than 24 h, and had no effect on the calcium response elicited by other peptides. None of these compounds effectively inhibited the growth of SCLC cell lines in [3H]dThd and cell growth assays in vitro. Similarly, the three cholecystokinin and AVP antagonists were highly specific for cholecystokinin and AVP, respectively, had widely varying potency, but had little inhibitory effect on SCLC growth in vitro. In contrast, the five substance P derivatives inhibited the calcium response to bombesin, AVP, bradykinin, and fettovine serum. None of these five antagonists were as potent as the six specific antagonists described above, but they were more effective in inhibiting the growth of SCLC cell lines in vitro. These substance P derivatives inhibited the growth of peptide-sensitive SCLC cell lines more efficiently than their inhibition of peptide-insensitive NSCLC or breast cancer cell lines. Relatively high concentrations of these substance P derivatives were required to inhibit in vitro growth, even in the absence of added peptide. It is likely that more potent broad spectrum antagonists, toxins, or radio-labeled stable antagonists will need to be developed for maximal clinical development of this type of anti-growth factor therapy.

INTRODUCTION

Bombesin and its human analogue, GRP,2 were reported to be growth factors for human fibroblasts (1), pulmonary epithelial cells (2), and lung cancers with neuroendocrine features, especially SCLC (3,5). The cellular response to these peptides is mediated by a signal transduction pathway in which the peptide receptor is coupled to G proteins in the cell membrane. Activation of the G protein leads to activation of phospholipase C, which simultaneously cleaves membrane lipids leading to: (a) formation of diacylglycerol (which may also be produced by activation of phospholipase D) and activation of protein kinase C; (b) hydrolysis of phosphatidylinositol bisphosphate to inositol trisphosphate with subsequent release of intracellular calcium (6,7). Subsequent signal events lead to transcription of fos, myc, and other genes which, in turn, lead to cell proliferation (8,9).

Recently, other neuropeptides including BK, CCK, AVP, neuropeptide Y, somatostatin, galanin, neurokinins A and B, and others were reported to serve as growth factors for human lung cancer cells by activating the same intracellular signal cascade (10,14). Several studies, including our own, showed that there was considerable heterogeneity of response to individual peptides among lung cancer cell lines (10,11,13). The heterogeneity was attributed to differences in receptor expression. Each receptor activated the signal pathway for only its specific peptide and was down-regulated in response to its peptide (10). These neuropeptides are felt to play a role in the pathogenesis and progression of neuroendocrine lung cancers because elevated peptide levels were reported in carcinogen-initiated hamster models (15) and in the bronchoalveolar lavage fluids of smokers (16) and lung cancer patients (17); exogenously administered bombesin promoted SCLC tumor growth in vitro (4,5) and in athymic nude mice (18); peptide production and receptors are present in bronchial epithelial cells and neuroendocrine tumor cells (19,20); and interference with the growth factor pathways inhibits tumor formation and progression (3,21).

Strategies to interfere with these autocrine and paracrine growth pathways were developed with both chemoprevention and therapeutic intent. Monoclonal antibodies to bombesin were shown to inhibit the growth of bombesin-responsive lung cancer cell lines in vitro (3). Subsequent studies in athymic nude mice demonstrated that these antibodies could inhibit in vivo growth but only when given continuously to bombesin-responsive cell heterotransplants and only when instilled shortly after heterotransplantation (3,22). A clinical trial in human lung cancer patients followed the mouse studies, and one clinical response was observed among the first 12 patients treated (23).

Another obvious strategy was to evaluate peptide antagonists. Specific peptide antagonists were developed which bound to specific receptors with varying affinities (24,27). In a few instances, these peptide antagonists were shown to have anti-proliferative effects on the growth of calcium-responsive but not nonresponsive cell lines in vitro (28,31). Although substance P did not appear to stimulate SCLC cell lines, some substance P antagonists were shown to inhibit the calcium signal induced by bombesin and inhibited growth of bombesin-responsive cells in vitro (28,30). High concentrations of the substance P antagonists were required for the antiproliferative effects, presumably because of low binding affinities or because this interference with the signal pathway occurs at the intracellular level (G proteins) which require higher concentrations. The potential clinical utility of these SPDs remains uncertain because of unknown...
serum stability and the lack of in vivo studies to determine whether sufficient serum concentrations can be achieved for antitumor effects.

Because of the therapeutic potential of neuropeptide antagonists coupled with the problems of affinity, specificity, heterogeneity, and serum stability, we performed a detailed analysis of multiple neuropeptide antagonists. To determine the specificity of peptide antagonists effects and to evaluate their antitumor potential, we evaluated the effects of a number of peptide analogues on calcium signal transduction and in vitro growth in human cancer cell lines. We also evaluated the serum stability of some of these peptide antagonists. Our results suggest that further preclinical studies should be conducted with serum-stable, broad spectrum antagonists and possibly with toxin-conjugated or radioabeled stable peptide analogues.

MATERIALS AND METHODS

Cell Lines. The human SCLC cell lines NCI-H6945, NCI-H510, NCI-H146, and NCI-H256 were kindly provided by Drs. John Minna and Adi Gazdar (National Cancer Institute, Bethesda, MD). The SCLC cell line NCI-H1658 was kindly provided by Dr. A. Koros (University of Pittsburgh, Pittsburgh, PA). The human NSCLC cell line A549 was obtained from the American Type Tissue Collection (Bethesda, MD). The breast adenocarcinoma cell lines ZR75 and T47DV were provided by Dr. K. Horwitz (University of Colorado Cancer Center, Denver, CO). The cell lines were grown in RPMI 1640 supplemented with HITES, ACL3 additives (32, 33), or with 10% heat-inactivated fetal bovine serum.

Peptides. The peptides [Tyr6]Bombesin, GRP 1–27, GRP 14–27, GRP 1–16, BK, cholecystokinin octapeptide (CCK 26–33), vasopressin [Arg8]AVP, [1-(ß-Mercapto-ß-cyclopentamethylenepropionic acid) 2-(O-methyl)homosynine]-Arg8-vasopressin, substance P, and the SPD [D-Arg1, D-Phe5, D-Trp7, 9, Leu11]substance P were obtained from Peninsula Laboratories (Belmont, CA). The SPD termed SPD 1–4 were synthesized by one of us (T. M.). Peptides S30, SC196, and S22 were synthesized in Serina Biotechnology at the University of Colorado School of Medicine (Denver, CO). The bombesin antagonist L-366175 and the CCK antagonists CCK-A1 (L364–718) and CCK-A2 (L365–260) were supplied by Merck Laboratories (West Point, PA). The BK antagonist CP-126 was supplied by Cortech, Inc. (Denver, CO).

The amino acid structures of these peptides and antagonists are provided in Table 1 and Fig. 1.

Table 1 Carboxy terminal amino acid sequence of various peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Carboxy terminal sequence</th>
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<tr>
<td>Bombesin</td>
<td>pGlu Gln Arg</td>
</tr>
<tr>
<td>GRP 14-27</td>
<td>Met Tyr Pro Arg Gly Asn His Trp Ala Val Gly His Leu Met NH2</td>
</tr>
<tr>
<td>S30</td>
<td>Ac Met Arg Pro Gly Asn His Trp Ala Val Gly DTrp Leu NH2</td>
</tr>
<tr>
<td>SC196</td>
<td>Ac Met Tyr Pro Arg Gly Asn His Asp Trp Ala Val Gly Leu NH2</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Arg Pro Pro Gly Phe Ser Pro Phe Arg COOH</td>
</tr>
<tr>
<td>CPI126</td>
<td>DArg Arg Pro Hyp Gly Phe Cys DPhi Leu Arg COOH</td>
</tr>
<tr>
<td>CCK 26-33</td>
<td>Asp pGlu Met Gly Trp Met Asp Phe NH2</td>
</tr>
<tr>
<td>AVP</td>
<td>Tyr Gln Pro Cys Pro Arg Gly NH2</td>
</tr>
<tr>
<td>SP</td>
<td>Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met NH2</td>
</tr>
<tr>
<td>SPD</td>
<td>DArg Pro Lys Pro DPhi Gln DTrp Phe DTrp Leu Leu NH2</td>
</tr>
<tr>
<td>SPD1</td>
<td>DArg Pro Lys Pro FDF Gln DTrp DDF Trp Gly Leu Leu NH2</td>
</tr>
<tr>
<td>SPD2</td>
<td>DArg Pro Lys Pro FDF Gln DFF DDF Trp FDW Gly Leu Leu NH2</td>
</tr>
<tr>
<td>SPD3</td>
<td>DArg Pro Lys Pro FDF Gln DFF FDW Gly Leu Leu NH2</td>
</tr>
<tr>
<td>SPD4</td>
<td>DArg Pro Lys Pro FDF Gln FDW FDW Gly Leu Met NH2</td>
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NEUROPEPTIDE ANALOGUE EFFECTS ON LUNG CANCER CELLS

L-366175(MP) Ac -His - Trp - Ala - Val - Gly - His - N -

CH₃

Cys—Tyr—Phe--Gln—Asn— ys—Pro—Arg—0ly—NH₂

AVP

RESULTS

Effects of Peptide Antagonists on Calcium Flux. To assess the effects of various peptide antagonists on [Ca²⁺], levels, human lung cancer cell lines were initially exposed to the peptide antagonist in varying concentrations. Shortly thereafter, the native peptide, in a concentration which provoked a maximal response, was added. An example is shown in Fig. 2. In this instance, an ineffective concentration of the BK antagonist CP126 (100 nM) was added to SHP-77 cells after 20 s (Fig. 2, A and B). The antagonist alone had no effect on [Ca²⁺], levels. After 60 s, BK (10 nM) was added. When the antagonist concentration was below threshold as in Fig. 2, A and B, the usual [Ca²⁺], response to BK occurred. At optimal concentra-

Fig. 2. The effects of a BK antagonist and BK on [Ca²⁺], levels in SHP-77 cells. A, the three-dimensional histogram of ratio (410:490) or [Ca²⁺], versus time and number of cells. The first arrow at 8 s shows the time of administration of CP126 (100 nM). There was no effect on [Ca²⁺], at the second arrow; at 28 s, BK (10 nm) was added, and there was a rapid increase in [Ca²⁺], which then returned toward baseline over the next 25 s. The BK response in this instance was identical to that produced by BK without prior CP126 admin-

istration, indicating that this concentration of CP126 had no inhibitory effect. B, the two-dimen-

sional representation of this experiment showing that 85% of the SHP-77 cells responded to BK. C and D, the experimental conditions were identical except that the CP126 concentration was 1 µM. This concentration completely inhibited the BK (10 nm)-induced [Ca²⁺], response.
effect on the [Ca$^{2+}$] levels in NCI-H345 and NCI-H510 cells. When administered before GRP 14–27 (100 nm), a concentration of S30 below 100 nm produced no inhibition, concentrations of 500 nm and 1 \mu m produced 20% inhibition, 4 \mu m produced 75% inhibition, and 10 \mu m and higher concentrations produced complete or near complete (\geq 90\%) inhibition of the GRP response. The fact that a concentration of S30 more than 10 times the GRP concentration was required for 50% inhibition and 100 times the GRP concentration was required for complete inhibition suggests that the affinity of S30 for the bombesin receptor is lower than the affinity of native bombesin or GRP. To assess the specificity of the peptide antagonism, other peptides were added in place of bombesin or GRP. S30 had no significant inhibitory effect on the [Ca$^{2+}$] response induced in lung cancer cells by CCK, BK, FBS or AVP (Table 2; Fig. 3B), suggesting that the inhibition of GRP was mediated by competitive inhibition at the receptor level. The S30 peptide has a \delta-Trp substitution at the His$^{12}$ position of bombesin and a N-Leu substitution at Met$^{14}$. These substitutions changed the peptide effect from agonist to antagonist and lowered the receptor affinity.

The SC196 bombesin antagonist is similar to other bombesin antagonists with a reduced peptide bond between Leu$^{13}$ and terminal Leu$^{14}$ position. This class of antagonist was reported to have greater binding affinity for the bombesin receptor (26). SC196 alone had no effect on the calcium levels in NCI-H345 and NCI-H510 cells over a wide concentration (1 nM-10 \mu M). The administration of SC196 prior to bombesin or GRP inhibited the [Ca$^{2+}$] response of bombesin or GRP (100 nm) in a concentration-dependent manner. SC196 concentrations of less than 100 nm had no effect on the [Ca$^{2+}$] response induced by GRP. The IC$_{50}$ was 100–200 nm, and complete or near complete inhibition was achieved by concentrations of 1 \mu M or above. The SC196 effects were also specific for bombesin/GRP because it had no effect on the [Ca$^{2+}$] response to CCK, BK, or AVP (Table 2).

The bombesin antagonist L366175 was reported to have an even higher affinity for the bombesin receptor than the other antagonists tested (Table 1; Ref. 34). Like SC196 and S30, L366175 alone had no effect on the [Ca$^{2+}$] level in NCI-H345 and NCI-H510 cells over a wide concentration range (1 nM-10 \mu M). The administration of L366175 prior to the administration of bombesin or GRP (100 nm) inhibited the GRP-induced [Ca$^{2+}$] response in a concentration-dependent manner. Concentrations of L366175 below 1 nm had no effect, but 1 \mu M L366175 partially inhibited (37.5\%) the GRP response. A concentration of only 50 nm produced a 75\% inhibition, and 100 nm or above completely inhibited the bombesin and GRP response. L366175 also had no effect on the [Ca$^{2+}$] response generated by CCK, AVP, or BK (Table 2).

We evaluated the effects of two nonpeptide CCK antagonists (35, 36) which we termed CCKA1 and CCKA2 (Fig. 1). Neither CCKA1 nor CCKA2 alone had any effect on the calcium levels in NCI-H345

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**Table 2. Effect of peptide antagonists on peptide-induced calcium responses**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>NCI-H345 bombesin*</th>
<th>NCI-H510 CCK*</th>
<th>NCI-H510 AVP*</th>
<th>NCI-H345 FBS*</th>
<th>SHP-77 BK*</th>
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<tbody>
<tr>
<td>S30 10 \mu M</td>
<td>37 ± 30</td>
<td>105 ± 1</td>
<td>102 ± 9</td>
<td>74 ± 7</td>
<td>99*</td>
</tr>
<tr>
<td>SC196 1 \mu M</td>
<td>7 ± 12</td>
<td>100 ± 3</td>
<td>86 ± 48</td>
<td>130 ± 42</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>L366175 100 nm</td>
<td>0 ± 0</td>
<td>107 ± 5</td>
<td>108 ± 40</td>
<td>91 ± 2</td>
<td>99 ± 3</td>
</tr>
<tr>
<td>SPD 10 \mu M</td>
<td>30 ± 25</td>
<td>72 ± 27</td>
<td>14 ± 23</td>
<td>68 ± 8.5</td>
<td>83*</td>
</tr>
<tr>
<td>SPD1 10 \mu M</td>
<td>40 ± 29</td>
<td>97*</td>
<td>64 ± 8</td>
<td>43 ± 3</td>
<td>67*</td>
</tr>
<tr>
<td>SPD2 10 \mu M</td>
<td>37 ± 26</td>
<td>98*</td>
<td>74 ± 2</td>
<td>52 ± 0</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>SPD3 10 \mu M</td>
<td>48 ± 35</td>
<td>105*</td>
<td>54 ± 13</td>
<td>47 ± 2</td>
<td>46*</td>
</tr>
<tr>
<td>SPD4 10 \mu M</td>
<td>45 ± 32</td>
<td>106*</td>
<td>74 ± 6</td>
<td>NT*</td>
<td>NT</td>
</tr>
<tr>
<td>CCKA1 10 \mu M</td>
<td>87 ± 20</td>
<td>28 ± 23</td>
<td>115 ± 33</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>CCKA2 10 nm</td>
<td>87 ± 12</td>
<td>22 ± 11</td>
<td>113 ± 33</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>AVPA1 4 \mu M</td>
<td>123 ± 20</td>
<td>116 ± 11</td>
<td>32 ± 45</td>
<td>NT</td>
<td>96*</td>
</tr>
</tbody>
</table>

* 100 nm.

* 10%.

* 1 nm.

* Experiment conducted only once.

* NT, not tested.

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**Fig. 3.** The effect of the bombesin antagonist S30 on [Ca$^{2+}$]$_{1}$, induced by bombesin, CCK, and AVP in SCLC cell lines. A, the % of NCI-H345 cells with an increase in [Ca$^{2+}$]$_{1}$ over time. At 25 s, either 0 nm (○), 500 nm (●), 1 \mu M (△), or 10 \mu M (▲) of S30 was added. S30 itself had no effect on the [Ca$^{2+}$]$_{1}$ levels. At 90 s, 50 nm bombesin was added. The 50 nm bombesin produced a response in 42\% of cells with no prior S30 (○), 35\% of cells with prior administration of 500 nm (●), or 1 \mu M S30 (△), and only 5\% of cells previously given 10 \mu M S3(A). B, at 20 s, either 0 nm S30 (○, △); or 5 \mu M S30 (●, ▲) was given and produced no change in resting calcium levels. At 90 s, CCK (100 nm) (○, △) and AVP (100 nm) (△, ▲) were added. The S30 had no effect on the CCK or AVP response.
or NCI-H510 cells over a concentration range of 1 nm to 10 μM. Both CCK antagonists inhibited the CCK and gastrin-induced effects on [Ca^{2+}]], levels in NCI-H345 and NCI-H510 cells as shown in Tables 2 and 3. The IC_{50} concentrations for the two CCK antagonists were 500 nm and 1 nm, respectively. The inhibition produced by these antagonists was specific to gastrin/CCK because neither CCKA1 (4 μM) nor CCKA2 (1 μM) had a significant inhibitory effect on the calcium response generated by GRP 1–27 or AVP in these SCLC cell lines (Table 2). We similarly found that an AVP antagonist (Fig. 1; Ref. 37) had specific inhibitory effects only on the calcium response generated by AVP (Table 3). The IC_{50} for the AVP antagonist was 10 μM for inhibiting the AVP response in NCI-H510 cells (Table 3).

We studied five SPDs because the [o-Arg^1, d-Phe^5, d-Trp^7-9, Leu^11]SPD was reported to block bombesin-induced responses and to inhibit proliferation of SCLC cell lines (28–30). None of the five SPDs alone affected the [Ca^{2+}]], levels in SCLC cell lines, whereas all five inhibited the calcium response generated by bombesin or GRP (Table 2). The IC_{50} for these five peptides ranged from 2 μM to 10 μM. In marked contrast to the other peptides studied, the inhibitory effects of these SPDs was not specific for one class of peptide agonist. As illustrated in Table 2, each of these SPDs at least partially inhibited the stimulatory effect of AVP (100 nM), FBS (10%) and BK (10 nM). However, none of these derivatives exhibited a significant inhibitory effect on the CCK (100 nM)-induced calcium response. The inhibitory effects of these SPDs on bombesin-induced stimulation varied between the different derivatives and was never complete (Table 2).

**Effect of Peptide Antagonists on Cell Growth.** We evaluated the effects of the peptide antagonists on cellular proliferation by [3H]dThd assays. Fig. 4A shows the effect of varying concentrations of four bombesin analogues/antagonists on the SCLC cell line NCI-H345. The inactive peptide GRP 1–16 had no effect at concentrations from 1 μM to 50 μM. The bombesin antagonist with the highest and lowest efficacies, L666175 and S30, respectively, inhibited the growth of NCI-H345 by about 50% but only at the highest concentration (50 μM). This concentration is considerably higher than the concentration required for complete inhibition of the calcium signal response. The SPD, which has a low efficacy but wide specificity, partially inhibited NCI-H345 growth at 20 μM and completely inhibited its growth at 40 μM (Fig. 4A). These concentrations were also much higher than required for inhibiting a [Ca^{2+}], response. The effects of the CCK and AVP antagonists on [3H]dThd incorporation in NCI-H345 cells were shown in Fig. 4B. There was little effect of both CCK antagonists at 1 μM or 10 μM, but 20 μM produced partial (25%-75%) inhibition. The AVP antagonist was slightly more potent with partial inhibition (~60%) at 10 μM.

The effects of the five SPDs (40 or 50 μM) on [3H]dThd incorporation in lung cancer and breast cancer cell lines are shown in Table 4. Qualitatively, the results were quite similar because no inhibition was noted at 10 nm to 1 μM for any of the five peptides on any of the cell lines. Partial inhibition (up to 40%) was noted in two of four SCLC cell lines (NCI-H69 and SHP77) but in no other cell lines at 10 μM. At a concentration of 20 μM, partial inhibition (8–78%) of each of the four SCLC cell lines but none of the NSCLC or breast cancer cell lines was noted with all five of the SPD. At the highest concentration (40 or 50 μM), each of the five SPD exhibited complete or partial inhibition for the four SCLC cell lines tested (Table 4). The SPD and SPD4 derivatives had the largest effect on all four SCLC cell lines. There was far less inhibition of the NSCLC (A549) and breast (T47D) adenocarcinoma cell lines, where the [3H]dThd incorporation ranged from 56 to 135% of control (Table 4). There were no

### Table 4 Effect of SPDs (40 or 50 μM) on [3H]dThd incorporation

<table>
<thead>
<tr>
<th>Derivative</th>
<th>NCI-H345</th>
<th>NCI-H510</th>
<th>NCI-H69</th>
<th>SHP-77</th>
<th>A549</th>
<th>T47D</th>
</tr>
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<tbody>
<tr>
<td>SPD</td>
<td>0%</td>
<td>4%</td>
<td>4%</td>
<td>1%</td>
<td>81%</td>
<td>77%</td>
</tr>
<tr>
<td>SPD1</td>
<td>79%</td>
<td>NTc</td>
<td>44b</td>
<td>50%</td>
<td>131%</td>
<td>104%</td>
</tr>
<tr>
<td>SPD2</td>
<td>1%b</td>
<td>NTc</td>
<td>6%</td>
<td>58%</td>
<td>98%</td>
<td>89%</td>
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<tr>
<td>SPD3</td>
<td>1%b</td>
<td>1%c</td>
<td>2%b</td>
<td>0%</td>
<td>86%</td>
<td>60%</td>
</tr>
<tr>
<td>SPD4</td>
<td>NTc</td>
<td>10%c</td>
<td>3%c</td>
<td>2%b</td>
<td>78%</td>
<td>60%</td>
</tr>
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</table>

* Significant at α = 0.05 (two-tail).
* Significant at α = 0.01 (two-tail).
* NT, not tested.

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3606
Fig. 5. Growth curves of six cell lines in the presence of varying concentrations of [d-Arg¹, d-Phe⁵, d-Trp⁷,⁹, Leu¹¹]substance P. NCI-H345, NCI-H146, and NCI-H510 are SCLC cell lines; A549 is a breast lung adenocarcinoma cell line, and T47DV and ZR75 are breast adenocarcinoma cell lines.
inhibitory effects on human skin fibroblasts at any concentration (data not shown).

The results of SPD on growth curves of the cell lines in serum-free HITES media were similar to the $[^3H]dThd$ results and are shown in Fig. 5. SPD at concentrations up to 20 μM had no significant effect on the breast adenocarcinoma cell lines T47DV and ZR75 and the NSCLC cell line A549. A SPD concentration of 10 μM partially inhibited the growth of the SCLC lines NCI-H345 and NCI-H510. At the 20 μM concentration, there was complete or near complete inhibition of all SCLC lines tested, and a 40 μM concentration completely inhibited all SCLC lines tested. This high concentration (40 μM) also partially inhibited the growth of the breast and lung adenocarcinoma cell lines, suggesting that the toxic effects could be nonspecific at high concentrations. Overall, the toxicity of these peptides was slightly greater in the growth curve assays than in the $[^3H]dThd$ assays, perhaps due to the more prolonged exposure to the antagonists.

Because clinical application of these peptide antagonists requires prolonged effective serum concentrations and because these peptides and their antagonists are often rapidly degraded by plasma proteases, chemical modifications were made in the antagonists to prevent serum degradation. To evaluate the stability of these peptides and peptide antagonists in serum, we incubated them in serum for varying times from 1 min to 24 h and evaluated the function of the native peptide and antagonists in the calcium assays (Table 5). For the BK and BK antagonist assays, we used SHP-77 cells as in Fig. 2. For the bombesin and bombesin antagonist assays, Indo-1AM-loaded NCI-H345 SCLC cells were first stimulated with FBS because it produces a calcium flux response. After serum stimulation, the cells are refractory to repeat serum effects for more than 24 h (10). When the $[Ca^{2+}]$, returned to resting values after 2 to 3 min, the peptide antagonist in serum was added. As anticipated, both bombesin and BK were inactivated completely within 15 min of incubation in serum (Table 5). The chemical modifications in the BK antagonist CP126 and the bombesin antagonists L366175, SPD, and SPDP allowed them to remain active in serum for more than 24 h, whereas the S30 bombesin antagonist lost 55% of its activity in 24 h (Table 5).

**DISCUSSION**

In these studies, we showed that there are several obstacles to overcome in developing peptide antagonists as anti-growth factor therapy for lung cancer: (a) the heterogeneity of peptide responsiveness (receptor expression) which allows lung cancer cells to respond to a number of peptide growth factors; (b) the low affinity of many antagonists, which leads to the requirement for high peptide concentrations; and (c) the rapid inactivation of many antagonists by serum peptidases, which makes systemic delivery difficult or impossible. We first evaluated a series of bombesin antagonists because of the established autocrine role of bombesin in lung cancer. We showed that certain chemical modifications could be made which would both increase the binding affinity and prolong the serum stability. For example, S30 had low potency with an IC_{50} of 5 μM for inhibition of bombesin-induced $[Ca^{2+}]$ response. S30 was far more stable in serum than was native bombesin but had lost 50% of its activity after a 24-h incubation in serum.

The SC196 bombesin antagonist was modified by reducing the peptide bond between Leu^{13} and Leu^{14} in S30. As shown in other model systems by Coy et al. (26), this reduced bond substitution markedly decreased the IC_{50} to 200 nM by increasing the binding affinity. The SC196 antagonist retained its full inhibitory properties for more than 24 h in serum. The IC_{50} was decreased even further by the substitutions at the carboxyl end of the peptide in L366175, where the IC_{50} was 50 nM. This compound also retained its activity in serum for at least 24 h. However, the ability of these highly potent and specific peptide antagonists to inhibit the growth of lung cancer cell lines was disappointing, presumably because heterogeneity of peptide responsiveness allowed greater growth factors to provide adequate growth stimulus. The concentration of the potent bombesin antagonists required to inhibit proliferation was in excess of 20 μM, which was similar to less potent and not so specific antagonists such as the SPD. High concentrations may produce nonspecific toxic effects and are probably impossible and/or impractical to achieve in humans.

There are conflicting data in the literature regarding the growth stimulatory effects of neuropeptides and inhibitory effects of peptide antagonists. Takuwa et al. (12) and Layton et al. (31) found little or no growth stimulatory or inhibitory effects of several neuropeptides and antagonists on SCLC cell lines. Bepler et al. (14) reported no growth stimulatory effects of AVP, neurotensin, or somatostatin in any growth assay but a small stimulatory effect for bombesin in soft agarose assays. We previously reported small stimulatory effects for bombesin in SCLC cells in $[^3H]dThd$, liquid growth and soft agarose assays, whereas BK, AVP, and CCK had no stimulating effects in any of the three assay systems (11). Rozengurt’s group has consistently shown growth stimulatory effects of neuropeptides and inhibitory effects of their antagonists in both $[^3H]dThd$ and soft agarose assays (15, 25, 28). In soft agarose assays, Staley et al. (38) and Mahmoud et al. (29) showed that bombesin antagonists inhibited the growth of SCLC cell lines. It is unclear whether the differences relate to the assay system (e.g., $[^3H]dThd$ versus soft agarose), methodological differences in the assays (e.g., the presence of albumin), the cell lines chosen, or other factors.

We and others showed that gastrin and CCK elicit calcium responses in SCLC cells through their interactions with CCK_{A} and CCK_{B} receptors (11, 13, 39, 40). The CCK and AVP antagonists which we studied were highly specific as reported previously in other systems (35–37, 39, 40). The CCKA1 (L364–718) antagonist preferentially affects CCK_{A} receptors, whereas CCKA2 (L365–260) preferentially interacts with CCK_{A} receptors (35–37, 39, 40). Our $[Ca^{2+}]$ flux assays showed that all three of these antagonists blocked the peptide-induced calcium response of only the expected peptide. CCKA2 was the most potent of the three antagonists with an IC_{50} of less than 1 nM. The two CCK antagonists were synthetic chemicals, not peptides, and were stable for long periods in serum. Other investigators reported that a synthetic pseudopeptide analogue of CCK-7 was found to be a potent agonist in the peripheral system and was a highly potent and selective antagonist in the striatum (41). Like the specific bombesin antagonists, high concentrations (20 μM) of the CCK antagonists which we studied were required for in vitro growth inhibition. This was most likely due to the specificity of the antagonist and the heterogeneity of the requirements of the cancer growth factor. It is unclear whether these concentrations can be achieved practically.
In vivo studies showed that SPDs have only modest antitumor effects on lung cancer cell lines when studied with pseudopeptide modifications at the carboxyl terminal amino acid nor with substitutions on the terminal amino group. These types of substitutions increased the potency of the bombesin derivatives which we studied. However, several pseudopeptide analogues of substance P or spandite (D-Arg1, D-Trp7,9, Leu11)SP, (D-Pro4, D-Trp7,9)SP-4—11, and (D-Pro4, D-Trp7,9, 11)SP4—11 are found to be competitive antagonists of a number of different peptides including cholecystokinin and bombesin (42). The SPD1, SPD2, SPD3, and SPD4 analogues that we studied behaved more closely to (D-Arg1, D-Phe9, DR-Trp7,9, 11)SP which inhibits bombesin, BK, and vasopressin but not CCK (43). Moreover, our studies were similar to those of others in showing that these peptides were capable of inhibiting the growth of SCLC cell lines but only at high concentrations (20 to 50 μM; Refs. 28—30).

In vivo studies showed that SPDs have only modest antitumor effects against SCLC xenografts (44).

Our studies showed that SPDs can be developed which are stable in serum for long periods. Our studies did not evaluate the metabolic fate of these peptides, but by using high performance liquid chromatography and other assays, others reported the metabolic half lines and metabolic products in serum-free conditions (45). Unfortunately, none of the SPDs which we studied was very potent because all required concentrations of at least 10 μM to inhibit calcium signal transduction and 40 μM to inhibit the growth of lung cancer cell lines. We did not study SPDs with pseudopeptide modifications at the carboxyl terminal amino acid nor with substitutions on the terminal amino group. These types of substitutions increased the potency of the bombesin derivatives which we studied. However, several pseudopeptide analogues of substance P or spandite were synthesized and found to act as more specific antagonists for SP receptors but with less potency than spandite (46). More potent SPDs should be synthesized and tested for their ability to block calcium signal transduction and lung cancer cell growth. We found that the growth inhibitory effects of the SPDs were greater on the SCLC cell lines than the NSCLC cell lines. This was predictable from the greater sensitivity of SCLC cell lines to neuropeptide stimulation which we and others demonstrated previously (10—13). Breast cancer cell lines were inhibited only at very high concentrations (40—50 μM), possibly due to nonspecific toxic effects.

The striking neuroendocrine features of many lung cancers and the importance of the signal transduction pathway induced by neuropeptides suggests that the development of specific or broad spectrum anti-growth factor compounds should be continued. Such anti-growth factor compounds have potential use in the chemoprevention of lung cancer as well as in its therapy. Increased levels of both bombesin-like peptides and gastrin were reported in the bronchoalveolar lavage of smokers and lung cancer patients (16, 17), suggesting an important role for these peptides in the early premalignant changes in the respiratory epithelium as well as in tumor progression. Our studies make it clear that such compounds developed for chemoprevention and therapy should be potent, stable in serum, and perhaps have a wide specificity. None of the compounds which we tested thus far meets all these criteria, but it is possible that such compounds can be developed rationally. Recent technology led to the development of BK antagonists with relatively high potency and a long half-life by the use of dimerization with a bisuccinimidoalkane (47). Furthermore, multimeric forms of peptide antagonists with improved activity were also reported (48). This dimerization or multimerization technology should also make it possible to link more than one peptide antagonist in the same molecule. Monoclonal antibodies to peptide growth factors and their receptors have shown some antitumor effects in vivo. These molecules alone or coupled to radioligands or toxins are difficult to deliver to human cancers because of their large size and their inability to avoid phagocytosis by the reticuloendothelial system. Peptide-toxin conjugates or radiolabeled peptides have a much smaller size and should be evaluated as antitumor agents for lung cancer. These studies suggest that such conjugates should bind to the widest number of cell lines and should be stable in serum.

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