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A vasoactive intestinal peptide antagonist inhibits non-small cell lung cancer growth

(vasoactive intestinal peptide receptors/cAMP)

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ABSTRACT **The most prevalent lung cancer, non-small cell lung cancer (NSCLC) has receptors for vasoactive intestinal peptide (VIP). Here the effects of a VIP antagonist (VIPhyb) on NSCLC growth were investigated. *In vivo*, when VIPhyb (10 μg, s.c.) was daily injected into nude mice, xenograft formation was significantly inhibited by ≈80%. *In vitro*, VIP (100 nM) stimulated colony formation ≈2-fold, whereas 1 μM VIPhyb inhibited colony formation by ≈50% when adenocarcinoma cell line NCI-H838 was used. The attenuation of tumor proliferation is receptor mediated, as VIPhyb inhibited specific ^{125}I-labeled VIP binding to cell lines NCI-H157 and NCI-H838 with an IC_{50} of 0.7 μM. VIP (10 nM) increased the cAMP levels 5-fold when cell line NCI-H838 was used, and 10 μM VIPhyb inhibited the increase in cAMP caused by VIP. Northern blot analysis and radioimmunoassays have shown VIP mRNA and VIP-like immunoreactivity in NSCLC cells. These data suggest that VIP may be a regulatory peptide in NSCLC and that VIPhyb is a VIP receptor antagonist that inhibits proliferation.**

Non-small cell lung cancer (NSCLC), which includes adenocarcinoma, large cell carcinoma, and squamous cell carcinoma, is the most prevalent type of lung cancer, killing ≈110,000 people in the United States annually (1). Even with current surgical and chemotherapeutic interventions, NSCLC patients have a median survival time of 5 years. Although the regulation of growth for NSCLC is poorly understood, previous investigations have shown that NSCLC cells possess receptor sites for vasoactive intestinal peptide (VIP; refs. 2 and 3), a substance with recognized effects on cellular proliferation and development (4–6).

VIP, a 28-amino acid polypeptide, is derived from a 170-amino acid precursor protein (7, 8). PreproVIP may be metabolized to VIP by trypsin-like enzymes, carboxypeptidase B-like enzymes, and peptidyl-α-monooxygenase (9–11). VIP, which is amidated at the C terminus, is localized to neurons and endocrine cells in the central nervous system (CNS) and periphery of normal animals. Upon CNS administration, VIP modulates neuronal activity, stimulates adenylate cyclase activity, and regulates cerebral blood flow (12). Also, VIP maintains survival of spinal cord neurons in primary culture (6). In the periphery, endogenous VIP is present in nerves supplying airway smooth muscle as well as glands and pulmonary vessels within the normal adult lung (13). VIP functions as a bronchodilator and relaxes pulmonary vascular smooth muscles (14–17). These actions may be mediated by the VIP receptors that have been detected in binding assays with plasma membranes derived from the rat, mouse, guinea pig, and human lung (18–20). By *in vitro* autoradiographic techniques and lung slices, these VIP receptors have been localized to the alveoli and epithelium of the rat lung and pulmonary artery smooth muscle and alveolar walls of the human lung (21, 22); VIP is deficient in patients with bronchial asthma (23).

In the malignant lung, high-affinity ^{125}I-labeled VIP binding sites were detected in small cell lung cancer (SCLC) and NSCLC cell lines (2, 3, 24). Also, high-affinity binding sites on SCLC were found for helodermin and pituitary adenylate cyclase-activating polypeptide (PACAP), which are both members of the VIP family in that they share structural similarity with VIP (25, 26). VIP elevates intracellular cAMP and increases the secretion rate of bombesin-like peptides from SCLC cells (27). Bombesin-like peptides stimulate SCLC but not NSCLC proliferation (28).

Recently, a VIP-receptor antagonist was synthesized that is a hybrid peptide of neurotensin and VIP consisting of an N-terminal Lys-Pro-Arg-Arg-Pro-Tyr (designed to increase membrane permeability) followed by the C-terminal 22 amino acids of VIP (29, 30). VIPhybrid (VIPhyb) is effective in blocking the actions of VIP in the CNS, but it is impotent at receptors present on lymphocytes (31–33), thereby suggesting regional VIP receptor heterogeneity and significant pharmacological specificity for this peptide. Here the effects of VIPhyb on NSCLC cells were investigated.

METHODS

Cell Culture. Human lung cancer cell lines (NCI-H23, -H157, -H226, -H322, -H460, -H522, -H727, -H838, -H1299, and -H1512) were cultured in serum-supplemented medium at 37°C [RPMI 1640 medium (Fisher Scientific) containing 10% heat-inactivated fetal bovine serum (GIBCO)]. The NSCLC cell lines were adherent and weekly were split 1:20 by using trypsin/EDTA (GIBCO). Routinely, the cells had >90% viability and were mycoplasma free. The cells were used when they were in the exponential growth phase.

Binding Studies. The ability of agents to inhibit ^{125}I-VIP binding was investigated. NCI-H23, -H157, -H727, or -H838 cells were harvested after treatment with trypsin/EDTA and 5×10^4 cells were added to 24-well plates, which were pretreated with 10 μg of human fibronectin (Sigma). After a monolayer of cells had formed (3 days), receptor binding studies were conducted. The cells were washed four times

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Abbreviations: NSCLC, non-small cell lung cancer; VIP, vasoactive intestinal peptide; CNS, central nervous system; SCLC, small cell lung cancer; PACAP, pituitary adenylate cyclase-activating polypeptide; BN/GRP, bombesin/gastrin-releasing peptide.

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with SIT medium (RPMI 1640 medium containing 30 nM Na_2SeO_3, 5 μg of insulin per ml, 10 μg of transferrin per ml; Sigma) and incubated with ^{125}I-VIP (10^5 cpm; specific activity, 2200 Ci/mmol; 1 Ci = 37 GBq) in 200 μl of receptor binding buffer (RPMI 1640 medium containing 1% bovine serum albumin and 1 mg of bacitracin per ml). After 30 min at 37°C, free radiolabeled peptide was removed and cells that contained bound peptide were dissolved in 0.2 M NaOH and assayed in a γ-counter. In a representative experiment with NSCLC cell line NCI-H838, total binding was 5876 ± 246 cpm, whereas nonspecific binding in the presence of 1 μM VIP was 1026 ± 375 cpm.

cAMP Assays. cAMP was assayed by radioimmunoassay as described (27). Cell lines NCI-H727, -H838, or -H1299 were harvested and resuspended in SIT medium containing 1% bovine serum albumin, 1 mg of bacitracin per ml, and 100 μM isobutylmethylxanthine. After 5 min, the reaction was quenched by addition of an equal volume (0.5 ml) of ethanol. The samples were mixed and frozen at −80°C until assay. The samples were acetylated before the radioimmunoassay.

***In Vitro* Growth Assays.** Growth assays were conducted with NCI-H226, -H727, -H838, or -H1299 cells and by the agarose cloning system described (28). The base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% fetal bovine serum in six-well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, the peptide(s) doubly concentrated, and 2×10^4 single viable cells. For each cell line and peptide concentration, triplicate wells were plated. After 2 weeks, 1 ml of 0.1% *p*-iodonitro tetrazolium violet was added and after 16 hr at 37°C the plates were screened for colony formation. The number of viable colonies >120 μm in diameter was counted.

***In Vivo* Growth Assays.** The ability of VIPhyb to inhibit xenograft formation in nude mice was investigated. Female athymic BALB/c nude mice, 4–5 weeks old, were housed in a pathogen-free temperature controlled isolation room and the diet consisted of autoclaved rodent chow and autoclaved water given ad libitum. NCI-H157, -H727, or -H838 cells (1×10^7) were injected s.c. into the right flank of each mouse. Palpable tumors were observed in ≈90% of the mice after 1 week. Phosphate-buffered saline (PBS) (100 μl), or VIPhyb (10 μg/100 μl) was injected s.c. daily. The tumor volume (height × width × depth) was determined with calipers twice weekly and recorded. When the tumor became necrotic, the growth studies were terminated. The tumor was then excised and weighed.

RNA Measurements and Radioimmunoassays. The VIP mRNA was determined by Northern blot analysis. Total NSCLC RNA was isolated by the guanidinium isothiocyanate or urea/lithium chloride method (9). Ten micrograms of denatured RNA was separated in a 0.66 M formaldehyde/1% agarose gel. The RNA was blotted onto a nitrocellulose membrane overnight and the membrane was hybridized with a complementary RNA probe for a human VIP-encoding exon (10). The probe was subcloned in the bacterial plasmid vector pGEM-1, labeled with [^{32}P]UTP according to the Promega Biotec protocol, and the blot was hybridized. After the blot was washed, the film exposure time was routinely 16 hr at −70°C.

For VIP radioimmunoassays, lung cancer cell lines were extracted by boiling in 2 M acetic acid, lyophilized, resuspended in phosphate-buffered saline containing 1% bovine serum albumin and rabbit anti-VIP antiserum (1:20,000) as well as 5000 cpm of ^{125}I-VIP (2200 Ci/mmol), and incubated for 16 hr at 4°C. Goat anti-rabbit antiserum was added at a dilution of 1:10 and normal rabbit serum was added at a dilution of 1:100; 12% polyethylene glycol was added to form a stable precipitate, which was collected by centrifugation at 1000 × *g* and assayed with a γ-counter. The sensitivity of the assay was 1 fmol of VIP and the antiserum had strong cross-reactivity with VIP and VIP-(10–28), but not to secretin, PACAP, glucagon, or thymosin α1.

RESULTS

VIP Stimulates NSCLC Proliferation *in Vitro*. The effects of VIP on NSCLC growth were investigated with a clonagenic assay using adenocarcinoma cell line NCI-H838. When colonies >120 μm in diameter were counted, 100 nM VIP significantly stimulated colony number, with 10 nM VIP slightly increasing growth (Fig. 1 *Top*). Similar data were observed for NCI-H226 (adenocarcinoma), NCI-H1299 (large cell carcinoma), and NCI-H727 (lung carcinoid) cell lines. Large viable colonies formed in the absence of additions (Fig. 1 *Middle*) and VIP increased the number and size of the colonies.

VIP Is Synthesized by NSCLC. The presence of VIP mRNA and VIP immunoreactivity was investigated. By using VIP-specific RNA probes (10, 11) and Northern blot hybridization, a major band at 2.1 kb and a minor band at 5 kb (Fig. 2) were detected in total RNA isolated from NCI-H727. Previous studies of rat brain and human tumors indicated that the 2.1- and 5-kb bands correspond to VIP mRNA and VIP RNA that was not properly spliced; the VIP gene has six introns (9–11).

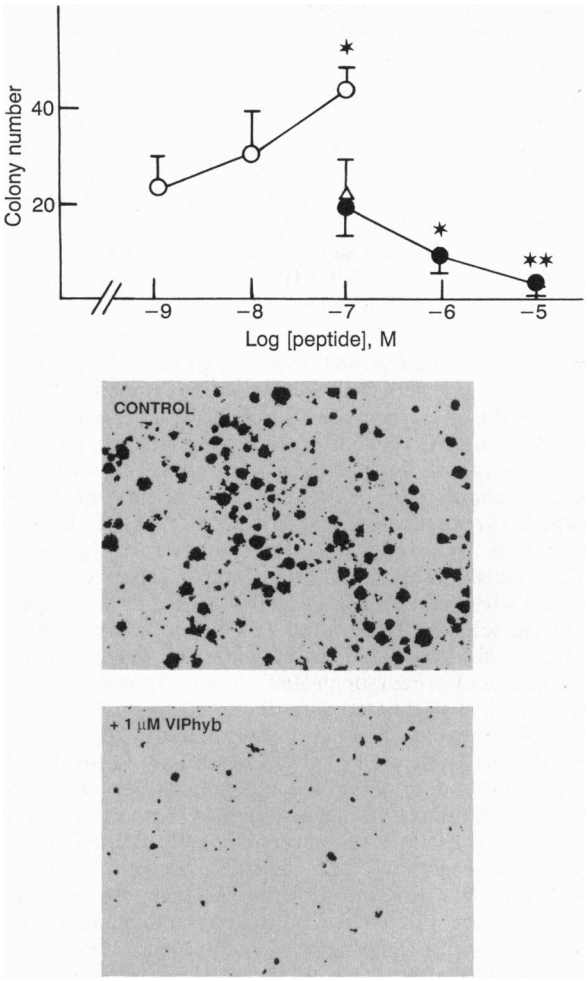

FIG. 1. Effect of VIPhyb on NSCLC growth *in vitro*. (*Top*) Effect of increasing doses of VIPhyb on NCI-H838 clonal growth was determined. Colony number was determined in the presence of increasing concentrations of VIP (○) or VIPhyb (●) and 100 nM VIP plus 1 μM VIPhyb (△). *, $P < 0.05$; **, $P < 0.01$. NCI-H838 colonies were grown in SIT medium (*Middle*) or in SIT medium containing 1 μM VIPhyb (*Bottom*). This experiment is representative of five others.

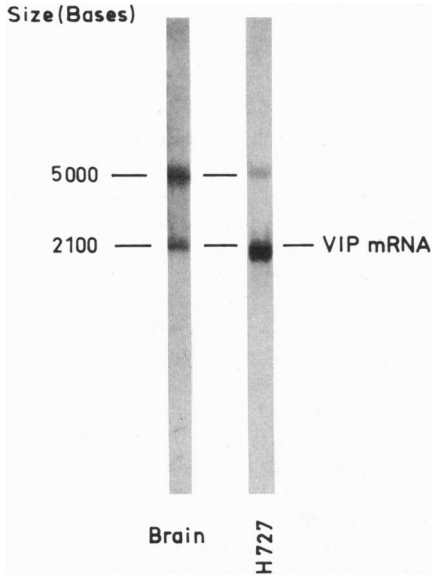

FIG. 2. Detection of VIP mRNA. Total RNA was prepared from NCI-H727 and 14-day-old rat brain and subjected to agarose gel electrophoresis and Northern blot hybridization with the [^{32}P]UTP VIP exon-specific RNA probe. A resulting autoradiogram is shown. This experiment is representative of two others.

In further investigations, VIP mRNA was detected in 8 of 11 NSCLC cell lines [e.g., NCI-H23, -H522, -H838, and -H1512 (adenocarcinoma); NCI-H157 (squamous cell carcinoma); NCI-H322 (bronchoalveolar); NCI-H460 (neuroendocrine); and NCI-H727 (lung carcinoid)]. In addition, NSCLC extracts from several NSCLC cell lines such as NCI-H838 or NCI-H727 contained immunoreactive VIP (data not shown; 0.1 pmol per mg of protein). Cell line NCI-H727 had the highest levels of 2.1-kb VIP mRNA of any lung cancer cell line examined.

A VIP Antagonist Inhibits NSCLC Growth *in Vitro*. The ability of VIPhyb to block VIP receptors was investigated. In the presence of 1 μM VIPhyb, the number and size of the NCI-H838 colonies grown in cell culture were reduced (Fig. 1 *Bottom*). Also, VIPhyb slightly decreased colony number at 100 nM and significantly reduced colony number at 1 and 10 μM (Fig. 1 *Top*). Similar data were obtained by using the cell lines NCI-H226 (adenocarcinoma), NCI-H1299 (large cell carcinoma), and NCI-H727 (lung carcinoid). Also, 100 nM VIP increased the number of NCI-H838 colonies from 21 to 44, and in the presence of VIP plus 1 μM VIPhyb, only 23 colonies formed. These data suggest that VIPhyb may antagonize the growth stimulation caused by endogenous or exogenous VIP.

To support the hypothesis that antagonist action was due to inhibition of VIP receptors on the tumor cells, radioligand binding studies were carried out. As shown in Fig. 3 *Top*, VIPhyb and VIP blocked specific ^{125}I-VIP binding to NSCLC cell line NCI-H838 in a concentration-dependent manner. The concentrations of VIPhyb, PACAP, and VIP required to inhibit 50% of the specific ^{125}I-VIP bound (IC_{50}) were 0.7 μM, 20 nM, and 7 nM. VIP-(10–28) was less potent and inhibited specific ^{125}I-VIP binding with an IC_{50} value of 5 μM. Similar data were obtained for NCI-H23 (adenocarcinoma), -H157 (squamous cell carcinoma), and -H727 (lung carcinoid) cells.

The effects of VIPhyb on second messenger production were investigated. VIP elevated the cAMP levels in a concentration-dependent manner (ED_{50} = 1 nM; Fig. 3 *Middle*). In contrast, VIPhyb (10 μM) completely inhibited the cAMP production elicited by 1 nM VIP (Fig. 3 *Middle*) and attenuated the increase in cAMP caused by 10 nM VIP with a half-maximal concentration of 6 μM (Fig. 3 *Bottom*). If 10 μM

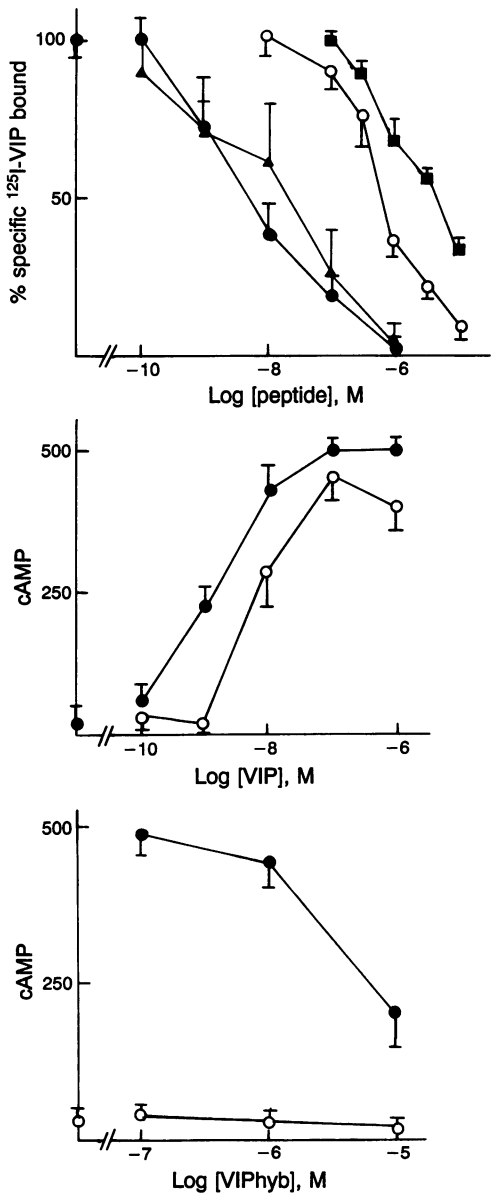

FIG. 3. Effect of VIPhyb on NSCLC VIP binding and cAMP production. (*Top*) Percentage specific ^{125}I-VIP binding to NCI-H838 was determined as a function of VIP (●), PACAP (▲), VIP-(10–28) (■), and VIPhyb (○) concentration. (*Middle*) cAMP was determined in the presence (○) or absence (●) of 10 μM VIPhyb as a function of VIP concentration. (*Bottom*) cAMP was determined in the presence (●) or absence (○) of 10 nM VIP as a function of VIPhyb concentration. Mean ± SE of four determinations is indicated. This experiment was repeated two times.

VIPhyb was added to NCI-H838 in the presence of increasing amounts of VIP, the dose–response curve was shifted to the right (ED_{50} = 5 nM; Fig. 3 *Middle*), consistent with the action of a competitive antagonist. Similar data were obtained by using cell lines NCI-H1299 and -H727. Finally, VIPhyb had no agonistic activity on cAMP production (Fig. 3 *Bottom*). Together these data support the conclusion that VIPhyb antagonizes VIP receptors on NSCLC cells.

VIPhyb Inhibits Lung Cancer Growth *in Vivo*. Also, the effects of VIPhyb were investigated on NSCLC xenograft formation. Fig. 4 shows that NSCLC xenografts formed after 1 week when cell line NCI-H727 was used. VIPhyb was then administered daily s.c. into the nude mice and the tumor volume was determined with calipers. In the control animals, tumor volume increased exponentially to 1898 mm^3 at week

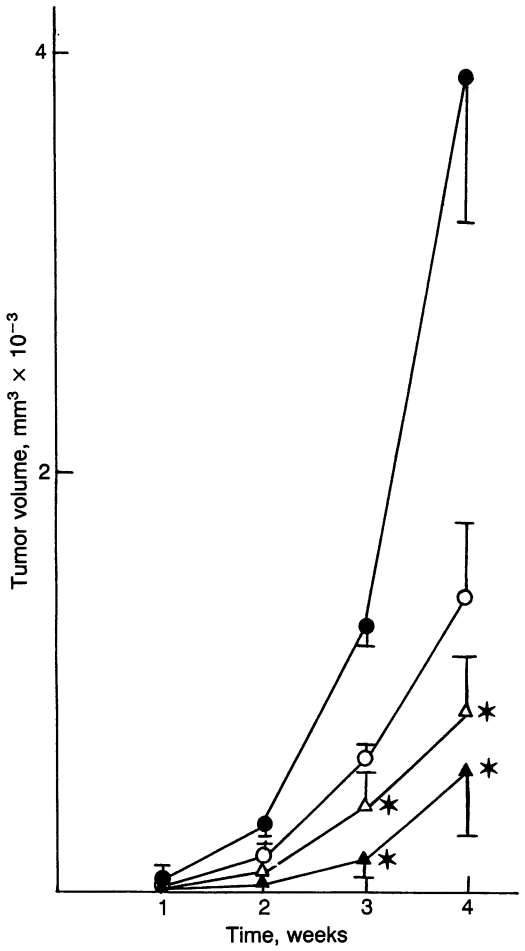

FIG. 4. Effect of VIPhyb on NSCLC growth *in vivo*. Female athymic BALB/c nude mice (4–5 weeks old) were injected with NCI-H727 cells (1×10^7) s.c. into the right flank. After 1 week, a palpable mass formed and the tumor-bearing mice were injected daily with 100 μl of PBS (●) or PBS containing 0.1 (○), 1 (△), or 10 (▲) μg of VIPhyb s.c. adjacent to the tumor nodule. Tumor volume (height × width × depth) was determined weekly with calipers and recorded. Mean ± SE of three determinations is indicated. *, $P < 0.05$ by Student's *t* test. This experiment is representative of four others.

3. VIPhyb inhibited tumor growth in a dose-dependent manner and at week 3 the mean tumor volumes were 433 and 255 mm^3 using 1 and 10 μg of VIPhyb, respectively. These data indicate that 0.1 μg of VIPhyb slowed NSCLC growth, whereas 1 and 10 μg significantly inhibited NSCLC xenograft formation. The effects of the VIP hybrid antagonist were reversible in that removal of the antagonist resulted in a tumor growth rate similar to that of control animals. If the tumors were excised at the end of the experiment, the mean tumor weight was significantly reduced in the nude mice injected with 1 and 10 μg of VIPhyb. In addition, Table 1 shows that with NCI-H157 (squamous cell carcinoma), daily administration of the VIPhyb (10 μg/day s.c.) significantly inhibited xenograft formation. At the end of the experiment, the total weight of nude mice treated with 10 μg of VIPhyb was similar, whereas the xenograft weight was significantly reduced.

Table 1. Effects of VIPhyb on NCI-H157 growth

Addition	Tumor weight, g	Animal weight, g
None	7.4 ± 1.35	29.45 ± 0.95
VIPhyb (10 μg)	4.0 ± 1.0*	27.0 ± 2.0

Tumor weight (mean ± SE of three determinations) is indicated. *$P < 0.05$.

DISCUSSION

The present study indicates that a VIP antagonist, VIPhyb, can attenuate the growth of lung cancer cells both in cell culture and *in vivo*. The specificity and potency of this peptide suggest that such a strategy may be effective at inhibiting the growth of these tumors by antagonizing the ability of VIP-like peptides that bind to VIP receptors.

The mechanisms by which VIP mediates its proliferative effects and VIPhyb exerts its antagonistic effects are receptor mediated. Thus, VIPhyb inhibited specific ^{125}I-VIP binding to NCI-H838 with an IC_{50} value of 0.7 μM and binds with ≈7-fold greater affinity than does VIP-(10–28). The affinity of VIPhyb for lung cancer receptors is ≈10-fold lower than its affinity for the CNS cAMP-associated VIP receptor but is much greater than that of the lymphoid cell VIP receptor, suggesting tissue specificity (31). In the embryonic peripheral nervous system, VIP stimulates mitosis at concentrations similar to those required to inhibit lung cancer proliferation (4). Recently, the peripheral VIP receptor was cloned and found to contain 459 amino acid residues and 7 hydrophobic domains (34). It has a structure reminiscent of other guanine nucleotide binding protein-coupled receptors such as the bombesin/gastrin-releasing peptide (BN/GRP) receptor (35, 36).

VIPhyb may inhibit binding to other receptors of the VIP family. ^{125}I-PACAP-27 binds with high affinity to lung cancer cell lines (26). Specific ^{125}I-PACAP-27 binding is inhibited with ≈2 orders of magnitude greater affinity by PACAP-27 than by VIP. Also, VIPhyb inhibits ^{125}I-PACAP-27 binding with approximately the same affinity as it does ^{125}I-VIP binding; VIPhyb has no effect on SCLC BN/GRP receptors. Therefore, VIPhyb may be useful at inhibiting PACAP in addition to VIP receptors. Also, helodermin receptors are on SCLC cells (25), and the effect of VIPhyb on these receptors is unknown.

The antagonistic effects of VIPhyb were also apparent at the level of VIP-stimulated cAMP formation. Approximately 6 μM VIPhyb was required to half-maximally inhibit the increase in cAMP caused by 10 nM VIP; VIP had no effects on phosphatidylinositol turnover at doses ranging from 0.1 to 1000 nM. This is approximately an order of magnitude more VIPhyb than was required to inhibit ^{125}I-VIP binding; however, the binding experiments used only 0.2 nM ^{125}I-VIP. Also, the ED_{50} for VIP to elevate cAMP increased from 1 to 5 nM in the presence of 10 μM VIPhyb. Because 74 nM VIPhyb was required to inhibit the cAMP increase caused by 1 μM VIP in the CNS (8), VIPhyb is less potent in NSCLC cells. VIP-(10–28) was approximately an order of magnitude less potent than VIPhyb at inhibiting VIP-stimulated cAMP (T.W.M., unpublished data).

NSCLC cells have amplification and increased expression of the c-*myc* and Ki-*ras* oncogenes (37, 38). NSCLC cells produce type α transforming growth factor (TGF-α) and have epidermal growth factor (EGF) receptors; the growth of NSCLC is inhibited by TGF-α and EGF receptor monoclonal antibodies (39–41). Lung cancer cells may proliferate because of the abundance of oncogenes and growth factors. The effects of VIP on growth factor gene expression remain to be investigated. Preliminary data indicate that VIP stimulates lung cancer c-*fos* mRNA levels and the increase in c-*fos* caused by VIP was inhibited by VIPhyb (M.D., unpublished data). VIP may stimulate early response genes by activation of protein kinase A, which in turn alters oncogene expression.

VIPhyb inhibited the growth of NSCLC. Other investigators have found that VIP stimulates (4–6) or inhibits (42) growth and development. Thus, the effects of VIP are complex and depend on cell type, dose, and assay used. Here VIPhyb inhibited and VIP stimulated the growth of NSCLC.

Also, purified VIP antiserum (10 μg/ml) and VIP-(10–28) (10 μM) inhibited NCI-H838 colony formation by ≈20%.

VIP mRNA and peptide were present in NSCLC. For NSCLC cell line NCI-H727, the 2.1-kb band is more intense than it is in the rat brain, whereas for rat brain the 5.0-kb band is more intense. It is possible that the NSCLC cell line may be more efficient than rat brain at splicing the preproVIP RNA. Surprisingly, the levels of immunoreactive VIP were very low in cell lines NCI-H838 and -H727 (0.1 pmol per mg of protein). It is possible that VIP (or a related peptide of the same precursor) is rapidly secreted and/or metabolized rather than stored. By using HPLC techniques, two major peaks of immunoreactivity were present with a NCI-H727 extract; one peak coeluted with synthetic VIP, whereas the other peak eluted before VIP and is a peptide of undetermined structure. The hydrophilic peak could be a proVIP-like peptide. Preliminary data (J. Fahrenkrug, personal communication) indicate that proVIP-like peptides are present in NSCLC extracts as well as conditioned medium and that the proVIP-like peptides are more abundant than VIP. VIP is synthesized as a 170-amino acid precursor protein that contains a single copy of VIP and the related peptide His-Met (PHM); VIP, PHM-like peptides, and C-terminal extensions of VIP-like peptides have appreciable biological activity (43–45). Therefore, VIP-like peptides are present in and secreted from NSCLC cells. Whether PACAP-like peptides are present in lung cancer cells remains to be investigated.

VIPhyb may also be effective in other tumors (e.g., mammary cancer; ref. 46) that use VIP as a growth factor. In SCLC, VIP elevated cAMP levels, resulting in increased secretion of BN/GRP (26). In turn, bombesin-like peptides bind to cell-surface receptors, elevate cytosolic calcium, and stimulate growth, which is inhibited by monoclonal antibodies against bombesin and bombesin receptor antagonists (28, 47). Preliminary data (T.W.M., unpublished data) indicate that VIPhyb also inhibits ^{125}I-VIP binding and VIP-stimulated cAMP formation, leading to an attenuation of SCLC growth. In contrast to SCLC, NSCLC cells lack receptors for BN/GRP, suggesting that VIP may mediate proliferative and secretagogue actions.

In summary, VIPhyb is a NSCLC VIP receptor antagonist that inhibits cancer growth *in vitro* and *in vivo*. These data suggest that VIP is an important regulatory peptide in NSCLC.

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