

Development of Simplified VIP Analogs with Receptor Selectivity and Stability for Human VPAC Receptors

HISATO IGARASHI, TETSUHIDE ITO, SAMUEL A. MANTEY, TAPAS K. PRADHAN,
WEI HOU, DAVID H. COY AND ROBERT T. JENSEN

*Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases,
National Institutes of Health (H.I., T.I., S.A.M., T.K.P., W.H., D.H.C., R.T.J.), Bethesda,
Maryland; and Peptide Research Laboratories, Department of Medicine, Tulane University
Health Sciences Center (D.H.C.), New Orleans, Louisiana (D.H.C.)*

Running title: Simplified VIP agonists

Corresponding author:

Dr. Robert T. Jensen

NIH/NIDDK/DDB

Bldg. 10, Rm. 9C-103

10 CENTER DR MSC 1804

BETHESDA MD 20892-1804

Tel: 301-496-4201; Fax: 301-402-0600; E-mail: robertj@bdg10.nidk.nih.gov

Number of: text pages – 18

tables – 3

figures – 6

references – 40

Word count: Abstract – 248

Introduction – 451

Discussion – 1,709

Abbreviations: VIP, vasoactive intestinal peptide; VPAC, VIP-PACAP receptors (for nomenclature see ref. (Harmar, et al., 1998); hVPAC₁, human VPAC₁ receptor subtype; hVPAC₂, human VPAC₂ receptor subtype; PANC1, human pancreatic cancer cell line; IBMX, 3-isobutyl-1-methyl xanthine; DMEN, Dulbecco's modified Eagle's medium; G418, geneticin; Ro-1553, Ac-His-Ser-Asp-Ala-Val-Phe-Thr-Glu-Asn-Tyr-Thr-Lys-Leu-Arg-Lys-Gln-Nle-Ala-Ala-Lys- cyclo[Lys-Tyr-Leu-Asn-Asp]-Leu-Lys-Lys-Gly-Gy-Thr-NH₂; PACAP, pituitary adenylate

cyclase-activating peptide; FBS, fetal bovine serum; BSA, bovine serum albumin; cAMP, cyclic
AMP

Recommended section: Gastrointestinal, Hepatic, Pulmonary and Renal

Abstract

Vasoactive intestinal peptide (VIP) is a widespread neurotransmitter whose physiological and pathophysiological actions are mediated by two receptor classes, VPAC₁ and VPAC₂. VIP is a 28-amino acid peptide that is rapidly degraded and simplified, metabolically stable analogs are needed. In this study we use information from studies of the VIP pharmacophore for VPAC₁/VPAC₂ to design nine simplified VIP analogs that could have high affinity and selectivity for each VPAC, or retained high affinity for both VPACs and were metabolically stable. From binding studies of their abilities to directly interact with hVPAC₁ (T47D cells, hVPAC₁ transfected cells) and hVPAC₂ (SupT₁ and VPAC₂-transfected cells) and to stimulate adenylate cyclase in each, two analogs, (Ala^{2, 8, 9, 11, 19, 22, 24, 25, 27, 28})VIP and (Ala^{2, 8, 9, 11, 19, 24, 28})VIP] were found to have >2000- and >600-fold selectivity for hVPAC₁. None of the nine analogs had hVPAC₂ selectivity. However, two simplified analogs [(Ala^{2, 8, 9, 16, 19, 24})VIP and (Ala^{2, 8, 9, 16, 19, 24, 25})VIP] retained high affinity and potency for both hVPACs. ¹²⁵I-[Ala^{2, 8, 9, 16, 19, 20, 21, 24, 25}]VIP was much more metabolically stable than ¹²⁵I-VIP. The availability of these simplified analogs of VIP, which are metabolically stable and have either hVPAC₁ selectivity or retain high affinity for both hVPACs, should be useful for exploring the role of VPAC subtypes in mediating VIP's actions as well as useful therapeutically and for exploring the usefulness of VIP receptor imaging of tumors and VIP receptor-mediated tumor cytotoxicity.

Introduction

VIP is a widely distributed neurotransmitter that is thought to play an important role in a number of physiological and pathological processes (Dockray, 1994;Gozes and Brenneman, 2000). VIP is proposed to play a role in a number of disease states (Gozes and Furman, 2004) including a role in growth of cancer cells (Gozes and Furman, 2004;Moody, 1996;Moody, et al., 2003); various central nervous system disorders (Dogrukol-Ak, et al., 2004;Gozes and Brenneman, 2000;Gozes and Furman, 2004); various inflammatory disorders such as rheumatoid arthritis (Gozes and Brenneman, 2000); various immunological disorders (Delgado, et al., 2004); and a role has been proposed for VIP in treatment of asthma (Groneberg, et al., 2001), impotence (Sandhu, et al., 1999;Kalsi, et al., 2002), and for treatment of septic shock (Kalsi, et al., 2002;Delgado, et al., 2004), central nervous system disorders (Dogrukol-Ak, et al., 2004;Gozes and Brenneman, 2000), and diabetes (Yung, et al., 2003).

In almost all cases, which VIP receptor subtype is mediating the action of VIP in these various conditions, is unclear. The actions of VIP are mediated by two receptor subtypes (VPAC₁ and VPAC₂), which have different pharmacology and distributions (Harmar, et al., 1998;Dockray, 1994). VIP has high affinity for both VIP receptor subtypes and therefore does not discriminate between the two VIP receptor subtypes (Harmar, et al., 1998). Furthermore, VIP is a 28-amino acid peptide, which undergoes rapid degradation *in vivo* with a half-life less than 1 min (Domschke, et al., 1978). Therefore, simplified VIP analogs that retain high affinity and have selectivity for one VIP receptor subtype, especially if metabolically stable, could be of value in investigating VIP's roles in physiological or pathological states as well as its use as a possible therapeutic agent. Furthermore, a metabolically stable, simplified VIP analog that retained high affinity for both VIP receptor subtypes could be useful for imaging tumors overexpressing VIP receptors as well as possibly for VIP receptor-directed anti-tumor treatment.

Recently we (Igarashi, et al., 2002a;Igarashi, et al., 2002b), and others (Nicole, et al., 2000) have performed alanine scanning as well as D-amino acid scanning of VIP (Igarashi, et al., 2002a;Igarashi, et al., 2002b) to define the VIP pharmacophore for the human VPAC₁ and human VPAC₂ receptors. These studies (Igarashi, et al., 2002a;Nicole, et al., 2000;Igarashi, et al., 2002b) provided information that could be helpful in the design of a simplified VIP analog that had either selective high affinity for one VPAC or which retained high affinity for both and yet might be metabolically stable. The latter point is supported by results of a previous study that demonstrated a poly-alaninated VIP analogue with high affinity for VPAC₁ was much more metabolically stable than VIP (Igarashi, et al., 2002b). However, in that study (Igarashi, et al., 2002b) the selectivity of this VIP analog for VPAC₁ (analog #2 in the present study) was not determined. Therefore, in the present study we used an analysis of these study results (Igarashi, et al., 2002a;Nicole, et al., 2000;Igarashi, et al., 2002b) on the VIP pharmacophore to design five additional VIP analogs with multiple alanine replacements that should have high affinity retained for VPAC₁ and three analogs that should have high affinity retained for VPAC₂ and assessed their affinities for each VPAC, their selectivity, and metabolic stability of selected ones.

Materials and Methods

Materials. PANC1 human pancreatic cancer cells and Sup T₁ human lymphoblastoma cells were obtained from American Type Culture Collection (Rockville, MD). Porcine vasoactive intestinal peptide (VIP) was from BACHEM Bioscience Inc., (King of Prussia, PA); Basal Medium Eagle (BME) amino acid mixture, BME vitamin solution, fetal bovine serum and Lipofectamine transfection reagent from Invitrogen (Carlsbad, CA); geneticin (G418 sulfate) from Mediatech Inc., (Herndon, VA); bacitracin, soybean trypsin inhibitor, 3-isobutyl-1-methylxanthine (IBMX) and alumina from Sigma (St. Louis, MO); AG50W-X4 resin from Bio-Rad (Richmond, VA); bovine serum albumin (BSA) fraction V from ICN Biomedicals (Aurora, OH); [¹²⁵I]VIP (2,200 Ci/mmol) and [¹²⁵I]PACAP(1-27) (2,200 Ci/mmol) from Perkin Elmer Life Science Products (Boston, MA); Na¹²⁵I (2,200 Ci/mmol) and [2-³H]adenine (22 Ci/mmol) from Amersham Pharmacia Biotech (Piscataway, NJ); 1, 3, 4, 6-Tetrachloro 3 α -, 6 α -diphenylglycouril (IODO-GEN) from Pierce Chemical Co., (Rockford, IL); N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) from Boehringer Mannheim Biochemicals, (Indianapolis, IN). Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium and HAM'S F-12K (HAM) medium from Biofluid, Inc., (Rockville, MD). The standard incubation solution contained 24.5 mM HEPES (pH 7.45), 98 mM NaCl, 6 mM KCl, 2 mM KH₂PO₄, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM CaCl₂, 1 mM MgCl₂, 1% (w/v) BSA, 0.2% (w/v) soybean trypsin inhibitor, 1% (v/v) amino acid mixture, and 1% (v/v) essential vitamin mixture.

Preparation of Peptides. Multiple alanine substituted VIP analogs (VPAC-AA-1 to 9, Table 1) were synthesized using standard solid phase methods as described previously (Sasaki and Coy, 1987; Igarashi, et al., 2002a). Homogeneity of the peptides was assessed by thin layer chromatography and analytical reverse-phase PHLC with the purity \geq 97% for each peptide.

Construction of PANC1 Cells Stably Transfected with hVPAC₂-R and hVPAC₂-R.

Construction of the human VPAC₁ receptor (hVPAC₁-R), and human VPAC₂ (hVPAC₂-R) receptor expression vector and construction of hVPAC₁-R or hVPAC₂-R stably transfected PANC1 cells (hVPAC₁-R or hVPAC₂-R/PANC1 cells) were described previously (Ito, et al., 2001; Igarashi, et al., 2002a).

Cell Culture. The hVPAC₁-R/PANC1 cells and hVPAC₂-R/PANC1 cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) antibiotics and 300 µg/ml G418. Sup T₁ human lymphoblastoma cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics, adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate as recommended by ATCC and were maintained in incubators at 37°C in an atmosphere of 5% CO₂ and 95 % air. T47D human breast cancer cells were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal bovine serum 1% (v/v) penicillin/streptomycin, insulin 0.2 IU/ml, and were maintained in incubators at 37°C in an atmosphere of 5% CO₂ and 95 % air.

Preparations of ¹²⁵I-Ro 25-1553. ¹²⁵I-Ro 25-1553 (a cyclic VIP analog selective for VPAC₂-R) (O'Donnell, et al., 1994a; O'Donnell, et al., 1994b; Gourlet, et al., 1997b) at a specific activity of 2200 Ci/mmol was prepared by a modification of the methods described previously (Zhou, et al., 1989). Briefly, 0.8 µg of IODO-GEN in chloroform was transferred to a vial, dried under a stream of nitrogen and washed with 100 µl of 0.5 M KH₂PO₄ (pH 8.0). To this vial, 20 µl of 0.5 M KH₂PO₄ (pH 8.0), 8 µg of peptide in 4 µl of water, and 2 mCi (20 µl) of Na¹²⁵I were added, mixed gently and incubated at room temperature for 6 min. The incubation was stopped by the addition of 100 µl of distilled water. The iodination mixture was applied to a Sep-Pak C18 (Waters Associates, Milford, MA), and free ¹²⁵I was eluted with 5 ml of water followed by 5

ml of 0.1% (v/v) trifluoroacetic acid. The radiolabeled peptides were eluted with 200 μ l of sequential elutions (x10) with 60% acetonitrile in 0.1% trifluoroacetic acid. The two or three fractions with the highest radioactivity were combined and purified on a reverse-phase, high performance liquid chromatography with a Vydac C18 column (0.46 x 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v) from 16-60% acetonitrile in 60 min, and 1-ml fractions were collected and assayed for radioactivity and receptor binding. The pH of the pooled fractions were adjusted to 7 using 0.2 M Tris (pH 9.5), and radioligands were stored in aliquots with 0.5% bovine serum albumin (w/v) at -20°C. ^{125}I -[Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP [VIP analog #2, Table 1 (Igarashi, et al., 2002a)] and ^{125}I -[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP (VIP analog #8, Table 1) were prepared using the same procedures.

Binding Studies. Binding of [^{125}I]VIP to hVPAC₁-R/PANC1 and T47D cells, and binding of [^{125}I]PACAP(1-27) to hVPAC₂-R/PANC1 were performed by incubation in standard incubation solution containing 0.05% (w/v) bacitracin for 60 min at room temperature and as described previously (Igarashi, et al., 2002b; Igarashi, et al., 2002a; Ito, et al., 2000). To assess VPAC₂-R affinities in Sup T₁ human lymphoblastoma cells, binding study was performed using [^{125}I]Ro 25-1553 in standard incubation solution containing 0.05% (w/v) bacitracin for 60 min at 37°C, because [^{125}I]VIP and [^{125}I]PACAP(1-27) were rapidly degraded in these cells even with protease inhibitors present. The separation of bound from free radioactivity was obtained by centrifugation of cells through 2% (w/v) BSA in standard incubation solution. The tubes were washed twice with 2% (w/v) BSA in standard incubation solution and radioactivity counted. Nonsaturable binding for [^{125}I]VIP, [^{125}I]PACAP(1-27) or [^{125}I]Ro 25-1553 was less than 5% of total binding.

For all peptides, the IC₅₀ was calculated, which was the concentration that gave half-maximally inhibition of that seen with a saturating concentration of VIP (1 μM). The IC₅₀ was calculated using the curve-fitting program Kaleidagraph.

cAMP Assay. T47D cells (0.05 X 10⁶ cells), hVPAC₁-R/PANC1(0.05 X 10⁶ cells) and hVPAC₂-R/PANC1 (0.05 X 10⁶ cells) were plated on 24-well plates and incubated for 48 h at 37°C with media containing 10% FBS (v/v). The media was then replaced with media supplemented with 2% FBS (v/v) and 2 μCi/ml [2-³H]adenine. Cells were incubated for an additional 48 h at 37°C. The media was removed and cells were incubated in the 500 μl of DMEM containing 1% (w/v) BSA and 0.5 mM IBMX with or without peptides at various concentrations for 1 h at 37°C. Reactions were terminated by the addition of 120 μl of cAMP stopping solution [2% SDS (w/v), 25 mM cAMP] followed by 1 ml of Tris (50 mM, pH 7.4). Samples were stored at -20 °C until analyzed. The amount of cAMP formation was determined using a modification of a method reported previously using Dowex AG 50W-X4 anion exchange resin and alumina (Igarashi, et al., 2002b;Benya, et al., 1994). For studying cAMP generation in Sup T₁ cells, 20 ml of medium containing 2.0 to 4.0 x 10⁶ cells/ml, supplemented with 2% FBS (v/v) and 2 μCi/ml [2-³H]adenine were incubated for 48 hrs at 37°C. Then the solution containing the cells were centrifuged and the cell pellet was washed with DMEM twice, followed by suspended with 50 ml DMEM containing 1% (w/v) BSA and 0.5 mM IBMX. 500 μl of this cell solution was added to each tube with or without peptides at various concentrations and incubated for 1 h at 37°C. The procedure of termination of the reaction and column processing procedure were the same as above. For all peptides, the EC₅₀ was calculated, which was the concentration of the peptide that gave half-maximal stimulation of a maximally effective concentration of VIP (1 μM). The EC₅₀ was calculated using the curve-fitting program Kaleidagraph.

Degradation Studies of Radiolabeled Ligands. ^{125}I -[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP, ^{125}I -[Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP, or ^{125}I -VIP corresponding to 500,000 CPM (250 pM) was incubated in 1 ml of standard incubation solution in the absence of bacitracin, with or without hVPAC₁-R/PANC cells (0.3×10^6 cells/ml) at 37°C for 7.5 min, or hVPAC₂-R/PANC cells (0.4×10^6 cells/ml) at 37°C for 15 min. After incubation aliquots were centrifuged and distribution of the radioactivity in the supernatants were analyzed by HPLC. Each supernatant (200,000 CPM) was injected onto a HPLC with a Vydac C-18 column. 2-ml fractions were collected and radioactivity determined.

Statistical Analysis. The results are mean \pm S.E.M. of three or more experiments. IC₅₀ and EC₅₀ were calculated using the curve-fitting program Kaleidagraph. Statistical comparisons were made using the Student's *t*-test.

Results

Development of Multi-alaninated VIP Analogs That Were Designed to Have a High Affinity for Human VPAC₁-R or VPAC₂-R (Fig. 1. In a previous study (Igarashi, et al., 2002a) we demonstrated a simplified multi-alaninated analog of VIP (analog #2, present study) could be synthesized that retained high affinity for rat, guinea pig and human VPAC₁-R. This analog also had increased stability compared to VIP for the hVPAC₁-R (Igarashi, et al., 2002a). However, no studies were performed on the selectivity or stability of this analog with hVPAC₂-R. In the present study we examined the VPAC-R selectivity of this analog and synthesized additional simplified, multi-alaninated analogs in an attempt to develop a highly selective VIP analog for hVPAC₁-R and hVPAC₂-R that might also be metabolically stable for either or both receptors.

Using the results of alanine scanning of VIP to define the amino acids essential for high affinity interaction with the hVPAC₁-R (Igarashi, et al., 2002a) and the hVPAC₂-R (Igarashi, et al., 2002b), we synthesized 8 additional multi-alaninated VIP analogs that, based on single alanine substitutions, should retain high affinity for the hVPAC₁-R or the hVPAC₂-R. Five new analogs (analog #s 1, 3-6; Fig. 1) were synthesized based on the VIP pharmacophore for hVPAC₁-R and three new analogs (analog #s 7-9; Fig. 1) based on the VIP pharmacophore of the hVPAC₂-R. In each case the multi-alaninated simplified analogs were made by making combinations of analogs in which, when alanine was substituted alone in a given position, there was a <10-fold decrease in affinity for either the VPAC₁ (Igarashi, et al., 2002a), or VPAC₂ receptor (Igarashi, et al., 2002b) (Fig. 1).

Affinity of Multi-alanine Substituted VIP Analogs Designed to Be hVPAC₁-R Agonists for hVPAC₁-R or hVPAC₂-R Containing Cells. Multi-alaninated VIP analog #s 1, 3-6 retained high affinity for the hVPAC₁-R, similar to VIP analog #2 (Fig. 2, Table 1). The

simplest, multi-alaninated VIP analog (VIP analog #4; Fig. 1) ([Ala^{2, 8, 9, 11, 19, 24, 27}]VIP) had a 6- to 7-fold decrease in the affinity compared to VIP, for the hVPAC₁-R containing cells. When two more alanine substitutions were added in positions 25 and 28, which resulted in the VIP analog #2, it had an equal affinity to VIP for the hVPAC₁-R, similar to that reported previously (Igarashi, et al., 2002a). An additional alanine replacement of either valine¹⁹ (analog #3) or Lys²¹ (analog #4), Tyr²² (analog #5) or Ile²⁶ (analog #26) had variable effects on hVPAC₁-R affinity (Fig. 2, top panel; Table 1). Analog #s 3, 5 and 6 had a 2- to 17-fold decrease in the affinity for the hVPAC₁-R compared to VIP and analog #2 (Fig. 2, top panel; Table 1).

To determine the possible VPAC-R selectivity of these analogs, their affinities for native hVPAC₂-R on Sup T₁ human lymphoblastoma cells and hVPAC₂-R stably transfected PANC1 cells were determined (Fig. 2, bottom panel; Table 1). VIP had a high affinity for the hVPAC₂-R containing cells; however, analog #s 1-6 had a lower affinity. Specifically, analog #1 and analog #2 had a 15- to 90-fold decrease in the affinity compared to VIP for the hVPAC₂-R. When additional alanine substitutions for Met¹⁷, Lys²¹, Tyr²² or Ile²⁶ were made to analog #2, the affinity for hVPAC₂-R became much lower, especially with the additional alanine substitution for Tyr²² and Ile²⁶ (Fig. 2, bottom panel; Table 1). The resultant analog #s 5 and 6 had a >4500-fold and 2500- to 3000-fold lower affinity than to VIP for hVPAC₂-R. In terms of hVPAC-R selectivity, VIP had a 4-fold higher affinity for hVPAC₁-R, whereas VIP analog #1 had a 36-fold higher selectivity for hVPAC₁-R over hVPAC₂-R and analog #2, a 150-fold selectivity for hVPAC₁-R (Table 1). The additional substitution of Met¹⁷ or Lys²¹ resulted in analogs #3 and #4, respectively, which had a 150- to 200-fold higher selectivity for hVPAC₁-R over hVPAC₂-R (Table 1). Alanine substitution of Tyr²² or Ile²⁶ in analog #2, which yielded VIP analogs #5 and #6, had the greatest selectivity for hVPAC₁-R over hVPAC₂-R, which were >2400-fold and 660-fold, respectively, (Table 1).

Affinity of Multi-alanine Substituted VIP Analogs Designed to Be hVPAC₂-R

Agonists for hVPAC₁-R or hVPAC₂-R Containing Cells. We synthesized three multi-alaninated VIP analogs that should retain high affinity for the hVPAC₂-R (VIP analogs #7 to #9, Fig. 1) and determined their abilities to interact with hVPAC₁-R receptors (Fig. 3, top panel; Table 1) and hVPAC₂-R containing cells (Fig. 3, bottom panel; Table 1). VIP analog #7 with 6 alanine substitutions (i.e., for Ser², Asp⁸, Asn⁹, Gln¹⁶, Val¹⁹ and Asn²⁴; Fig. 1) as well as VIP analog #8 which had an additional alanine substitution for Ser²⁵ (Fig. 1), had a similar high affinity to VIP for the hVPAC₂-R (Fig. 3, bottom panel; Table 1). However, when two additional alanine substitutions for Lys²⁰ and Lys²¹ were added to analog #8, the resultant analog #9 had a 3 to 8-fold decrease in affinity for hVPAC₂-R compared to VIP (Fig. 3, bottom panel; Table 1). We also determined their affinities for hVPAC₁-R containing cells (Fig. 3, top panel; Table 1) and found that analog #7 and analog #8 showed a 2- to 3-fold and a 5- to 7-fold decrease, respectively, in affinity for hVPAC₁-R compared to VIP. In contrast, analog #9 had a 50-fold decrease in the affinity compared to VIP for hVPAC₁-R (Fig. 3, top panel; Table 1). In terms of VPAC-R selectivity, each of these 3 analogs (#s 7, 8, 9) had a 2- to 4-fold higher affinity for hVPAC₂-R than hVPAC₁-R, while VIP had a 3.7-fold higher selectivity for hVPAC₁-R than hVPAC₂-R (Table 1, Fig. 3).

Potency of Multi-alanine-Substituted VIP Analogs Designed to Be hVPAC₁-R

Agonists for hVPAC₁-R or hVPAC₂-R Activation. Activation of adenylate cyclase is the principal intracellular mediator of the action of VPAC₁ and VPAC₂ receptors (Nicole, et al., 2000; Ulrich, II, et al., 1998; Zhou, et al., 1989). To investigate whether these multi-alaninated VIP analogs functioned as agonists and if so, their potency and efficacy for hVPAC₁-R and hVPAC₂-R activation, we first determined the ability of VIP analogs #1 to #6, which were hVPAC₁-R-preferring by binding studies, to stimulate cAMP generation in hVPAC₁-R and

hVPAC₂-R containing cells (Fig. 4, Table 2). Each of the 6 analogs had similar efficacy to VIP in stimulating cAMP generation through the hVPAC₁-R (Fig. 4, top panel). VIP had a high potency for stimulating cAMP generation in hVPAC₁-R containing cells with EC₅₀'s of 2.5 to 2.7 nM and caused a maximal stimulation with 100 nM concentration (Table 2, Fig. 4). Except for VIP analogs #1 and #4, which showed a 2- to 4-fold decrease in potency for activating VPAC₁-R compared to VIP, each of the other 4 VPAC₁-R-preferring analogs (#s 2, 3, 5, 6) retained a high potency for the VPAC₁-R (Fig. 4, top panel; Table 2).

VIP had a high potency for stimulating cAMP generation via the hVPAC₂-R, with an EC₅₀ of 5 nM and caused maximal stimulation at 100-300 nM concentration (Fig. 4, bottom panel). Three of the VPAC₁-R-preferring analogs (#s 1, 3, 4) had sufficient potency so that their efficacy could be determined and was shown to be equal to VIP at the VPAC₂-R (Fig. 4, bottom panel). VIP analogs #1 to #6 had >90-fold lower potency than VIP for stimulating cAMP generation in hVPAC₂-R cells. Analog #5 had the lowest potency, demonstrating >3000-fold lower potency than VIP (Fig. 4, bottom panel; Table 2). In terms of their selectivity for activating hVPAC₁-R or hVPAC₂-R, VIP analogs #1 to #6 had >100-fold higher selectivity for hVPAC₁-R over hVPAC₂-R, while VIP had a 2.0-fold selectivity for hVPAC₁-R over hVPAC₂-R (Table 2). Based on potency, VIP analog #5 had the greater selectivity for stimulating cAMP generation, having a 15600-fold higher selectivity for hVPAC₁-R over hVPAC₂-R (Table 2).

Potency of Multi-alanine Substituted VIP Analogs (Analog #s 7-9) Designed to Have Higher Affinity for the hVPAC₂-R for hVPAC₁-R or hVPAC₂-R Activation. VIP analogs #7 to #9 had equal efficacy to VIP for activating either hVPAC₁R (Fig. 5, top panel) or hVPAC₂-R cells (Fig. 5, bottom panel). Each of these three analogs had a similar potency to VIP for stimulating cAMP generation in VPAC₁-R cells (Fig. 5, top panel; Table 3). In contrast, none of these three analogs had higher potency than VIP for stimulating cAMP generation in hVPAC₂-R

cells (Fig. 5, bottom panel; Table 3). In terms of relative potency for VPAC-R activation, none of these three analogs had selectivity for hVPAC₂-R over hVPAC₁-R (Table 3).

Stability of VIP and Multi-alaninated VIP Analogs Incubated With hVPAC₁-R/PANC1 Cells or hVPAC₂-R/PANC1 Cells. In a previous study (Igarashi, et al., 2002a), we demonstrated analog #2 ([Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP) was more resistant to degradation than VIP by hVPAC₁-R/PANC1 cells. However, in the present study we demonstrate the VIP analog #2 has less than a 200-fold selectivity for the hVPAC₁-R (Table 1) and thus would not be useful for *in vivo* receptor studies attempting to localize both hVPAC₁R and hVPAC₂-R in tumors. It is unknown whether the other multi-alaninated analogs which retained high affinity for both hVPAC-R subtypes described in the present study (Tables 1, 2) and could thus be useful for *in vivo* receptor characterization of both VPAC-R subtypes, were also resistant to degradation. To address this question we prepared ¹²⁵I-analog #8 ([¹²⁵I-[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP) and ¹²⁵I-analog #2 ([¹²⁵I-[Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP) and compared the amount of degradation during an incubation with hVPAC₁-R/PANC1 cells or hVPAC₂-R/PANC1 cells to that seen with ¹²⁵I-VIP. As seen in Figure 6 (left panel), more than 70% of ¹²⁵I-VIP was degraded during incubation with hVPAC₁-R/PANC1 cells, while only 20% of ¹²⁵I-analog #8 was degraded. As reported previously (Igarashi, et al., 2002a), no degradation of ¹²⁵I-analog #2 was seen under the same conditions, suggesting that analog #8 had a greater stability than VIP incubated with hVPAC₁-R/PANC1 cells, but was less metabolically stable than analog #2. Figure 6 (right panel) demonstrates that more than 70% of ¹²⁵I-VIP was degraded during an incubation with hVPAC₂-R/PANC1 cells, however only 20% of ¹²⁵I-analog #8 was degraded, showing that analog #8 also had a greater stability than VIP during incubation with hVPAC₂-R/PANC1 cells.

Discussion

The purpose of the present study was to attempt to identify simplified VIP analogs that either have high selectivity and high affinity for one subtype of VIP receptor, or that were metabolically stable and retained high affinity for both VIP receptor subtypes. In a previous study (Igarashi, et al., 2002a) we identified a simplified analog of VIP, [Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}] (analog #2 in present study), which retained high affinity for hVPAC₁ and which was synthesized after analyzing the VIP pharmacophore for VPAC₁ in human, rat and guinea pig. In the present study we confirmed that [Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}] has a similar high affinity to VIP for the human VPAC₁ in both native hVPAC₁-containing cells (T47D cells) and hVPAC₁-transfected PANC1 cells; however, we further demonstrate this simplified VIP analog has relatively low selectivity (<170-fold) for hVPAC₁ over hVPAC₂. Therefore, [Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}] does not fulfill our goal of identifying a highly selective agonist for either VPAC. Furthermore, even though it is metabolically stable (Igarashi, et al., 2002a), its lower affinity for hVPAC₂ (100-500 nM) makes it unsuitable as a high affinity ligand for both VPACs, our second goal. In the present study we have successfully identified simplified VIP analogs that have high selectivity and retain high affinity for the hVPAC₁. Two of the five new, simplified VIP analogs, [Ala^{2, 8, 9, 11, 19, 22, 24, 25, 27, 28}]VIP (analog #5) and [Ala^{2, 8, 9, 11, 19, 24-28}]VIP (analog #6), had >2400-fold and 600-fold higher binding affinities, respectively, for hVPAC₁ over hVPAC₂ receptors. Furthermore, each of these two analogs was a fully efficacious agonist at the VPAC₁ receptor and analog #5 had >15,000-fold greater potency for activating the hVPAC₁ compared to the hVPAC₂, and analog #6 had >1200-fold greater potency for hVPAC₁ over hVPAC₂. This discrepancy between potency and binding affinity for the VPAC₁ compared to native VIP, likely is at least partially due to their greater metabolic stability because each of these analogs have multi-alaninated substitutions similar to analogs #8 and #2 in the present study and analog #2 in

a previous study (Igarashi, et al., 2002a), each of which has enhanced metabolic stability. These results demonstrate that both the simplified VIP analogs #6 and #5 may have greater selectivity than the 65-fold selectivity of [Leu²²]VIP for hVPAC₁ over hVPAC₂ (Gourlet, et al., 1998; Bhargava, et al., 2002), and at least comparable and maybe greater than the widely used VPAC₁ selective agonist (Gourlet, et al., 1997a), [Lys¹⁵, Arg¹⁶, Leu²⁷]VIP(1-7)-GRF(8-27), which is reported to have a 53- to 169-fold selectivity for rVPAC₁ over rVPAC₂ transfected into CHO cells in one study (Ito, et al., 2000), but a 15,000-fold selectivity in another study (Gourlet, et al., 1997a), and a 300- to 30,000-fold selectivity in hVPAC₁/hVPAC₂-containing cells (Gourlet, et al., 1997a; Igarashi, et al., 2002b).

In contrast to our results with the hVPAC₁, none of the three simplified VIP analogs synthesized, based on the analysis of the VIP pharmacophore for the VPAC₂ (Igarashi, et al., 2002b), demonstrated selectivity for the hVPAC₂. However, two of these three simplified VIP analogs [(Ala^{2, 8, 9, 16, 19, 24})VIP (analog #7) and [(Ala^{2, 8, 9, 16, 19, 24, 25})VIP (analog #8)] retained high affinity (5.6 and 7.1 nM) for the hVPAC₂, in contrast to each of the six simplified VIP analogs (analog #1-6) designed to have high affinity for VPAC₁, each of which had low affinity for hVPAC₂ (468 - >30,000 nM). These results demonstrate that using the designed strategy applied in the present study, simplified analogs of VIP could be made which retained high affinity for hVPAC₂. The lack of selectivity of these two high affinity simplified VIP analogs (analog #7, #8) for VPAC₂ is in contrast to findings in other studies using different strategies which have reported finding peptides that have selectivity for VPAC₂. The VIP-related peptide, helodermin, is reported to have 15-fold higher affinity for hVPAC₂ over hVPAC₁ (Gourlet, et al., 1997b). Ro-25-1553, a cyclic analog of VIP with a lactam ring (O'Donnell, et al., 1994a), is reported to have 75- to 600-fold selectivity for hVPAC₂ over hVPAC₁ (Ito, et al., 2000; Igarashi, et al., 2002a; Moreno, et al., 2000; Gourlet, et al., 1997b), 246- to 4300-fold for rVPAC₂ over

rVPAC₁ (Ito, et al., 2000), and hexanoyl [Ala¹⁹, Lys^{27, 28}]VIP, to have an 800-fold selectivity for hVPAC₂ over hVPAC₁ (Langer, et al., 2004).

A review of the previous studies of the VIP pharmacophore for the VPAC₁ and VPAC₂ (Igarashi, et al., 2002a; Igarashi, et al., 2002b; Nicole, et al., 2000) provides some insights into why the approach used in the study likely resulted in selective VPAC₁ but not selective VPAC₂ agonists. In these previous studies (Igarashi, et al., 2002b; Nicole, et al., 2000; Igarashi, et al., 2002a; Gourlet, et al., 1998) using alanine-scanning to identify the VIP pharmacophore for hVPACs, a number of amino acids, particularly Thr¹¹, Tyr²², Asn²⁴, Leu²⁷ and Asn²⁸ were more important for VPAC₂ than hVPAC₁ affinity. In these studies (Igarashi, et al., 2002a; Igarashi, et al., 2002b; Nicole, et al., 2000) and others (Gourlet, et al., 1998) the presence of Tyr²² in VIP was much more important for high affinity for VPAC₂ than VPAC₁ with the result that a single substitution of alanine in position 22 of VIP had the most profound effect on VIP's ability to interact with each VPAC subtype of any single alanine substitution. In our study analog #5 ([Ala^{2, 8, 9, 11, 18, 19, 22, 24, 25, 27, 28}]VIP), which had the greatest selectivity for hVPAC₁, was the only simplified analog to contain this substitution. The Ala²² substitution was not included in the other five proposed VPAC₁ selective agonists (analogs #1-4, 6) because it has been shown to cause a 4-fold decrease in hVPAC₁ affinity (Igarashi, et al., 2002a; Igarashi, et al., 2002b), which we wanted to avoid if possible. The single substitution of alanine for Thr¹¹ or Leu²⁷ in VIP also caused a >15-fold decrease in VPAC₂ affinity with minimal changes in VPAC₁ affinity (Igarashi, et al., 2002a; Igarashi, et al., 2002b). Both of these substitutions were included in each of the six proposed simplified VPAC₁ selective agonists; however, they only resulted in a 35- to 196-fold selectivity in analogs #1-4. Furthermore, the single substitution of Ala for Met¹⁷, Lys²¹, Ile²⁶ or Asn²⁸ in VIP caused only a 2.5- to 7.3-fold decrease in VPAC₂ affinity with minimal effects on VPAC₁ affinity (Igarashi, et al., 2002a; Igarashi, et al., 2002b). Similarly, when they were

included in the simplified proposed VPAC₁ selective agonists (analogs #2-4, 6), they generally had only a modest effect on increasing VPAC₁ selectivity. In contrast to the results with VPAC₁ in studies of the VIP pharmacophore for the VPAC₂ (Igarashi, et al., 2002a; Nicole, et al., 2000; Igarashi, et al., 2002b), no single alanine substitution or D-amino acid substitution in VIP resulted in much greater decrease in affinity for the hVPAC₁ than the hVPAC₂. VIP itself had a 4-fold greater affinity for hVPAC₁ than VPAC₂. Single alanine substitution for Gln¹⁶ or Lys²⁰ in VIP resulted in a 3- to 4-fold greater decrease in affinity for VPAC₁ than VPAC₂ (Igarashi, et al., 2002a; Igarashi, et al., 2002b). However, inclusion of these substitutions in analogs #7-9 resulted in only a 3- to 4-fold decrease in affinity of the substituted VIP analogs (analogs #7-9) for VPAC₁, with the result these simplified analogs had nearly equal affinity for both hVPAC₁ and hVPAC₂ and therefore were not selective.

The second goal of this study was to attempt to identify a simplified VIP analog that retained high affinity for each VPAC subtype and that also might be metabolically stable. Such VIP analogs could be particularly useful for imaging of tumors overexpressing VIP receptors or for VIP receptor-directed antitumor treatment. Previous studies (Domschke, et al., 1978; Bryant, et al., 1976) have demonstrated VIP is rapidly degraded *in vivo*, having a half-life less than one minute (Domschke, et al., 1978; Bryant, et al., 1976) in man. It has been proposed (Domschke, et al., 1978; Bryant, et al., 1976) that VIP is primarily degraded by being cleaved primarily at Ser²⁵-Ile²⁶ or at Thr⁷-Asp⁸ residues to yield the major products VIP(1-25) and VIP(26-28) and the minor products VIP(1-7) and VIP(8-28). All of these products are either inactive or have very low affinity for the VPAC receptors (Bolin, et al., 1995). Furthermore, another study (O'Donnell, et al., 1991) suggests an alanine replacement of Val¹⁹ in VIP may increase VIP's resistance to degradation. In that study (O'Donnell, et al., 1991) which investigated the effect of alanine substitutions into the VIP analogue, Ac-[Lys¹², Nle¹², Val²⁶, Thr²⁸]VIP on the duration of

bronchodilator activity, it was reported that the substitution of Val¹⁹ by an Ala¹⁹ extended the duration of effect by more than 17 times. We therefore anticipated that our analogues which all had with substitutions at positions 8,19,25 and/or 26 of VIP should have enhanced stability. This possibility was further supported by our previous study (Igarashi, et al., 2002a) in which we found that the multi-alaninated analogue, [Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP (analogue #2), was much more resistant than VIP to degradation. A number of our results support the conclusion that we successfully achieved the second aim in the present study. First, in binding studies both analog #7 ([Ala^{2, 8, 9, 16, 19, 24}]VIP) and analog #8 ([Ala^{2, 8, 9, 16, 19, 24, 25}]VIP) retained high affinity for both VPAC₁ and VPAC₂. Second, both analogs #7 and #8 were fully efficacious agonists at both VPACs, and each retained high potency for activating each VPAC and stimulating adenylate cyclase activity. Third, radiolabeled analog #8 demonstrated none to minimal degradation by hVPAC₁ or hVPAC₂ cells, whereas ¹²⁵I-VIP was degraded >70% by both, demonstrating analog #8 was metabolically stable with cells containing both VPAC subtypes. Furthermore, with VPAC₁-containing cells, ¹²⁵I-[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP (¹²⁵I-analog #8), was only slightly less metabolically stable than ¹²⁵I-[Ala^{2, 8, 9, 11, 18, 19, 24, 25, 27, 28}]VIP (analogue #2), which was previously reported (Igarashi, et al., 2002a) to be much more metabolically stable than VIP with hVPAC₁-containing cells.

In conclusion, analyzing the results of studies of the VIP pharmacophore for high affinity interaction with the hVPAC₁ or hVPAC₂ (Igarashi, et al., 2002a; Igarashi, et al., 2002b; Nicole, et al., 2000), we synthesized nine simplified, poly-alaninated analogs of VIP to attempt to develop high affinity VIP analogs that were either selective for one of the two VPAC subtypes or that functioned as high affinity agonists for each VPAC and that would be metabolically stable. Our results demonstrate that [Ala^{2, 8, 9, 11, 19, 22, 24, 25, 27, 28}]VIP (analogue #5) has high affinity and >2000-fold selectivity for hVPAC₁ over hVPAC₂. No selective VPAC₂ agonists were identified.

However, [Ala^{2, 8, 9, 16, 19, 24, 25}]VIP (analog #8) had high affinity and potency for both VPAC subtype and was much more metabolically stable than VIP in cells containing each VPAC subtype. These simplified, metabolically stable analogs should be useful for investigating the role of VPAC₁ in biological and pathological processes, for enhanced imaging of tumors overexpressing VIP receptors using VIP receptor scintigraphy (Virgolini, 1997;Thakur, et al., 2004;Rao, et al., 2001;Thakur, et al., 2000;Bhargava, et al., 2002), as well as for possible VIP receptor-directed antitumor treatment for tumors overexpressing VPACs (Moody, et al., 2004;Gotthardt, et al., 2004;Ou, et al., 2005).

References

- Benya RV, Fathi Z, Pradhan T, Battey JF, Kusui T and Jensen RT (1994) Gastrin-releasing peptide receptor-induced internalization, down-regulation, desensitization and growth: Possible role of cAMP. *Mol Pharmacol* **46**(2):235-245.
- Bhargava S, Licha K, Knaute T, Ebert B, Becker A, Grotzinger C, Hassenius C, Wiedenmann B, Schneider-Mergener J and Volkmer-Engert R (2002) A complete substitutional analysis of VIP for better tumor imaging properties. *J Mol Recognit* **15**:145-153.
- Bolin DR, Michalewsky J, Wasserman MA and O'Donnell M (1995) Design and development of a vasoactive intestinal peptide analog as a novel therapeutic for bronchial asthma. *Biopolymers* **37**:57-66.
- Bryant MG, Polak JM, Modlin I, Bloom SR, Albuquerque RH and Pearse AGE (1976) Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter substance. *Lancet* 991-993.
- Delgado M, Pozo D and Ganea D (2004) The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacol Rev* **56**:249-290.
- Dockray GJ (1994) Vasoactive intestinal polypeptide and related peptides, in *Gut Peptides* (Walsh JH and Dockray GJ eds) pp 447-472, Raven Press, New York.
- Dogrukol-Ak D, Tore F and Tuncel N (2004) Passage of VIP/PACAP/secretin family across the blood-brain barrier: therapeutic effects. *Curr Pharm Des* **10**:1325-1340.

Domschke S, Domschke W, Bloom SR, Mitznegg P, Mitchell SJ, Lux G and Strunz U (1978) Vasoactive intestinal peptide in man: pharmacokinetics, metabolic and circulatory effects. *Gut* **19**:1049-1053.

Gotthardt M, Boermann OC, Behr TM, Behe MP and Oyen WJ (2004) Development and clinical application of peptide-based radiopharmaceuticals. *Curr Pharm Des* **10**:2951-2963.

Gourlet P, Vandermeers A, Vertongen P, Rathe J, DeNeef P, Cnudde J, Waelbroeck M and Robberecht P (1997a) Development of high affinity selective VIP1 receptor agonists. *Peptides* **18**:1539-1545.

Gourlet P, Vandermeers-Piret MC, Rathe J, De Neef P, Cnudde J, Robberecht P and Waelbroeck M (1998) Vasoactive intestinal peptide modification at position 22 allows discrimination between receptor subtypes. *Eur J Pharmacol* **348**:95-99.

Gourlet P, Vertongen P, Vandermeers A, Vandermeers-Piret MC, Rathe J, DeNeef P, Waelbroeck M and Robberecht P (1997b) The long-acting vasoactive intestinal polypeptide agonist RO 25-1553 is highly selective of the VIP2 receptor subclass. *Peptides* **18**:403-408.

Gozes I and Brenneman DE (2000) A new concept in the pharmacology of neuroprotection. *J Mol Neurosci* **14**:61-68.

Gozes I and Furman S (2004) Clinical endocrinology and metabolism. Potential clinical applications of vasoactive intestinal peptide: a selected update. *Best Pract Res Clin Endocrinol Metab* **18**:623-640.

Groneberg DA, Springer J and Fischer A (2001) Vasoactive intestinal polypeptide as mediator of asthma. *Pulm Pharmacol Ther* **14**:391-401.

Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA and Waschek JA (1998) International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* **50**:265-270.

Igarashi H, Ito T, Hou W, Mantey SA, Pradhan TK, Ulrich CD, II, Hocart SJ, Coy DH and Jensen RT (2002a) Elucidation of vasoactive intestinal peptide pharmacophore for VPAC₁ receptors in human, rat and guinea pig. *JPET* **301**:37-50.

Igarashi H, Ito T, Pradhan TK, Mantey SA, Hou W, Coy DH and Jensen RT (2002b) Elucidation of the vasoactive intestinal peptide pharmacophore for VPAC₂ receptors in human and rat, and comparison to the pharmacophore for VPAC₁ receptors. *J Pharmacol Exp Ther* **302**:445-460.

Ito T, Hou W, Katsuno T, Igarashi H, Pradhan TK, Mantey SA, Coy DH and Jensen RT (2000) Rat and guinea pig pancreatic acini possess both VIP₁ and VIP₂ receptors, which mediate enzyme secretion. *Am J Physiol (Gastrointest Liver Physiol 1)* **278**:G64-G74.

Ito T, Igarashi H, Pradhan TK, Hou W, Mantey SA, Taylor JE, Murphy WA, Coy DH and Jensen RT (2001) GI side-effects of a possible therapeutic GRF analogue in monkeys are likely due to VIP receptor agonist activity. *Peptides* **22**:1139-1151.

Kalsi JS, Celtek S, Muneer A, Kell PD, Ralph DJ and Minhas S (2002) Current oral treatments for erectile dysfunction. *Expert Opin Pharmacother* **11**:1613-1629.

Langer I, Gregoire F, Nachtergaeel I, De Neef P, Vertongen P and Robberecht P (2004) Hexanoylation of a VPAC₂ receptor-preferring ligand markedly increased its selectivity and potency. *Peptides* **25**:275-278.

Moody T (1996) Peptides and growth factors in non-small cell lung cancer. *Peptides* **17**:545-555.

Moody TW, Czerwinski G, Tarasova NI, Moody DL and Michejda CJ (2004) The development of VIP-ellipticine conjugates. *Regul Pept* **123**:187-192.

Moody TW, Hill JM and Jensen RT (2003) VIP as a trophic factor in the CNS and cancer cells. *Peptides* **24**:163-177.

Moreno D, Gourlet P, De Neef P, Cnudde J, Waelbroeck M and Robberecht P (2000) Development of selective agonists and antagonists for the human vasoactive intestinal polypeptide VPAC₂ receptor. *Peptides* **21**:1543-1549.

Nicole P, Lins L, Rouyer-Fessard C, Drouot C, Fulcrand P, Thomas A, Couvineau A, Martinez J, Brasseur R and Laburthe M (2000) Identification of key residues for interaction of vasoactive intestinal peptide with human VPAC₁ and VPAC₂ receptors and development of a highly selective VPAC₁ receptor agonist. *J Biol Chem* **275**:24003-24012.

O'Donnell M, Garippa RJ, O'Neill NC, Bolin DR and Cottrell JM (1991) Structure-activity studies of vasoactive intestinal polypeptide. *J Biol Chem* **266**:6389-6392.

O'Donnell M, Garippa RJ, Rinaldi N, Selig WM, Simko B, Renzetti L, Tannu SA, Wasserman MA, Welton A and Bolin DR (1994a) Ro 25-1553: a novel, long-acting vasoactive intestinal peptide agonist. Part I: In vitro and in vivo bronchodilator studies. *J Pharmacol Exp Ther* **270**:1282-1288.

O'Donnell M, Garippa RJ, Rinaldi N, Selig WM, Tocker JE, Tannu SA, Wasserman MA, Welton A and Bolin DR (1994b) Ro 25-1553: a novel, long-acting vasoactive intestinal peptide agonist.

Part II: Effect on in vitro and in vivo models of pulmonary anaphylaxis. *J Pharmacol Exp Ther* **270**:1289-1294.

Ou X, Tan T, He L, Li Y, Li J and Kuang A (2005) Antitumor effects of radioiodinated antisense oligonucleotide mediated by VIP receptor. *Cancer Gene Ther* **12**:313-320.

Rao PS, Thakur ML, Pallela V, Patti R, Reddy K, Li H, Sharma S, Pham HL, Diggles L, Minami C and Marcus CS (2001) ^{99m}Tc labeled VIP analog: evaluation for imaging colorectal cancer. *Nucl Med Biol* **28**:445-450.

Sandhu D, Curless E, Dean J, Hackett G, Liu S, Savage D, Oakes R and Frenzt G (1999) A double blind, placebo controlled study of intracavernosal vasoactive intestinal polypeptide and phenotolamine mesylate in a novel auto-injector for the treatment of non-psychogenic erectile dysfunction. *Int J Impot Res* **11**:91-97.

Sasaki Y and Coy DH (1987) Solid phase synthesis of peptides containing the CH₂NH peptide bond isostere. *Peptides* **8**:119-121.

Thakur ML, Aruva MR, Garipey J, Acton P, Rattan S, Prasad S, Wickstrom E and Alavi A (2004) PET imaging of oncogene overexpression using ⁶⁴Cu-vasoactive intestinal peptide (VIP) analog: comparison with ^{99m}Tc-VIP analog. *J Nucl Med* **45**:1381-1389.

Thakur ML, Marcus CS, Saeed S, Pallela V, Minami C, Diggles L, Pham HL, Ahdoot R, Kalinowski EA and Moody T (2000) Imaging tumors in humans with Tc-^{99m}-VIP. *Ann N Y Acad Sci* **921**:37-44.

Ulrich CD, II, Holtmann M and Miller LJ (1998) Secretin and vasoactive intestinal peptide receptors: members of a unique family of G protein-coupled receptors. *Gastroenterology* **114**:382-397.

Virgolini I (1997) Receptor nuclear medicine: vasointestinal peptide and somatostatin receptor scintigraphy for diagnosis and treatment of tumour patients. *Eur J Clin Invest* **27**:793-800.

Yung LS, Dela Cruz F, Hamren S, Zhu J, Tsutsumi M, Bloom JW, Caudle M, Rocznik S, Todd T, Lemoine L, MacDougall M, Shanafelt AB and Pan CQ (2003) Generation of highly selective VPAC2 receptor agonists by high throughput mutagenesis of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide. *J Biol Chem* **278**:10273-10281.

Zhou ZC, Gardner JD and Jensen RT (1989) Interaction of peptides related to VIP and secretin with guinea pig pancreatic acini. *Am J Physiol* **256**:G283-G290.

Figure Legends

Figure 1: Structure of the nine multi-alaninated VIP analogs studied. VIP is a 28-amino acid peptide with alanines in positions 4 and 18. The results of alanine scan of VIP for hVPAC₁-R or hVPAC₂-R/PANC1 cells, T47D human breast cancer cells and Sup T1 human lymphoblastoma cells that we have reported previously are summarized (Igarashi, et al., 2002a; Igarashi, et al., 2002b). The fold change in affinity compared to native VIP with a single alanine substitution in each of the indicated positions in VIP is shown from these studies (Igarashi, et al., 2002a; Igarashi, et al., 2002b). NC: <2.0-fold decrease or increase in the affinity compared to VIP; *: ≥10 but <100-fold decrease in the affinity compared to VIP; **: ≥100-fold decrease in the affinity compared to VIP and for a 2- to 9.9-fold decrease the exact fold decrease is shown. The position of the alanine substitutions for each analog (labeled Ala) is shown and unchanged amino acids from VIP are indicated by -. VIP analogs #1 to #6 were designed to possibly function as simplified, high affinity hVPAC₁-R agonists, and VIP analogs #7 to #9 as simplified, possible high affinity hVPAC₂-R agonists.

Figure 2: Abilities of VIP and multi-alaninated VIP analogs designed to be high affinity hVPAC₁-R agonists to inhibit binding of [¹²⁵I]peptides to hVPAC₁-R (top panel) or hVPAC₂-R (bottom panel) containing cells. *Top panel:* T47D human breast cancer cells (*left panel*) which possess native VPAC₁-R (1.2 x 10⁶ cells/ml) and human VPAC₁-R stably transfected PANC1 cells (0.2 x 10⁶ cells/ml) (*right panel*) were incubated for 60 min at room temperature with 75 pM [¹²⁵I]VIP alone or with the indicated concentrations of unlabeled peptides. *Bottom panel:* Sup T₁ human lymphoblastoma cells (*left panel*) which possess native VPAC₂ receptor (2.5 x 10⁶ cells/ml) were incubated for 60 min at 37°C with 75 pM [¹²⁵I]Ro 25-1553 alone or with the indicated concentrations of unlabeled peptides. Human VPAC₂ receptor stably transfected PANC1 cells (0.1 x 10⁶ cells/ml) (*right panel*) were incubated for 60 min at

room temperature with 75 pM [¹²⁵I]PACAP(1-27) alone or with the indicated concentrations of unlabeled peptides. In *both panels* results are expressed as the percentage of the saturable binding of [¹²⁵I]peptide observed in absence of competing peptide. In each experiment, each value was determined in duplicate and results given are means ± S.E.M. from at least three separate experiments. Structures of the different VIP analogs are shown in Figure 1.

Figure 3: **Abilities of VIP and multi-alaninated VIP analogs designed to be high affinity hVPAC₂-R agonists to inhibit binding of [¹²⁵I]peptides to hVPAC₁-R (*top panel*) or hVPAC₂-R cells (*bottom panel*).** The experimental conditions and results were the same as described in Fig. 2 legend. In each experiment, each value was determined in duplicate and results given are means ± S.E.M. from at least three separate experiments. Structures of the different VIP analogs are shown in Figure 1.

Figure 4: Abilities of VIP and multi-alaninated VIP analogs designed to be high affinity hVPAC₁-R agonists to stimulate cAMP accumulation in hVPAC₁-R (*top panel*) or hVPAC₂-R (*bottom panel*) containing cells. Top panel: cAMP was performed using T47D cells (*left panel*) or hVPAC₁-R/PANC1 cells (*right panel*) with or without various concentrations (0.01 nM-1 μM) of the indicated multi-alaninated VIP analogs or native VIP as described in Material and Methods. Results are expressed as the percentage of the maximal stimulation of cAMP accumulation caused by 1 μM VIP. Maximal stimulation of cAMP accumulation caused by 1 μM VIP was: T47D cells, 144 ± 48-fold; and hVPAC₁-R/PANC1 cells, 32.6 ± 4.9-fold over control. In each experiment each value was determined in duplicate and values given are means ± S.E.M. from at least three separate experiments. Bottom panel: cAMP was performed using Sup T₁ cells (*left panel*) or hVPAC₂-R/PANC1 cells (*right panel*) with or without various concentrations (0.01 nM-30 μM) of the indicated multi-alaninated VIP analogs or native VIP as described in Material and Methods. Results are expressed as the percentage of the maximal

stimulation of cAMP accumulation caused by 1 μ M VIP. Maximal stimulation of cAMP accumulation caused by 1 μ M VIP was; Sup T₁ cells; 7.1 ± 1.1 fold and hVPAC₂-R/PANC1 cells; 50.5 ± 9.5 fold over control. In each experiment each value was determined in duplicate and values given are means \pm S.E.M. from at least three separate experiments.

Figure 5: Abilities of VIP and multi-alaninated VIP analogs designed to be high affinity hVPAC₂-R agonists to stimulate cAMP accumulation in hVPAC₁-R (*top panel*) or hVPAC₂-R (*bottom panel*) containing cells. cAMP was performed as described in Figure 4 legend and the Material and Methods. Results are expressed as the percentage of the maximal stimulation of cAMP accumulation caused by 1 μ M VIP. In each experiment each value was determined in duplicate and values given are means \pm S.E.M. from at least three separate experiments.

Figure 6: Stability of the multi-alaninated VIP analog, ¹²⁵I-[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP and ¹²⁵I-[Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP compared to ¹²⁵I-VIP. Left panel: The degradation of ¹²⁵I-VIP, ¹²⁵I-[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP (VIP analog #8) or ¹²⁵I-[Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP (VIP analog #2) by hVPAC₁-R/PANC1 cells is shown. Shown are the HPLC elution profiles of supernatants after incubation of ¹²⁵I-VIP (*top panel*), ¹²⁵I-[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP (*middle panel*) or ¹²⁵I-[Ala^{2, 8, 9, 11, 19, 24, 25, 27, 28}]VIP (*bottom panel*) (500,000 cpm/ml) with or without hVPAC₁-R/PANC1 cells (0.3×10^6 /ml) in standard incubation solution for 7.5 min at 37°C. After incubation, supernatants containing 200,000 cpm were analyzed by HPLC. Fractions (2-ml) were collected, and radioactivity was determined. Arrows indicate the point of elution of intact ¹²⁵I-peptide. Right panel: The degradation of ¹²⁵I-VIP or ¹²⁵I-[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP (VIP analog #8) by hVPAC₂-R/PANC1 cells is shown. Shown are the HPLC elution profiles of supernatants after incubation of ¹²⁵I-VIP (*top panel*) or ¹²⁵I-[Ala^{2, 8, 9, 16, 19, 24, 25}]VIP (*bottom panel*) (500,000 cpm/ml) with or without hVPAC₂-R/PANC1 cells (0.4×10^6 /ml) in standard incubation solution for 15 min at 37°C. After incubation, supernatants containing 200,000 cpm were analyzed by

HPLC. Fractions (2-ml) were collected, and radioactivity was determined. Arrows indicate the point of elution of intact ^{125}I -peptide.

Table 1: The affinity of VIP and simplified multi-alaninated VIP analogs designed to be selective hVPAC₁-R agonists

| Proposed hVPAC-R Selective | Binding affinity (IC ₅₀ : nM) | | | | hVPAC-R selectivity (fold) |
|--|--|---------------------------------|--|---------------------------------|--|
| | hVPAC ₁ -R /T47D | hVPAC ₁ -R /PANC1 | hVPAC ₂ -R /Sup T ₁ | hVPAC ₂ -R /PANC1 | |
| Agonist (Analog #) | | | | | |
| hVPAC₁-R - Selective | | | | | hVPAC₁-R - selectivity |
| VIP | 1.6 ± 0.1 | 1.7 ± 0.1 | 5.3 ± 0.1 | 6.9 ± 0.3 | 3.7 |
| #1 | 10.4 ± 1.4 | 12.5 ± 1.3 | 507 ± 16 | 313 ± 28 | 35.8 |
| #2 | 1.9 ± 0.2 | 1.5 ± 0.2 | 468 ± 10 | 105 ± 14 | 169 |
| #3 | 26.9 ± 1.1 | 21.9 ± 2.5 | 1698 ± 289 | 6026 ± 423 | 158 |
| #4 | 2.7 ± 0.1 | 8.3 ± 0.8 | 1281 ± 84 | 871 ± 74 | 196 |
| #5 | 11.5 ± 0.9 | 13.5 ± 0.8 | >30000 | >30000 | >2400 |
| #6 | 25.7 ± 2.3 | 25.7 ± 3.3 | 13180 ± 1828 | 20890 ± 1821 | 663 |
| hVPAC₂-R - Selective | | | | | hVPAC₂-R - Selectivity |
| #7 | 3.7 ± 0.4 | 5.6 ± 0.4 | 2.3 ± 0.2 | 5.6 ± 0.4 | 1.2 |
| #8 | 10.6 ± 0.5 | 7.1 ± 0.9 | 3.7 ± 0.4 | 7.1 ± 0.9 | 1.6 |
| #9 | 79.4 ± 5.7 | 93.5 ± 10.1 | 18.6 ± 2.0 | 93.5 ± 10.1 | 1.5 |

The indicated cell type was incubated with 75 pM [¹²⁵I]peptide and various concentrations of the unlabeled alanine-substituted VIP analog as described in Figure 2 and 3 legends, and *Materials and Methods*. The IC₅₀ was the concentration causing half-maximal inhibition of the saturable binding caused

by 1 μ M VIP calculated using the curve-fitting program Kaleidagraph. In each experiment each value was determined in duplicate and values given are means \pm S.E.M. from at least three separate experiments. The selectivity was calculated using the mean value of the IC₅₀ in the two different VPAC cells.

Table 2: The potency of VIP and multi-alaminated VIP analogs designed to be hVPAC₁-R agonists for stimulating cAMP generation in hVPAC₁-R or hVPAC₂-R containing cells.

| Proposed hVPAC ₁ -R agonist | Potency for cAMP generation (EC ₅₀ : nM) | | | | hVPAC ₁ -R selectivity (fold) |
|--|---|---------------------------------|--|---------------------------------|--|
| | hVPAC ₁ -R /T47D | hVPAC ₁ -R /PANC1 | hVPAC ₂ -R /Sup T ₁ | hVPAC ₂ -R /PANC1 | |
| VIP | 2.5 ± 0.1 | 2.7 ± 0.3 | 5.2 ± 0.5 | 5.0 ± 0.3 | 2.0 |
| VIP #1 | 10.0 ± 1.8 | 7.4 ± 0.4 | 447 ± 64 | 2754 ± 621 | 184 |
| analog #2 | 2.9 ± 0.3 | 5.6 ± 0.6 | 1029 ± 181 | 1334 ± 140 | 285 |
| #3 | 2.9 ± 0.2 | 5.6 ± 0.9 | 575 ± 31 | 3447 ± 519 | 473 |
| #4 | 8.1 ± 0.9 | 5.5 ± 0.6 | 668 ± 92 | 912 ± 109 | 116 |
| #5 | 0.68 ± 0.07 | 1.9 ± 0.3 | 23710 ± 4363 | 16560 ± 3432 | 15609 |
| #6 | 3.2 ± 0.3 | 3.4 ± 0.5 | 3379 ± 350 | 5026 ± 668 | 1273 |

The cAMP assay was performed as described in Figure 4 and 5 legends, and in the *Materials and Methods*. The EC₅₀ was the concentration causing half-maximal stimulation of the value caused by 1 μM VIP, which was calculated using the curve-fitting program Kaleidagraph. In each experiment each value was determined in duplicate and values given are means ± S.E.M. from at least three separate experiments. The selectivity was calculated using the mean value of EC₅₀ in the two different VPAC-containing cells.

Table 3: The potency of VIP and multi-alaninated VIP analogs designed to be hVPAC₂-R agonists for stimulating cAMP generation in hVPAC₁-R or hVPAC₂-R containing cells.

| Proposed hVPAC ₁ -R agonist | Potency for cAMP generation (EC ₅₀ : nM) | | | | hVPAC ₂ -R selectivity (fold) |
|--|---|---------------------------------|--|---------------------------------|--|
| | hVPAC ₁ -R /T47D | hVPAC ₁ -R /PANC1 | hVPAC ₂ -R /Sup T ₁ | hVPAC ₂ -R /PANC1 | |
| VIP | 2.5 ± 0.1 | 2.7 ± 0.3 | 5.2 ± 0.5 | 5.0 ± 0.3 | 0.51 |
| VIP #7 | 0.79 ± 0.11 | 7.9 ± 0.7 | 50.1 ± 6.5 | 11.5 ± 1.8 | 0.14 |
| analog #8 | 1.0 ± 0.1 | 7.2 ± 0.6 | 26.3 ± 3.2 | 25.1 ± 1.5 | 0.16 |
| #9 | 2.2 ± 0.2 | 3.8 ± 0.3 | 25.1 ± 3.8 | 7.6 ± 1.1 | 0.18 |

See Table 2 legend for cAMP details and methods of calculating values. In each experiment each value was determined in duplicate and values given are means ± S.E.M. from at least three separate experiments. The selectivity was calculated using the mean value of EC₅₀ in the two different VPAC-containing cells.

Fig. 1

Possible VPAC₁ Selective Agonists

| VIP | 1 His | 2 Ser | 3 Asp | 4 Ala | 5 Val | 6 Phe | 7 Thr | 8 Asp | 9 Asn | 10 Tyr | 11 Thr | 12 Arg | 13 Leu | 14 Arg | 15 Lys | 16 Gln | 17 Met | 18 Ala | 19 Val | 20 Lys | 21 Lys | 22 Tyr | 23 Leu | 24 Asn | 25 Ser | 26 Ile | 27 Leu | 28 Asn | |
|--|-----------|------------------------|----------|----------|----------|----------|----------|------------------------|------------------------|-----------|-------------------------|-----------|-----------|-----------|-----------|-----------|-------------------------|-----------|-------------------------|-----------|-------------------------|-------------------------|-----------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-----|
| hVPAC ₁ -R / PANC1 | * | 2.1↓ | ** | | * | ** | * | NC | NC | * | NC | * | 4.2↓ | ** | 9.5↓ | 3.9↓ | 2.6↓ | | 2.4↓ | 7.7↓ | 2.9↓ | 4.2↓ | ** | NC | 2.0↓ | 6.6↓ | NC | NC | NC |
| hVPAC ₁ -R / T47D | * | NC | * | | * | ** | * | NC | NC | * | NC | * | 2.1↓ | * | 9.9↓ | 4.1↓ | 2.4↓ | | NC | 8.0↓ | 3.2↓ | 4.4↓ | * | NC | 2.4↓ | 4.9↓ | NC | NC | NC |
| Proposed hVPAC ₁ Agonists | A1 | - | Ala | - | - | - | - | Ala | Ala | - | Ala | - | - | - | - | - | - | - | Ala | - | - | - | - | Ala | - | - | Ala | - | Ala |
| | A2 | - | Ala | - | - | - | - | Ala | Ala | - | Ala | - | - | - | - | - | - | - | Ala | - | - | - | - | Ala | Ala | - | Ala | - | Ala |
| | A3 | - | Ala | - | - | - | - | Ala | Ala | - | Ala | - | - | - | - | - | - | Ala | - | Ala | - | - | - | Ala | Ala | - | Ala | - | Ala |
| | A4 | - | Ala | - | - | - | - | Ala | Ala | - | Ala | - | - | - | - | - | - | - | Ala | - | Ala | - | - | Ala | Ala | - | Ala | - | Ala |
| | A5 | - | Ala | - | - | - | - | Ala | Ala | - | Ala | - | - | - | - | - | - | - | Ala | - | - | Ala | - | Ala | Ala | - | Ala | - | Ala |
| | A6 | - | Ala | - | - | - | - | Ala | Ala | - | Ala | - | - | - | - | - | - | - | Ala | - | - | - | - | Ala | Ala | Ala | Ala | Ala | Ala |

Possible VPAC₂ Selective Agonists

| VIP | 1 His | 2 Ser | 3 Asp | 4 Ala | 5 Val | 6 Phe | 7 Thr | 8 Asp | 9 Asn | 10 Tyr | 11 Thr | 12 Arg | 13 Leu | 14 Arg | 15 Lys | 16 Gln | 17 Met | 18 Ala | 19 Val | 20 Lys | 21 Lys | 22 Tyr | 23 Leu | 24 Asn | 25 Ser | 26 Ile | 27 Leu | 28 Asn |
|--|-----------|------------------------|----------|----------|----------|----------|----------|------------------------|------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-------------------------|-----------|-----------|-------------------------|-------------------------|-------------------------|-----------|-----------|-------------------------|-------------------------|-----------|-----------|-----------|
| hVPAC ₂ -R / PANC1 | * | NC | ** | | 7.0↓ | ** | ** | NC | NC | ** | * | * | * | * | 5.9↓ | NC | 4.8↓ | | NC | 1.8↓ | 2.5↓ | ** | ** | 2.0↓ | NC | 7.1↓ | * | 3.0↓ |
| hVPAC ₂ -R / SupT ₁ | ** | NC | ** | | * | ** | ** | 2.5↓ | NC | ** | ** | * | 9.9↓ | * | 7.2↓ | NC | * | | NC | 4.1↓ | 2.4↓ | ** | ** | 3.5↓ | 2.9↓ | 5.3↓ | * | 5.1↓ |
| Proposed hVPAC ₂ Agonists | A7 | - | Ala | - | - | - | - | Ala | Ala | - | - | - | - | - | - | Ala | - | - | Ala | - | - | - | - | Ala | - | - | - | - |
| | A8 | - | Ala | - | - | - | - | Ala | Ala | - | - | - | - | - | - | Ala | - | - | Ala | - | - | - | - | Ala | Ala | - | - | - |
| | A9 | - | Ala | - | - | - | - | Ala | Ala | - | - | - | - | - | - | Ala | - | - | Ala | Ala | Ala | - | - | Ala | Ala | - | - | - |

Downloaded from iper.assh-pub.com at ASPET on April 20, 2024

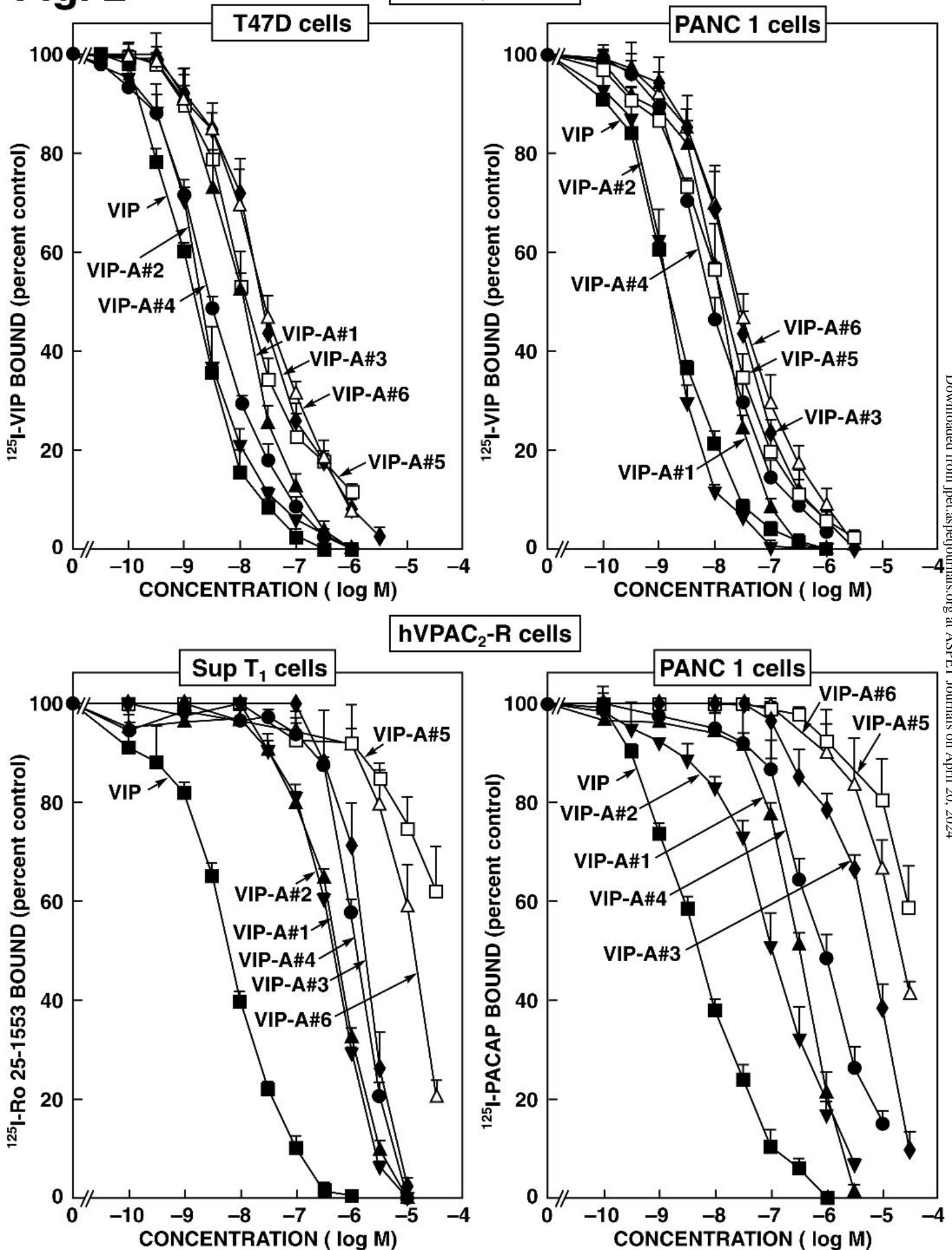
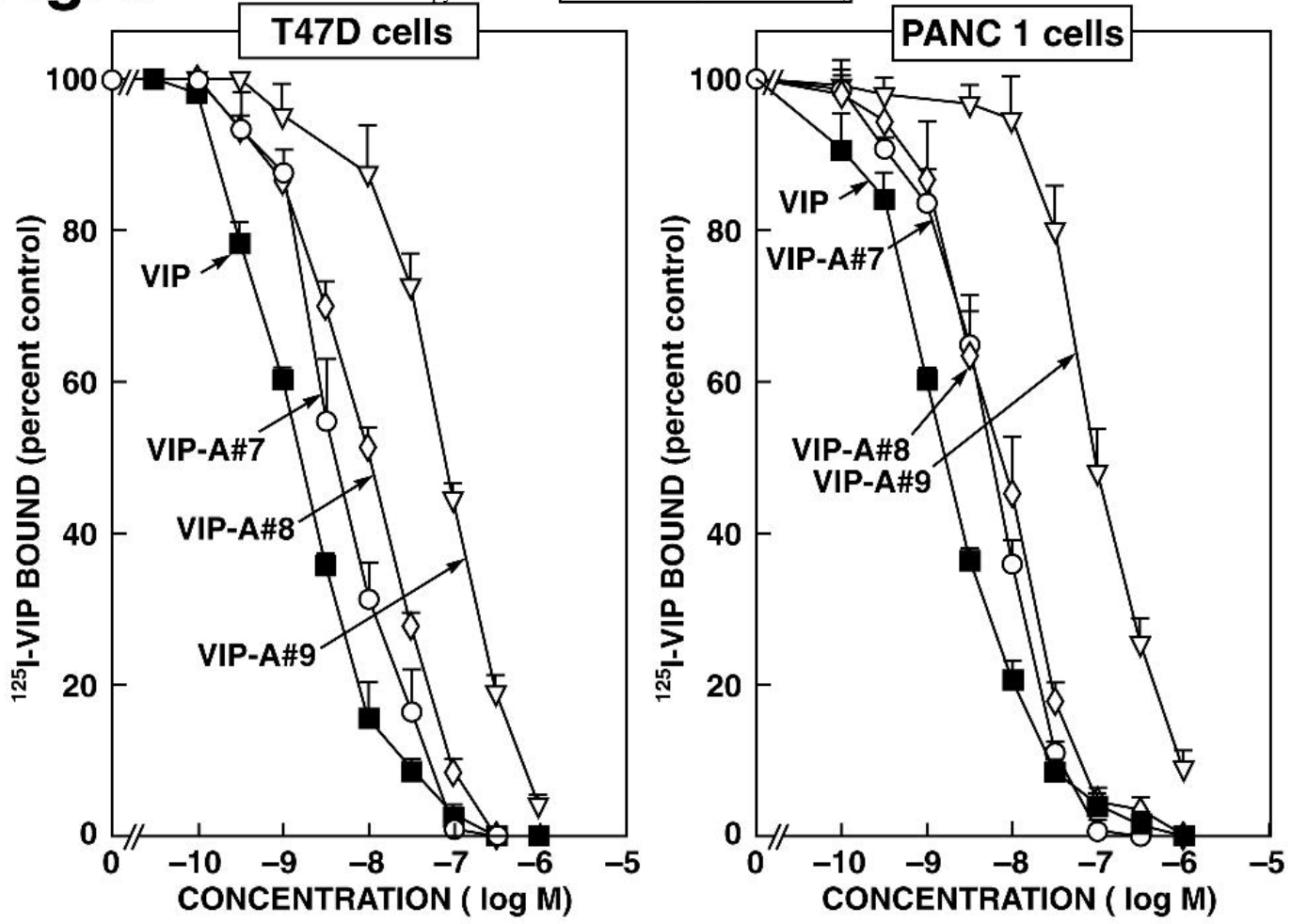
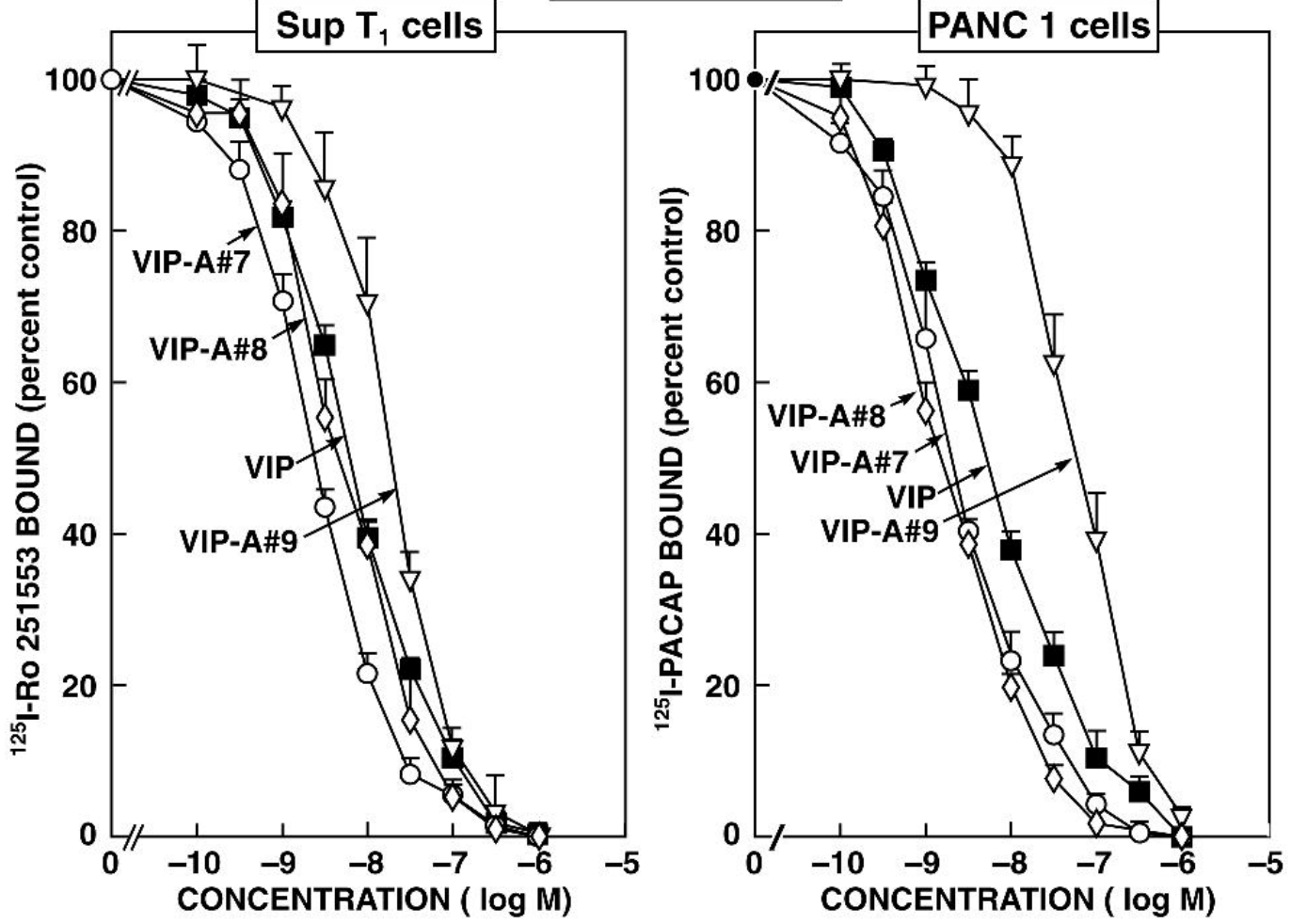
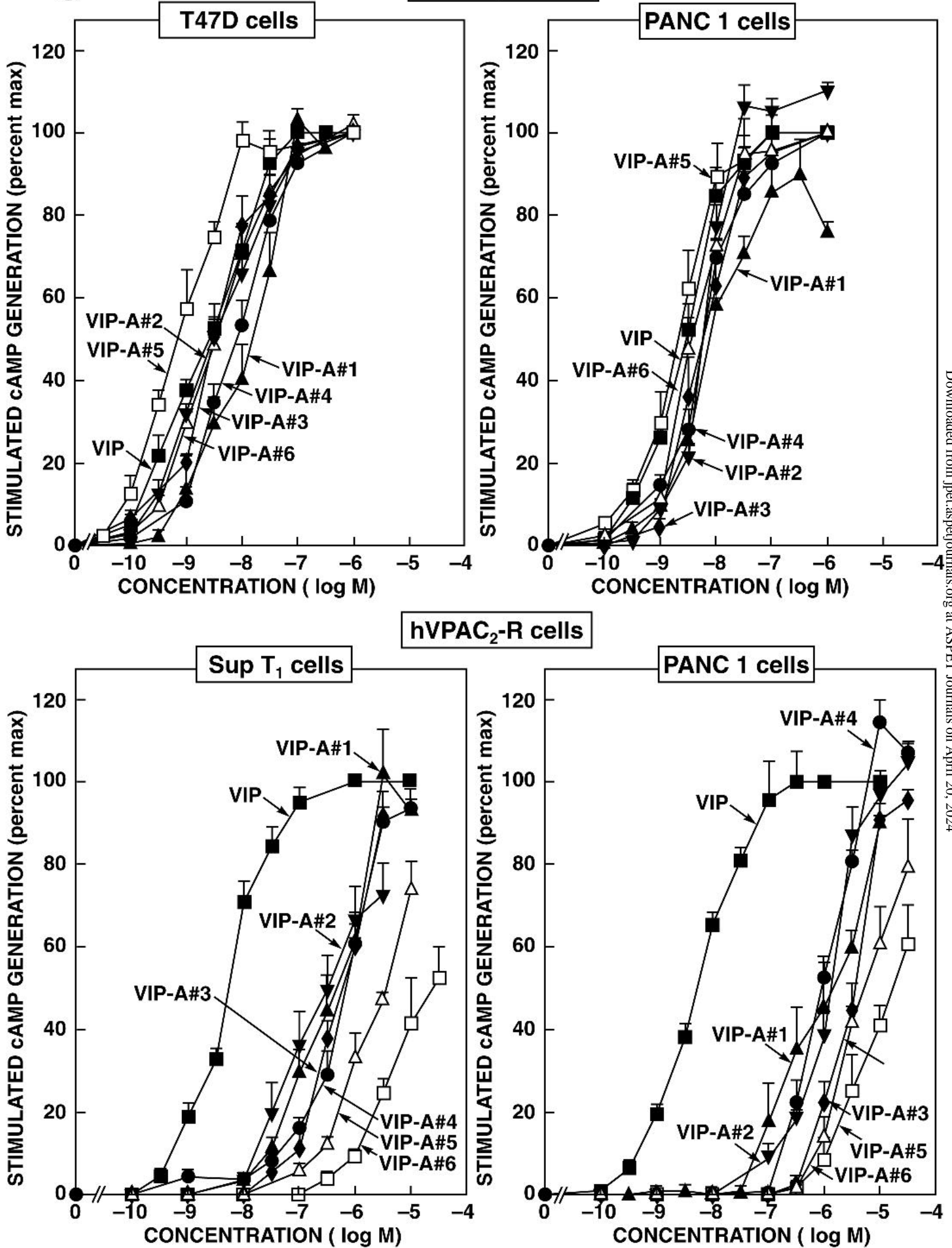
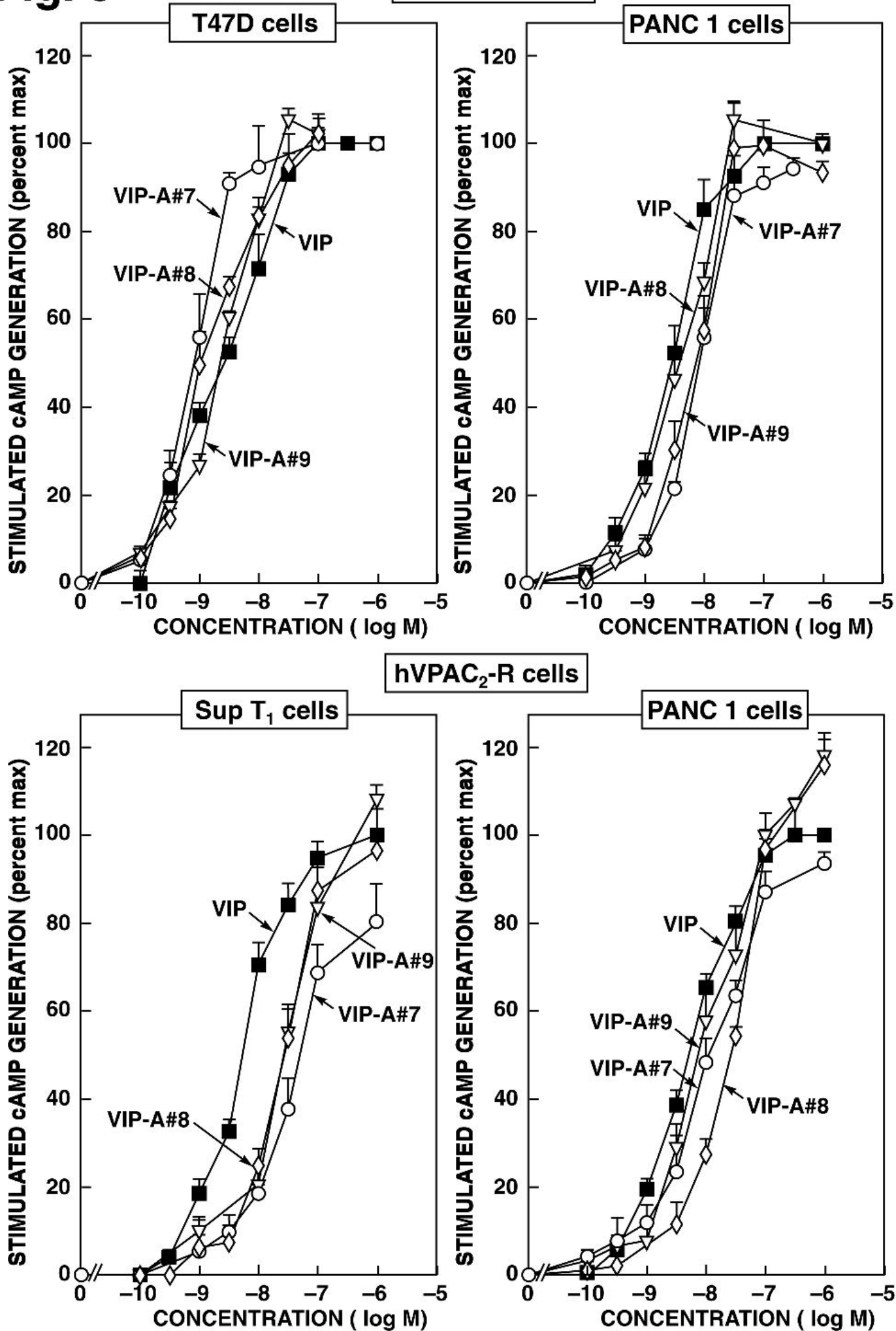
Fig. 2

Fig. 3**hVPAC₂-R cells****hVPAC₂-R cells**





hVPAC₁-R / PANC1 cells

hVPAC₂-R / PANC1 cells

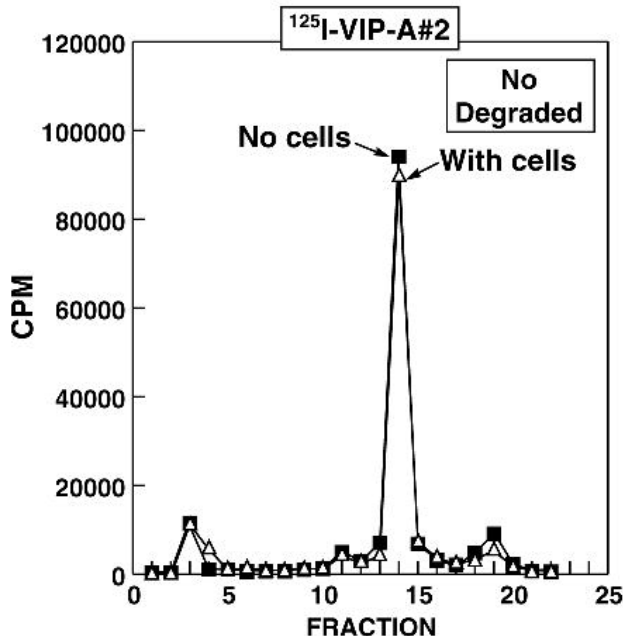
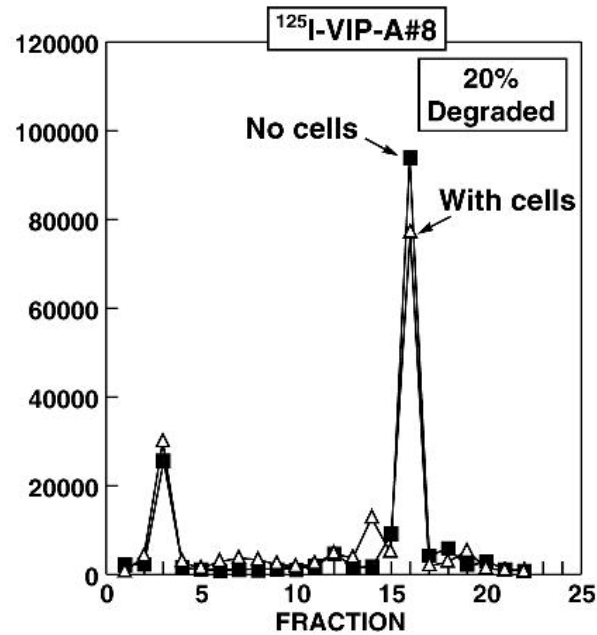
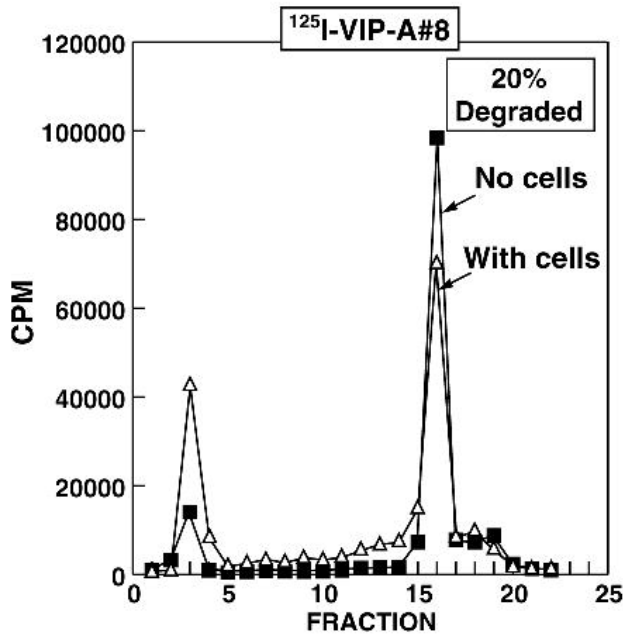
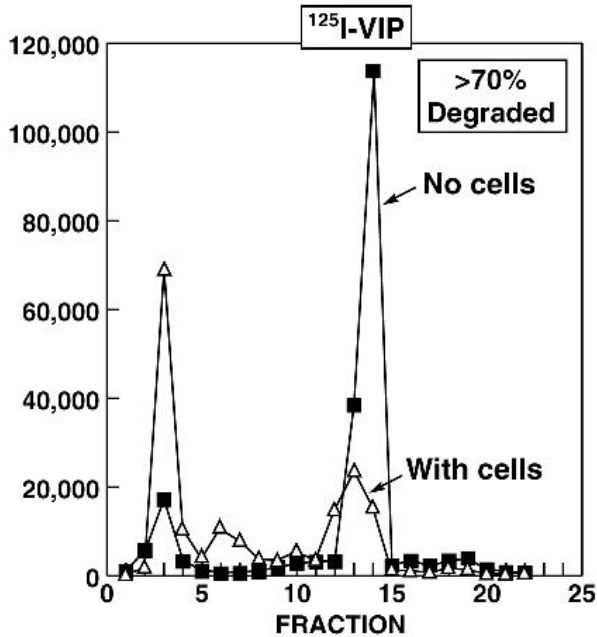
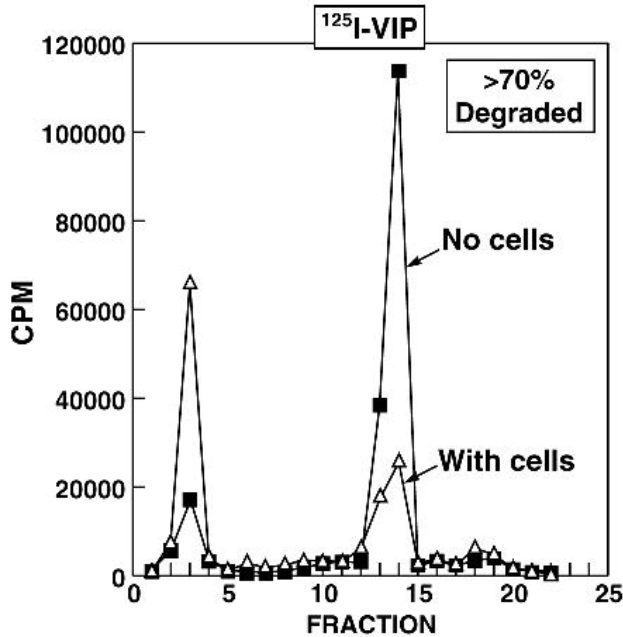


Fig. 6