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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.)

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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.niddk.nih.gov**

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Abbreviations: VIP, vasoactive intestinal peptide; VPAC, VIP-PACAP receptors (for nomenclature see ref. (Harmar, et al., 1998); $hVPAC_1$, human $VPAC_1$ receptor subtype; $hVPAC_2$, human $VPAC_2$ 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-NH_2; PACAP, pituitary adenylate

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

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Abstract

Vasoactive intestinal peptide (VIP) is a widespread neurotransmitter whose physiological and pathophysiological actions are mediated by two receptor classes, $VPAC_1$ and $VPAC_2$. 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 $VPAC_1$/$VPAC_2$ 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 $hVPAC_1$ (T47D cells, $hVPAC_1$ transfected cells) and $hVPAC_2$ ($SupT_1$ and $VPAC_2$-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 $hVPAC_1$. None of the nine analogs had $hVPAC_2$ 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. ^{125}I-[$Ala^{2, 8, 9, 16, 19, 20, 21, 24, 25}$]VIP was much more metabolically stable than ^{125}I-VIP. The availability of these simplified analogs of VIP, which are metabolically stable and have either $hVPAC_1$ 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 cytoxicity.

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 ($VPAC_1$ and $VPAC_2$), 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 $VPAC_1$ and human $VPAC_2$ 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 $VPAC_1$ 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 $VPAC_1$ (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 $VPAC_1$ and three analogs that should have high affinity retained for $VPAC_2$ 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 T_1 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); [^{125}I]VIP (2,200 Ci/mmol) and [^{125}I]PACAP(1-27) (2,200 Ci/mmol) from Perkin Elmer Life Science Products (Boston, MA); Na^{125}I (2,200 Ci/mmol) and [2-^{3}H]adenine (22 Ci/mmol) from Amersham Pharmacia Biotech (Piscataway, NJ); 1, 3, 4, 6-Tetrachloro 3α-, 6α-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 KH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 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.

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Construction of PANC1 Cells Stably Transfected with $hVPAC_2$-R and $hVPAC_2$-R.

Construction of the human $VPAC_1$ receptor ($hVPAC_1$-R), and human $VPAC_2$ ($hVPAC_2$-R) receptor expression vector and construction of $hVPAC_1$-R or $hVPAC_2$-R stably transfected PANC1 cells ($hVPAC_1$-R or $hVPAC_2$-R/PANC1 cells) were described previously (Ito, et al., 2001;Igarashi, et al., 2002a).

Cell Culture. The $hVPAC_1$-R/PANC1 cells and $hVPAC_2$-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 T_1 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^{o}C in an atmosphere of 5% CO_2 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^{o}C in an atmosphere of 5% CO_2 and 95 % air.

Preparations of ^{125}I-Ro 25-1553. ^{125}I-Ro 25-1553 (a cyclic VIP analog selective for $VPAC_2$-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 KH_2PO_4 (pH 8.0). To this vial, 20 µl of 0.5 M KH_2PO_4 (pH 8.0), 8 µg of peptide in 4 µl of water, and 2 mCi (20 µl) of $Na^{125}I$ 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 ^{125}I was eluted with 5 ml of water followed by 5

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ml of 0.1% (v/v) trifluoroacetic acid. The radiolabeled peptides were eluted with 200 µl 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^{o}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 hVPAC$_1$-R/PANC1 and T47D cells, and binding of [^{125}I]PACAP(1-27) to hVPAC$_2$-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 VPAC$_2$-R affinities in Sup T$_1$ 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^{o}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 IC_{50} was calculated, which was the concentration that gave half-maximally inhibition of that seen with a saturating concentration of VIP (1 μM). The IC_{50} was calculated using the curve-fitting program Kaleidagraph.

cAMP Assay. T47D cells (0.05 X 10^6 cells), $hVPAC_1$-R/PANC1(0.05 X 10^6 cells) and $hVPAC_2$-R/PANC1 (0.05 X 10^6 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-^{3}H]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 T_1 cells, 20 ml of medium containing 2.0 to 4.0 x 10^6 cells/ml, supplemented with 2% FBS (v/v) and 2 μCi/ml [2-^{3}H]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 EC_{50} was calculated, which was the concentration of the peptide that gave half-maximal stimulation of a maximally effective concentration of VIP (1 μM). The EC_{50} was calculated using the curve-fitting program Kaleidagraph.

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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 hVPAC$_1$-R/PANC cells (0.3 x 10^6 cells/ml) at 37^{o}C for 7.5 min, or hVPAC$_2$-R/PANC cells (0.4 x 10^6 cells/ml) at 37^{o}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. IC_{50} and EC_{50} were calculated using the curve-fitting program Kaleidagraph. Statistical comparisons were made using the Student's *t*-test.

Results

Development of Muti-alaninated VIP Analogs That Were Designed to Have a High Affinity for Human $VPAC_1$-R or $VPAC_2$-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 $VPAC_1$-R. This analog also had increased stability compared to VIP for the $hVPAC_1$-R (Igarashi, et al., 2002a). However, no studies were performed on the selectivity or stability of this analog with $hVPAC_2$-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 $hVPAC_1$-R and $hVPAC_2$-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 $hVPAC_1$-R (Igarashi, et al., 2002a) and the $hVPAC_2$-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 $hVPAC_1$-R or the $hVPAC_2$-R. Five new analogs (analog #s 1, 3-6; Fig. 1) were synthesized based on the VIP pharmacophore for $hVPAC_1$-R and three new analogs (analog #s 7-9; Fig. 1) based on the VIP pharmacophore of the $hVPAC_2$-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 $hVPAC_1$-R Agonists for $hVPAC_1$-R or $hVPAC_2$-R Containing Cells. Multi-alaninated VIP analog #s 1, 3-6 retained high affinity for the $hVPAC_1$-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 hVPAC$_1$-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 hVPAC$_1$-R, similar to that reported previously (Igarashi, et al., 2002a). An additional alanine replacement of either valine^{19} (analog #3) or Lys^{21} (analog #4), Tyr^{22} (analog #5) or Ile^{26} (analog #26) had variable effects on hVPAC$_1$-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 hVPAC$_1$-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 hVPAC$_2$-R on Sup T$_1$ human lymphoblastoma cells and hVPAC$_2$-R stably transfected PANC1 cells were determined (Fig. 2, bottom panel; Table 1). VIP had a high affinity for the hVPAC$_2$-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 hVPAC$_2$-R. When additional alanine substitutions for Met^{17}, Lys^{21}, Tyr^{22} or Ile^{26} were made to analog #2, the affinity for hVPAC$_2$-R became much lower, especially with the additional alanine substitution for Tyr^{22} and Ile^{26} (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 hVPAC$_2$-R. In terms of hVPAC-R selectivity, VIP had a 4-fold higher affinity for hVPAC$_1$-R, whereas VIP analog #1 had a 36-fold higher selectivity for hVPAC$_1$-R over hVPAC$_2$-R and analog #2, a 150-fold selectivity for hVPAC$_1$-R (Table 1). The additional substitution of Met^{17} or Lys^{21} resulted in analogs #3 and #4, respectively, which had a 150- to 200-fold higher selectivity for hVPAC$_1$-R over hVPAC$_2$-R (Table 1). Alanine substitution of Tyr^{22} or Ile^{26} in analog #2, which yielded VIP analogs #5 and #6, had the greatest selectivity for hVPAC$_1$-R over hVPAC$_2$-R, which were >2400-fold and 660-fold, respectively, (Table 1).

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Affinity of Multi-alanine Substituted VIP Analogs Designed to Be hVPAC$_2$-R Agonists for hVPAC$_1$-R or hVPAC$_2$-R Containing Cells. We synthesized three multi-alaninated VIP analogs that should retain high affinity for the hVPAC$_2$-R (VIP analogs #7 to #9, Fig. 1) and determined their abilities to interact with hVPAC$_1$-R receptors (Fig. 3, top panel; Table 1) and hVPAC$_2$-R containing cells (Fig. 3, bottom panel; Table 1). VIP analog #7 with 6 alanine substitutions (i.e., for Ser^2, Asp^8, Asn^9, Gln^{16}, Val^{19} and Asn^{24}; Fig. 1) as well as VIP analog #8 which had an additional alanine substitution for Ser^{25} (Fig. 1), had a similar high affinity to VIP for the hVPAC$_2$-R (Fig. 3, bottom panel; Table 1). However, when two additional alanine substitutions for Lys^{20} and Lys^{21} were added to analog #8, the resultant analog #9 had a 3 to 8-fold decrease in affinity for hVPAC$_2$-R compared to VIP (Fig. 3, bottom panel; Table 1). We also determined their affinities for hVPAC$_1$-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 hVPAC$_1$-R compared to VIP. In contrast, analog #9 had a 50-fold decrease in the affinity compared to VIP for hVPAC$_1$-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 hVPAC$_2$-R than hVPAC$_1$-R, while VIP had a 3.7-fold higher selectivity for hVPAC$_1$-R than hVPAC$_2$-R (Table 1, Fig. 3).

Potency of Multi-alanine-Substituted VIP Analogs Designed to Be hVPAC$_1$-R Agonists for hVPAC$_1$-R or hVPAC$_2$-R Activation. Activation of adenylate cyclase is the principal intracellular mediator of the action of VPAC$_1$ and VPAC$_2$ 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 hVPAC$_1$-R and hVPAC$_2$-R activation, we first determined the ability of VIP analogs #1 to #6, which were hVPAC$_1$-R-preferring by binding studies, to stimulate cAMP generation in hVPAC$_1$-R and

hVPAC$_2$-R containing cells (Fig. 4, Table 2). Each of the 6 analogs had similar efficacy to VIP in stimulating cAMP generation through the hVPAC$_1$-R (Fig. 4, top panel). VIP had a high potency for stimulating cAMP generation in hVPAC$_1$-R containing cells with EC$_{50}$'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 VPAC$_1$-R compared to VIP, each of the other 4 VPAC$_1$-R-preferring analogs (#s 2, 3, 5, 6) retained a high potency for the VPAC$_1$-R (Fig. 4, top panel; Table 2).

VIP had a high potency for stimulating cAMP generation via the hVPAC$_2$-R, with an EC$_{50}$ of 5 nM and caused maximal stimulation at 100-300 nM concentration (Fig. 4, bottom panel). Three of the VPAC$_1$-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 VPAC$_2$-R (Fig. 4, bottom panel). VIP analogs #1 to #6 had >90-fold lower potency than VIP for stimulating cAMP generation in hVPAC$_2$-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 hVPAC$_1$-R or hVPAC$_2$-R, VIP analogs #1 to #6 had >100-fold higher selectivity for hVPAC$_1$-R over hVPAC$_2$-R, while VIP had a 2.0-fold selectivity for hVPAC$_1$-R over hVPAC$_2$-R (Table 2). Based on potency, VIP analog #5 had the greater selectivity for stimulating cAMP generation, having a 15600-fold higher selectivity for hVPAC$_1$-R over hVPAC$_2$-R (Table 2).

Potency of Multi-alanine Substituted VIP Analogs (Analog #s 7-9) Designed to Have Higher Affinity for the hVPAC$_2$-R for hVPAC$_1$-R or hVPAC$_2$-R Activation. VIP analogs #7 to #9 had equal efficacy to VIP for activating either hVPAC$_1$R (Fig. 5, top panel) or hVPAC$_2$-R cells (Fig. 5, bottom panel). Each of these three analogs had a similar potency to VIP for stimulating cAMP generation in VPAC$_1$-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 hVPAC$_2$-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 hVPAC$_2$-R over hVPAC$_1$-R (Table 3).

Stability of VIP and Multi-alaninated VIP Analogs Incubated With hVPAC$_1$-R/PANC1 Cells or hVPAC$_2$-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 hVPAC$_1$-R/PANC1 cells. However, in the present study we demonstrate the VIP analog #2 has less than a 200-fold selectivity for the hVPAC$_1$-R (Table 1) and thus would not be useful for *in vivo* receptor studies attempting to localize both hVPAC$_1$R and hVPAC$_2$-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 ^{125}I-analog #8 ([^{125}I-[Ala$^{2, 8, 9, 16, 19, 24, 25}$]VIP) and ^{125}I-analog #2 ([^{125}I-[Ala$^{2, 8, 9, 11, 19, 24, 25, 27, 28}$]VIP) and compared the amount of degradation during an incubation with hVPAC$_1$-R/PANC1 cells or hVPAC$_2$-R/PANC1 cells to that seen with ^{125}I-VIP. As seen in Figure 6 (left panel), more than 70% of ^{125}I-VIP was degraded during incubation with hVPAC$_1$-R/PANC1 cells, while only 20% of ^{125}I-analog #8 was degraded. As reported previously (Igarashi, et al., 2002a), no degradation of ^{125}I-analog #2 was seen under the same conditions, suggesting that analog #8 had a greater stability than VIP incubated with hVPAC$_1$-R/PANC1 cells, but was less metabolically stable than analog #2. Figure 6 (right panel) demonstrates that more than 70% of ^{125}I-VIP was degraded during an incubation with hVPAC$_2$-R/PANC1 cells, however only 20% of ^{125}I-analog #8 was degraded, showing that analog #8 also had a greater stability than VIP during incubation with hVPAC$_2$-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 hVPAC$_1$ and which was synthesized after analyzing the VIP pharmacophore for VPAC$_1$ 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 VPAC$_1$ in both native hVPAC$_1$-containing cells (T47D cells) and hVPAC$_1$-transfected PANC1 cells; however, we further demonstrate this simplified VIP analog has relatively low selectivity (<170-fold) for hVPAC$_1$ over hVPAC$_2$. 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 hVPAC$_2$ (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 hVPAC$_1$. Two of the five new, simplified VIP analogs, [Ala$^{2, 8, 9, 11, 19, 22, 24, 25, 27, 28}$]VIP (analog #5) and [Ala$^{2, 8, 9, 11, 19, 24\text{-}28}$]VIP (analog #6), had >2400-fold and 600-fold higher binding affinities, respectively, for hVPAC$_1$ over hVPAC$_2$ receptors. Furthermore, each of these two analogs was a fully efficacious agonist at the VPAC$_1$ receptor and analog #5 had >15,000-fold greater potency for activating the hVPAC$_1$ compared to the hVPAC$_2$, and analog #6 had >1200-fold greater potency for hVPAC$_1$ over hVPAC$_2$. This discrepancy between potency and binding affinity for the VPAC$_1$ 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 [Leu^{22}]VIP for hVPAC$_1$ over hVPAC$_2$ (Gourlet, et al., 1998;Bhargava, et al., 2002), and at least comparable and maybe greater than the widely used VPAC$_1$ selective agonist (Gourlet, et al., 1997a), [Lys^{15}, Arg^{16}, Leu^{27}]VIP(1-7)-GRF(8-27), which is reported to have a 53- to 169-fold selectivity for rVPAC$_1$ over rVPAC$_2$ 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 hVPAC$_1$/hVPAC$_2$-containing cells (Gourlet, et al., 1997a;Igarashi, et al., 2002b).

In contrast to our results with the hVPAC$_1$, none of the three simplified VIP analogs synthesized, based on the analysis of the VIP pharmacophore for the VPAC$_2$ (Igarashi, et al., 2002b), demonstrated selectivity for the hVPAC$_2$. 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 hVPAC$_2$, in contrast to each of the six simplified VIP analogs (analogs #1-6) designed to have high affinity for VPAC$_1$, each of which had low affinity for hVPAC$_2$ (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 hVPAC$_2$. The lack of selectivity of these two high affinity simplified VIP analogs (analogs #7, #8) for VPAC$_2$ is in contrast to findings in other studies using different strategies which have reported finding peptides that have selectivity for VPAC$_2$. The VIP-related peptide, helodermin, is reported to have 15-fold higher affinity for hVPAC$_2$ over hVPAC$_1$ (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 hVPAC$_2$ over hVPAC$_1$ (Ito, et al., 2000;Igarashi, et al., 2002a;Moreno, et al., 2000;Gourlet, et al., 1997b), 246- to 4300-fold for rVPAC$_2$ over

rVPAC$_1$ (Ito, et al., 2000), and hexanoyl [Ala^{19}, Lys$^{27, 28}$]VIP, to have an 800-fold selectivity for hVPAC$_2$ over hVPAC$_1$ (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 VPAC$_1$ but not selective VPAC$_2$ 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 Thr^{11}, Tyr^{22}, Asn^{24}, Leu^{27} and Asn^{28} were more important for VPAC$_2$ than hVPAC$_1$ 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 Tyr^{22} in VIP was much more important for high affinity for VPAC$_2$ than VPAC$_1$ 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 hVPAC$_1$, was the only simplified analog to contain this substitution. The Ala^{22} substitution was not included in the other five proposed VPAC$_1$ selective agonists (analogs #1-4, 6) because it has been shown to cause a 4-fold decrease in hVPAC$_1$ affinity (Igarashi, et al., 2002a;Igarashi, et al., 2002b), which we wanted to avoid if possible. The single substitution of alanine for Thr^{11} or Leu^{27} in VIP also caused a >15-fold decrease in VPAC$_2$ affinity with minimal changes in VPAC$_1$ affinity (Igarashi, et al., 2002a;Igarashi, et al., 2002b). Both of these substitutions were included in each of the six proposed simplified VPAC$_1$ selective agonists; however, they only resulted in a 35- to 196-fold selectivity in analogs #1-4. Furthermore, the single substitution of Ala for Met^{17}, Lys^{21}, Ile^{26} or Asn^{28} in VIP caused only a 2.5- to 7.3-fold decrease in VPAC$_2$ affinity with minimal effects on VPAC$_1$ affinity (Igarashi, et al., 2002a;Igarashi, et al., 2002b). Similarly, when they were

included in the simplified proposed $VPAC_1$ selective agonists (analogs #2-4, 6), they generally had only a modest effect on increasing $VPAC_1$ selectivity. In contrast to the results with $VPAC_1$ in studies of the VIP pharmacophore for the $VPAC_2$ (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 $hVPAC_1$ than the $hVPAC_2$. VIP itself had a 4-fold greater affinity for $hVPAC_1$ than $VPAC_2$. Single alanine substitution for Gln^{16} or Lys^{20} in VIP resulted in a 3- to 4-fold greater decrease in affinity for $VPAC_1$ than $VPAC_2$ (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 $VPAC_1$, with the result these simplified analogs had nearly equal affinity for both $hVPAC_1$ and $hVPAC_2$ 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 Ser^{25}-Ile^{26} or at Thr^{7}-Asp^{8} 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 Val^{19} 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-[Lys^{12}, Nle^{12}, Val^{26}, Thr^{28}]VIP on the duration of

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bronchodilator activity, it was reported that the substitution of Val^{19} by an Ala^{19} 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 $VPAC_1$ and $VPAC_2$. 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 $hVPAC_1$ or $hVPAC_2$ cells, whereas ^{125}I-VIP was degraded >70% by both, demonstrating analog #8 was metabolically stable with cells containing both VPAC subtypes. Furthermore, with $VPAC_1$-containing cells, ^{125}I-[$Ala^{2, 8, 9, 16, 19, 24, 25}$]VIP (^{125}I-analog #8), was only slightly less metabolically stable than ^{125}I-[$Ala^{2, 8, 9, 11, 18, 19, 24, 25, 27, 28}$]VIP (analog #2), which was previously reported (Igarashi, et al., 2002a) to be much more metabolically stable than VIP with $hVPAC_1$-containing cells.

In conclusion, analyzing the results of studies of the VIP pharmacophore for high affinity interaction with the $hVPAC_1$ or $hVPAC_2$ (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 (analog #5) has high affinity and >2000-fold selectivity for $hVPAC_1$ over $hVPAC_2$. 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 $VPAC_1$ 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).

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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 $hVPAC_1$-R or $hVPAC_2$-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 $hVPAC_1$-R agonists, and VIP analogs #7 to #9 as simplified, possible high affinity $hVPAC_2$-R agonists.

Figure 2: **Abilities of VIP and multi-alaninated VIP analogs designed to be high affinity $hVPAC_1$-R agonists to inhibit binding of [^{125}I]peptides to $hVPAC_1$-R *(top panel)* or $hVPAC_2$-R *(bottom panel)* containing cells.** *Top panel:* T47D human breast cancer cells (*left panel*) which possess native $VPAC_1$-R (1.2 x 10^6 cells/ml) and human $VPAC_1$-R stably transfected PANC1 cells (0.2 x 10^6 cells/ml) (*right panel*) were incubated for 60 min at room temperature with 75 pM [^{125}I]VIP alone or with the indicated concentrations of unlabeled peptides. *Bottom panel:* Sup T_1 human lymphoblastoma cells (*left panel*) which possess native $VPAC_2$ receptor (2.5 x 10^6 cells/ml) were incubated for 60 min at 37°C with 75 pM [^{125}I]Ro 25-1553 alone or with the indicated concentrations of unlabeled peptides. Human $VPAC_2$ receptor stably transfected PANC1 cells (0.1 x 10^6 cells/ml) (*right panel*) were incubated for 60 min at

room temperature with 75 pM [^{125}I]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 [^{125}I]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 hVPAC$_2$-R agonists to inhibit binding of [^{125}I]peptides to hVPAC$_1$-R *(top panel)* or hVPAC$_2$-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 hVPAC$_1$-R agonists to stimulate cAMP accumulation in hVPAC$_1$-R *(top panel)* or hVPAC$_2$-R *(bottom panel)* containing cells. *Top panel:* cAMP was performed using T47D cells (*left panel*) or hVPAC$_1$-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 hVPAC$_1$-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 T$_1$ cells (*left panel*) or hVPAC$_2$-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 μM VIP. Maximal stimulation of cAMP accumulation caused by 1 μM VIP was; Sup T_1 cells; 7.1 ± 1.1 fold and hVPAC$_2$-R/PANC1 cells; 50.5 ± 9.5 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.

Figure 5: **Abilities of VIP and multi-alaninated VIP analogs designed to be high affinity hVPAC$_2$-R agonists to stimulate cAMP accumulation in hVPAC$_1$-R *(top panel)* or hVPAC$_2$-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 μM VIP. In each experiment each value was determined in duplicate and values given are means ± S.E.M. from at least three separate experiments.

Figure 6: **Stability of the multi-alaninated VIP analog, ^{125}I-[Ala$^{2, 8, 9, 16, 19, 24, 25}$]VIP and ^{125}I-[Ala$^{2, 8, 9, 11, 19, 24, 25, 27, 28}$]VIP compared to ^{125}I-VIP.** *Left panel:* The degradation of ^{125}I-VIP, ^{125}I-[Ala$^{2, 8, 9, 16, 19, 24, 25}$]VIP (VIP analog #8) or ^{125}I-[Ala$^{2, 8, 9, 11, 19, 24, 25, 27, 28}$]VIP (VIP analog #2) by hVPAC$_1$-R/PANC1 cells is shown. Shown are the HPLC elution profiles of supernatants after incubation of ^{125}I-VIP *(top panel)*, ^{125}I-[Ala$^{2, 8, 9, 16, 19, 24, 25}$]VIP *(middle panel)* or ^{125}I-[Ala$^{2, 8, 9, 11, 19, 24, 25, 27, 28}$]VIP *(bottom panel)* (500,000 cpm/ml) with or without hVPAC$_1$-R/PANC1 cells (0.3 x10^6/ml) in standard incubation solution for 7.5 min at 37^{o}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. *Right panel:* The degradation of ^{125}I-VIP or ^{125}I- [Ala$^{2, 8, 9, 16, 19, 24, 25}$]VIP (VIP analog #8) by hVPAC$_2$-R/PANC1 cells is shown. Shown are the HPLC elution profiles of supernatants after incubation of ^{125}I-VIP *(top panel)* or ^{125}I-[Ala$^{2, 8, 9, 16, 19, 24, 25}$]VIP *(bottom panel)* (500,000 cpm/ml) with or without hVPAC$_2$-R/PANC1 cells (0.4 x10^6/ml) in standard incubation solution for 15 min at 37^{o}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.

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Table 1: The affinity of VIP and simplified multi-alaninated VIP analogs designed to be selective hVPAC$_1$-R agonists

Proposed hVPAC-R Selective Agonist (Analog #)	Binding affinity (IC_{50}: nM) hVPAC$_1$-R /T47D	hVPAC$_1$-R /PANC1	hVPAC$_2$-R /Sup T$_1$	hVPAC$_2$-R /PANC1	hVPAC-R selectivity (fold)
hVPAC$_1$-R - Selective					**hVPAC$_1$-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
hVPAC$_2$-R - Selective					**hVPAC$_2$-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 [^{125}I]peptide and various concentrations of the unlabeled alanine-substituted VIP analog as described in Figure 2 and 3 legends, and *Materials and Methods*. The IC_{50} was the concentration causing half-maximal inhibition of the saturable binding caused

by 1 μM VIP 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 the IC_{50} in the two different VPAC cells.

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Table 2: The potency of VIP and multi-alaninated VIP analogs designed to be $hVPAC_1$-R agonists for stimulating cAMP generation in $hVPAC_1$-R or $hVPAC_2$-R containing cells.

Proposed $hVPAC_1$-R agonist		Potency for cAMP generation (EC_{50}: nM) $hVPAC_1$-R /T47D	$hVPAC_1$-R /PANC1	$hVPAC_2$-R /Sup T_1	$hVPAC_2$-R /PANC1	$hVPAC_1$-R selectivity (fold)
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 EC_{50} 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 EC_{50} in the two different VPAC-containing cells.

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Table 3: The potency of VIP and multi-alaninated VIP analogs designed to be $hVPAC_2$-R agonists for stimulating cAMP generation in $hVPAC_1$-R or $hVPAC_2$-R containing cells.

Proposed $hVPAC_1$-R agonist		Potency for cAMP generation (EC_{50}: nM) $hVPAC_1$-R /T47D	$hVPAC_1$-R /PANC1	$hVPAC_2$-R /Sup T_1	$hVPAC_2$-R /PANC1	$hVPAC_2$-R selectivity (fold)
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 EC_{50} in the two different VPAC-containing cells.

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Fig. 1

Possible $VPAC_1$ 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
$hVPAC_1$-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
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

Possible $VPAC_2$ 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↓
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	–	–	–

Fig. 2

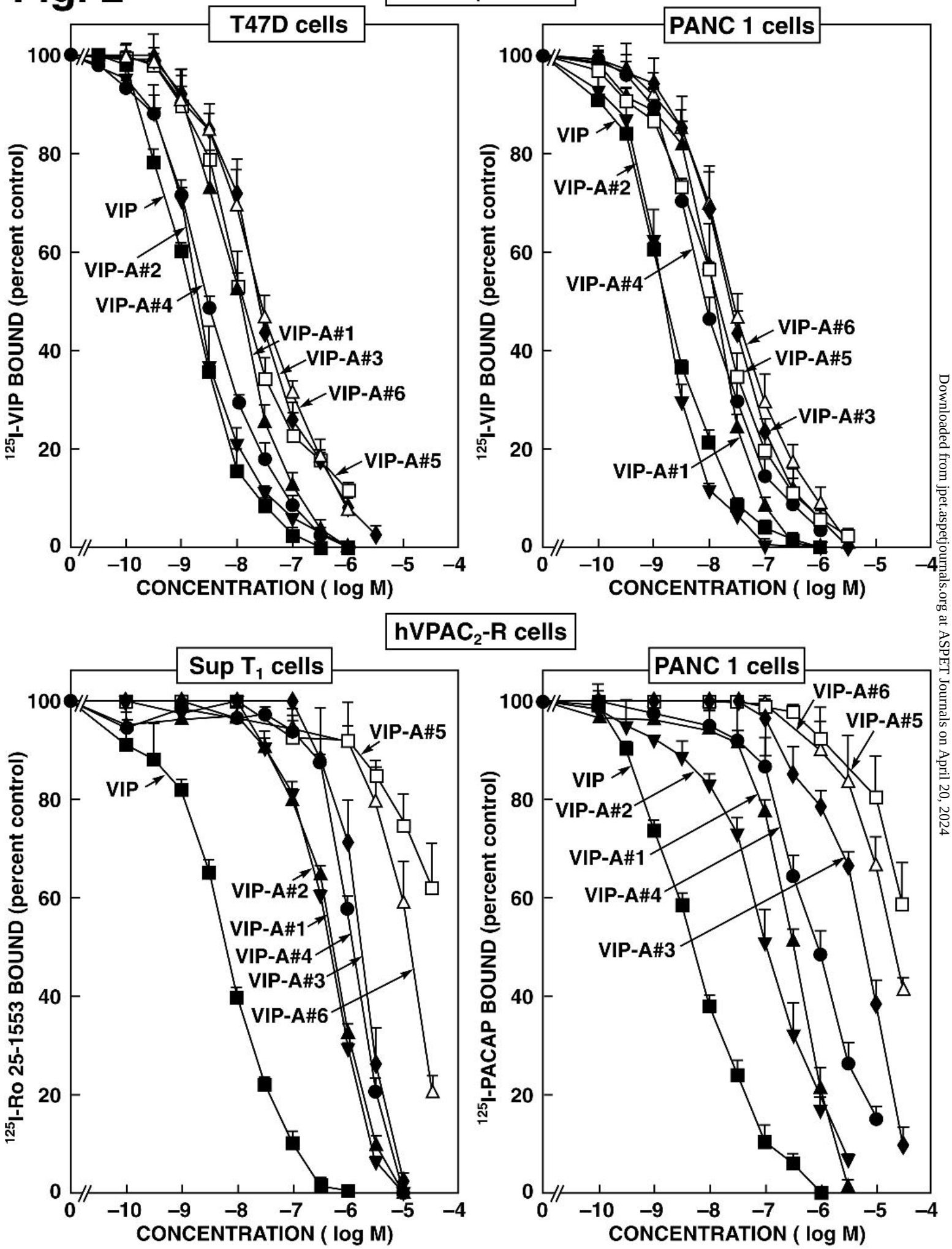

Fig. 3

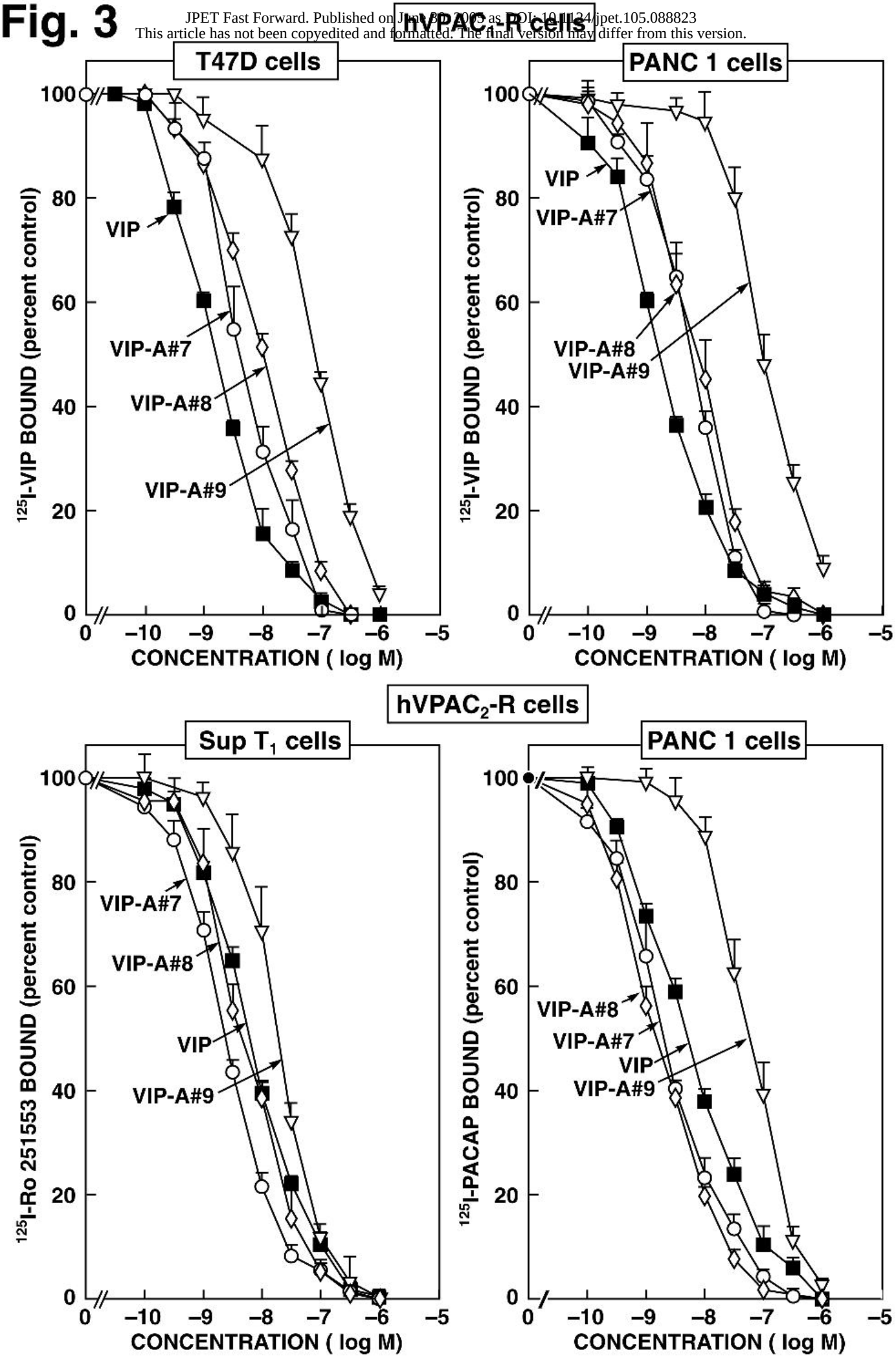

Fig. 4

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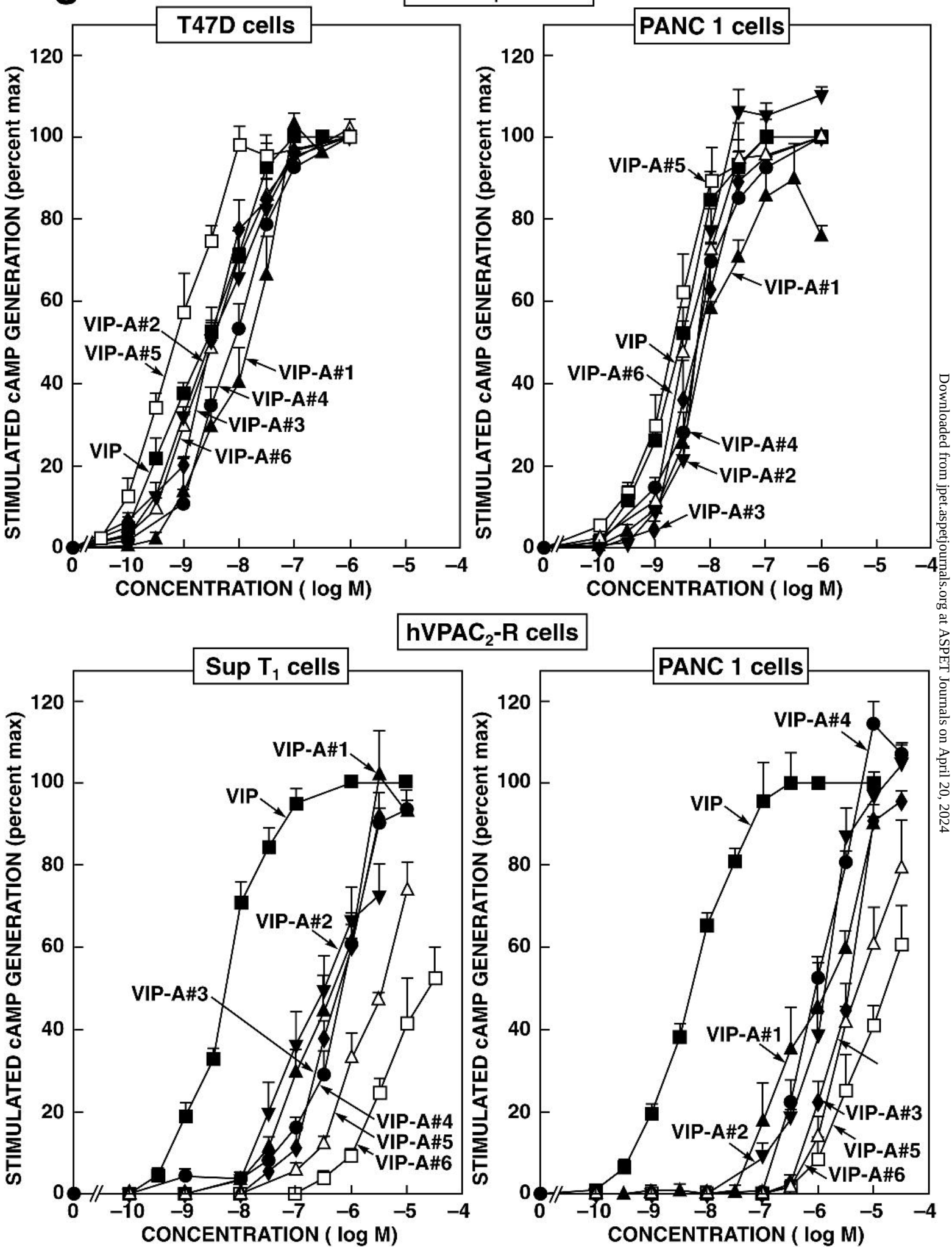

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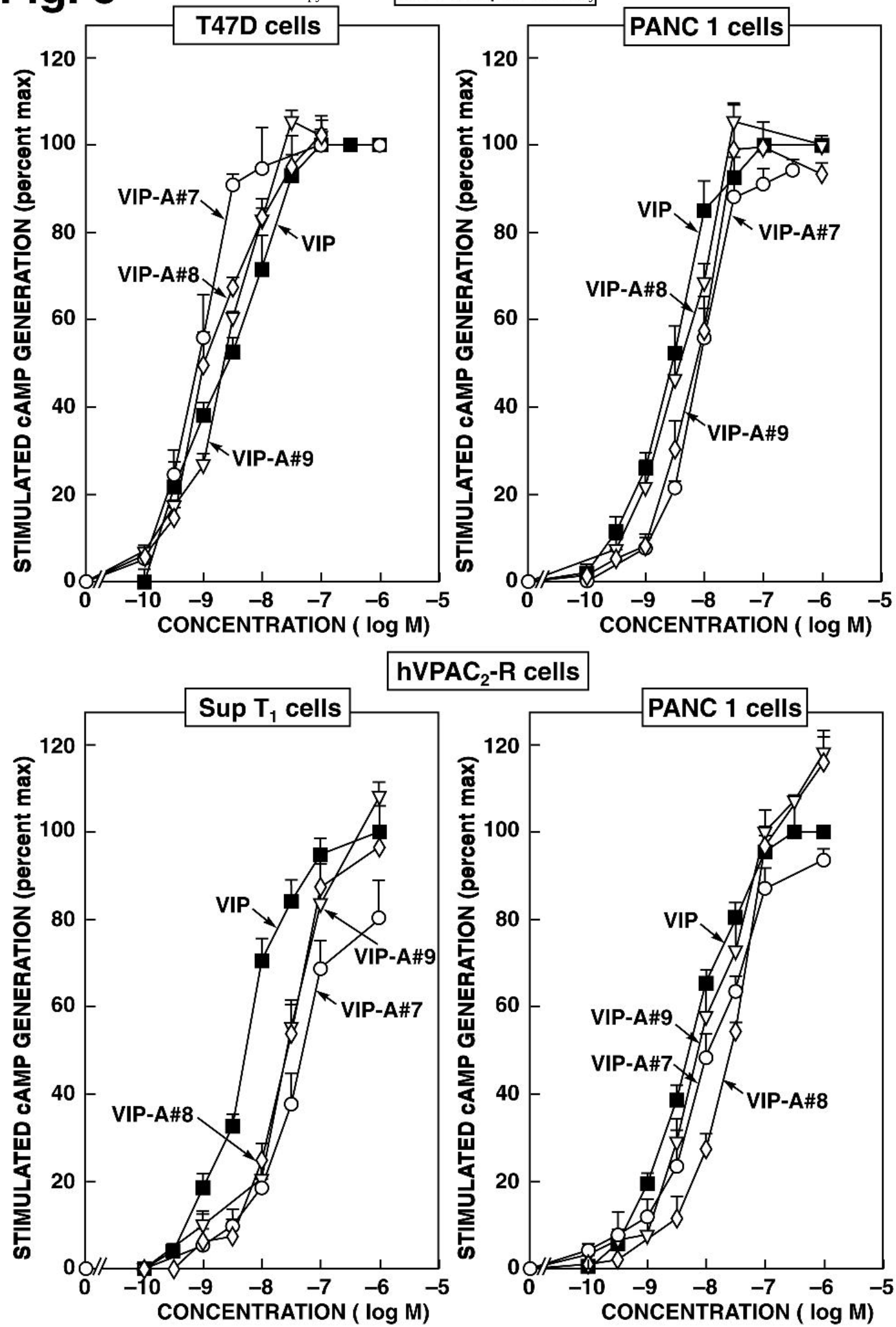
Fig. 5



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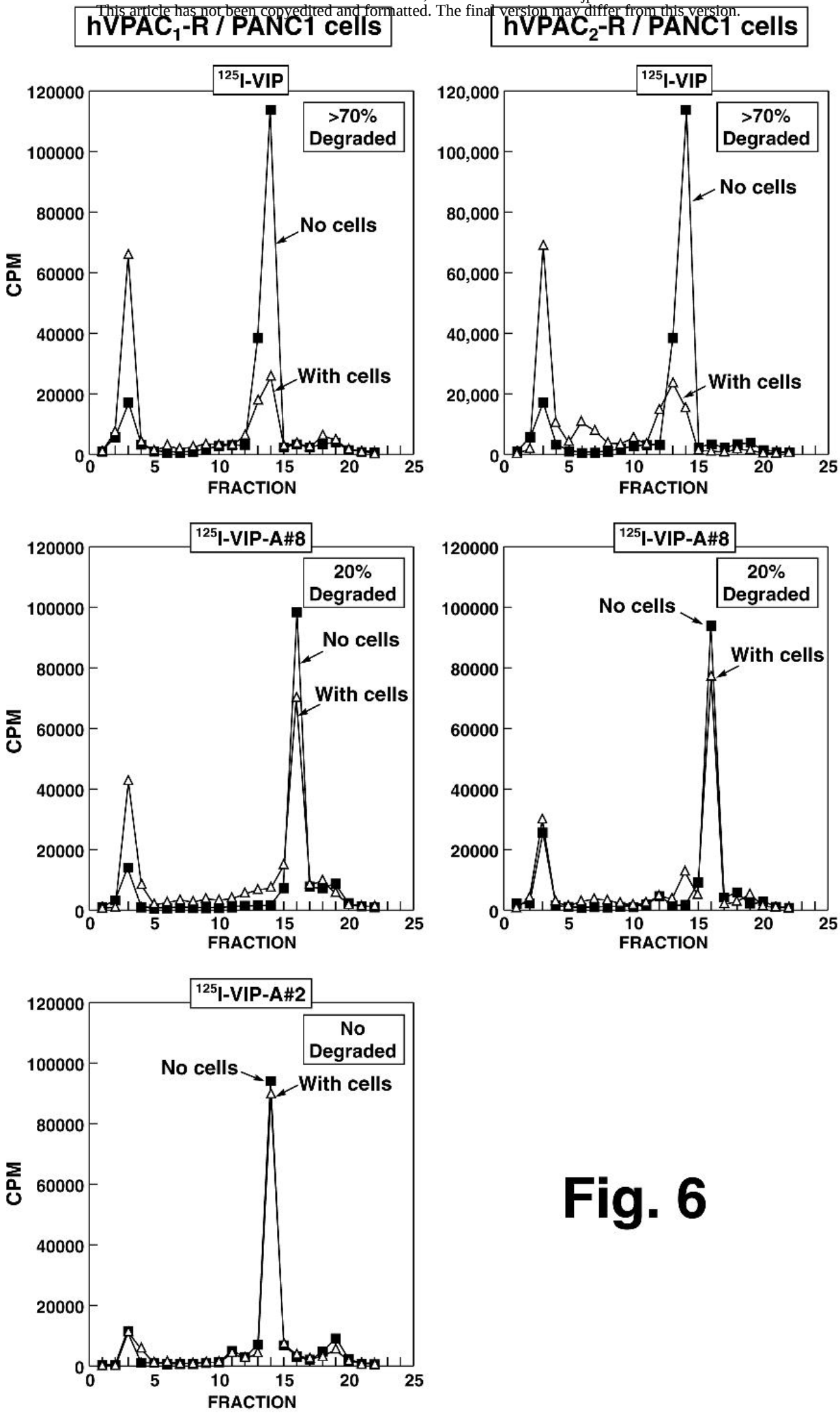

Fig. 6