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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-078328- -00003-0000

Fecha: 2008-07-17 16:56:02 Dep. 2020 NUECREACIONE
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALI
Act. 538 PETICION Folios: 2

Trámite : 011
Evento : 379
Actuación : 538

Referencia : Expediente No. 06.078.328
Asunto : Solicitud de Patente para **AGONISTAS DEL
RECEPTOR (VPAC2) DEL PEPTIDO ACTIVADOR DE
LA ADELINATO CICLASA HIPOFISARIA (PACAP) Y
SUS METODOS DE USO FARMACOLOGICO**
Solicitante : **BAYER PHARMACEUTICALS CORPORATION**
Clase : A61K 038/000, A61K 038/016
Publicada en : La Gaceta No. 587 Del 18 de enero de 2007
Fecha de Solicitud : 9 de agosto de 2006

1. Yo, Emilio FERRERO, identificado como aparece al pie de mi firma, actuando como apoderado en el asunto de la referencia, atentamente, solicito se efectúe el examen de patentabilidad de la solicitud en referencia presentada de acuerdo con el Capítulo II del PCT y según lo establecido en el artículo 44 de la Decisión 486 de la Comisión de la Comunidad Andina. Para tal efecto presento junto con este escrito el recibo por valor de \$210.000.00, que corresponde a la tasa fijada según lo dispuesto en la resolución No. 44372 de 26 de diciembre de 2007.

2. Ruego atentamente a la Señora Jefe se sirva ordenar que se anexe este recibo para que se efectúe dicho examen y se continúe con el trámite de la solicitud.

Señora Jefe,

EMILIO FERRERO
T.P.A. No. 31.929

COLO-14125-841-011-9
ASG\NNJ\ID-130752\WPC14125
No. M-2008-130752\ASG

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 900176089-2

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IMPORTE DE CADA : 00 - 45,976
: JUNIO 9 DE 2008

CONSIGNACION

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CAVELIER	CONSIGNACION	BANCO DE BOGOTA	062754387	2678328	09/06/2008	89,445,000.00

CONCEPTO

REPARTICION	CONCEPTO	TOTAL CONCEPTO
1 30000-01-01 SOLICITUDES	114 PTC CAP-II EXAMEN DE PATENTIBILIDAD	210,000.00
	TOTAL :	210,000.00

SUM: DOSCIENTOS DIEZ MIL PESOS

RESPONSE :

RECIBO DE PAGO AL EXPEDIENTE No.

14125-841-011-9.

DIVISIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 06-78328

EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCION

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 587 DE

FECHA 18 DE ENERO DE 2008 EL TÉRMINO HÁBIL SEÑALADO POR LA LEY

EXPIRA EL 16 DE ABRIL DE 2008

NOTA: Dentro del plazo de los seis (6) meses siguientes a partir de la fecha de publicación en la gaceta de Propiedad Industrial, deberá solicitar y cancelar el examen de patentabilidad so pena de que la solicitud caiga en abandono, de acuerdo con lo establecido en el Artículo 44 de la Decisión 486/2000.

SE PRESENTARON OPOSICIONES POR PARTE DE TERCEROS.

SI _____ **NO** _____ **X** _____

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Generation of Highly Selective VPAC2 Receptor Agonists by High Throughput Mutagenesis of Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-activating Peptide*

Received for publication, November 22, 2002
Published, JBC Papers in Press, January 13, 2003, DOI 10.1074/jbc.M211945200

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Pituitary adenylate cyclase-activating peptide (PACAP) has a specific receptor PAC1 and shares two receptors VPAC1 and VPAC2 with vasoactive intestinal peptide (VIP). VPAC2 activation enhances glucose-induced insulin release while VPAC1 activation elevates glucose output. To generate a large pool of VPAC2 selective agonists for the treatment of type 2 diabetes, structure-activity relationship studies were performed on PACAP, VIP, and a VPAC2 selective VIP analog. Chemical modifications on this analog that prevent recombinant expression were sequentially removed to show that a recombinant peptide would retain VPAC2 selectivity. An efficient recombinant expression system was then developed to produce and screen hundreds of mutant peptides. The 11 mutations found on the VIP analog were systematically replaced with VIP or PACAP sequences. Three of these mutations, V19A, L27K, and N28K, were sufficient to provide most of the VPAC2 selectivity. C-terminal extension with the KRY sequence from PACAP38 led to potent VPAC2 agonists with improved selectivity (100–1000-fold). Saturation mutagenesis at positions 19, 27, 29, and 30 of VIP and charge-scanning mutagenesis of PACAP27 generated additional VPAC2 selective agonists. We have generated the first set of recombinant VPAC2 selective agonists described, which exhibit activity profiles that suggest therapeutic utility in the treatment of diabetes.

Pituitary adenylate cyclase-activating polypeptide (PACAP),¹ originally isolated from ovine hypothalamus (1) by following pituitary adenylate cyclase activation, belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP) family of peptides (2). These peptides are expressed as part of larger proteins that are processed by proteolysis followed by C-

terminal amidation to generate the mature amidated peptides (Fig. 1). PACAP exists as a 38-residue form (PACAP38), and as a shorter form corresponding to the N-terminal 27 amino acids of PACAP38 (PACAP27). Both forms of PACAP bind to and activate the G-protein-coupled receptors PAC1, VPAC1, and VPAC2, whereas the related 28-mer peptide VIP only recognizes VPAC1 and VPAC2 (3). Activation of multiple receptors by PACAP or VIP has broad physiological effects on nervous, endocrine, cardiovascular, reproductive, muscular, and immune systems (4). Thus, clinical applications will require selective activation of a particular receptor to minimize potential side effects mediated by the other receptors. For example, we have previously demonstrated that VPAC2 activation induces glucose-dependent insulin secretion without the undesired side effects mediated by VPAC1 such as watery diarrhea (5). Therefore, to provide a potential therapy for type 2 diabetes, VPAC2 selectivity is an absolute requirement.

We sought to identify key determinants of VPAC2 selectivity and generate a peptide with the minimum number of mutations. However, structure-function relationship studies on VIP and PACAP (6–15) have been restricted by the number of peptides that can be tested due to limitations of peptide synthesis. Recombinant expression of these peptides has been difficult because they are rapidly degraded during expression and thus require expression as a fusion with a protein followed by specific proteolytic cleavage to release the peptide. Previous attempts using this approach have yielded limited success because the released peptide carried additional amino acids that reduced activity (16, 17). In the current study, we have developed an efficient recombinant expression system that can produce peptides without any exogenous amino acids. This has allowed us to perform an extensive structure-activity relationship study of PACAP, VIP, and a VPAC2 selective analog (18) and we report the first recombinant production of VPAC2 selective agonists.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—Chemically synthesized peptides PACAP27, PACAP38, VIP, R3P1 to R3P17, R3P33, R3P55, R3P60, R3P66, and PAC1 to PAC27 were supplied by Sigma Genosys (The Woodlands, TX). The cyclized peptide Ro 25-1553 was supplied by SynPep (Dublin, CA). All peptides were shown to be >95% pure by reverse-phase analytical high performance liquid chromatography and confirmed to have the predicted mass by mass spectrometry.

Recombinant Mutagenesis—Most mutant DNA were generated by site-directed PCR mutagenesis using VIP or R3P3, as the template, and oligonucleotides containing codons corresponding to the desired mutations (19). The VIP and R3P3 templates were created by hybridizing two synthesized overlapping DNA fragments (Invitrogen) encoding the

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¹ The abbreviations used are: PACAP, pituitary adenylate cyclase-activating peptide; VIP, vasoactive intestinal peptide; HPLC, high performance liquid chromatography; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HFBA, heptafluorobutyric acid.

TABLE I

Mutagenesis of VIP to generate highly selective VPAC2 mutant peptides with minimal number of mutations

Lead peptides from each round of mutagenesis is highlighted by its round number. Underlined histidine (H) symbolizes N-terminal acylation while asterisk (*) represents C-terminal amidation. Underlined leucine (L) corresponds to norleucine. Ro 25-1553, is cyclized between K21 and D25. Mutations from VIP are shaded. IC_{50} is determined to be the concentration of peptide at which there is a 50% decrease in binding of radiolabeled PACAP27 to the cell membrane as a result of competition from the peptide. EC_{50} is defined as the concentration of the peptide at which 50% of maximum PACAP27-induced cAMP accumulation in CHO or RIN cells transfected with the appropriate receptor is reached. Data are the mean $\pm$ S.E. of at least three experiments performed in triplicates. Ratio is a measure of selectivity, or preference for VPAC2 over VPAC1, and is derived from the ratio of IC_{50} or EC_{50} at VPAC1 versus that at VPAC2. None of these VIP-based peptides displayed any PAC1 binding or caused cAMP accumulation.

Peptide	Sequence	Competition binding, IC_{50} (nM)			CAMP Accumulation, EC_{50} (nM)			
		VPAC ₁	VPAC ₂	Ratio	VPAC ₁	VPAC ₂	Ratio	RIN
VIP	HSDAVFTDNYTLRQMAVKKYLNSILN*	7 $\pm$ 2	1.4 $\pm$ 0.6	0.2	0.2 $\pm$ 0.04	0.2 $\pm$ 0.03	1	5 $\pm$ 1
Ro25-1553	HSDAVFTDNYTLRQMAVKKYLNSILN*	20 $\pm$ 10	1200 $\pm$ 20	60	0.3 $\pm$ 0.1	>100	>300	
R3P1	HSDAVFTDNYTLRQMAVKKYLNSILN*	20 $\pm$ 3	1600 $\pm$ 400	80	2 $\pm$ 0.8	>100	>30	
R3P2	HSDAVFTDNYTLRQMAVKKYLNSILN*	100 $\pm$ 60	1400 $\pm$ 300	14	3 $\pm$ 1	>1000	>300	
1-R3P3	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 6	800 $\pm$ 200	20	0.8 $\pm$ 0.1	100 $\pm$ 20	130	60 $\pm$ 10
R3P5	HSDAVFTDNYTLRQMAVKKYLNSILN*	140 $\pm$ 60	1900 $\pm$ 400	14	5 $\pm$ 2	200 $\pm$ 50	40	
R3P7	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 10	40 $\pm$ 20	1	3 $\pm$ 1	3 $\pm$ 0.1	1	
R3P8	HSDAVFTDNYTLRQMAVKKYLNSILN*	30 $\pm$ 10	180 $\pm$ 20	6	1 $\pm$ 0.3	10 $\pm$ 2	10	
R3P9	HSDAVFTDNYTLRQMAVKKYLNSILN*	60 $\pm$ 20	600 $\pm$ 200	10	1 $\pm$ 0.6	40 $\pm$ 2	40	
R3P10	HSDAVFTDNYTLRQMAVKKYLNSILN*	80 $\pm$ 6	12 $\pm$ 4	0.2	6 $\pm$ 4	4 $\pm$ 0.2	1	
R3P11	HSDAVFTDNYTLRQMAVKKYLNSILN*	20 $\pm$ 1	45 $\pm$ 4	2	1 $\pm$ 0.6	10 $\pm$ 3	10	
2-R3P12	HSDAVFTDNYTLRQMAVKKYLNSILN*	100 $\pm$ 20	2500 $\pm$ 300	25	1 $\pm$ 0.7	300 $\pm$ 100	300	
R3P13	HSDAVFTDNYTLRQMAVKKYLNSILN*	50 $\pm$ 10	300 $\pm$ 90	6	1.3 $\pm$ 0.9	150 $\pm$ 20	120	
R3P14	HSDAVFTDNYTLRQMAVKKYLNSILN*	70 $\pm$ 20	140 $\pm$ 30	2	17 $\pm$ 8	50 $\pm$ 20	3	
R3P20	HSDAVFTDNYTLRQMAVKKYLNSILN*	42 $\pm$ 9	1400 $\pm$ 300	33	1 $\pm$ 0.4	800 $\pm$ 300	800	
R3P21	HSDAVFTDNYTLRQMAVKKYLNSILN*	90 $\pm$ 45	360 $\pm$ 20	4	1 $\pm$ 0.3	80 $\pm$ 30	80	
R3P22	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 8	400 $\pm$ 90	10	0.7 $\pm$ 0.4	140 $\pm$ 50	200	
R3P23	HSDAVFTDNYTLRQMAVKKYLNSILN*	10 $\pm$ 7	10 $\pm$ 3	1	1 $\pm$ 0.6	20 $\pm$ 7	20	
R3P24	HSDAVFTDNYTLRQMAVKKYLNSILN*	20 $\pm$ 3	600 $\pm$ 70	30	0.6 $\pm$ 0.3	30 $\pm$ 10	50	
R3P25	HSDAVFTDNYTLRQMAVKKYLNSILN*	60 $\pm$ 20	300 $\pm$ 30	5	0.4 $\pm$ 0.2	30 $\pm$ 10	80	
R3P26	HSDAVFTDNYTLRQMAVKKYLNSILN*	50 $\pm$ 20	700 $\pm$ 30	14	0.4 $\pm$ 0.1	200 $\pm$ 70	500	
R3P27	HSDAVFTDNYTLRQMAVKKYLNSILN*	5 $\pm$ 4	8 $\pm$ 4	2	0.4 $\pm$ 0.3	3 $\pm$ 2	8	
R3P28	HSDAVFTDNYTLRQMAVKKYLNSILN*	30 $\pm$ 10	13 $\pm$ 6	0.4	4 $\pm$ 3	3 $\pm$ 2	1	
R3P29	HSDAVFTDNYTLRQMAVKKYLNSILN*	100 $\pm$ 40	300 $\pm$ 100	3	0.5 $\pm$ 0.1	50 $\pm$ 30	100	
R3P30	HSDAVFTDNYTLRQMAVKKYLNSILN*	50 $\pm$ 20	300 $\pm$ 70	6	0.2 $\pm$ 0.1	40 $\pm$ 30	200	
R3P31	HSDAVFTDNYTLRQMAVKKYLNSILN*	100 $\pm$ 20	4000 $\pm$ 3000	40	0.5 $\pm$ 0.2	400 $\pm$ 300	800	
R3P32	HSDAVFTDNYTLRQMAVKKYLNSILN*	50 $\pm$ 30	1000 $\pm$ 80	20	0.5 $\pm$ 0.2	400 $\pm$ 200	800	
R3P33	HSDAVFTDNYTLRQMAVKKYLNSILN*	70 $\pm$ 30	900 $\pm$ 200	13	0.5 $\pm$ 0.2	400 $\pm$ 300	800	
R3P34	HSDAVFTDNYTLRQMAVKKYLNSILN*	50 $\pm$ 20	1000 $\pm$ 50	20	1 $\pm$ 0.1	300 $\pm$ 200	300	
R3P35	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 20	1000 $\pm$ 400	25	1 $\pm$ 0.7	400 $\pm$ 300	400	
4-R3P36	HSDAVFTDNYTLRQMAVKKYLNSILN*	60 $\pm$ 30	9000 $\pm$ 800	150	0.4 $\pm$ 0.1	200 $\pm$ 50	500	18 $\pm$ 4
R3P41	HSDAVFTDNYTLRQMAVKKYLNSILN*	100 $\pm$ 30	300 $\pm$ 90	3	1 $\pm$ 0.6	50 $\pm$ 30	50	
R3P42	HSDAVFTDNYTLRQMAVKKYLNSILN*	30 $\pm$ 10	14 $\pm$ 4	0.5	0.3 $\pm$ 0.1	0.5 $\pm$ 0.2	2	
R3P43	HSDAVFTDNYTLRQMAVKKYLNSILN*	10 $\pm$ 3	10 $\pm$ 4	1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	1	
R3P44	HSDAVFTDNYTLRQMAVKKYLNSILN*	500 $\pm$ 100	100 $\pm$ 30	0.2	2 $\pm$ 1	10 $\pm$ 2	5	
R3P45	HSDAVFTDNYTLRQMAVKKYLNSILN*	>1000	>1000	1	>1000	>1000	1	
R3P46	HSDAVFTDNYTLRQMAVKKYLNSILN*	30 $\pm$ 9	10 $\pm$ 3	0.3	0.2 $\pm$ 0.1	0.4 $\pm$ 0.1	2	
R3P47	HSDAVFTDNYTLRQMAVKKYLNSILN*	14 $\pm$ 3	14 $\pm$ 4	1	0.2 $\pm$ 0.1	0.8 $\pm$ 0.3	4	
R3P48	HSDAVFTDNYTLRQMAVKKYLNSILN*	30 $\pm$ 4	10 $\pm$ 2	0.3	0.2 $\pm$ 0.1	1 $\pm$ 0.3	5	
R3P49	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 10	120 $\pm$ 40	3	0.3 $\pm$ 0.1	9 $\pm$ 2	30	
R3P50	HSDAVFTDNYTLRQMAVKKYLNSILN*	20 $\pm$ 3	20 $\pm$ 2	1	0.2 $\pm$ 0.1	0.8 $\pm$ 0.3	4	
1-R3P51	HSDAVFTDNYTLRQMAVKKYLNSILN*	20 $\pm$ 6	400 $\pm$ 30	20	0.2 $\pm$ 0.1	30 $\pm$ 5	150	20 $\pm$ 6
R3P53	HSDAVFTDNYTLRQMAVKKYLNSILN*	60 $\pm$ 10	9000 $\pm$ 1000	150	0.2 $\pm$ 0.1	500 $\pm$ 10	2300	9 $\pm$ 1
R3P54	HSDAVFTDNYTLRQMAVKKYLNSILN*	600 $\pm$ 200	>1000	>2	0.5 $\pm$ 0.1	>100	>200	
R3P55	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 6	7000 $\pm$ 2000	180	0.2 $\pm$ 0.1	100 $\pm$ 10	500	11 $\pm$ 1
R3P56	HSDAVFTDNYTLRQMAVKKYLNSILN*	500 $\pm$ 200	>1000	>2	0.4 $\pm$ 0.1	40 $\pm$ 5	100	
R3P57	HSDAVFTDNYTLRQMAVKKYLNSILN*	250 $\pm$ 100	>1000	>4	0.2 $\pm$ 0.4	40 $\pm$ 30	200	12 $\pm$ 1
R3P58	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 10	600 $\pm$ 400	15	0.3 $\pm$ 0.1	60 $\pm$ 20	200	9 $\pm$ 5
R3P59	HSDAVFTDNYTLRQMAVKKYLNSILN*	70 $\pm$ 5	700 $\pm$ 300	10	0.2 $\pm$ 0.1	10 $\pm$ 3	50	7 $\pm$ 1
5-R3P63	HSDAVFTDNYTLRQMAVKKYLNSILN*	40 $\pm$ 4	3200 $\pm$ 1000	80	0.2 $\pm$ 0.1	100 $\pm$ 30	500	

Competition Binding Assay and cAMP Scintillation Proximity Assay (SPA)—PACAP competition binding and cAMP accumulation assay were performed as described previously (5).

RESULTS

VPAC2 Selective Agonist Can Be a Recombinant Peptide (Round 1 Mutagenesis)—The only available VPAC2 selective agonists are two cyclized synthetic analogs of VIP (18, 20). To determine whether a recombinant VPAC2-selective peptide can be made, the four chemical modifications of one of them, Ro 25-1553 (21), were sequentially removed and tested in radioligand competition binding and cAMP activation assay (see Table I, R3P1 to R3P3). All three peptides, including the recombinant expression compatible R3P3, retained good VPAC2

agonist activity and selectivity in the binding and activation assay. Therefore, a peptide with high potency and selectivity toward VPAC2 can be made recombinantly.

Recombinant Expression of Peptides—VIP mutant peptides were expressed as C-terminal fusions to glutathione S-transferase with a Factor Xa recognition site inserted immediately N-terminal to the desired peptide sequence. Factor Xa cleavage yielded recombinant peptides that were identical to their synthetic counterparts. Both synthetic and recombinant R3P3, R3P9, R3P12, R3P33, R3P55, and R3P60 peptides were generated. In every case, recombinantly produced peptides were shown to be identical by mass spectrometry, amino acid analysis, competition binding assay (for example, see R3P3 in Fig.

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TABLE II
Mutagenesis of PACAP to generate VPAC2 selective mutant peptides

Asterisk (*) represents C-terminal amidation. Mutations from PACAP are shaded. IC₅₀ is determined as peptide concentration at which there is a 50% decrease in binding of radiolabeled PACAP27 to the cell membrane as a result of competition by the peptide. EC₅₀ is defined as the concentration of the peptide at which 50% of maximum PACAP27 cAMP accumulation in CHO cells transfected with the appropriate receptor is reached. Data are the mean of at least two experiments performed in triplicates. The standard errors were typically less than 30% of the mean.

Peptide	Sequence	Competition binding, IC ₅₀ (nM)			cAMP Induction, EC ₅₀ (nM)		
		VPAC ₂	VPAC ₁	PAC ₁	VPAC ₂	VPAC ₁	PAC ₁
PACAP27	HSDGIFTDYSRYRQMAVKKYLA AVL*	2	1	10	0.2	0.2	0.2
PACAP38	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	4	7	5	1	3	0.3
R3P6	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	500	2000	300	90	200	0.4
R3P15	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	80	250	90	5	12	0.06
R3P16	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	70	280	60	10	15	0.03
R3P17	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	90	110	30	2	5	0.14
R3P37	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	400	1500	200	3	87	0.6
R3P38	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	100	300	200	2.5	50	9
R3P39	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	200	60	60	0.5	4	0.05
R3P40	HSDGIFTDYSRYRQMAVKKYLA AVLGKRYKRVKMK*	120	70	80	0.5	4	0.05
PAC1	HSDGIFTDYSRYRQMAVKKYLA AVL*	10	20	>10000			
PAC2	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	>10000	>10000			
PAC3	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	>10000	>10000			
PAC4	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	1400	>10000			
PAC5	HSDGIFTDYSRYRQMAVKKYLA AVL*	500	200	>10000			
PAC6	HSDGIFTDYSRYRQMAVKKYLA AVL*	>3000	>3000	>3000			
PAC7	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	>10000	>10000			
PAC8	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	7000	>10000			
PAC9	HSDGIFTDYSRYRQMAVKKYLA AVL*	2600	40	260			
PAC10	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	>10000	>10000			
PAC11	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	200	770			
PAC12	HSDGIFTDYSRYRQMAVKKYLA AVL*	140	300	>10000			
PAC13	HSDGIFTDYSRYRQMAVKKYLA AVL*	1900	80	5200			
PAC14	HSDGIFTDYSRYRQMAVKKYLA AVL*	7500	7500	10000			
PAC15	HSDGIFTDYSRYRQMAVKKYLA AVL*	10	120	50			
PAC16	HSDGIFTDYSRYRQMAVKKYLA AVL*	110	30	>3000			
PAC17	HSDGIFTDYSRYRQMAVKKYLA AVL*	600	30	70			
PAC18	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	30	300			
PAC19	HSDGIFTDYSRYRQMAVKKYLA AVL*	800	400	1600			
PAC20	HSDGIFTDYSRYRQMAVKKYLA AVL*	360	200	800			
PAC21	HSDGIFTDYSRYRQMAVKKYLA AVL*	40	10	60			
PAC22	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	50	>10000			
PAC23	HSDGIFTDYSRYRQMAVKKYLA AVL*	>10000	4000	>10000			
PAC24	HSDGIFTDYSRYRQMAVKKYLA AVL*	30	30	80			
PAC25	HSDGIFTDYSRYRQMAVKKYLA AVL*	140	20	90			
PAC26	HSDGIFTDYSRYRQMAVKKYLA AVL*	130	13	260			
PAC27	HSDGIFTDYSRYRQMAVKKYLA AVL*	80	140	80			

(Ala-19 and Lys-27) of the original 11 mutations found in Ro 25-1553, yet it was more selective in the binding assay (180-fold versus 60-fold, respectively). Sequence comparison of R3P53 and R3P55 suggested that the Lys-28 and Tyr-31 mutations may not be crucial for the dramatic selectivity displayed since neither mutation was found in both peptides (see Table I). The four common mutations Ala-19, Lys-27, Lys-29, and Arg-30 were introduced into VIP to generate R3P65, which exhibited similar VPAC2 selectivity and potency as R3P53 and R3P55. Further reductions in the number of mutations did not result in peptides with comparable selectivity (data not shown), suggesting that all four mutations were needed to ensure maximum selectivity. Thus, we have created a highly VPAC2 selective and potent peptide that differs from VIP by only four amino acids (see Fig. 4).

High Throughput Saturation Mutagenesis of VPAC2 Selective Positions—To understand which amino acids maximally favor VPAC2 selectivity at each of the four critical VPAC2 selective positions 19, 27, 29, and 30, saturation mutagenesis at each position was performed using peptide R3P65 as a template followed by recombining the most selective mutations. This data showed that position 19 was most sensitive to substitutions in the cAMP assay (see Table III). Large, bulky, and hydrophobic amino acids at position 19 in general resulted

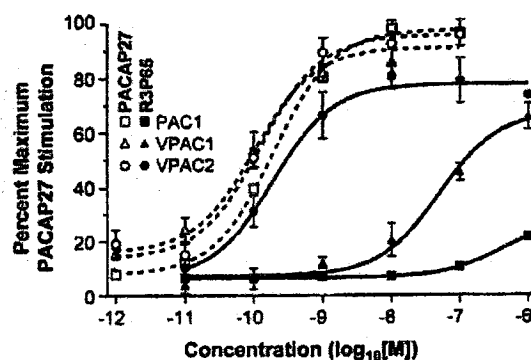


FIG. 4. cAMP assay of PACAP27, VIP, and VPAC2 selective agonist R3P65. CHO cells transfected with PAC1, VPAC1, or VPAC2 were treated with increasing concentrations of PACAP27 or R3P65, followed by the measurement of accumulated cAMP. Basal and maximal-stimulated cAMP levels generally were 1 and 5 pmol per well. The data shown are representative of at least three similar experiments. Each data point represents the average of triplicate data with the S.E. plotted.

in a loss of selectivity. In contrast, most amino acids were well tolerated for VPAC2 selectivity at position 27, with proline, arginine, and glutamine being somewhat more selective than

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TABLE IV
Combinatorial mutagenesis of R3P65 at positions 19, 27, 29, and 30

EC₅₀ is defined as the concentration of the peptide at which 50% of maximum PACAP27 cAMP accumulation in CHO cells transfected with the appropriate receptor is reached. Data are the mean ± S.E. of at least three experiments performed in triplicates. Ratio is a measure of selectivity, or preference for VPAC2 over VPAC1, and is derived from the ratio of EC₅₀ at VPAC1 versus that at VPAC2. None of these VIP-based peptides displayed any PAC1 binding or activation.

Peptide	Mutant Sequences	VPAC ₁ EC ₅₀	VPAC ₂ EC ₅₀	Ratio
R3P65	HSDAVFTDNYTRLRKQMAAKKYLSIKNR	0.6 ± 0.1	80 ± 9	130
R3P174	HSDAVFTDNYTRLRKQMAAKKYLSIKNR	0.2 ± 0.1	18 ± 4	90
R3P175	HSDAVFTDNYTRLRKQMAAKKYLSIKNR	2 ± 0.8	400 ± 100	200
R3P176	HSDAVFTDNYTRLRKQMAAKKYLSIKNR	2 ± 1	300 ± 100	150
R3P177	HSDAVFTDNYTRLRKQMAAKKYLSIKNR	1.6 ± 0.9	110 ± 40	70
R3P178	HSDAVFTDNYTRLRKQMAAKKYLSIKNR	0.8 ± 0.4	80 ± 50	100
R3P179	HSDAVFTDNYTRLRKQMAAKKYLSIKNR	1.5 ± 0.9	230 ± 80	150
R3P180	HSDAVFTDNYTRLRKQMAAKKYLSIPNR	7 ± 3	>100	>15
R3P181	HSDAVFTDNYTRLRKQMAAKKYLSIPNR	5 ± 1	300 ± 30	60
R3P182	HSDAVFTDNYTRLRKQMAAKKYLSIPNR	3 ± 0.6	300 ± 130	100
R3P183	HSDAVFTDNYTRLRKQMAAKKYLSIPNR	5 ± 0.4	>150	>30
R3P184	HSDAVFTDNYTRLRKQMAAKKYLSIQNR	0.37 ± 0.1	10 ± 1	30
R3P185	HSDAVFTDNYTRLRKQMAAKKYLSIQNR	4.5 ± 1.3	200 ± 20	40
R3P186	HSDAVFTDNYTRLRKQMAAKKYLSIQNR	1.6 ± 0.2	70 ± 10	40
R3P187	HSDAVFTDNYTRLRKQMAAKKYLSIQNR	1.6 ± 0.1	>130	>80
R3P188	HSDAVFTDNYTRLRKQMAAKKYLSIQNR	2.2 ± 0.1	150 ± 50	70
R3P189	HSDAVFTDNYTRLRKQMAAKKYLSIRNR	0.04 ± 0.01	1.3 ± 0.6	30
R3P190	HSDAVFTDNYTRLRKQMAAKKYLSIRNR	2 ± 0.6	200 ± 100	100
R3P191	HSDAVFTDNYTRLRKQMAAKKYLSIRNR	3 ± 0.4	>200	>70
R3P192	HSDAVFTDNYTRLRKQMAAKKYLSIRNR	0.6 ± 0.1	40 ± 20	70
R3P193	HSDAVFTDNYTRLRKQMAAKKYLSIRNR	2 ± 0.1	200 ± 90	100

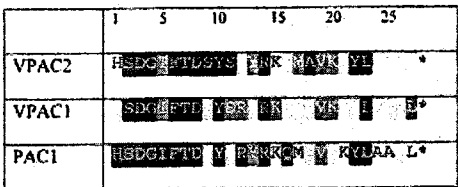


FIG. 5. Effect of introducing positively charged amino acids into neutral positions or reversal of positive charges on PACAP27 binding of VPAC2, VPAC1, and PAC1. The reduction in receptor affinity is measured as the fold increase in IC₅₀ as defined by the IC₅₀ ratio of the mutant peptide versus PACAP27, with <10-fold increase in IC₅₀ at the particular position unshaded; 10 to <100-fold, lightly shaded; 100 to <1000-fold, darkly shaded; and ≥1000-fold, completely blacked out.

1553, Nle-17, Ala-19, and Asp-25 are also found in the VPAC1 selective agonist [Lys-15, Arg-16, Leu-27]VIP-(1-7)/GRF-(8-27). Because these three mutations do not prevent the VPAC1 agonist from binding tightly to VPAC1, they are probably not involved in VPAC2 selectivity. The minor modification of Y10 with a methyl group found in Ro 25-1392 is most likely not important for VPAC2 selectivity because Ro 25-1553 does not have this modification. Thus, Lys-27 and Lys-28, the remaining two mutations found in both VPAC2 selective analogs, are the most probable candidates to contribute to VPAC2 selectivity.

To understand the structure-activity relationship of VPAC2 selectivity of Ro 25-1553 and to generate novel VPAC2 selective agonists with the minimal number of mutations that can be produced in recombination, we performed several rounds of site-directed mutagenesis. All four chemical modifications found in Ro 25-1553 that would prevent recombinant expression were determined to be unnecessary for achieving high VPAC2 selectivity. A highly efficient recombinant expression system was then developed to allow a practical and rapid screen of hundreds of mutant peptides. Systematic amino acid replacement of Ro 25-1553 back to the VIP sequence revealed

that only three out of the eleven mutations were needed to provide comparable VPAC2 selectivity as exhibited by Ro 25-1553. Two of the three mutations, L27K and N28K, were predicted by the sequence alignment analysis (see Fig. 1). Combining the L27K and N28K mutations with PACAP38 residues 29 and 30 greatly improved selectivity. Saturation and combinatorial mutagenesis of the resulting VIP mutant peptide containing the four key mutations, V19A, L27K, 29K, and 30R, yielded dozens of additional highly selective VPAC2 agonists. Scanning mutagenesis of PACAP27 identified critical binding and activation determinants for each PACAP receptor and resulted in additional VPAC2 selective mutant peptides.

Previous structure-activity relationship studies of the secretin/glucagon/VIP family have been limited to synthetic analogs as it has been difficult to generate fully functional peptides by recombinant means (16, 17). PACAP and VIP have been especially difficult to express because of the high number of basic amino acids, including two dibasic sites that may be particularly sensitive to proteolysis during expression. We have successfully expressed in *E. coli* PACAP and VIP mutant peptides using a fusion GST-Factor Xa recognition site-peptide construct, which resulted in dramatically lower peptide degradation than direct expression of free PACAP and VIP (data not shown), presumably because of protection provided by the globular GST protein. The GST tag also permitted a one-step purification of the fusion protein and Factor Xa processing resulted in the release of free peptide without any extraneous sequence. The efficiency and ease of use of this expression and purification strategy permitted the generation of over 200 mutant peptides. Production and analysis of the large number of VIP and PACAP mutant peptides resulted in the identification of critical positions for conferring VPAC2 agonist selectivity as well as selection of specific amino acids within these positions that gave maximal selectivity.

A recent limited analysis of VPAC2 binding selectivity of Ro 25-1553 with several analogs of VIP and Ro 25-1553 (29) was

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.

Abogado
Emilio Ferrero

ASUNTO Radicación 06-78328
 Trámite 011
 Evento 379
 Actuación 519
 Oficio
 Folios 003

E 0 4455

Patente de Invención ☒ Patente de Modelo de Utilidad ☐

Vía Nacional ☐

Vía PCT (fase nacional) ☒ Cap. I ☐ Cap. II ☒

No. Sol. Internacional PCT/US2005/002609
Pub. Internacional WO 2005/072385

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

<u>Descripción:</u>	Folios: 131 a 174	09/08/2006
<u>Reivindicaciones:</u>	Folios: 175 a 180	09/08/2006
<u>Figuras:</u>	Folios: 182 a 198	09/08/2006
<u>Secuencias:</u>	Folios: 199 a 248	09/08/2006
<u>Prioridad:</u>	US 60/539,550	27/01/2004
	US 60/566,499	29/04/2004

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de las prioridades reivindicadas.

3. Objeto de la invención

Título de la solicitud: **"Agonistas del receptor (VPAC2) del péptido activador de la adenilato ciclasa hipofisaria (PACAP) y sus métodos de uso farmacológico"**.

El objeto de la presente solicitud es suministrar un polipéptido útil para la liberación de insulina de las células β -pancreáticas en una forma dependiente de la glucosa.
El problema planteado en esta solicitud es la necesidad de suministrar un medicamento alternativo de tipo secretagogo para la diabetes tipo 2, que aumente la sensibilidad de los tejidos hacia la insulina y que no genere resistencia en el paciente.

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7. Unidad de invención (Art 25 D486)

La solicitud carece de unidad de invención por cuanto los 148 péptidos reclamados en la reivindicación 1 carecen de concepto común inventivo. Lo anterior teniendo en cuenta que la estructura común de dichos péptidos y su actividad ya era conocida en el estado del arte, ver por ejemplo WO 2004/006839, Yung S.,*et.al* (2003), EP 0536741.

Tal como manifestó la autoridad internacional, la presente solicitud tiene 149 grupos de invención, definidos de la siguiente forma:

Grupo 1. Polipéptido de SEQ N°1, fragmentos y derivados equivalentes, composición que lo contiene, anticuerpo que se une, métodos de tratamiento y uso (reivindicaciones 1-5, 9-53 parcialmente). Anticipado por WO 2004/006839.

Grupo 2 a 148. Polipéptido de SEQ N°2 a SEQ N°148 fragmentos y derivados equivalentes, anticuerpo que se une, composición que lo contiene, métodos de tratamiento y uso (reivindicaciones 1-5, 9-53 parcialmente). Algunos grupos anticipados por Yung S,*et.al* (2003).

Grupo 149. Anticuerpo que se une a polietilenglicol (reivindicación 6)

De esta manera, se recomienda al solicitante realizar las divisiones que considere necesarias a su solicitud o restringir la misma a una única invención, teniendo en cuenta la materia sustentada en la descripción e indicar la opción de su preferencia.

8. Determinación del Estado de la Técnica

Para determinar el estado de la técnica se ha tenido en cuenta la fecha de presentación de la solicitud prioritaria reivindicada con la solicitud: 27/01/2004.

Teniendo en cuenta la fecha indicada se realizó búsqueda en el estado de la técnica de documentos relacionados con la solicitud, encontrándose los siguientes documentos relevantes a la misma:

Nº	Documento	Título	Fecha de prioridad
D1	WO 2004/006839	"Pituitary adenylate cyclase activating peptide (PACAP) receptor (VPAC2) agonists and their pharmacological methods of use"	22/01/2004
D2	Yung S., et.al. The journal of biological chemistry, vol. 278, No. 12, pp. 10273-10281.	"Generation of Highly Selective VPAC2 Receptor Agonists by High Throughput Mutagenesis of Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-activating Peptide"	March 21, 2003
D3	EP 0536741	"Cyclic VIP analogs"	14/04/1993
D4	WO 01/23420	"Pituitary adenylate cyclase activating peptide (PACAP) receptor 3 (R3) agonists and their pharmacological methods of use"	05/04/2001

Resumen de los documentos citados:

D1: La invención divulga nuevos péptidos que funcionan in-vivo como agonistas del receptor VPAC2 (resumen).

<http://www.wipo.int/patentscope/search/en/search.jsf> ingresar WO2004006839.

D2: Se divulga un grupo de agonistas selectivos de VPAC2 para el tratamiento de diabetes tipo 2, estudios de relación estructura-actividad fueron realizados en PACAP, VIP y VPAC2 (resumen, folio 270).

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Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D2	D3	D4
polipéptido	Polipéptidos agonistas del receptor VPAC2	Polipéptidos VIP	Polipéptidos agonistas del receptor VPAC2
SEQ N° 1 a 148 y los fragmentos, derivados y variantes de este funcionalmente equivalentes	PACAP27 , PACAP38 y muchos otros (fig1, tabla 1-4)	SEQ N°68 (similar a la SEQ N°5, 116 solicitud)	SEQ N° R3P66, R3P71, R3P66, R3P29, R3P21 (similar a las SEQ N°1,2,4,5, 112, 113, 115, 116 de la solicitud)

En el estado de la técnica se conocen varias alternativas de polipéptidos con actividad agonista al receptor VPAC2, en todas estas anterioridades se divulgan múltiples opciones de polipéptidos:

- El documento de Yung (D2) divulga polipéptidos agonistas del receptor VPAC2 mostrando diversas alternativas para los mismos, e identificando las sustituciones aminoácidas útiles para modificar su actividad (tablas 1-4);
- El documento EP0536741 (D3), divulga polipéptidos VIP de los cuales se indica su actividad, su uso para el tratamiento de diversas enfermedades incluida asma, composiciones farmacéuticas que contienen dichos péptidos (resumen; pág 29 línea 41). Este documento divulga ente otras las secuencia N°68 que tiene una alta similitud con los péptidos de las secuencias N° 5 y 116 de la solicitud;
- El documento WO 01/23420 (D4), divulga péptidos con función in-vivo para estimular la liberación de insulina de las células beta pancreáticas. Dichos polipéptidos son empleados para el tratamiento de pacientes con diabetes tipo 2 y son seleccionados de polipéptidos relacionados al grupo VIP/PACAP (resumen). También se divulgan composiciones que contienen dichos polipéptidos (pág 17), kits, anticuerpos para detectarlos (pág 5 y 34) y métodos de tratamiento para diversas enfermedades relacionadas (pág 16-17). Particularmente, D4 divulga polipéptidos de alta similitud a los polipéptidos de la solicitud de SEQ ID N° 1,2,4,5,112,113,115,116.

La diferencia entre D2-D4 y la materia reclamada en la solicitud en estudio, es que algunas de las secuencias de polipéptidos reclamadas en la solicitud son diferentes a las divulgadas en cualquiera de estos documentos.

El efecto de este cambio no resulta aparente. Por tanto, a la luz de D2, D3 ó D4 el problema técnico objetivo resuelto por la solicitud es suministrar polipéptidos alternativos agonistas del receptor VPAC2.

Sin embargo, las modificaciones que dan lugar a los polipéptidos reclamados en la solicitud son producto de sustituciones de 1 a 4 aminoácidos del péptido original divulgado y dichas modificaciones en esas posiciones de la secuencia ya habían sido previstas por estos documentos. Por ejemplo en D2 se divulgan varias posiciones con diferentes opciones de sustitución (ver pág 10277).

De esta manera, a partir de las divulgaciones realizadas en D2, D3 ó D4 la persona del oficio normalmente versada en la materia hubiera podido desarrollar la invención, por lo que la misma carece de nivel inventivo e incumple el artículo 18 de la D486.