



No. 06-128571-00003-0000

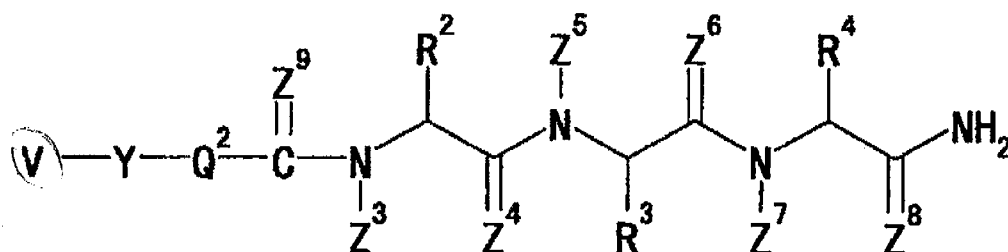
Fecha: 2008-12-01 16:43:12 Dep. 2020 NUECREACION
Ira. 11 PATENTE/II Lvs: 378 FASENACIONAL
Act. 329 CTOINFORMACION Fojos: 38**FORMULARIO UNICO DE CORRECCIONES Y MODIFICACIONES**

1	SOLICITUD DE: ☐ Corrección de Error Material ☒ Modificación a una solicitud	
2 SOLICITANTE	Nombre: TAKEDA PHARMACEUTICAL COMPANY LIMITED. Sociedad constituida y existente bajo las leyes de Japón. Dirección: 1-1, Doshomachi 4-chome, Chuo-ku, Osaka-shi, 541045 Osaka, Japón Domicilio: Osaka-shi, Osaka, Japón Teléfono: 546 09 70 Fax: 249 40 70 E-mail: clientes@camyp.com	IDENTIFICACION C.C. ☐ NIT ☐ C.E. ☐ Otro ☐ Número: _____
3 REPRESENTANTE O APODERADO	Nombre: Emilio FERRERO Dirección: Carrera 4ª No. 72-35 Teléfono: 347 36 11 Fax: 217 92 11 E-mail: cavelier@cavelier.com	IDENTIFICACION C.C. ☒ NIT ☐ C.E. ☐ Otro ☐ Número: 438.019 TP 31.929
4	Número de expediente: 06.128.571	
5	Descripción de la corrección o modificación solicitada: Presento nuevo capítulo reivindicatorio compuesto por 78 reivindicaciones y pido respetuosamente a Su Despacho que las tenga en cuenta al momento de realizar el examen de patentabilidad de la presente solicitud de Patente.	
6	Comprobante de pago No. 08-91.607 Fecha: 25 de noviembre de 2008	
7	Anexos ☒ Comprobante de pago de la tasa por \$103.000, por concepto de modificaciones. ☐ Poderes, si fuere el caso ☐ Documento que acredita la existencia y representación legal de las partes, cuando sean personas jurídicas.	
8	NOMBRE: EMILIO FERRERO	FIRMA:  C.C. 438.019 de Usaquén T.P.A. 31.929

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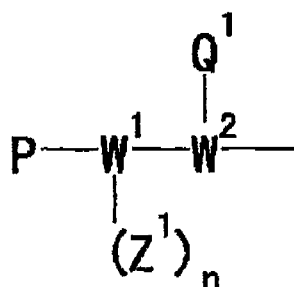
REIVINDICACIONES

1. Un derivado de metastina (II) caracterizado por que está representado por la fórmula:

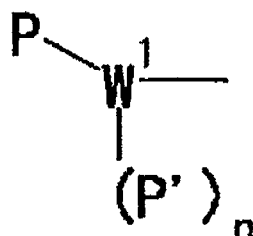


[en donde;

V representa un grupo representado por la fórmula:



o un grupo representado por la fórmula:



n representa 0 ó 1;

W¹ representa N, CH ó O (con la condición que cuando W¹ es N ó CH, n representa 1 y cuando W¹ es O, n representa 0);

W² representa N ó CH;

Z¹, Z³, Z⁵ y Z⁷ cada uno representa un átomo de hidrógeno o un grupo alquilo C₁₋₃;

Z⁴, Z⁶ y Z⁸ cada uno representa átomo de hidrógeno, O ó S;

R² representa (1) átomo de hidrógeno ó (2) un grupo alquilo C₁₋₁₀ cíclico o lineal, o (3) un grupo alquilo C₁₋₁₀ que

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consiste de un grupo alquilo cíclico y un grupo de alquilo lineal, o (4) un grupo alquilo C_{1-8} opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de un grupo carbamoilo opcionalmente sustituido, un grupo hidroxilo opcionalmente sustituido y un grupo cíclico aromático opcionalmente sustituido;

R^3 representa (1) un grupo alquilo C_{1-8} que posee un grupo básico opcionalmente sustituido y opcionalmente posee un sustituyente adicional, (2) un grupo aralquilo que posee un grupo básico opcionalmente sustituido y opcionalmente posee un sustituyente adicional, (3) un grupo alquilo C_{1-4} que tiene un grupo hidrocarburo cíclico no aromático de cantidad de átomos de carbono no mayor que 7 el cual tiene un grupo básico opcionalmente sustituido, y opcionalmente posee un sustituyente adicional, o (4) un grupo alquilo C_{1-4} que tiene un grupo heterocíclico no aromático de cantidad de átomos de carbono no mayor que 7 el cual tiene un grupo básico opcionalmente sustituido, y opcionalmente posee un sustituyente adicional;

R^4 representa un grupo alquilo C_{1-4} , el cual puede ser opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de (1) un grupo hidrocarburo aromático C_{6-12} opcionalmente sustituido, (2) un grupo heterocíclico aromático opcionalmente sustituido de 5 a 14 miembros que consiste de 1 a 7 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (3) un grupo cíclico fusionado aromático C_{8-14} opcionalmente sustituido, (4) un grupo heterocíclico

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fusionado aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 3 a 11 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (5) un grupo hidrocarburo cíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7, y (6) un grupo heterocíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7;

Q^1 representa un grupo alquilo C_{1-4} que puede ser opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de (1) un grupo hidrocarburo aromático C_{6-12} opcionalmente sustituido, (2) un grupo heterocíclico aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 1 a 7 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (3) un grupo cíclico fusionado aromático C_{8-14} opcionalmente sustituido, (4) un grupo heterocíclico fusionado aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 3 a 11 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (5) un grupo hidrocarburo cíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7, y (6) un grupo heterocíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7;

Q^2 representa (1) CH_2 que puede ser opcionalmente sustituido con un grupo alquilo C_{1-4} opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de

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grupo carbamoilo y grupo hidroxilo, (2) NH, el cual puede ser opcionalmente sustituido con un grupo alquilo C₁₋₄ opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de grupo carbamoilo y grupo hidroxilo, o (3) O;

Y representa un grupo representado por la fórmula:
-CONH-, -CSNH-, -CH₂NH-, -NHCO-, -CH₂O-, -CH₂S-, -COO-, -CSO-
ó -CH₂CH₂- que puede ser opcionalmente sustituido con un grupo alquilo C₁₋₆; y,

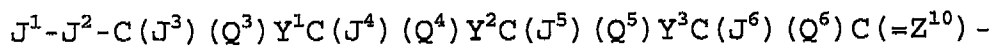
Z⁹ representa átomo de hidrógeno, O ó S; y,

P y P', los cuales pueden ser iguales o diferentes, cada uno puede formar un anillo combinando P y P' ó P y Q¹ juntos representan:

(1) átomo de hidrógeno;

(2) un residuo de aminoácido opcional limitado de manera continua o discontinua del C terminal de la secuencia de aminoácido 1-48 en la secuencia de aminoácido representada por SEQ ID NO: 1;

(3) un grupo representado por la fórmula:



(en donde:

J¹ representa (a) átomo de hidrógeno o (b) (i) un grupo acilo C₁₋₁₅, (ii) un grupo alquilo C₁₋₁₅, (iii) un grupo arilo C₆₋₁₄, (iv) grupo carbamoilo, (v) grupo carboxilo, (vi) grupo sulfino, (vii) grupo amidino, (viii) grupo glioxiloilo o (ix) grupo amino, cuos grupos pueden ser opcionalmente sustituidos con un sustituyente que contiene de un grupo cíclico opcionalmente sustituido;

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J² representa (1) NH opcionalmente sustituido con un grupo alquilo C₁₋₆, (2) CH₂ opcionalmente sustituido con un grupo alquilo C₁₋₆, (3) O ó (4) S;

J³ a J⁶ representa átomo de hidrógeno o un grupo alquilo C₁₋₃;

Q³ a Q⁶ representa un grupo alquilo C₁₋₄ el cual puede tener un sustituyente opcionalmente seleccionado del grupo que consiste de:

(1) un grupo hidrocarburo aromático C₆₋₁₂ opcionalmente sustituido,

(2) un grupo heterocíclico aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 1 a 7 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre,

(3) un grupo cíclico fusionado aromático C₈₋₁₄ opcionalmente sustituido,

(4) un grupo heterocíclico fusionado aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 3 a 11 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre,

(5) un grupo hidrocarburo cíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7,

(6) un grupo heterocíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7,

(7) un grupo amino opcionalmente sustituido,

(8) un grupo guanidino opcionalmente sustituido,

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- (9) un grupo hidroxilo opcionalmente sustituido,
 - (10) un grupo carboxilo opcionalmente sustituidos,
 - (11) un grupo carbamoilo opcionalmente sustituido, y
 - (12) un grupo sulfhidrilo opcionalmente sustituido,
- o átomo de hidrógeno.;

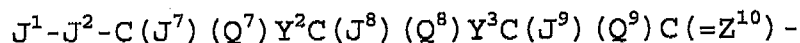
Pueden combinarse entre sí J^3 y Q^3 , J^4 y Q^4 , J^5 y Q^5 ó J^6 y Q^6 juntos, o, pueden combinarse entre sí J^2 y Q^3 , Y^1 y Q^4 , Y^2 y Q^5 , ó Y^3 y Q^6 , para formar un anillo;

Y^1 a Y^3 representa un grupo representado por la fórmula:

$-\text{CON}(J^{13})-$, $-\text{CSN}(J^{13})-$, $-\text{C}(J^{14})\text{N}(J^{13})-$ ó $-\text{N}(J^{13})\text{CO}-$ (en donde J^{13} y J^{14} cada uno representa átomo de hidrógeno o un grupo alquilo C_{1-3}); y,

Z^{10} representa átomo de hidrógeno, O ó S);

(4) un grupo representado por la fórmula:



(en donde:

J^1 y J^2 , cada uno posee el mismo significado según lo descripto anteriormente;

J^7 a J^9 poseen el mismo significado que para J^3 ;

Q^7 a Q^9 poseen el mismo significado que para Q^3 ;

Y^2 y Y^3 cada uno posee el mismo significado según lo descripto anteriormente;

Z^{10} posee el mismo significado según lo descripto anteriormente;

Pueden combinarse entre sí J^7 y Q^7 , J^8 y Q^8 ó J^9 y Q^9 , ó, pueden combinarse entre sí J^2 y Q^7 , Y^2 y Q^8 o Y^3 y Q^9 , para formar un anillo);

(5) un grupo representado por la fórmula:

$$J^1 - J^2 - C(J^{10}) (Q^{10}) Y^3 C(J^{11}) (Q^{11}) C(=Z^{10}) -$$

(en donde:

J^1 y J^2 poseen el mismo significado según lo descripto anteriormente representa;

J^{10} y J^{11} poseen el mismo significado que para J^3 ;

Q^{10} y Q^{11} poseen el mismo significado que para Q^3 ;

Y^3 posee el mismo significado según lo descripto anteriormente;

Z^{10} posee el mismo significado según lo descripto anteriormente; y

Pueden combinarse entre sí J^{10} y Q^{10} ó J^{11} y Q^{11} , o pueden combinarse entre sí J^2 y Q^{10} ó Y^3 y Q^{11} , para formar un anillo);

(6) un grupo representado por la fórmula:

$$J^1 - J^2 - C(J^{12}) (Q^{12}) C(=Z^{10}) -$$

(en donde;

J^1 y J^2 poseen el mismo significado según lo descripto anteriormente;

J^{12} posee el mismo significado de J^3 ;

Q^{12} posee el mismo significado de Q^3 ;

Z^{10} posee el mismo significado según lo descripto anteriormente; y,

Pueden combinarse entre sí J^{12} y Q^{12} , o pueden combinarse entre sí J^2 y Q^{12} , para formar un anillo); ó,

(7) un grupo representado por la fórmula:.

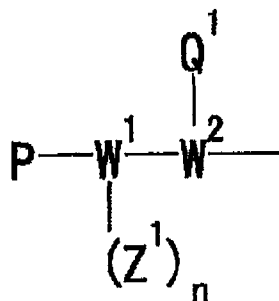
$$J^1 -$$

(en donde:

J^1 posee el mismo significado según lo descripto

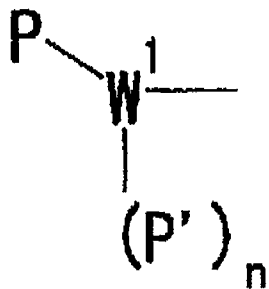
anteriormente)] (con la condición que un péptido que consiste en la secuencia de aminoácido de 1-54, 2-54, 3-54, 4-54, 5-54, 6-54, 7-54, 8-54, 9-54, 10-54, 11-54, 12-54, 13-54, 14-54, 15-54, 16-54, 17-54, 18-54, 19-54, 20-54, 21-54, 22-54, 23-54, 24-54, 25-54, 26-54, 27-54, 28-54, 29-54, 30-54, 31-54, 32-54, 33-54, 34-54, 35-54, 36-54, 37-54, 38-54, 39-54, 40-54, 41-54, 42-54, 43-54, 44-54, 45-54, 46-54, 47-54, 48-54 ó 49-54 en la secuencia de aminoácido representada por SEQ ID NO.: 1 sea excluida), o una sal del mismo.

2. Un derivado de metastina (II) de acuerdo con la reivindicación 1, caracterizado por que V es un grupo representado por la fórmula:



(en donde cada símbolo posee el mismo significado como el definido en reivindicación 1), o una sal del mismo.

3. Un derivado de metastina (II) de acuerdo con la reivindicación 1, caracterizado por que V es un grupo representado por la fórmula:



(en donde cada símbolo posee el mismo significado como el definido en la reivindicación 1), o una sal del mismo.

4. Una prodroga caracterizada por que es un derivado

de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo.

5. Un compuesto farmacéutico caracterizado por que comprende el derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

6. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para suprimir la metástasis del cáncer o un agente para suprimir el crecimiento del cáncer.

7. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para prevenir o tratar el cáncer.

8. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para controlar la función placentaria.

9. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para prevenir o tratar coriocarcinoma, mola hidatídica, mola invasiva, aborto, hipoplasia fetal, metabolismo de glucosa anormal, metabolismo de lípido anormal o inducción al parto.

10. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para mejorar la función gonadal.

11. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para prevenir o tratar el cáncer dependiente de hormona, infertilidad, endometriosis, pubertad precoz o mioma de

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útero.

12. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para inducir o estimular la ovulación.

13. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente secretagogo de hormona gonadotrópica o un agente secretagogo de hormona sexual.

*14. Un compuesto farmacéutico de acuerdo con la reivindicación 5, caracterizado por que es un agente para prevenir o tratar la enfermedad de Alzheimer o deterioro cognoscitivo moderado.

*15. Un método para suprimir metástasis de cáncer o crecimiento de cáncer, caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

*16. Un método para prevenir o tratar cáncer caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

*17. Un método para controlar la función placentar caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

18. Un método para prevenir o tratar coriocarcinoma,

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mola hidatídica, mol invasiva, aborto, hipoplasia fetal, metabolismo de glucosa anormal, metabolismo de lípidos anormal o inducción al parto, caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

19. Un método para mejorar la función gonadal caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

20. Un método para prevenir o tratar cáncer dependiente de hormona, infertilidad, endometriosis, pubertad precoz o mioma de útero, caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

21. Un método para inducir o estimular la ovulación, caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

22. Un método para promover la secreción de hormona gonadotrópica o promover la secreción de hormona sexual caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

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23. Un método para prevenir o tratar la enfermedad de Alzheimer o el deterioro cognoscitivo moderado caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo.

24. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente para suprimir la metástasis del cáncer o un agente para suprimir el crecimiento del cáncer.

25. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente para prevenir o tratar el cáncer.

26. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente para controlar la función placentar.

27. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente para prevenir o tratar coriocarcinoma, mola hidatídica, mola invasiva, aborto, hipoplasia fetal, metabolismo de glucosa anormal, metabolismo de lípido anormal o inducción al parto.

28. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una

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prodroga del mismo caracterizada por que es para fabricar un agente para mejorar la función gonadal.

29. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente para prevenir o tratar el cáncer dependiente de hormona, infertilidad, endometriosis, pubertad precoz o mioma de útero.

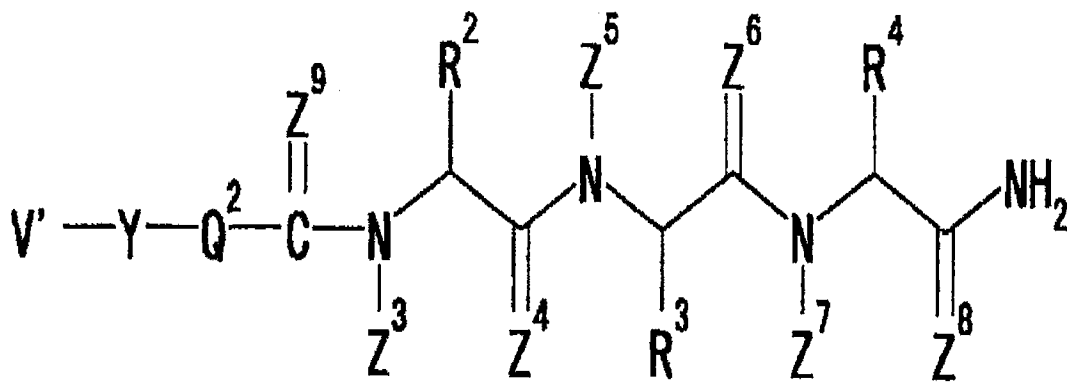
30. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente para inducir o estimular la ovulación.

* 31. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente secretagogo de hormona gonadotrópica o un agente secretagogo de hormona sexual.

* 32. Una utilización del derivado de metastina (II) de acuerdo con la reivindicación 1 o una sal del mismo, o una prodroga del mismo caracterizada por que es para fabricar un agente para prevenir o tratar la enfermedad de Alzheimer o el deterioro cognoscitivo moderado.

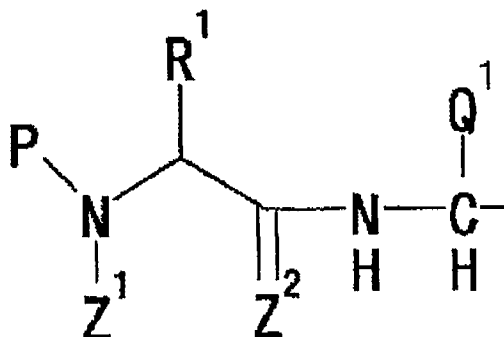
33. Un agente para suprimir la secreción de hormona gonadotrópica o un agente para suprimir la secreción de hormona sexual caracterizado por que comprende el derivado de metastina (III) representado por la fórmula:

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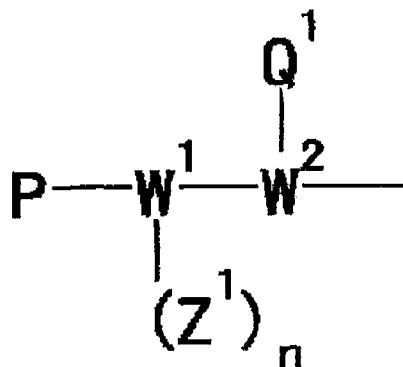
[en donde:

V' representa un grupo representado por la fórmula:

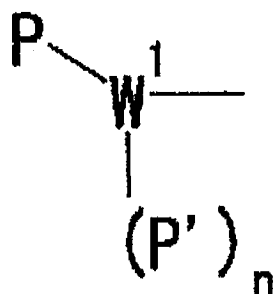


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Sociedad

un grupo representado por la fórmula:



o un grupo representado por la fórmula:



n representa 0 ó 1;

W¹ representa N, CH ó O (con la condición que W¹ es N ó CH, n representa 1, y cuando W¹ es O, n representa 0);

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W^2 representa N ó CH;

Z^1 , Z^3 , Z^5 y Z^7 cada uno representa átomo de hidrógeno o un grupo alquilo C_{1-3} ;

Z^2 , Z^4 , Z^6 y Z^8 cada uno representa átomo de hidrógeno, O ó S;.

R^1 representa (1) átomo de hidrógeno, (2) un grupo alquilo C_{1-8} opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de un grupo carbamoilo opcionalmente sustituido, un grupo hidroxilo opcionalmente sustituido y un grupo cíclico aromático opcionalmente sustituido, (3) un grupo alquilo C_{1-10} lineal o cíclico o (4) un grupo alquilo C_{1-10} que consiste de un grupo alquilo cíclico y un grupo alquilo lineal, o (5) un grupo cíclico aromático opcionalmente sustituido;

R^2 representa (1) átomo de hidrógeno o (2) un grupo alquilo C_{1-10} lineal o cíclico o (3) un grupo alquilo C_{1-10} que consiste en un grupo alquilo cíclico y un grupo alquilo lineal, o (4) un grupo alquilo C_{1-8} opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de un grupo carbamoilo opcionalmente sustituido, un grupo hidroxilo opcionalmente sustituido y un grupo cíclico aromático opcionalmente sustituido;

R^3 representa (1) un grupo alquilo C_{1-8} que posee un grupo básico opcionalmente sustituido y posee opcionalmente un sustituyente adicional, (2) un grupo aralquilo que posee un grupo básico opcionalmente sustituido y posee opcionalmente un sustituyente adicional, (3) un grupo alquilo C_{1-4} que no tiene un grupo hidrocarburo cíclico no aromático

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de una cantidad de átomos de carbono no mayor que 7 el cual tiene un grupo básico opcionalmente sustituido, y opcionalmente posee un sustituyente adicional, o (4) un grupo alquilo C_{1-4} que tiene un grupo heterocíclico no aromático de una cantidad de átomos de carbono no mayor que 7 el cual tiene un grupo básico opcionalmente sustituido, y opcionalmente posee un sustituyente adicional;

R^4 representa un grupo alquilo C_{1-4} que puede ser opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de (1) un grupo hidrocarburo aromático C_{6-12} opcionalmente sustituido, (2) un grupo heterocíclico aromático opcionalmente sustituido de 5 a 14 miembros que consiste de 1 a 7 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (3) un grupo cíclico fusionado aromático C_{8-14} opcionalmente sustituido, (4) un grupo heterocíclico fusionado aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 3 a 11 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (5) un grupo hidrocarburo cíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7, y (6) un grupo heterocíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7;.

Q^1 representa un grupo alquilo C_{1-4} que puede ser opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de (1) un grupo hidrocarburo aromático C_{6-12} opcionalmente sustituido, (2) un grupo heterocíclico

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aromático opcionalmente sustituido de 5 a 14 miembros que consiste de 1 a 7 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (3) un grupo cíclico fusionado aromático C₈₋₁₄ opcionalmente sustituido, (4) un grupo heterocíclico fusionado aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 3 a 11 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre, (5) un grupo hidrocarburo cíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7, y (6) un grupo eterocíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7;

Q² representa (1) CH₂ que puede ser opcionalmente sustituido con un grupo alquilo C₁₋₄ opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de grupo carbamoilo y grupo hidroxilo, (2) NH que puede ser opcionalmente sustituido con un grupo alquilo C₁₋₄ opcionalmente sustituido con un sustituyente seleccionado del grupo que consiste de grupo carbamoilo y grupo hidroxilo, o (3) O;

Y representa un grupo representado por la fórmula:

-CONH-, -CSNH-, -CH₂NH-, -NHCO-, -CH₂O-, -CH₂S-, -COO-, -CSO-
ó -CH₂CH₂- que puede ser opcionalmente sustituido con un grupo alquilo C₁₋₆; y,

Z⁹ representa átomo de hidrógeno, O ó S; y,

P y P', los cuales pueden ser iguales o diferentes, cada uno puede formar un anillo por combinación entre sí de P y P'

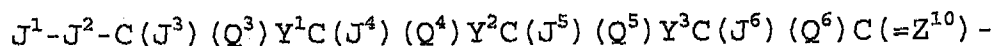
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ó P y Q¹ y representa:

(1) átomo de hidrógeno;

(2) un residuo de aminoácido opcional continua o discontinuamente limitado del C terminal de la secuencia de aminoácido 1-48 en la secuencia de aminoácido representada por SEQ ID NO: 1;

(3) un grupo representado por la fórmula:



(en donde:

J¹ representa (a) átomo de hidrógeno o (b) (i) un grupo acilo C₁₋₁₅, (ii) un grupo alquilo C₁₋₁₅, (iii) un grupo arilo C₆₋₁₄, (iv) grupo carbamoilo, (v) grupo carboxilo, (vi) grupo sulfino, (vii) grupo amidino, (viii) grupo glioxiloilo ó (ix) grupo amino, tales grupos pueden ser opcionalmente sustituidos con un sustituyente que contiene un grupo cíclico opcionalmente sustituido;

J² representa (1) NH opcionalmente sustituido con un grupo alquilo C₁₋₆, (2) CH₂ opcionalmente sustituido con un grupo alquilo C₁₋₆, (3) O ó (4) S;

J³ a J⁶ cada uno representa átomo de hidrógeno o un grupo alquilo C₁₋₃;

Q³ a Q⁶ cada uno representa un grupo alquilo C₁₋₄ que puede tener un sustituyente opcionalmente seleccionado del grupo que consiste de:

(1) un grupo hidrocarburo aromático C₆₋₁₂ opcionalmente sustituido,

(2) un grupo heterocíclico aromático opcionalmente sustituido de 5 a 14 miembros que consiste de 1 a 7 átomos de

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carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre,

(3) un grupo cíclico fusionado aromático C₈₋₁₄ opcionalmente sustituido,

(4) un grupo heterocíclico fusionado aromático de 5 a 14 miembros opcionalmente sustituido que consiste de 3 a 11 átomos de carbono y heteroátomos seleccionados del grupo que consiste de átomos de nitrógeno, oxígeno y azufre,

(5) un grupo hidrocarburo cíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7,

(6) un grupo heterocíclico no aromático opcionalmente sustituido que posee una cantidad de átomos de carbono no mayor que 7,

(7) un grupo amino opcionalmente sustituido,

(8) un grupo guanidino opcionalmente sustituido,

(9) un grupo hidroxilo opcionalmente sustituido,

(10) un grupo carboxilo opcionalmente sustituido,

(11) un grupo carbamoilo opcionalmente sustituido, y

(12) un grupo sulfhidrilo opcionalmente sustituido,

o átomo de hidrógeno;

Pueden combinarse entre sí J³ y Q³, J⁴ y Q⁴, J⁵ y Q⁵ ó J⁶ y Q⁶, o, pueden combinarse entre sí Z¹ y R¹, J² y Q³, Y¹ y Q⁴, Y² y Q⁵, o Y³ y Q⁶, para formar un anillo;

Y¹ a Y³ cada uno representa un grupo representado por la fórmula:

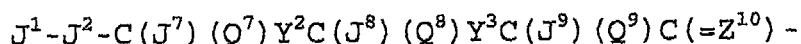
-CON(J¹³)-, -CSN(J¹³)-, -C(J¹⁴)N(J¹³)- ó -N(J¹³)CO- (en

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donde J^{13} y J^{14} cada uno representa átomo de hidrógeno o un grupo alquilo C_{1-3} ; y,

Z^{10} representa átomo de hidrógeno, O ó S);

(4) un grupo representado por la fórmula:



(en donde:

J^1 y J^2 , cada uno posee el mismo significado según lo descripto anteriormente;

J^7 a J^9 poseen el mismo significado que para J^3 ;

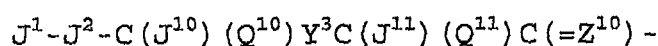
Q^7 a Q^9 poseen el mismo significado que para Q^3 ;

Y^2 y Y^3 cada uno posee el mismo significado según lo descripto anteriormente;

Z^{10} posee el mismo significado según lo descripto anteriormente;

Pueden combinarse entre sí J^7 y Q^7 , J^8 y Q^8 ó J^9 y Q^9 , o, pueden combinarse entre sí J^2 y Q^7 , Y^2 y Q^8 ó Y^3 y Q^9 , para formar un anillo);

(5) un grupo representado por la fórmula:



(en donde:

J^1 y J^2 poseen el mismo significado según lo descripto anteriormente representa;

J^{10} y J^{11} poseen el mismo significado que para J^3 ;

Q^{10} y Q^{11} poseen el mismo significado que para Q^3 ;

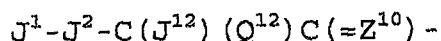
Y^3 posee el mismo significado según lo descripto anteriormente;

Z^{10} posee el mismo significado según lo descripto anteriormente; y,

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Pueden combinarse entre sí J^{10} y Q^{10} ó J^{11} y Q^{11} , o pueden combinarse entre sí J^2 y Q^{10} ó Y^3 y Q^{11} , para formar un anillo);

(6) un grupo representado por la fórmula:



(en donde;

J^1 y J^2 poseen el mismo significado según lo descripto anteriormente;

J^{12} posee el mismo significado de J^3 ;

Q^{12} posee el mismo significado de Q^3 ;

Z^{10} posee el mismo significado según lo descripto anteriormente; y

Pueden combinarse entre sí J^{12} y Q^{12} , o pueden combinarse entre sí J^2 y Q^{12} , para formar un anillo); o,

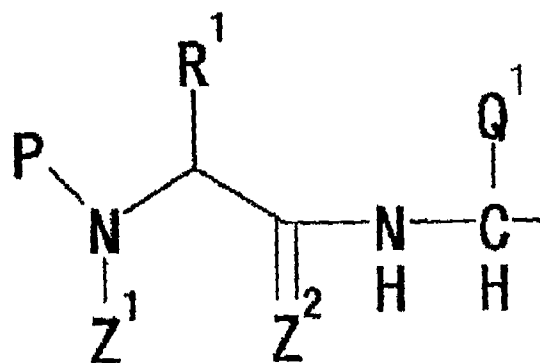
(7) un grupo representado por la fórmula:

J^1- (en donde J^1 posee el significado según lo descripto anteriormente)] o una sal del mismo, o una prodroga del mismo.

34. Un agente de acuerdo con la reivindicación 33, caracterizado por que el derivado de metastina (III) es derivado de metastina (II) de acuerdo con la reivindicación 1.

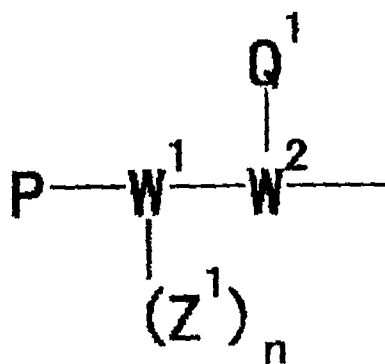
35. Un agente de acuerdo con la reivindicación 33, caracterizado por que V' es un grupo representado por la fórmula:

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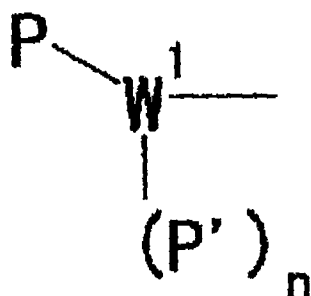
(en donde cada símbolo posee el mismo significado como el definido en reivindicación 33).

36. Un agente de acuerdo con la reivindicación 33, caracterizado por que V' es un grupo representado por la fórmula:



(en donde cada símbolo posee el mismo significado como el definido en reivindicación 33).

37. Un agente de acuerdo con la reivindicación 33, caracterizado por que V' es un grupo representado por la fórmula:



(en donde cada símbolo posee el mismo significado como el definido en reivindicación 33).

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38. Un agente de acuerdo con las reivindicaciones 33 a 37, caracterizado por que es un agente para regular en disminución la hormona gonadotrópica u hormona sexual. US

39. Un agente de acuerdo con las reivindicaciones 33 a 37, caracterizado por que es un agente para regular en disminución la proteína OT7T175 humana (receptor de metastina) que consiste en la secuencia de aminoácido representada por SEQ ID NO: 9. US

40. Un agente de acuerdo con las reivindicaciones 33 a 39, caracterizado por que es un agente para prevenir o tratar el cáncer dependiente de hormona. (Caract por un result) US

41. Un método para suprimir la secreción de hormona gonadotrópica o suprimir la secreción de hormona sexual caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina (III) de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del mismo. US

42. Un método para regular en disminución la hormona gonadotrópica u hormona sexual caracterizado por que comprende administrando a un mamífero una dosis eficaz del derivado de metastina de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del mismo.

43. Un método para regular en disminución la proteína OT7T175 humana (receptor de metastina) que consiste en la secuencia de aminoácido representada por SEQ ID NO: 9, caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del

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mismo.

✓44. Un método para prevenir o tratar cáncer dependiente de hormona caracterizado por que comprende administrar a un mamífero una dosis eficaz del derivado de metastina de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del mismo.

45. Una utilización del derivado de metastina de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del mismo, caracterizada por que es para fabricar un agente para suprimir la secreción de hormona gonadotrópica o un agente para suprimir la secreción de hormona sexual.

46. Una utilización del derivado de metastina de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del mismo, caracterizada por que es para fabricar un agente para regular en disminución la hormona gonadotrópica u hormona sexual.

47. Una utilización del derivado de metastina de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del mismo, caracterizada por que es para fabricar un agente para regular en disminución la proteína OT7T175 humana (receptor de metastina) que consiste en la secuencia de aminoácido representada por SEQ ID NO: 9.

✱48. Una utilización del derivado de metastina de acuerdo con la reivindicación 33 o una sal del mismo, o una prodroga del mismo, caracterizada por que es para fabricar un agente para prevenir o tratar el cáncer dependiente de hormona.

49. Un derivado de metastina representado por la

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fórmula:

XX0-XX2-XX3-XX4-XX5-XX6-AzaGly-XX8-XX9-XX10-NH₂

(en donde:

XX0 representa formilo, alcanilo C₁₋₆,
ciclopropancarbonilo, 6-(acetil-D-arginilamino)caproilo, 6-
((R)-2,3-diaminopropionilamino)caproilo, 6-(D-
norleucilamino)caproilo, 4-(D-arginilamino)butirilo, 3-(4-
hidroxifenil)propionilo, glicilo, tirosilo, acetilglicilo,
acetiltirosilo, D-tirosilo, acetil-D-tirosilo, piroglutamilo,
3-(piridin-3-il)propionilo, adipoilo ó 6-aminocaproilo;

XX2 representa Tyr, D-Tyr, D-Ala, D-Leu, D-Phe, D-Lys D-
Trp ó brazo de enlace;

XX3 representa Trp, Pro, 4-piridilalanina, Tic, D-Trp,
D-Ala, D-Leu, D-Phe, D-Lys, D-Glu D-2-piridilalanina, D-3-
piridilalanina ó D-4-piridilalanina;

XX4 representa Asn, ácido 2-amino-3-ureidopropiónico,
ácido N^β-formildiaminopropiónico ó ácido N^β-
acetildiaminopropiónico;

XX5 representa Ser, Thr ó Val;

XX6 representa Phe, Tyr, Trp, Tyr(Me), Thi, Nal(2), Cha,
4-piridilalanina ó 4-fluorofenilalanina;

AzaGly representa azaglicina;

XX8 representa Leu, Nva ó Val;

XX9 representa Arg, Orn, Arg(Me) ó Arg(symMe₂);

XX10 representa Phe, Trp, 2-naftilalanina, 2-
tienilalanina, tirosina ó 4-fluorofenilalanina), ó una sal
del mismo.

50. Un compuesto caracterizado por que su fórmula es:

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D-Tyr-D-Pya (4) -Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -Phe-NH₂

(Compuesto No. 305),

D-Tyr-D-Pya (4) -Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 385),

D-Tyr-D-Pya (4) -Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 501),

Benzoil-Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No.

509),

D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto

No. 512),

Ac-D-Tyr-D-Pya (4) -Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -Phe-NH₂

(Compuesto No. 516),

D-Tyr-D-Trp-Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto

No. 540),

D-Arg-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 541),

Benzoil-Asn-Ser-Phe (4F) -AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto

No. 545),

D-Tyr-D-Pya (4) -Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -

Pheψ(CSNH)NH₂ (Compuesto No. 548),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 550),

Ac-D-Arg-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-

NH₂ (Compuesto No. 551),

D-Dap-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 552),

D-Nle-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 553),

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D-Arg- γ -Abu-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 555),

Ac-D-Tyr-D-Trp-Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 558),

3-(4-Hidroxifenil)propionil-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 559),

Ac-D-Tyr-D-Pya (4) -Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 562),

Ac-D-Tyr-D-Trp-Asn-Val-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 564),

Ciclopropancarboxil-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 566),

Butiril-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 567),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Phe-NH₂ (Compuesto No. 571),

Ac-D-Tyr-D-Trp-Alb-Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 579),

Ac-D-Tyr-D-Trp-Asn-Ser (Me) -Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 580),

Ac-D-Tyr-D-Trp-Dap (Ac) -Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 584),

Ac-D-Tyr-D-Trp-Dap (For) -Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 585),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Nal (2) -NH₂ (Compuesto No. 589),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Thi-NH₂ (Compuesto No. 590),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Tyr-NH₂

(Compuesto No. 591),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Phe (4F) -NH₂

(Compuesto No. 592),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Orn-Trp-NH₂ (Compuesto No. 599),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg-Trp-NH₂ (Compuesto No. 600),

Ac-D-NMeTyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 602),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (symMe₂) -Trp-NH₂

(Compuesto No. 608),

For-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 612),

Propionil-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 613),

Ac-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 615),

Ac-D-Ala-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 616),

Ac-D-Leu-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 617),

Ac-D-Phe-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 618),

Ac-D-Lys-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 621),

Ac-D-Tyr-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 623),

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Ac-D-Tyr-D-Ala-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 625),

Ac-D-Tyr-D-Leu-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 626),

Ac-D-Tyr-D-Phe-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 627),

Ac-D-Tyr-D-Lys-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 629),

Ac-D-Tyr-D-Glu-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 630),

Ac-D-Tyr-D-Trp-Asn-Thr-Pya (4) -AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 635),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe (4F) -AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 637),

Ac-D-Tyr-D-Pya (4) -Asn-Thr-Phe (4F) -AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 638),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Val-Arg (Me) -Trp-NH₂

(Compuesto No. 642),

Gly-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 648),

Ac-Gly-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 649),

D-Tyr-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 650),

Ac-D-Tyr-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 651),

pGlu-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 652),

296 797

Ac-D-Tyr-Pro-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 657),

Ac-D-Tyr-D-Pya (2) -Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 658),

Ac-D-Tyr-D-Pya (3) -Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 660),

Ac-D-Tyr-Tic-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 662),

Ac-D-Trp-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 663),

Ac-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 666),

Hexanoil-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 667),

3-Piridinpropionil-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 670),

Adipoil-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 671),

Ac-D-Tyr-NMeTrp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 672),

6-Aminocaproil-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 674), o sales del mismo.

51. Un compuesto caracterizado por que su fórmula es:

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 550),

Ac-D-Arg-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 551),

D-Dap-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

797 798

(Compuesto No. 552),

Ac-D-Tyr-D-Trp-Asn-Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 558),

3-(4-Hidroxifenil)propionil-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 559),

Ac-D-Tyr-D-Pya (4) -Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 562),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Phe-NH₂

(Compuesto No. 571),

Ac-D-Tyr-D-Trp-Alb-Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 579),

Ac-D-Tyr-D-Trp-Dap (For) -Ser-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 585),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Nal (2) -NH₂

(Compuesto No. 589),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Phe (4F) -NH₂

(Compuesto No. 592),

For-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 612),

Propionil-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 613),

Ac-D-Phe-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 618),

Ac-D-Tyr-D-Phe-Asn-Thr-Phe-AzaGly-Leu-Arg (Me) -Trp-NH₂

(Compuesto No. 627),

Ac-D-Tyr-D-Trp-Asn-Thr-Phe (4F) -AzaGly-Leu-Arg (Me) -Trp-NH₂ (Compuesto No. 637),

Ac-D-Tyr-D-Pya (4) -Asn-Thr-Phe (4F) -AzaGly-Leu-Arg (Me) -

798

Trp-NH₂ (Compuesto No. 638),

Ac-D-Tyr-D-Pya(2)-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂
(Compuesto No. 658),

Ac-D-Tyr-D-Pya(3)-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂
(Compuesto No. 660),

Ac-D-Trp-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂
(Compuesto No. 663), o sales de los mismos.

52. Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 550) o sales de los mismos.

53. Ac-D-Arg-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 551) o sales de los mismos.

54. D-Dap-Acp-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 552) o sales de los mismos.

55. (NEW) Ac-D-Tyr-D-Trp-Asn-Ser-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 558) o sales de los mismos.

56. 3-(4-Hydroxyphenyl)propionyl-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 559) o sales de los mismos.

57. Ac-D-Tyr-D-Pya(4)-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 562) o sales de los mismos.

58. Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me)-Phe-NH₂ (Compuesto No. 571) o sales de los mismos.

59. Ac-D-Tyr-D-Trp-Alb-Ser-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 579) o sales de los mismos.

60. Ac-D-Tyr-D-Trp-Dap(For)-Ser-Phe-AzaGly-Leu-Arg(Me)-Trp-NH₂ (Compuesto No. 585) o sales de los mismos.

61. Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -
Nal(2)-NH₂ (Compuesto No. 589) o sales de los mismos.
62. Ac-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -
Phe(4F)-NH₂ (Compuesto No. 592) o sales de los mismos.
63. For-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -Trp-
NH₂ (Compuesto No. 612) o sales de los mismos.
64. Propionyl-D-Tyr-D-Trp-Asn-Thr-Phe-AzaGly-Leu-
Arg(Me) -Trp-NH₂ (Compuesto No. 613) o sales de los mismos.
65. Ac-D-Phe-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -Trp-
NH₂ (Compuesto No. 618) o sales de los mismos.
66. Ac-D-Tyr-D-Phe-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -Trp-
NH₂ (Compuesto No. 627) o sales de los mismos.
67. Ac-D-Tyr-D-Trp-Asn-Thr-Phe(4F)-AzaGly-Leu-Arg(Me) -
Trp-NH₂ (Compuesto No. 637) o sales de los mismos.
68. Ac-D-Tyr-D-Pya(4)-Asn-Thr-Phe(4F)-AzaGly-Leu-
Arg(Me) -Trp-NH₂ (Compuesto No. 638) o sales de los mismos.
69. Ac-D-Tyr-D-Pya(2)-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -
Trp-NH₂ (Compuesto No. 658) o sales de los mismos.
70. Ac-D-Tyr-D-Pya(3)-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -
Trp-NH₂ (Compuesto No. 660) o sales de los mismos.
71. Ac-D-Trp-D-Trp-Asn-Thr-Phe-AzaGly-Leu-Arg(Me) -Trp-
NH₂ (Compuesto No. 663) o sales de los mismos.
- ✓ 72. Un método para mejorar la estabilidad en sangre,
caracterizado por que comprende introducir uno o dos grupos
alquilo en la cadena lateral de Arg en el péptido que
contiene Arg.
- * 73. Un método para mejorar la estabilidad en sangre,
caracterizado por que comprende introducir un grupo alquilo

800 701

en la cadena lateral de Arg en el péptido que contiene Arg.

* 74. Un método para mejorar la estabilidad en sangre, caracterizado por que comprende convertir la cadena lateral de Arg en el péptido que contiene Arg en N^o-Arg alquilado.

* 75. Un método de acuerdo con las reivindicaciones 72 a 74, caracterizado por que el grupo alquilo es un grupo alquilo C₁₋₄.

* 76. Un método de acuerdo con las reivindicaciones 72 a 74, caracterizado por que el grupo alquilo es un grupo metilo.

* 77. Un método de acuerdo con las reivindicaciones 72 a 74, en donde el péptido que contiene Arg es un péptido que posee un péptido parcial caracterizado por que posee la estructura -Arg-XXX-, en donde XXX representa un aminoácido que posee opcionalmente un grupo anillo aromático sustituido en la cadena lateral.

78. Un método de acuerdo con la reivindicación 77, caracterizado por que XXX es Phe, Trp ó Tyr.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 80017608

RECIBO OFICIAL DE CAJA : 08 - 91,607
FECHA : NOVIEMBRE 25 DE 2008



******* CONSIGNACION *******

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	VP. PAGO
CAVELIER ABOGADOS	CONSIGNACION	BANCO DE BOGOTA	062754387	10528372	24/11/2008	43,000,000.00

******* C O N C E P T O *******

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICA	
		12 MARCAS Y LEHAS COMERCIALES	103,000.00
TOTAL :			\$ 103,000.00

SON: CIENTO TRES MIL PESOS

RESPONSABLE :

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

Cdo 14656-891-015-4

DIVISIÓN DE NUEVAS CREACIONES

RADICACIÓN EXPEDIENTE No. 06-128571

EL EXTRACTO DE LA PRESENTE SOLICITUD DE PATENTE DE INVENCION

FUE PUBLICADO EN LA GACETA DE PROPIEDAD INDUSTRIAL No 583 DE

FECHA 18 DE DICIEMBRE DE 2007 EL TÉRMINO HÁBIL SEÑALADO POR LA

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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

Expression of *KiSS-1*, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta[☆]

Yasuko Terao^a, Satoshi Kumano^b, Yoshihiro Takatsu^b, Masahiko Hattori^c,
Atsushi Nishimura^c, Tetsuya Ohtaki^b, Yasushi Shintani^{a,*}

^aPharmacology Research Laboratories I, Pharmaceutical Research Division, Takeda Chemical Industries, Ltd., 17-85,
Jusohonmachi 2-chome, Yodogawa, Osaka 532-8686, Japan

^bDiscovery Research Laboratories I, Pharmaceutical Research Division, Takeda Chemical Industries,
Ltd., 10 Wadai, Tsukuba, Ibaraki 300-4293, Japan

^cDiscovery Research Laboratories II, Pharmaceutical Research Division, Takeda Chemical Industries,
Ltd., 10 Wadai, Tsukuba, Ibaraki 300-4293, Japan

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Abstract

Metastin is encoded by a putative human metastasis suppressor gene *KiSS-1*, and is the cognate ligand of a G-protein-coupled receptor designated OT7T175. To study the physiological function(s) of metastin, we cloned rat and mouse *KiSS-1* cDNAs both encoding 130-amino acid *KiSS-1* proteins. Sequence analysis suggested that processing of the rat and mouse *KiSS-1* proteins produces 52-amino-acid peptides, each with an amidated carboxyl terminal and with a single possible disulfide bond, corresponding to rat and mouse metastins. The carboxyl-terminal sequence of metastin, known to be essential for functional receptor interaction, was found to be highly conserved among humans and rodents. Real-time PCR analysis indicated that rat *KiSS-1* mRNA showed the highest expression level in the cecum and colon. Since *KiSS-1* mRNA and metastin are known to be abundant in human placenta, we further studied the localization of *KiSS-1* and OT7T175 mRNAs in rat placenta by in situ hybridization. *KiSS-1* and OT7T175 mRNAs were specifically detected in trophoblast giant cells at embryonic day 12.5, and the transcripts in the cells gradually decreased during placental maturation. These results suggest that metastin/OT7T175 signaling may participate in implantation of the mammalian embryo, placenta formation, and maintenance of pregnancy.
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Keywords: *KiSS-1*; Metastin; OT7T175; Placenta; Trophoblast giant cell; Implantation

1. Introduction

We have cloned a human orphan G-protein-coupled receptor (GPCR) designated OT7T175, which shares

Abbreviations: GPCR, G-protein-coupled receptor; CHO, Chinese hamster ovary; RACE, rapid amplification of cDNA end; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; TGCs, trophoblast giant cells; MMP-9, matrix metalloproteinase-9 or 92-kDa type IV collagenase; EVT, extravillous trophoblast cells; hCG, human chorionic gonadotropin; LH, luteinizing hormone; uPA, urokinase-type plasminogen activator; ECM, extracellular matrix; DIG, Digoxigenin.

* The nucleotide sequence reported in this paper has been submitted to GenBank under accession no. AY182231 and AY196983.

* Corresponding author. Tel.: +81-6-6300-6164; fax: +81-6-6300-6306.

E-mail address: Shintani_Yasushi@takeda.co.jp (Y. Shintani).

39.2% amino acid homology with the galanin receptor [1]. Chinese hamster ovary (CHO) cells expressing recombinant human OT7T175 (CHO/h175), however, showed no functional response to galanin [2] or galanin-like peptide [3]. Subsequently, we isolated a novel peptide from human placenta that binds specifically to OT7T175 with high affinity and potently induces an increase in the intracellular calcium ion concentration in CHO/h175 cells [1]. The peptide was designated metastin [1], since it was found to be a carboxy-terminally amidated peptide with 54 amino acid residues encoded by a putative metastasis suppressor gene, *KiSS-1* [4–6]. Kotani et al. [7] also reported the isolation of the same *KiSS-1*-gene-derived peptide, designated kisspeptin, as an endogenous ligand for GPR54. Muir et al. [8] confirmed that AXOR12, a GPCR that is very similar to human OT7T175 except for a single amino acid

change near the carboxy-terminal, responds to the peptides derived from KiSS-1 protein.

KiSS-1 has been reported to be a metastasis suppressor gene that suppresses the metastasis of human melanoma [5] and breast carcinoma [9] without affecting tumorigenicity. However, the functional mechanisms of KiSS-1 have not been elucidated. To clarify whether metastatin suppresses metastasis via the OT7T175 receptor, we constructed an OT7T175-transfected B16-BL6 melanoma. Continuous administration of metastatin to mice suppressed pulmonary metastasis of the OT7T175-overexpressing melanomas, thus confirming that metastatin is able to suppress the metastasis of some tumor cells through the OT7T175 receptor [1].

We recently found that the immunoreactive (ir)-metastatin concentration in human plasma increases dramatically in pregnancy and is derived mainly from the placenta by using our newly developed, sensitive, and specific two-site enzyme immunoassay [10]. Metastatin mRNA and immunoreactivity was also found to be localized to the syncytiotrophoblasts from histochemical studies. Considering the localization of metastatin in syncytiotrophoblasts and the dramatic elevation of plasma ir-metastatin, it is possible that metastatin may be involved in the regulation of trophoblast invasion. To investigate the developmental relationship between KiSS-1/OT7T175 expression and placenta maturation and to clarify the role of metastatin during implantation, we cloned the rat and mouse KiSS-1 genes, and examined the localization of rat KiSS-1 and OT7T175 mRNAs in rat placenta during three stages at embryonic days 12.5, 15.5, and 18.5 by in situ hybridization. Though Stafford et al. [11] recently cloned the mouse KiSS1 gene, and their sequence was completely identical to ours. We have also found an additional in-frame ATG codon upstream of the ATG codon conserved in the human, rat, and mouse forms, and the four-amino-acid-deleted variant of mouse KiSS1 proteins which have not previously been described. We found that both mRNAs were specifically expressed in trophoblast giant cells in rat placenta on embryonic day 12.5, and that their expressions gradually decreased during placental maturation, suggesting an important role of the metastatin/OT7T175 pathway in placentation.

2. Materials and methods

2.1. Mouse KiSS-1 cDNA cloning

Putative mouse KiSS-1 cDNA fragments were cloned using a 15.5-day mouse embryo superscript cDNA library (Gibco BRL) as a template and primers designed on the basis of the human KiSS-1 sequence as follows: 5'-TAGACCTGCCCTTCCTCCCAGA-3' and 5'-CTGCTGGCCTGTGGATCCAGGCTT-3' by the thermal cycling condition with a denaturation step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s,

and 72 °C for 1.5 min, with the final extension step at 72 °C for 7 min.

The PCR products with each expected size were subcloned into a pCR2.1-TOPO plasmid (Invitrogen), and sequenced using a DNA sequencer 377 (Applied Biosystems). Using the putative KiSS-1 cDNA, the full-length cDNAs were cloned by a gene trapper cDNA positive selection system (Life Technologies) with two biotinylated primers (5'-TATGGGGAGCCGCTGGCAAAAGTG-3', and 5'-AAGCCTGGATCCACAGGCCAGCAG-3') and the above cDNA library according to the manufacturer's instructions. After the cloning, two mouse KiSS-1 open reading frames deduced from the above sequences were amplified from two cDNA libraries: a 15.5-day mouse embryo superscript cDNA library (Gibco BRL) and a E15.5 mouse embryo marathon ready cDNA library (Clontech) using the following primers: 5'-TGCAGGAGAGTGAAGATTAAATCCCCA-3' and 5'-TGCACAAGTC-TAGAAGCTCCCTGC-3', by the thermal cycling condition with a denaturation step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min, with the final extension step at 72 °C for 7 min and with the sequences being confirmed.

2.2. Rat KiSS-1 cDNA cloning

Total RNA was extracted from the placentas of pregnant Wistar rats (9 weeks old; 18 days of pregnancy) using TRIZOL reagent (Gibco BRL), purified into poly(A)+ RNA, and was reverse-transcribed into the first-strand cDNA using a superscript preamplification system (Life Technologies). A rat KiSS-1 partial fragment was obtained by degenerate PCR using *rTaq* DNA polymerase, the placental cDNA, and the following primers designed on the basis of the human and mouse KiSS-1 coding sequences: 5'-TTCTTGGCAGCTRCTGCTTYTCCTCTGTG-3' and 5'-GAAGCGCAGGCCGAAGGAGTTCCA-3', by the thermal cycling condition with a denaturation step at 94 °C for 5 min, followed by 9 cycles at 94 °C for 20 s, 72 °C for 20 s and 72 °C for 30 s with reduction of the annealing temperature by 0.5 °C with each cycle, 30 cycles at 94 °C for 20 s, 61.8 °C for 20 s, and 72 °C for 30 s, with the final extension step at 72 °C for 7 min.

The RACE reactions were performed using a Marathon-Ready cDNA kit according to manufacturer's instructions (Clontech). First, the PCR reaction for 3' - and 5' -RACE were performed with the anchor primer AP-1 and gene-specific primers 288-10F (5'-TTGGGGAGCCACTGGCAAAAATGGCACC-3') for 3' -RACE, AP-1, and 288-254R (5'-TGACATGTCCTTCTCGCGCTGCACCAGC-3') for 5' -RACE, respectively, *rTaq* DNA polymerase, and rat liver cDNA by the thermal cycling condition with a denaturation step at 94 °C for 60 s, followed by 5 cycles at 94 °C for 30 s and 72 °C for 2 min, 5 cycles at 94 °C for 30 s and 70 °C for 2 min, 25 cycles at 94 °C for 20 s and 68 °C for 2 min, with the final extension step at 68 °C for 10 min. Nested PCR was performed using amplified

products as a template with the anchor primer AP-2 and gene-specific primers 288-41F (5'-GGTGAACCCT-GAACCCACAGGCCAACAG-3') for 3'-RACE, AP-2, and 288-44R (5'-GGACTGTTGGCCTGTGGGTT-CAGGGTTC-3') for 5'-RACE, respectively. DNA fragments were cloned to the pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen) and the sequence was then analyzed.

RT-PCR was performed with the gene-specific primers rKiSS-364 F (5'-CGTCTCAGCCTCTGGACAC-CCTGTGGATCTGCC-3') and rKiSS-859R (5'-TGG-CGACAGCATTGCTTTATTGCACAAGTCTA-3'), Pfu DNA polymerase, and rat liver cDNA by the thermal cycling condition with a denaturation step at 94 °C for 60 s, followed by 3 cycles at 94 °C for 20 s and 72 °C for 2 min, 3 cycles at 94 °C for 20 s and 70 °C for 2 min, 3 cycles at 94 °C for 20 s and 68 °C for 2 min, 30 cycles at 94 °C for 20 s, 63 °C for 30 s and 68 °C for 2 min, with the final extension step at 68 °C for 10 min in the presence of 1% dimethylsulfoxide.

The nucleotide sequences of the rat and mouse *KiSS-1* cDNAs reported here have been submitted to the GenBank™/EBI Data Bank with accession numbers AY196983 and AY182231, respectively.

2.3. Real-time PCR

Total RNAs were extracted from multiple tissues of 7- to 8-week-old Wistar rats using Trizol reagent (Gibco BRL), and poly(A)⁺ RNAs were isolated from the pooled RNAs using a message maker reagent assembly (Gibco BRL). The first strand cDNAs were synthesized using a superscript preamplification system (Gibco BRL). The quantities of rat *KiSS-1* and *OT7T175* mRNAs in each tissue were determined by real-time PCR in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using each of the specific primers and fluorescence-labeled probes as follows: 5'-CATGCAGACCGTCACCAATTT-3', 5'-GCAGCACA-GAAGGAAAGTGACA-3', and 5'-(FAM)-TACATCGC-TAACCTGGCGGCCACA-(TAMRA)-3' for rat *OT7T175*; and 5'-GGCAAAAATGGCACCTGTG-3', 5'-GCCAGGCATTAACGAGTTCCT-3', and 5'-(FAM)-AACCCTGAACCCACAGGCCAACAGTCC-(TAMRA)-3' for rat *KiSS-1*.

2.4. In situ hybridization

PCR was performed using a rat uterine Marathon-Ready cDNA (Clontech) and the following primers: 5'-CGTCTCAGCCTCTGGACACCCTGTG-3' (A) and 5'-CCTGCCTTGGCCTCTGCCATGGG-3' (B), (A) and 5'-TGGCGATGTTCGGCGAGCGCGCAG-3' (C), and (B) and 5'-ACTGCGCGCTCGCCGAACATCGCCA-3' (D) to make 489-bp, 251-bp, and 262-bp *KiSS-1* partial DNA fragments, respectively, and using a rat brain Marathon-Ready cDNA (Clontech) and the following primers: 5'-

ATGGCCGCAGAGGCGACGTTGGGT-3' (E) and 5'-CCACCCAGATGCTAAGGCTGACAGTC-3' (F), (E) and 5'-GCAGCACAGAAGGAAAGTGACA-3' (G), and (F) and 5'-CATGCAGACCGTCACCAATT-3' (H) to make 505-bp, 285-bp, and 290-bp *OT7T175* partial DNA fragments, respectively. These PCR products were cloned into a pCR2.1-TOPO plasmid, and their sequences and insert directions were confirmed by sequence analysis. Digoxigenin (DIG)-labeled sense and antisense cRNA probes were synthesized with the above clones using a T7 RNA synthesis kit (NipponGene), SP6 RNA synthesis kit (NipponGene), and DIG-11-UTP (Roche). To prepare rat tissue sections, placentas in utero were dissected from Wistar rats (Charles River) on embryonic day 12.5, 15.5, or 18.5, fixed in 4% paraformaldehyde-containing phosphate buffer, and embedded in paraffin. Four-micron-thick sections were cut and mounted onto silane-coated glass slides (Matsunami Glass), dewaxed, and digested with 10 µg/ml proteinase K (Roche) for permeabilization. After refixation, sections were treated with 0.2 N HCl for 10 min and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min with vigorous shaking, and were then dehydrated in a graded series of ethanol. DIG-cRNA probes were denatured and incubated with each section at 50 °C for 16 h in a humid chamber. After hybridization, sections were washed and treated with 1 µg/ml RNase A (Sigma) at 37 °C for 5 min. Sections were further washed using SSC buffer and blocked in blocking solution (DIG Wash and Block Buffer Set; Roche) for 30 min. Immunological detection was performed using alkaline phosphatase-conjugated-anti-DIG antibody (Sigma), and processed for colorimetric detection using nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The reaction was terminated at 10–16 h and mounted in mounting solution (Clearmount™, Zymed Laboratories). Hematoxylin and eosin (H & E) staining was also performed to evaluate placenta formation.

2.5. Miscellaneous

A stable CHO cell line (CHO/rOT7T175) that expresses rat *OT7T175* (rOT7T175) was established by transfection of rOT7T175/pAKKO expression vector to CHO/dhfr-cells [1]. CHO/rOT7T175 and CHO/hOT7T175 [1] cells were challenged with metastin and metastin C-terminal peptides in a FLIPR system (Molecular Devices) [1] to determine the increase in intracellular calcium ion concentration in response to peptides.

3. Results

3.1. Molecular cloning of mouse and rat *KiSS-1* cDNAs

We have cloned the full-length *KiSS-1* cDNA from mouse embryonic cDNA libraries. Mouse *KiSS-1* cDNA

1 tggacgaaaaattccacacgtgactttctgcaggagagtgagattaaatccccaagct 60
 61 gtgaatgatatctgagattttggcgttgatgtctgcagcctgagtcctggaggagactgt 120
 * M Y L R F G V D V C S L S P W K E T V
 121 agacctgccccttctccagaatgatctcaatggcttcttggcagctgctgcttctcc 180
 D L P L P P R M I S M A S W Q L L L L L
 181 ctgtgtcgccacctatggggagcgtgagcaaaagtggaaccttgggaagcctgggac 240
 C V A T Y G E P L A K V A P L V K P G S
 241 cacaggccagcagtcgggacccaggaactcgttaatgctgggaaaaggatcgcggtta 300
 T G Q Q S G P Q E L V N A W E K E S R Y
 301 tgcagagagcaagcctgggtctgcagggtcgccgctcgtaggtcgtcgccatgcccgc 360
 A E S K P G S A G L R A R R S S P C P P
 361 ggttgaggggcccgccggggccagcggccctgtgtgctcccgagtcgctgctgctcc 420
 V E G P A G R Q R P L C A S R S R L I P
 421 tggcccccgggagcgggtgctggtgcagcgggagaaggacctgtccacctacaactggaa 480
 A P R G A V L V Q R E K D L S T Y N W N
 481 ctctctggcctggtctacggcaggagcaggcggcgccggcggcggcggcggcggcggc 540
 S F G L R Y G R R Q A A R A A R G
 541 gggctgaggtgggtggttagaggccaaggcaggagcgtttctagacttgtgcaataaagc 600
 601 aatgtgcgctcttacagtggttatttttccctgactcctgcgagatgggacaggtcc 660
 661 tcttctccctcagttgctcttttctgattcaagatgctcctgggtgcaaagtcgggtgc 720
 721 tttttaactactagttaaggaatgtcccgcttctgttgaataaagggtggagaaac 780
 781 tcggccactga

Fig. 1. Nucleotide sequence and deduced amino acid sequence of mouse *KISS-1* cDNA. The terminating codons (TGA) are indicated by an asterisk. The deduced amino acid sequence is shown in single-letter codes below codons of the *KISS-1* open reading frame. In the amino acid sequence, a putative coding region upstream from the second ATG initiation codon of the *KISS-1* sequence is boxed. The 12-bp nucleotides spliced out in a variant of *KISS-1* cDNA are underlined. The full cDNA sequence including these 12 bp can be obtained from GenBank™ (accession number AY182231).

has an open reading frame composed of 468-bp nucleotides (156 amino acids) as shown in Fig. 1. A deletion of 12 bp in the coding sequence has been found in some clones (Fig. 1). These could represent a splicing variant, since the deletion has never been found in mouse genomic sequences. Meanwhile, we have cloned rat *KISS-1* cDNA by taking a degenerate PCR approach including 5'- and 3'-RACE techniques. The rat *KISS-1* cDNA sequence was single as far as we could examine, and contained a coding sequence of 390-bp nucleotides (130 amino acids).

A comparison of the coding sequences of *KISS-1* cDNAs revealed that mouse *KISS-1* has an additional in-frame ATG codon upstream of the ATG codon conserved in the human, rat, and mouse forms. Kozack's rule (GCCCTCAatG) [12] cannot clearly suggest whether the first ATG (GCTGTGA-

atgT) or the second ATG (TCCCAGAatgA) is the more probable initiation codon. Protein translation from the first ATG will result in an amino-terminal extension of 26 amino acid residues, MYLRFVGVDVCSLSPWKETVDLPLPPR (Fig. 1), compared with the rat and human *KISS-1* proteins. Alternatively, if the second ATG is taken as the initiation codon, the mouse *KISS-1* protein will have 130 amino acid residues like that of rat (Fig. 2). Therefore, the second ATG presumably serves as the initiation codon of mouse *KISS-1* protein.

3.2. Rat and mouse metastins

We previously reported that human *KISS-1* protein has a putative signal peptide, M¹NSLVSWQLLLFLCATHFG¹⁹.

Human <i>KISS-1</i>	1	MNSLVSWQLLLFLCATHFGEPLKVASVGNRP	TGQQLSLGLLAPGQS	50
Mouse <i>KISS-1</i>	1	MISMASHQLLLLCVATYGEPLAKVAPLVKPGSTGQSGPQELVNAWEKE		50
Rat <i>KISS-1</i>	1	MISLASWQLLLLCVASFGEPLAKMAPVVNPPTSQSGPQELVNAWQKG		50

Human <i>KISS-1</i>	51	LPCTERKPAATARL-SRRGTSLSPPPESSGSRQPGLSAPHSRQIPAPQG		99
Mouse <i>KISS-1</i>	51	SRYAESKPGSAG-LRARRSSPCP-PVEGPAGRQRPLCAS-RSRLIPAPRG		97
Rat <i>KISS-1</i>	51	PRYAESKPGAAG-LRARRTSPCP-PVENPTGHRPPCAT-RSRLIPAPRG		97

Human <i>KISS-1</i>	100	AVLVQREKDLPTNTHNRRGLRGRKREAPGNHGRSAGRGWGAGAGQ		145
Mouse <i>KISS-1</i>	98	AVLVQREKDLSTYNNRGLRGRQARAARG		130
Rat <i>KISS-1</i>	98	SVLVQREKDSATYNNRGLRGRQARAARG		130

Fig. 2. Alignment of human, mouse, and rat *KISS-1* proteins deduced from the cDNA sequences. Identical (*) and conservatively substituted (.) amino acids are shown. Gaps (-) have been introduced to maximize the alignment. Putative signal sequences of the *KISS-1* proteins are underlined, human and rodent metastins are boxed, and the amino acid sequences corresponding to human metastin (45–54) are shaded.

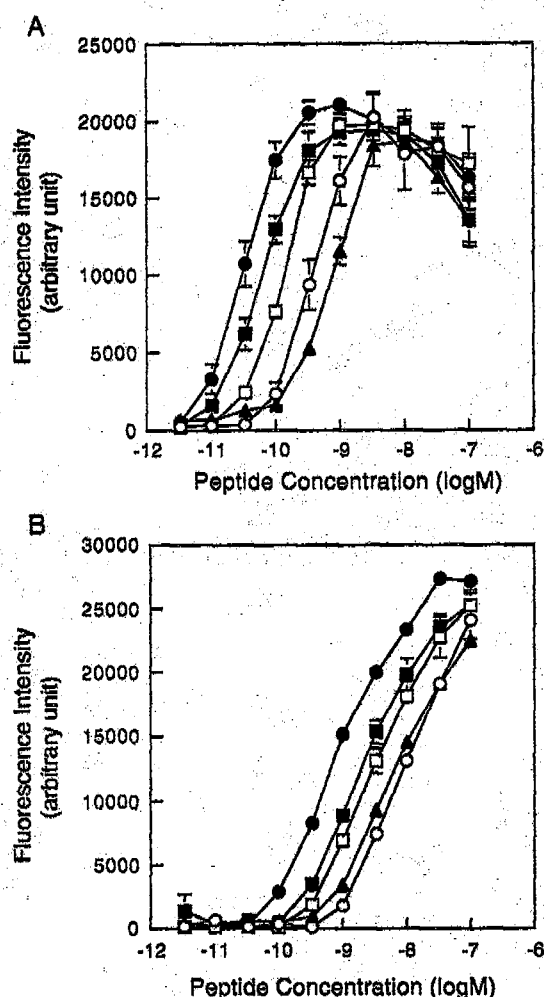


Fig. 3. FLIPR assay of metastin and metastin fragment peptides. CHO cells expressing hOT7T175 (A) and rOT7T175 (B) were challenged with human metastin (1–54) (○), metastin (40–54) (□), metastin (45–54) (●), [Tyr⁵⁴]-metastin (45–54) (■), or metastin (46–54) (▲). The maximum fluorescence change in each dose was plotted versus the peptide concentration.

and two potential processing sites for prohormone convertases, R⁶⁶R⁶⁷ and (G¹²²K¹²³R¹²⁴) [1]. The processing of human KiSS-1 protein followed by an amide transfer from G¹²² to F¹²¹ produces a carboxy-terminally amidated peptide with 54 amino acid residues, which was designated human metastin [1]. Analysis of the protein has revealed that the signal peptides are highly conserved among the species (Fig. 2). The website SignalP server predicted that possible extension of the 26 amino acid residues in mouse KiSS-1 protein would not affect the cleavage position of the signal peptide. Furthermore, two potential processing sites, R⁶⁶R⁶⁷ and G¹²⁰R¹²¹R¹²² (for G¹²²K¹²³R¹²⁴ in human), are conserved in rodents. Processing of the mouse and rat KiSS-1 proteins in the same manner as the human KiSS-1 protein would liberate a carboxy-terminally amidated peptide with 52 amino acid residues, i.e., mouse and rat metastin, respectively. It should also be noted that this rodent metastin may have a disulfide bond at the N-terminal region.

The similarity of the mouse and rat KiSS-1 proteins is as high as 82%, whereas that of mouse and human KiSS-1 proteins is only 46%. Furthermore, the similarity of mouse and rat metastin and that of mouse and human metastin are 83% and 59%, respectively. The lower similarity between mouse and human metastin is mainly due to diversity in the amino terminal. On the other hand, when comparing the carboxy-terminal 10 amino acid residues of metastin, which are the minimal essential sequence for the high-affinity receptor-binding and maximal receptor-activation [1], all the amino acid residues except the carboxy-terminal residue (F in human or Y in mouse and rat) are conserved among these species (Fig. 2).

We next tested whether metastin and metastin fragment peptides activate the human and rat metastin receptors, hOT7T175 and rOT7T175, using a FLIPR that follows the time course of fluorescence intensity for the calcium ion indicator Fluo-3. Metastin and fragment peptides including the rodent-type metastin C-terminal decapeptide, YNWNFGLRY-amide designated [Tyr⁵⁴]-metastin (45–54), potentially induced a robust increase in intracellular calcium concentration in hOT7T175-expressing CHO cells (as partly reported in Ref. [1]). The maximum fluorescence change in each dose was plotted to compare the potency of each peptide. As shown in Fig. 3, the rank order of potency in activating hOT7T175 was metastin (45–54) > [Tyr⁵⁴]-metastin (45–54) > metastin (40–54) > metastin (1–54) > metastin (46–54). Also, [Tyr⁵⁴]-metastin (45–54) potentially activated the rat metastin receptor, rOT7T175. The rank order in activating rOT7T175 was similar to that found in hOT7T175.

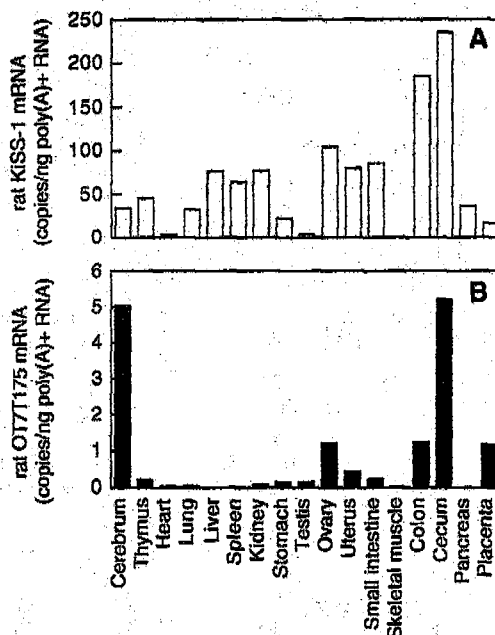


Fig. 4. Real-time PCR analysis of rat KiSS-1 (A) and OT7T175 (B) transcripts in various tissues. The quantity of each transcript in rat tissues was calculated as the copy number per 1 ng of poly(A)+RNA used for cDNA synthesis.

These results suggest that the rodent *KiSS-1* gene encodes rodent metastin that is a cognate ligand for *rOT7T175*. The activity of rat whole metastin peptide with 52 amino acid residues should be determined in future studies.

3.3. Tissue distribution of *KiSS-1* and *OT7T175* mRNAs in rats

To investigate the physiological role(s) of the metastin/*OT7T175* signaling system, we next examined the tissue distribution of *KiSS-1* and *OT7T175* mRNAs in rats using real-time PCR analysis (Fig. 4). The highest levels of *KiSS-1* mRNA were found in the cecum and colon. Moderate levels were present in the liver, spleen, kidney, ovary, uterus, and small intestine and low levels were evident in

the stomach, pancreas, and placenta. On the other hand, the highest expression levels of rat *OT7T175* were found in the cerebrum and cecum. Moderate expression levels were evident in the ovary, colon, and placenta and low levels were evident in the uterus, small intestine, and thymus. Although we and others have reported that human *KiSS-1* mRNA is expressed most abundantly in the placenta, and that human *OT7T175* mRNA is found predominantly in the placenta and pancreas [1,8], both rat *KiSS-1* and *OT7T175* mRNAs were expressed only moderately in the placenta.

3.4. Expression of *KiSS-1* and *OT7T175* mRNAs in rat trophoblast giant cells

Since there is a possibility that the expression of *KiSS-1* and *OT7T175* mRNAs in the rat placenta might be regulated tightly in specific cell lineages or at certain maturation stages, we further investigated their expression patterns in detail using in situ hybridization. Since the placenta changes its conformation and function during the period from implantation to exclusion after birth [13,14], we examined rat placentas in utero at three stages at embryonic days (E) 12.5, 15.5, and 18.5. As shown in Fig. 5, *KiSS-1* gene expression was observed in trophoblast giant cells, the placenta-derived cell lineage aligned at the boundary between the uterus and placenta, at E12.5 (Fig. 5B and C). *OT7T175* mRNA expression was also detected in trophoblast giant cells at E12.5, however, its hybridization signal was faint and was only observed in some of these cells (Fig. 5H). The *KiSS-1* transcripts in the cells gradually decreased during placental maturation, as shown in Fig. 5, and the signal was not detectable in the cells at E18.5 (Fig. 5G).

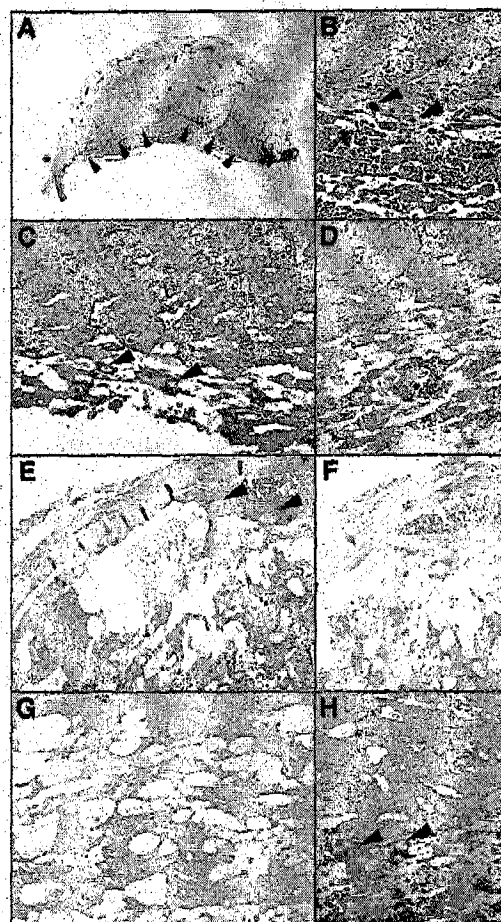


Fig. 5. Morphological changes of the placenta in utero and the distribution of *KiSS-1* and *OT7T175* transcripts in rat trophoblast giant cells. Histological examination at E12.5 (A, B) of rat placenta in utero showed trophoblast giant cells (TGCs) at the boundary between the uterus and placenta (H & E staining). *KiSS-1* signals were strong in TGCs at E12.5 (C), weak at E15.5 (E), and undetectable at E18.5 (G). No hybridization was detected with *KiSS-1* sense probes at E12.5 (D) and E15.5 (F). *OT7T175* mRNA was weakly expressed in TGCs at E12.5 (H) (original magnification $\times 1$ (A), $\times 100$ (B–H)). TGCs are indicated by black arrowheads. (For color see online version).

4. Discussion

In this study, we succeeded in isolating rat and mouse *KiSS-1* cDNAs, and, in addition, found a variant of a mouse *KiSS-1* transcript that lacks a 12-bp short coding sequence. Although Stafford et al. [11] recently cloned the mouse *KiSS1* gene, and their sequence was completely identical to ours, they did not describe the additional in-frame ATG codon upstream of the ATG codon conserved in the human, rat, and mouse forms, and the 12-bp deletion variant of the mouse *KiSS1* gene. However, the physiological significance of this variant should be further clarified, since a similar deletion has never been found in humans and rats. We speculate that this short 12-bp sequence might be erroneously recognized as an intron and spliced out. Amino acid sequence analysis has revealed that the secretory signal peptide [15] and two prohormone convertase cleavage sites [16] of the human *KiSS-1* protein are conserved in the mouse and rat *KiSS-1* proteins. The homologies of the *KiSS-1* proteins between human and mouse, and between mouse and rat are 46% and 82%, respectively, but increase to 59%

and 83%, respectively, when limited to the metastin peptides. Furthermore, the C-terminal metastin sequence that is essential for receptor binding and receptor activation [1], Y⁴⁵ NWNSFGLRF⁵⁴ (or Y⁵² in rodents)-amide, is almost (except for the C-terminal residue) conserved among humans and rodents. We further confirmed that a rodent-type metastin C-terminal peptide, YNWNSFGLRY-amide, potently activates both of the human and rat metastin receptors, hOT7T175 and rOT7T175. The activity of rat and mouse whole metastin peptide with 52 amino acid residues has not been tested, since it is relatively difficult to synthesize these big peptides with a possible disulfide bond. However, the present result indicates that human metastin or metastin C-terminal peptides can substitute for the rat metastin whole molecule in exploring the physiological roles of metastin in rats. On the other hand, the nucleotide sequence of mouse OT7T175 gene that we cloned previously was identical to that of mouse KiSS1 receptor reported by Stafford et al. [11]. The rat OT7T175 sequence has been published in our previous paper [1] and GPR54, a rat receptor nearly identical to rat OT7T175, has been reported by Lee et al. [17]. According to these sequences, mouse, rat, and human OT7T175 proteins share high homology in the NH₂-terminal domain and the seven-terminal membrane regions, and the homologies of the OT7T175 proteins between human and mouse, and mouse and rat are 85% (from the Stafford paper) and 95%, respectively.

The placenta is composed of an inner vascular network covered by an outer epithelium of trophoblast cells, all designed to promote the delivery of nutrients to the fetus. Rodent mature placenta is composed of multiple trophoblast lineages, such as spongiotrophoblast, syncytiotrophoblasts, and trophoblast giant cells (TGCs). Although all of these cells are derived from trophoblast, there are differences in the tissue covering the blastocyst in a monolayer form, their morphologies, positions in the placenta, and physiological and endocrinological functions [13,14]. A proportion of trophoblasts differentiate into TGCs, which appear during the initial period of gestation, mainly during blastocyst formation, through an unusual type of cell cycle known as endoreduplication [18]. They first become attached to the uterine wall as an implantation cue, reach their maximum size, and exhibit conspicuous synthetic and invasive activity during placentation [19]. In the present study, we investigated the spatiotemporal patterns of *KiSS-1* and *OT7T175* expression in rat placenta (Fig. 5C). Although *KiSS-1* mRNA was detectable in TGCs, the level of the mRNA decreased dramatically during placentation (Fig. 5E and G). *OT7T175* mRNA was detected faintly in the TGC layer at E12.5 (Fig. 5H), and had disappeared by E15.5 (data not shown). Therefore, metastin/OT7T175 signaling may act as a growth and differentiation factor at a relatively early stage of gestation. Recently, we found that the immunoreactive (ir)-metastin concentration in human plasma increases dramatically in pregnancy and is derived mainly from the placenta by using our newly developed, sensitive, and

specific two-site enzyme immunoassay [10]. *Metastin* mRNA and immunoreactivity was also found to be localized to the syncytiotrophoblasts from histochemical studies. Considering the localization of metastin in syncytiotrophoblasts and the dramatic elevation of plasma ir-metastin, it is possible that metastin may be involved in the regulation of trophoblast invasion. However, Janneau et al. [20] reported that human *KiSS-1* expression levels did not significantly differ between the early and term human placentas, while expression levels of the *KiSS-1* receptor gene were higher in early placentas than in term placentas. They speculated that the expression of these genes was detected in extravillous invasive cytotrophoblasts which are the subclass of trophoblast cells that invade into the deciduas. The trophoblast cell lineage is simply divided into two major, terminally differentiated cell types: an "invasive trophoblast" cell subtype such as extravillous cytotrophoblast cells in humans and trophoblast giant cells in mice, and a "transport trophoblast" cell subtype that is a syncytium (syncytiotrophoblast) in humans and mice, as described by Cross et al. [21]. Since the trophoblast cell lineages in rat placentas are thought to be basically the same as those in mouse placentas, the human extravillous cytotrophoblast cells described by Janneau et al. can be considered to be the same functional type of cells as trophoblast giant cells in rat placenta. However, as described in our previous paper [10], we identified *metastin* mRNA and immunoreactivity to human syncytiotrophoblasts by histochemical studies of human placenta. The cells are not of the same cell lineage as trophoblast giant cells in the rat. Since we were unable to detect immunoreactive signals by antibodies against rat *KiSS-1* proteins, probably because of their low efficiency, it is still unclear whether the molecule would also express in syncytiotrophoblast cells in rat placenta. Therefore, it should be confirmed whether *KiSS1* mRNA is expressed in a "transport trophoblast" cell subtype like syncytiotrophoblasts. Further, while the *KiSS-1* and *OT7T175* hybridization signals in rat placentas gradually decreased during placenta maturation, as shown in Fig. 5, we cannot explain the discrepancy of the expression period between human and rat placentas. However, it may be related to the strict regulation of expression of several invasion and maturation-related secreted proteins, including not only metastin but also many other regulating peptides, such as CRH [22,23], neuropeptide Y [24], leptin [25], and neurokinin B [26], which are all produced in the placenta and released into the maternal plasma. We should further clarify the differences in the physiological role of metastin from other peptides in the maturation of the placenta.

We previously reported that metastin at 10–100 nM significantly suppresses motility in both chemotaxis and wound healing assays, and inhibits CHO/hOT7T175 cell spreading, monolayer growth, and colony formation in agar by regulating the formation of the cytoskeleton [27]. Yan et al. [28] recently reported that *KiSS-1*-overexpressing HT1080 human fibrosarcomas demonstrate lower 92-kDa

Type IV collagenase (MMP-9) expression and proteinase activity, and substantially lower invasiveness. They indicated that KiSS-1 is able to diminish MMP-9 expression by down-regulating NF- κ B binding to the promoter. Meanwhile, matrix proteinases are believed to play a role in the tissue remodeling accompanying decidualization in the endometrium and in embryo invasion during implantation [29]. Furthermore, TGCs are known to show their invasive activity, and to secrete MMP-9 and urokinase-type plasminogen activator (uPA) during embryo implantation and placenta formation [30]. Therefore, metastin/OT7T175 signaling may suppress their invasive phenotype in an autocrine manner by regulating the expression of remodeling-related proteins such as MMP-9.

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Metastin and its variant forms suppress migration of pancreatic cancer cells

Toshihiko Masui,^a Ryuichiro Doi,^{a,*} Tomohiko Mori,^a Eiji Toyoda,^a Masayuki Koizumi,^a Kazuhiro Kami,^a Daisuke Ito,^a Stephen C. Peiper,^b James R. Broach,^c Shinya Oishi,^d Ayumu Niida,^d Nobutaka Fujii,^d and Masayuki Imamura^a

^a Department of Surgery and Surgical Basic Science, Kyoto University, Kyoto, Japan

^b Department of Pathology, Medical College of Georgia, USA

^c Department of Molecular Biology, Princeton University, USA

^d Department of Bioorganic Medicinal Chemistry, Kyoto University, Kyoto, Japan

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Abstract

Metastin, a post-translationally modified variant of KiSS1, was recently identified as an endogenous peptide agonist for a novel G-protein coupled receptor, hOT7T175 (AXOR12, GPR54). In this study, we analyzed the role of KiSS1 and hOT7T175 in both pancreatic cancer tissues and pancreatic cancer cell lines. Furthermore, we synthesized novel short variant forms of metastin and tested the inhibitory effect of those variants on in vitro cell functions that are relevant to metastasis. Pancreatic cancer tissues showed significantly lower expression of KiSS1 mRNA than normal tissues ($p = 0.018$), while cancer tissues showed significantly higher expression of hOT7T175 mRNA than normal pancreatic tissues ($p = 0.027$). In human pancreatic cancer cell lines, KiSS1 mRNA was highly expressed in 2 out of 6 pancreatic cancer cell lines, while hOT7T175 mRNA was expressed in all cell lines at various degrees. PANC-1 cells showed the highest expression of hOT7T175. Exogenous metastin did not suppress cell proliferation but significantly reduced the in vitro migration of PANC-1 cells ($p < 0.01$). Metastin induced activation of ERK1 in PANC-1 and AsPC-1 cells. Finally, we synthesized 3 novel short variant forms of metastin, FM053a2TFA, FM059a2TFA, and FM052a4TFA. These metastin variants significantly suppressed the migration of PANC-1 cells and activated ERK1. These data suggest that the metastin receptor, hOT7T175, is one of the promising targets for suppression of metastasis, and that small metastin variants could be an anti-metastatic agent to pancreatic cancer.

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Keywords: KiSS1; hOT7T175; Metastin; Pancreatic cancer; Migration; Metastasis

The KiSS1 peptide was originally identified as being differentially up-regulated in C8161 melanoma cells that have been rendered to have non-metastatic function by microcell mediated transfer of human chromosome 6 [1]. Transfection of *KiSS1* into human melanoma and breast carcinoma cells prevents these cells from metastasizing without an effect on cell proliferation [2]. Furthermore, the KiSS1 product has been shown to repress 92-kDa type 4 collagenase (MMP-9) expression by affecting NF- κ B binding to the promoter [3]. The KiSS1

product was found to be expressed in normal human placenta, testis, brain, pancreas, and liver [4].

Recently, it was shown that the human metastasis suppressor gene *KiSS1* encodes a COOH-terminally amidated peptide with 54 amino acid residues, and that this peptide is a ligand of a novel human G-protein coupled receptor (AXOR12 and hOT7T175) which couples primarily to Gq/11 [4–7]. The receptor has a high degree of homology (81% amino acid identity) to the rat orphan heptahelical receptor, GPR54 [8], indicating that these two receptors are orthologs. The peptide ligand, named as metastin, enhances the expression and activity of focal adhesion kinase, and attenuates pulmonary metastasis of hOT7T175 transfected

* Corresponding author. Fax: +81-75-751-4390.

E-mail address: doir@kuhp.kyoto-u.ac.jp (R. Doi).

B16-BL6 melanomas *in vivo* [5]. In another experiment, metastin inhibits chemotaxis and invasion of hOT7T175 transfected Chinese hamster ovary cells (CHO cells) *in vitro* with the activation of phospholipase C, arachidonic acid release, and phosphorylation of ERK [6,7]. These indicate that metastin-hOT7T175 axis may act as an anti-metastatic system. The characteristics of inhibitory effects on cancer cell metastasis without affecting cellular growth properties of normal cells make the metastin receptor to be an attractive target for cancer therapy.

The *KiSS1* is located on human chromosome 1q32–q41 [9]. However, evidences from subsequent experiments suggest that the expression of *KiSS1* is regulated by a gene(s) located in the region between 6q16.3 and q23 [1]. In pancreatic cancer, losses of 6q, 8p, 9p, 17p, and 18q are frequently observed and those alterations tend to cause lymph node and distant metastases, which suggests a suppressor gene(s) important for pancreatic cancer metastasis may exist in these regions [10–12]. Therefore, pancreatic cancer, has good reasons to downregulate *KiSS1* expression. Moreover, in other cancers such as ovarian cancer, breast cancer, and thyroid papillary cancer, over-expression of hOT7T175 has been demonstrated, although *KiSS1* is less frequently expressed in the tumor tissue [4,5]. Until now, however, expressions of *KiSS1* and hOT7T175, and their function have not been investigated in pancreatic cancer. The purpose of the present study was to determine if *KiSS1* and its receptor hOT7T175 are expressed in pancreatic cancer tissues, and to analyze the effect of exogenous metastin on pancreatic cancer cell lines with different expression levels of hOT7T175. Finally, we newly synthesized short variant forms of metastin and tested the inhibitory effect of those variants on *in vitro* cell functions that are relevant to metastasis.

Materials and methods

Cell culture. Pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-2, CFPAC-1, PANC-1, and SUIT-2 were purchased from the American Type Culture Collection. Cells were cultured as monolayers in the appropriate medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humid atmosphere of 5% CO₂/95% air. As for AsPC-1 and PANC-1, upon reaching 80% confluence, the medium was removed, the cells were washed in phosphate-buffered saline (PBS) and treated with various concentrations of metastin (Takeda Chemical Industries, Tsukuba, Japan) with 10% fetal bovine serum, and protein was isolated 15 min later as described below.

Patients and tumor samples. Pancreatic cancer tissues obtained from 30 patients who underwent pancreatectomy at our Department between January 1998 and June 2001 were used. Patients with other pancreatic malignancies, such as intraductal papillary mucinous adenocarcinoma, acinar cell carcinoma, and endocrine tumor, were excluded. Informed consent was obtained from each patient according to the institutional guidelines. Samples for mRNA expression were immediately frozen in liquid nitrogen at the time of surgery and stored at –80 °C.

Peptide synthesis. Fifty-four amino acid peptide, metastin, was kindly provided from Dr. T. Ohtaki, Takeda Chemical Industries [5]. We synthesized 3 short peptide variants of metastin, which were defined as FM053a2TFA, FM059a2TFA, and FM052a4TFA. The sequences of these peptides are as follows:

FM053a2TFA: Gu-Amb-Phe-Gly-Leu-Arg-Trp-NH₂.

FM059a2TFA: Ac-Trp-Asn-Arg-Phe-Gly-Leu-Arg-Trp-NH₂.

FM052a4TFA: Bis(Py)-Amb-Phe-Gly-Leu-Arg-Trp-NH₂.

These peptides were selected by screening from various truncated forms of *KiSS1* peptide and modified peptides for gaining almost the same internal signals with metastin through the receptor using reporter gene assay in yeast.

All reagents for peptide synthesis were purchased from Watanabe Chemical Industries (Hiroshima, Japan), NovaBiochem (Darmstadt, Germany), Nacalai Tesque (Kyoto, Japan), and Wako Pure Chemical Industries (Osaka, Japan). Ion-spray mass spectra were obtained with a Sciex APIIII triple quadrupole mass spectrometer. Protected peptide-resins were manually constructed on Fmoc-NH-SAL resin by Fmoc-based solid-phase peptide synthesis. Trt for Asn and Pbf for Arg were employed for side-chain protection. In the synthesis of FM052a and FM053a, amino-group modifications were performed after coupling of 4-(aminomethyl)benzoic acid, respectively. Reductive amination using pyridine-2-aldehyde and NaBH₃ (OAc) provided the protected resin for FM052a. Treatment with 1H-pyrazole-1-carboxamide hydrochloride and *N,N*-diisopropylethylamine gave the resin for FM053a. Deprotection/cleavage by treatment of the peptide resins with a mixture of TFA-thioanisole-*m*-cresol-H₂O-1,2-ethanedithiol-triisopropylsilane (80:5:5:5:2.5:2.5, v/v) followed by purification by reverse phase HPLC (Cosmosil 5C18-ARII column, Nacalai Tesque, Japan, 20 × 250 mm) yielded the peptides. FM059a was prepared by *N*-terminal acetylation of the protected octapeptide, subsequent deprotection/cleavage, and HPLC purification as well.

Quantitative RT-PCR. To monitor gene expression, we used quantitative real-time RT-PCR analysis [13–15]. Briefly, within the amplicon defined by a gene-specific PCR primer pair, an oligonucleotide probe labeled with 2 fluorescent dyes is created and designated as TaqMan probe. As long as the probe is intact, the emission of the reporter dye (6-carboxy-fluorescein, FAM) at the 5'-end is quenched by the second fluorescence dye (6-carboxy-tetramethyl-rhodamine, TAMRA) at the 3'-end. During the extension phase of PCR, the polymerase cleaves the TaqMan probe, resulting in a release of the reporter dye. The increasing amount of reporter dye emission is detected by an automated sequence detector combined with analysis software (ABI Prism 7700 Sequence Detection System; PE Applied Biosystems). The conditions of the reaction were according to the manufacturer's protocol. Five microliters of cDNA (reverse transcription mixture) with 25 µl TaqMan Universal PCR Master Mix (PE Applied Biosystems) and oligonucleotides at a final concentration of 0.3 µM for primers and 0.2 µM for the TaqMan hybridization probe were analyzed in a 50-µl volume.

The following primers and TaqMan probes were used for analysis. The *KiSS1* specific primers were

5'-ACTCACTGGTTTCTTGGCAGC-3' (upstream primer),

5'-ACCTTTTCTAATGGCTCCCCA-3' (downstream primer), and

5' (FAM)-ACTGCTTTCCTCTGTGCCACCCACT-(TAMRA)3' (TaqMan probe).

The hOT7T175 specific primers were

5'-CGACTTCATGTGCAAGTTCGTC-3' (upstream primer),

5'-CACACTCATGGCGGTCAGAG-3' (downstream primer), and

5' (FAM)-ACTACATCCAGCAGGTCTCGGTGCAGG-(TAMRA)3' (TaqMan probe).

The thermal cycle parameters were 95 °C for 10 min (for heat activation of Taq-Polymerase), followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Assessment of GAPDH RNA for quality and normalization was done with the TaqMan GAPDH Control Reagent Kit (PE Applied Biosystems) which utilizes standard TaqMan probe chemistry.

Protein extraction and Western blotting. Cells were collected into microtubes with a cell scraper and lysed for 60 min in phosphorylation-inhibitory RIPA buffer containing 50 mM Hepes (pH 7.0), 250 mM NaCl, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 20 μ g/ml gabexate mesilate, and then the lysate was sonicated for 10 s. Total extracts were cleaned by centrifugation at 12,000 rpm for 10 min at 4°C and the supernatants were collected. Protein concentrations were measured using a protein assay kit (Tonein-TP, Otsuka Pharmaceutical, Tokyo, Japan). The lysates were resuspended in one volume of the gel loading buffer which contained 50 mM Tris-HCl (pH 6.7), 4% SDS, 0.02% bromophenol blue, 20% glycerol and 4% 2-mercaptoethanol, and then boiled at 95°C for 90 s. The extracted protein was subjected to Western blotting, as previously described [16]. In brief, 30- μ g aliquots of protein were size-fractionated to a single dimension by SDS-PAGE (12% gels) and transblotted to a 0.45- μ m polyvinylidene difluoride membrane (Bio-Rad, Richmond, CA) in a semidry electroblot apparatus (Bio-Rad, Richmond, CA). The blots were then washed 3 times with TBST buffer and incubated for 2 h at RT in the first antibody solution containing anti-phospho-ERK antibody (pTEpY, Promega, Madison, WI), anti-phospho38 MAPK (pTgPY, Promega, Madison, WI) or anti-phosphoJNK (pTPPY, Promega, Madison, WI), 0.2% I-block (Promega, Madison, WI). After 3 washings in TBST buffer, the blots were incubated for 1 h at RT with horseradish peroxidase-conjugated anti-rabbit IgG at a 1:2000 dilution with TBST buffer. After 3 washings in TBST buffer, membranes were treated with enhanced chemiluminescence reagents (Amersham Life Sciences, Amersham, UK) according to the manufacturer's protocol. Membranes were exposed to X-ray film for 50–60 s. Protein expression was measured by ATTO densitometer system AE-6920M (ATTO Corporation, Tokyo Japan) and the quantity was expressed numerically. The quantity of the target protein was divided by that of β -actin and relative intensities were calculated.

Cell proliferation assay. AsPC-1 and PANC-1 cells (1×10^4 cells/3 cm diameter dish) were seeded in 10% FBS medium and incubated with increasing doses of metastatin for 48 and 96 h. Cells were trypsinized and cell numbers were counted using hemacytometer.

Cell migration assay and Matrigel invasion assays. A polyvinylpyrrolidone-free polycarbonate framed filter (8 μ m pores) was set in a chamber (Corning Coster, Cambridge, MA). Cells (2×10^6 cells in 200 μ l RPMI1640 for AsPC-1 and in 200 μ l DMEM for PANC-1)

and designated concentrations of peptide were added to the upper chamber and incubated at 37°C for 12 h to allow migration to the lower chamber, which contained 10% FBS/RPMI1640 for AsPC-1 or 5% FBS/DMEM for PANC-1 as a chemoattractant. After removing non-migrating cells with a cotton swab from the upper surface of the membrane, cells on the lower surface were fixed, stained with Diff-Quick (International Reagent, Kobe, Japan). For quantification, cells were counted under a microscope in 5 predetermined fields at 200 $\times$.

Cells and peptide (2×10^6 cells in 200 μ l RPMI1640 for AsPC-1 and in 200 μ l DMEM for PANC-1) were added to a Matrigel-coated Transwell (8 μ m pores, Becton-Dickinson Labware, Bedford, MA) and incubated at 37°C for 12 h versus a lower chamber containing 10% FBS/RPMI1640 for AsPC-1 or 5% FBS/DMEM for PANC-1. After removing the Matrigel and cells from the upper surface of the membrane, cells on the lower surface were fixed, stained with Diff-Quick and the number quantified as well. Invasion index was defined as the number of invaded cells per that of migrated cells.

Statistical analyses. The comparative statistical evaluations among groups in the densitometry or in the migratory activity were first performed by a two-way analysis of variance for repeated measures, followed by a post hoc Tukey test. To compare the mRNA levels in pancreatic tissues, Wilcoxon's rank sum test was performed. All assays were performed 3 times independently. Statistical analyses were done using JMP statistical software (version 3.02). Probability value of <0.05 was considered significant.

Results

Expression of KiSS1 and hOT7T175 in pancreatic cancer tissues

First, we measured the mRNA expression levels of KiSS1 and hOT7T175 in 30 pancreatic cancer tissues and in 5 adjacent normal pancreatic tissues. All the normal pancreatic tissues (5/5) and 14/30 of pancreatic ductal carcinoma tissues expressed KiSS1 mRNA (Fig. 1A). The expression level of KiSS1 mRNA in

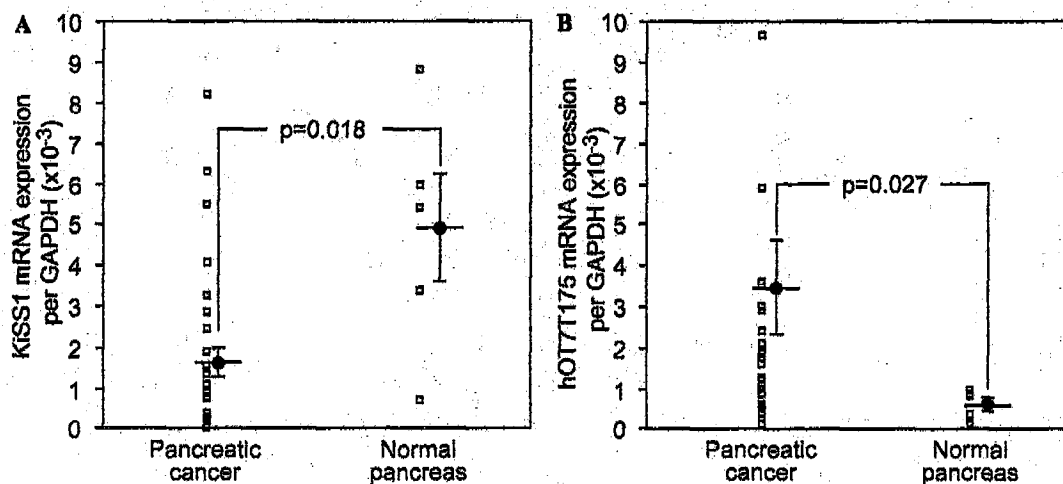


Fig. 1. Expression of KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer tissues. The KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer ($n = 30$) and normal pancreatic tissues ($n = 5$) were measured by real-time RT-PCR. The level of KiSS1 mRNA in pancreatic cancer tissues was significantly lower than that of normal pancreatic tissues ($p = 0.018$). The level of hOT7T175 mRNA in pancreatic cancer tissues was significantly higher than that of normal pancreatic tissues ($p = 0.027$).

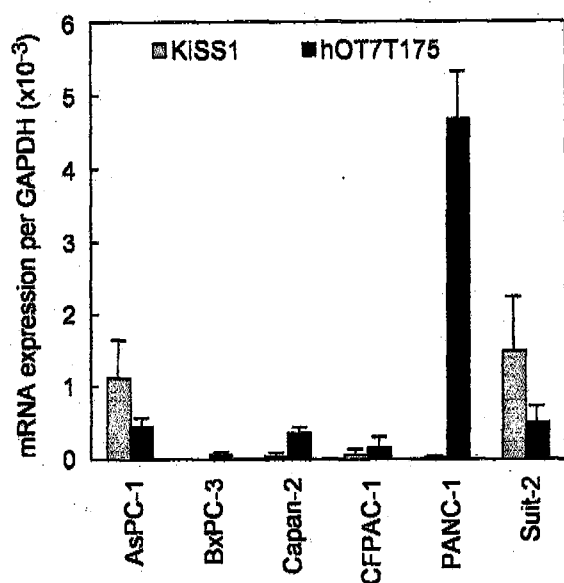


Fig. 2. Expression of KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer cell lines. The KiSS1 mRNA and hOT7T175 mRNA in pancreatic cancer cells were measured by real-time RT-PCR. AsPC-1 and SUI-2 showed high level of metastin mRNA expression and other 4 cell lines showed very low level of expression. In contrast, all cell lines expressed hOT7T175 mRNA. PANC-1 cell most highly expressed hOT7T175 mRNA. Measurements were repeated three times and data are expressed as means $\pm$ SEM.

pancreatic cancer tissues was significantly lower than normal pancreatic tissues ($p = 0.018$). In contrast, all the pancreatic cancer tissues (30/30) and normal pancreatic tissues (5/5) expressed KiSS1 receptor hOT7T175 mRNA, and the expression level of hOT7T175 mRNA in pancreatic cancer tissues was significantly higher than

that of normal tissues ($p = 0.027$) (Fig. 1B). We tested paired samples from 5 patients (cancer and normal tissues from each patient). The expression of hOT7T175 was higher in cancer tissue than the adjacent normal pancreatic tissue in all the 5 paired samples.

Expression of KiSS1 and hOT7T175 in pancreatic cancer cell lines

We next measured the expression of KiSS1 mRNA and hOT7T175 mRNA in 6 pancreatic cancer cell lines (Fig. 2). Among 6 pancreatic cancer cell lines, AsPC-1 and SUI-2 showed high level of KiSS1 mRNA expression and the other 4 cell lines showed a very low level of expression. In contrast, all cell lines expressed hOT7T175 mRNA at various degrees. PANC-1 cell most strongly expressed hOT7T175 mRNA. According to these results, we chose AsPC-1 cell line as a representative of high KiSS1 and low hOT7T175, and chose PANC-1 cell line as that of low KiSS1 and high hOT7T175. We used these two cell lines in the following experiments.

Effects of exogenous metastin on proliferation, migration, and invasion through endogenous metastin receptor

We examined the effect of exogenous metastin on pancreatic cancer cell proliferation. Metastin peptide was added to AsPC-1 and PANC-1 cells in the phase of exponential growth at final concentrations of 0, 0.1, 1, and 10 μ M for 48 and 96 h. The addition of metastin had no effects on cell proliferation of AsPC-1 and PANC-1.

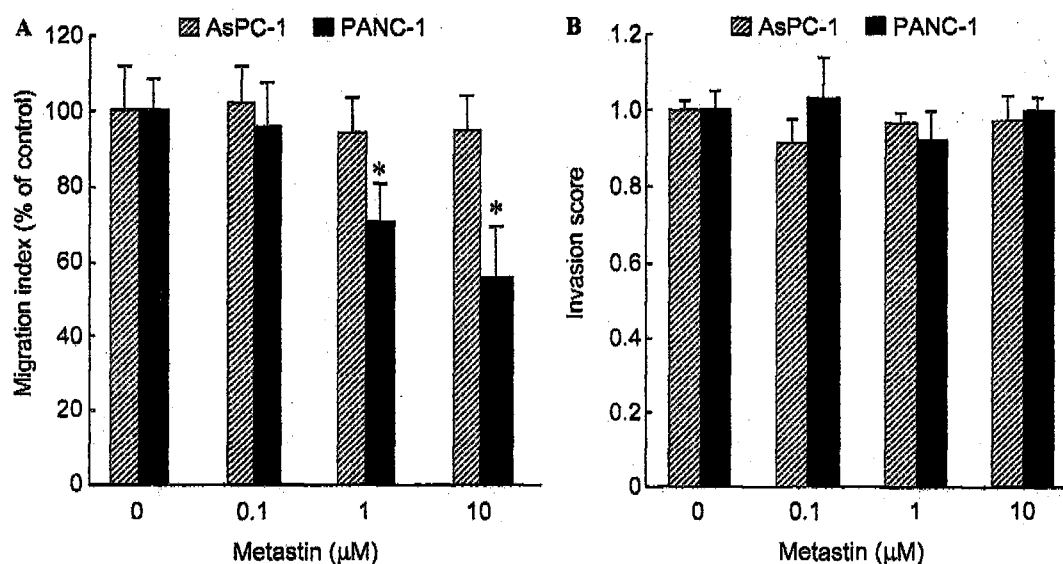


Fig. 3. Effects of exogenous metastin on migration and invasion of pancreatic cancer cells. (A) Effect of metastin on migration of pancreatic cancer cells. Cells were treated with various concentrations of metastin for 12 h. Metastin at 1 and 10 μ M significantly suppressed migratory activity of PANC-1 cells. (B) Effect of metastin on invasion of pancreatic cancer cells. Cells were treated with various concentrations of metastin for 12 h. Invasion activity was not affected by tested concentrations of metastin in both AsPC-1 and PANC-1. Experiments were repeated three times and data are expressed as means $\pm$ SEM. * Represents $p < 0.05$ against control.

Next, we tested the effect of metastatin on migration and invasion of these cell lines. The migration of AsPC-1 was not significantly affected by metastatin, while PANC-1 was significantly inhibited by metastatin at 1 and 10 μM ($p < 0.05$) (Fig. 3A). The invasion of the two cell lines was not significantly affected by metastatin (Fig. 3B).

Effects of metastatin on MAPK activation in AsPC-1 and PANC-1 cells

We analyzed the activation of MAPK by metastatin. Cancer cells in exponential phase were incubated in serum containing medium and then transferred to 1% BSA medium with metastatin as described in the section of migration and invasion assay. After incubation with metastatin for 15 min, ERK1/2, p38, and JNK1/2 were investigated by immunoblotting (Fig. 4). Protein expression was measured by ATTO densito-analyzer system AE-6920M (ATTO Corporation, Tokyo Japan) and the relative intensity was expressed numerically (Fig. 5). Metastatin induced a significant increase of pERK1 in AsPC-1 cells at 1 and 10 μM and in PANC-1 at 0.1–10 μM (Fig. 5A). Metastatin induced a significant increase of pp38 in PANC-1 cells at 10 μM (Fig. 5B).

Effects of short variant forms of metastatin on proliferation and migration of hOT7T175 expressing pancreatic cancer cells

We analyzed the effects of metastatin and newly synthesized short peptides, FM053a2TFA, FM059a2TFA,

and FM052a4TFA, on PANC-1 cells which highly express hOT7T175. We found that the cell growth was not affected by these 3 peptides. In migration assay, metastatin, FM059a2TFA, and FM052a4TFA significantly inhibited the migration of PANC-1 cells (Fig. 6). We examined the activation of ERK1/2, p38, and JNK by these variant forms and found that ERK1 and p38 were activated by metastatin and all variant forms of metastatin (Fig. 7).

Discussion

In this study, we have demonstrated for the first time that hOT7T175 is expressed in pancreatic cancer tissues, but KiSS1 is less expressed when compared to normal pancreatic tissues. These results are in agreement with the analysis by other investigators in ovarian cancer, breast cancer, and colon cancer [2,5]. Several reports indicated that KiSS1 and its receptor hOT7T175 are also highly expressed in placenta [4,5]. The placenta is an invasive tissue, and there are similarities in the behavior of invading cancer cells and that of invading placenta cells [17]. It is possible that KiSS1 and hOT7T175 may constitute a common mechanism in both of these processes, whereas the correlations of clinicopathological factors such as distant metastasis and invasion with KiSS1/hOT7T175 function have not been clearly proved yet.

We next demonstrated that PANC-1 cells, which express hOT7T175, showed significant suppression of cell migration with concomitant activation of ERK1 but not

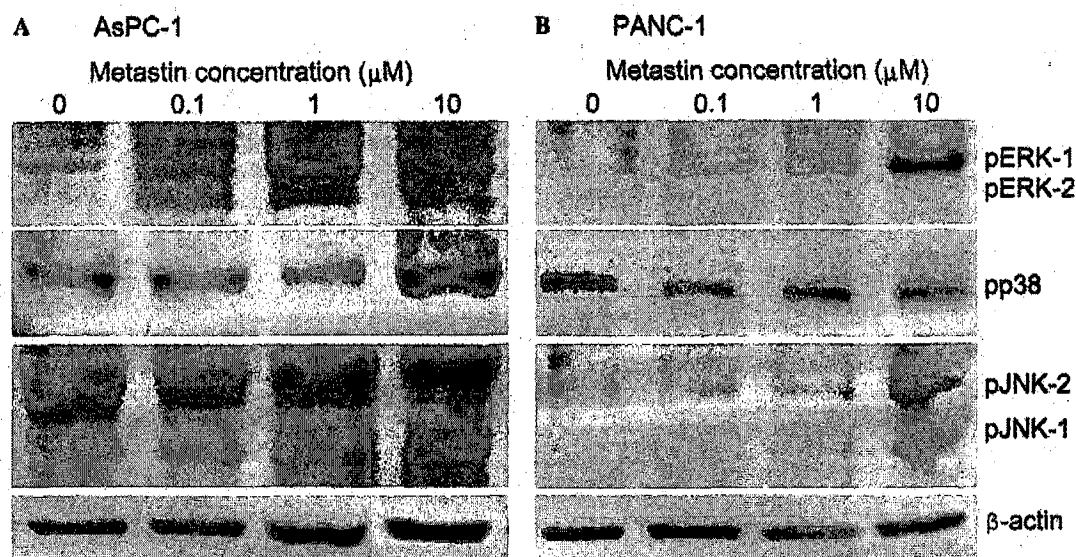


Fig. 4. Effects of metastatin on MAPK activation in AsPC-1 and PANC-1 cells. AsPC-1 and PANC-1 cells were treated with various concentrations of metastatin for 15 min. Western blot analysis identified double band corresponding to phosphorylated ERK1 (pERK1) and phosphorylated ERK2 (pERK2), single band of phosphorylated p38 (pp38), and double band showing phosphorylated JNK1 (pJNK1) and phosphorylated JNK2 (pJNK2). pERK1 was augmented in AsPC-1 by metastatin at 1 and 10 μM and in PANC-1 by metastatin at 0.1, 1, and 10 μM . p38 was augmented in PANC-1 by metastatin at 10 μM .

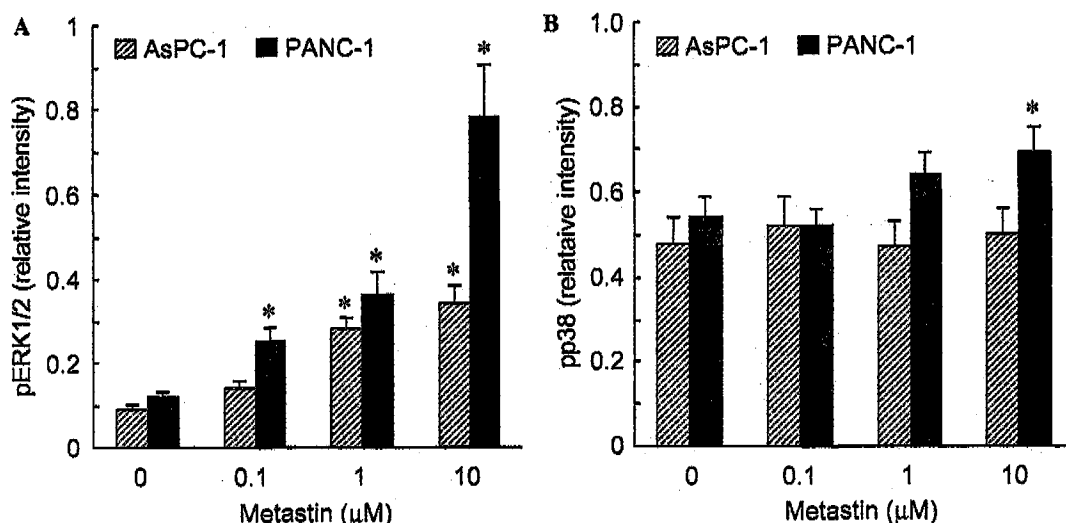


Fig. 5. Effects of metastatin on pERK1/2 and of pp38 in AsPC-1 and in PANC-1 cells. AsPC-1 and PANC-1 cells were treated with various concentrations of metastatin for 15 min. Protein expression of pERK1/2 or pp38 was measured by a densito-analyzer system and the quantity was expressed numerically. The quantity of the protein was divided by that of β -actin and the relative intensities were calculated. Metastatin induced a significant increase of pERK1 in AsPC-1 cells at 1 and 10 μ M and in PANC-1 at 0.1–10 μ M (A). Metastatin induced a significant increase of pp38 in PANC-1 cells at 10 μ M (B). Experiments were repeated three times and data are expressed as means $\pm$ SEM. * Represents $p < 0.05$ against control.

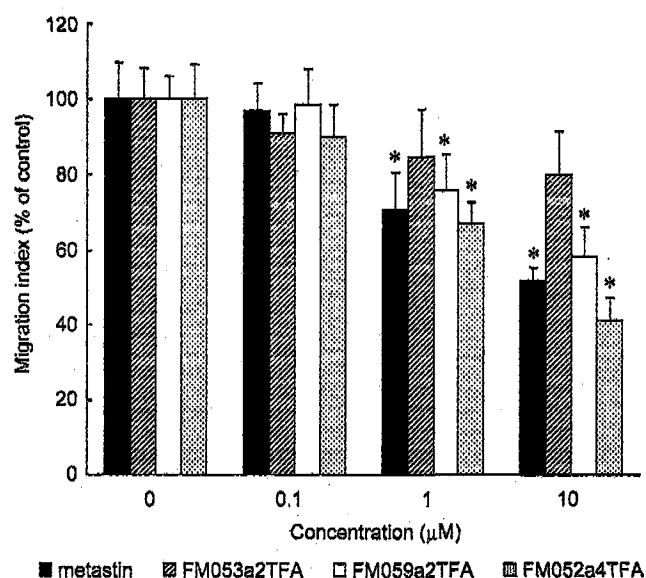


Fig. 6. Effects of short variant forms of metastatin on migration of hOT7T175 expressing pancreatic cancer cells. The effect of newly synthesized short variant forms of metastatin on migration of PANC-1 cells was evaluated. Metastatin, FM059a2TFA, and FM052a4TFA significantly inhibited the migration activity of PANC-1 cells. Experiments were repeated three times and data are expressed as means $\pm$ SEM. * Represents $p < 0.05$ against control.

of JNK1/2 in response to exogenous metastatin, while AsPC-1 cells with low expression of hOT7T175 revealed comparatively less response. Of note, the cell growth suppression was not observed both in PANC-1 and in AsPC-1, that is consistent with the previous results in melanoma and in breast cancer cells [1,2]. In contrast,

in other types of cells, KiSS1 product was reported to activate ERKs and to inhibit cell proliferation [7]. These controversial results suggest that the proliferative characteristics were not a property shared by this receptor. Rather this, ERK activation may be involved in suppression of the tumor cell motility. In our study, suppression of the motility of PANC-1 cells was concomitant with the activation of ERK pathway. Moreover, as demonstrated by the newly synthesized short peptide treatment, the rate of ERK activation is in proportion to the suppression of migration.

Activation of MAP kinase and p38 has been described in hOT7T175 transfected Chinese hamster ovary cells (CHO cells) [7]. However, it has been shown that only MAP kinase but not p38 was activated with metastatin treatment in anaplastic thyroid cancer cells ARO, which endogenously express hOT7T175 [18]. This discrepancy may be partly accounted by the expression level of hOT7T175 and may be by the cell specificity. In our experiment, PANC-1 cells with high expression of hOT7T175 showed activation of ERK1 and p38, while AsPC-1 cells with less hOT7T175 expression did not. These results may indicate the necessity of strong metastatin-hOT7T175 signals on p38 activation in cancer cells.

We found metastatin did not suppress invasion of PANC-1 and AsPC-1 cells, although several authors have reported that metastatin suppresses invasion of melanoma and breast cancer cells [2,5]. The inhibitory effect on invasion could be explained by the report that showed that KiSS1 represses the invasion of HT1080 cells through decreased type 4 collagenase (MMP-9) expression and downregulation of NF- κ B [3]. In pancreatic cancer tissues, we previously reported that active

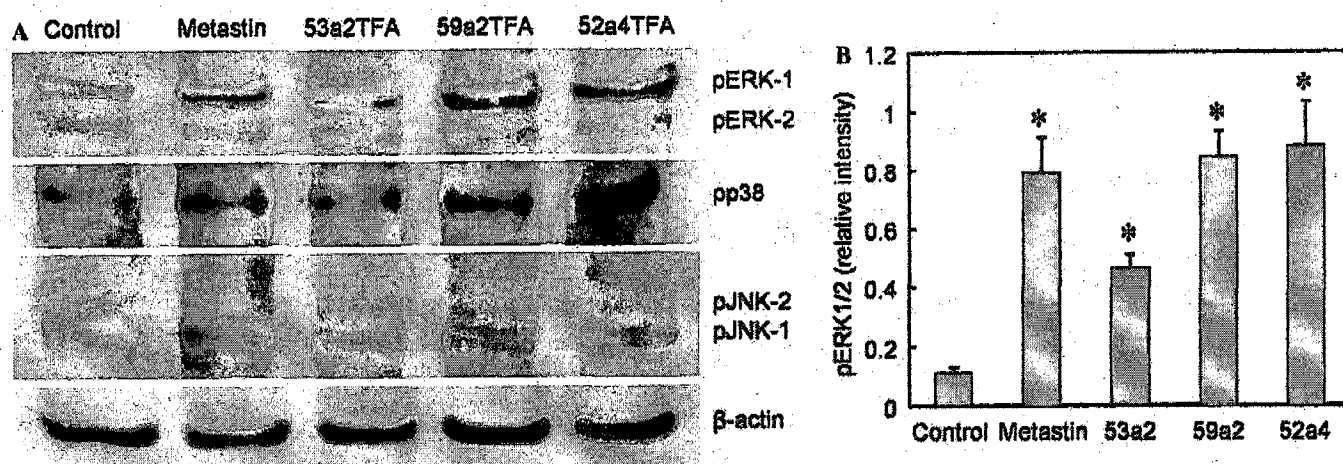


Fig. 7. Effects of short variant forms of metastatin on MAPK activation in hOT7T175 expressing pancreatic cancer cells. (A) The activation of ERK1/2, p38, and JNK ERK1 activation by metastatin and short variant forms of metastatin was observed in PANC-1 cells. (B) pERK1 expression was measured by a densito-analyzer system, the quantity of the protein was divided by that of β -actin, and the relative intensities were calculated. In PANC-1 cells, ERK1 was significantly activated by metastatin and all variant forms of metastatin. Experiments were repeated three times and data are expressed as means $\pm$ SEM. * Represents $p < 0.01$ against control.

form of MMP-2 was detected in all samples; however, active form of MMP-9 was seen in only 21% of the samples [19]. Because latent forms of MMP-2 and MMP-9 were expressed in all pancreatic cancer tissues, MMP-9 may not mainly contribute to cancer invasion when compared to MMP-2. This might be one of the reasons why metastatin did not affect invasion of PANC-1 and AsPC-1 cells. To our knowledge, there has been no other investigation on migration or invasion of cancer cells which endogenously express hOT7T175.

Our newly synthesized short variant forms of metastatin showed significant suppression on motility of PANC-1 cells. These peptides are composed of 6–9 amino acids, suggesting that at most 10 amino acids of the C-terminus of metastatin-54 will be sufficient for its binding affinity and function. However, FM053a2TFA is less effective in suppressing migration of PANC-1 cells when compared to other two compounds or metastatin-54. Interestingly, the suppressive activity was in proportion to the ERK activation rates. Much remains to be understood about how effective they will block migration and how the peptides could be stably delivered to the tumors. The effective short peptide has an advantage that it would not cause immune responses if it could be given to patients orally.

In conclusion, we demonstrated that pancreatic cancer tissues express hOT7T175 and low expression of KiSS1 when compared to normal pancreatic tissues. The exogenous metastatin and the variant forms of metastatin suppress migration of hOT7T175-expressing pancreatic cancer cells and activate ERK1 and p38. Our results suggest that hOT7T175 may be one of the promising targets against cancer cell functions that are relevant to metastasis, and that short variant forms of metastatin could be an anti-metastatic agent to pancreatic cancer.

Acknowledgment

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Design of Selective Peptidomimetic Agonists for the Human Orphan Receptor BRS-3^{*}

Dirk Weber,[†] Claudia Berger,[‡] Peter Eickelmann,^{*,§} Jochen Antel,[†] and Horst Kessler^{*,†}

Institut für Organische Chemie und Biochemie, Technische Universität München, Lichtenbergstrasse 4, 85747 Garching, Germany, and Solvay Pharmaceuticals GmbH, Hans-Böckler-Allee 20, 30173 Hannover, Germany

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New tool substances may help to unravel the physiological role of the human orphan receptor BRS-3 and its possible use as a drug target for the treatment of obesity and cancer. In continuation of our work on BRS-3, the solid- and solution-phase synthesis of a library of low molecular weight peptidomimetic agonists based on the recently developed short peptide agonist **4** is described. Functional potencies of the compounds were determined measuring calcium mobilization in a fluorometric imaging plate reader (FLIPR) assay. Focusing on the N-terminus, the D-Phe-Gln moiety of **4** was modified in a combinatorial SAR-oriented medicinal chemistry approach. With the incorporation of N-arylated glycine and alanine building blocks azaglycine, piperazine, or piperidine and the synthesis of semicarbazides and semicarbazones, a number of highly potent and selective compounds with a reduced number of peptide bonds were obtained, which also should have enhanced metabolic stability.

Introduction

G-protein-coupled receptors (GPCRs) represent the largest and most important class of all drug targets.¹ Currently, worldwide more than 50% of all drugs are GPCR-based and their annual sales exceeded 30 billion U.S. dollars in 2001.² Owing to this proven track of being excellent drug targets, it is commonly assumed that orphan GPCRs, which emerged from genomic research, will offer a similar aptitude in the future.^{3,4} About 1–3% of our genome encode approximately 1000 GPCRs, a number that was until recently predicted to be much larger.⁵ Excluding sensory receptors, there are presently only about 150 orphan GPCRs.⁴ Although the orphan receptor strategy and reverse pharmacology approach⁶ have led to the identification of many natural ligands in recent years, the proportion of available orphan receptor sequences that have been "deorphanized" in this manner remains relatively small. New approaches such as the constitutively activating receptor technology (CART) are gaining increasing attention,⁷ also because it is now a generally accepted concept that various ligands can bind to different binding sites to have different effects on GPCRs.^{7–9} The identification of a natural ligand is an important step in the target validation of orphan GPCRs,¹⁰ however, in the case of the orphan receptor bombesin receptor subtype 3 (BRS-3), a naturally occurring high-affinity ligand is still unknown. The BRS-3 receptor was assigned to the bombesin (Bn) receptor family because of its high sequence homology with the two mammalian bombesin receptors neuromedin B receptor (NMB-R) and gastrin-releasing peptide receptor (GRP-R).^{11,12}

Data obtained from the knock-out mouse model implicated BRS-3 with the regulation of energy balance, body weight, and blood pressure because mice lacking functional BRS-3 developed mild obesity, hypertension, and diabetes.¹³ Second, it has been observed that the BRS-3 receptor is expressed on several human carcinoma cell lines.^{11,14} In general, bombesin-like peptides (BLPs) are involved in the growth regulation of various cancers, especially small-cell lung cancer (SCLC) cell lines.¹⁵ Expression of BRS-3 in mammals, as determined by distribution of BRS-3 mRNA, is species-dependent and limited^{16,17} compared to the widely expressed NMB-R and GRP-R.¹⁸ Very recently, BRS-3 was connected with the treatment of neurological disorders.¹⁹ Despite the development of the high-affinity ligand [D-Tyr⁶,β-Ala¹¹,Phe¹³,Nle¹⁴]Bn(6–14) (**1**) and its analogues,²⁰ which allowed receptor pharmacology studies,^{20,21} more tool substances are needed to find out about the possible usefulness of BRS-3 as a drug target. Recently, we and others^{22,23} performed structure–activity relationship studies of **1** mainly by utilizing its ability to mobilize intracellular calcium in BRS-3 transfected CHOα-16 cells in a FLIPR assay. With the obtained information, we applied a strategy similar to the "peptoid" approach,²⁴ which was used to design peptidomimetic antagonists for several GPCRs^{25–27} and subsequent C-terminal optimization to develop the selective short peptide agonist **4** (Table 1 and Figure 1) for the human BRS-3 receptor.²² In continuation of this work, we describe the solid- and solution-phase synthesis of a library of low molecular weight peptidomimetic BRS-3 agonists based on the short peptide agonist **4**, which should have enhanced metabolic stability. Besides the incorporation of peptoid building blocks,^{28–32} the synthesized BRS-3 agonists include a number of azapeptides,³³ a class of compounds that was successfully developed into drugs,^{33–35} semicarbazides, semicarbazones, and compounds containing a piperazine/piperidine scaffold (Figure 1).

^{*} Dedicated to Prof. George M. Sheldrick on the occasion of his 60th birthday.

[†] To whom correspondence should be addressed. Phone: ++49+89/289-13300. Fax: ++49+89/289-13210. E-mail: kessler@ch.tum.de.

[‡] Technische Universität München.

[§] Solvay Pharmaceuticals GmbH.

[§] Present address: Boehringer Ingelheim Pharma KG, Biberach an der Riss, Germany.

Table 1. Functional Potencies from 4 to 30 Independent Concentration–Response Curves of [D-Phe⁶,β-Ala¹¹,Phe¹³,Nle¹⁴]Bn(6–14) (1), Endogenous Ligands NMB (2) and GRP (3), and the Synthetic Agonist 4 in CHO Cells Transfected with the Human Bombesin Receptors NMB-R, GRP-R, and BRS-3^a

compd	peptide	EC ₅₀ [nM]		
		NMB-R	GRP-R	BRS-3
1	[D-Phe ⁶ ,β-Ala ¹¹ ,Phe ¹³ ,Nle ¹⁴]Bn(6–14)	310 (260–380)	84 (69–100)	151 (134–170)
2	NMB	120 (91–150)	41 (36–47)	4500 (4100–4900)
3	GRP	3200 (2400–4400)	34 (24–49)	6400 (6100–6700)
4	[Qw-1-(2-phenylethyl)amide	inactive	inactive	710 (590–860)

^a For further experimental details see Table 2 or Supporting Information.

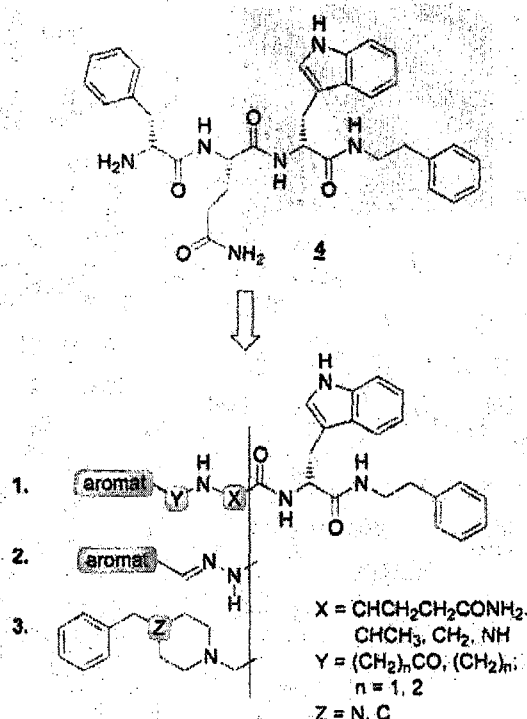


Figure 1. Development of peptidomimetic BRS-3 agonists from lead structure 4. The marked region of structure 4 shows the previously optimized part of the molecule, which was left unchanged in this study.

Chemistry

General Strategy. Recently, we described the design of the short peptide agonist H-D-Phe-Gln-D-Trp-1-(2-phenylethyl)amide (4).²² Previous results suggested that the side chain of the Gln residue is not essential for functional activity; however, all three aromatic moieties are essential.²² Furthermore, a stereochemical change of the N-terminal D-Phe did not affect functional potency.²² Consequently, our work was directed toward the N-terminal part of the molecule while leaving the C-terminal part D-Trp-1-(2-phenylethyl)amide, which already has been optimized,²² unchanged. We tried to modify the residues D-Phe-Gln step by step, to elaborate the structural features of this part of the agonist required for biological activity, to reduce it to a minimum fragment, and moreover to replace it with proteolytically more stable surrogates.

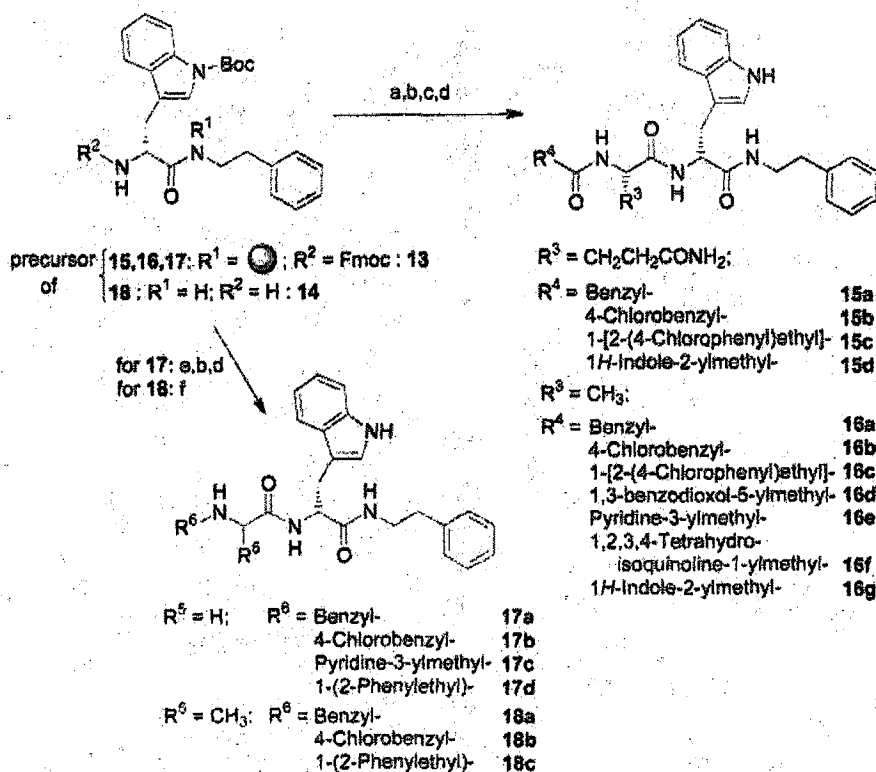
Removal of N-Terminal NH₂ and Side Chain of the Gln (Compounds 15a–d, 16a–g). First, to remove the N-terminal chirality and increase lipophilicity, peptides with removed N-terminal amino function (15a–d) were prepared (Scheme 1).³⁶ For synthesis, FMPE resin was reductively aminated and loaded with Fmoc-D-Trp-OH to yield 13 in a procedure similar to the one

previously described.³⁷ Chain extension including the coupling of the N-terminal building blocks 7a–c,^{38–41} was carried out using standard Fmoc protocols⁴² with TBTU/HOBt/DIEA activation.⁴³ Peptides with an additional substitution of Gln by Ala (16a–g) were prepared similarly (Scheme 1).

Insertion of N-Substituted Glycine/Alanine (Compounds 17a–d and 18a–c). In the second step, the D-Phe-Gln unit was replaced with N-substituted glycines and alanines. All Fmoc-protected peptidomonomers, except 11c, were synthesized in two steps. First, 2-(arylalkylamino)acetic acid ethyl esters (8a,b,d) and 2-(arylalkylamino)propionic acid ethyl esters (10a–c) were prepared in a modified, previously described procedure.⁴⁴ Then, the esters were hydrolyzed with aqueous NaOH and Fmoc was introduced without intermediate purification to yield building blocks 11a,b,d and also 12a–c, which were obtained as racemic mixtures. 2-{Pyridine-3-ylmethyl}[(9H-fluorene-9-ylmethoxy)carbonyl]aminoacetic acid (11c) was synthesized in three steps⁴⁵ via the *tert*-butyl ester 8c, which was Fmoc-protected to give 9. Final cleavage of the *tert*-butyl group was carried out with TFA/TIPS (10:1) (v/v). Assembly of the peptidomimetic hybrids 17a–d was carried out on solid support³⁶ using TBTU/HOBt/DIEA activation⁴³ (Scheme 1). For synthesis of compounds 18a–c, Fmoc-protected peptidomonomers 12a–c were coupled to 14 in solution with HATU/HOAt/collidine activation⁴⁶ (Scheme 1). Cleavage of the Boc-protecting group from D-Trp turned out to be incomplete after treatment with TFA/TIPS (10:1) (v/v) at 0 °C. ESI mass spectral data indicated that the carbaminic acid was stable under these highly acidolytic water-free conditions.⁴⁷ Destruction of the carbaminic acid was achieved by treatment with DMSO/H₂O/HOAc (8:1:1) (v/v). Compounds 18a–c were tested as racemic mixtures.

Synthesis of Azapeptides 21a–e. In the next step, Gln was replaced by azaglycine. Numerous routes have been described for the synthesis of azapeptides in solution,³³ but besides the synthesis of azatides,⁴⁸ only a few were reported for the preparation on solid support.^{49–51} Other methods suffer from major drawbacks such as hydantoin formation and a slow reaction rate⁵⁰ or give rise to considerable amounts of byproducts such as the in situ activation of Fmoc-hydrazine with triphosgene.^{51,52a} To overcome these drawbacks, we incorporated azaglycine in the solid-phase synthesis of azapeptides 21a–e using an excess of freshly prepared 5-(9H-fluorene-9-ylmethoxy)-1,3,4-oxadiazol-2(3H)-one (19).⁵² Therefore, 13 was Fmoc-deprotected and reacted with 19, which was obtained from the activation of Fmoc-hydrazine with an excess of a solution of phosgene

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Scheme 1^a

^a Reagents: (a) (only 13, 20% piperidine/NMP), Fmoc-Gln(Trt)-OH or Fmoc-Ala-OH, TBTU/HOBt/DIEA, NMP; (b) 20% piperidine/NMP; (c) carboxylic acid building blocks 7a–g (compounds 7a–e were purchased from commercial sources), TBTU/HOBt/DIEA, NMP; (d) TFA/TIPS/H₂O (18:1:1); (e) [arylkyl-[(9H-fluoren-9-ylmethoxy)carbonyl]amino]acetic acid (11a–d), TBTU/HOBt/DIEA, NMP; (f) (1) 2-[arylkyl-[(9H-fluoren-9-ylmethoxy)carbonyl]amino]propionic acid (12a–c), HATU/HOAt/collidine, DMF, (2) TFA/CH₂Cl₂/TIPS (10:10:1), (3) DMSO/HOAc/H₂O (8:1:1), (4) 20% piperidine/DMF (65–87%).

in toluene as previously described⁵² (Scheme 2). Diastereomers 21d1 and 21d2 were separated by HPLC.

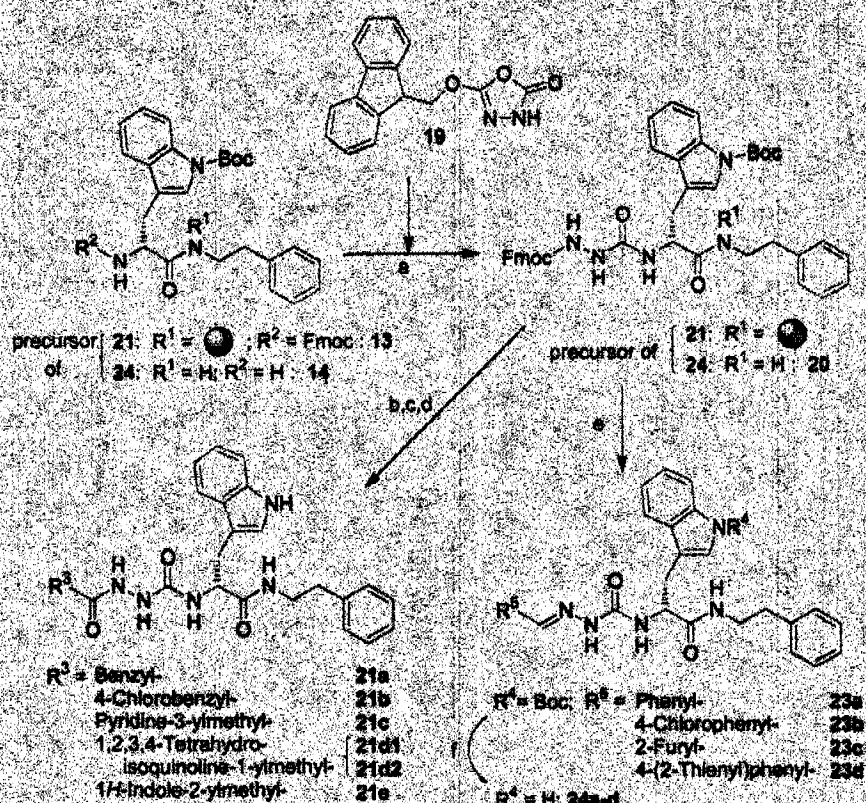
Synthesis of Semicarbazones 24a–d. Analogues of the azapeptides 21 lacking an N-terminal peptide bond and a C–N double bond were prepared in solution (Scheme 2). Acylation of amine 14 with freshly prepared oxadiazolone 19 in DMF at room temperature resulted in aza compound 20, which was then Fmoc-protected and without intermediate purification reacted with aldehydes 22a–d in THF at room temperature. Imine bond formation was slow probably because of residual basicity; however, since the desired compounds 23a–d were obtained in acceptable yields, optimization of this process was not pursued. Aldehydes 22a–c were purchased from commercial sources, whereas 4-(2-thienyl)benzaldehyde (22d) was synthesized from 4-bromobenzaldehyde via Suzuki coupling.⁵³ Final Boc deprotection under first strong and then mild acidic conditions gave semicarbazones 24a–d.

Synthesis of Semicarbazides 28a–c. The synthesis of analogues of azapeptides 21 lacking an N-terminal peptide bond afforded the preparation of suitably protected azaamino acid constituents (Scheme 3). Therefore, Boc-hydrazine was reacted to the Boc-protected hydrazones 25a–c with the appropriate aldehydes in THF.⁵⁴ Contrary to previous reports, no conversion was observed when reduction of 25a was attempted through hydrogenation over 5% Pd/C in THF as a solvent at room temperature,⁵⁴ both at atmospheric pressure or at 50 atm. However, reduction with sodium cyanoborohydride⁵⁵ afforded the desired Boc-protected arylalkylhydrazines 26a–c in good yields. It was further planned

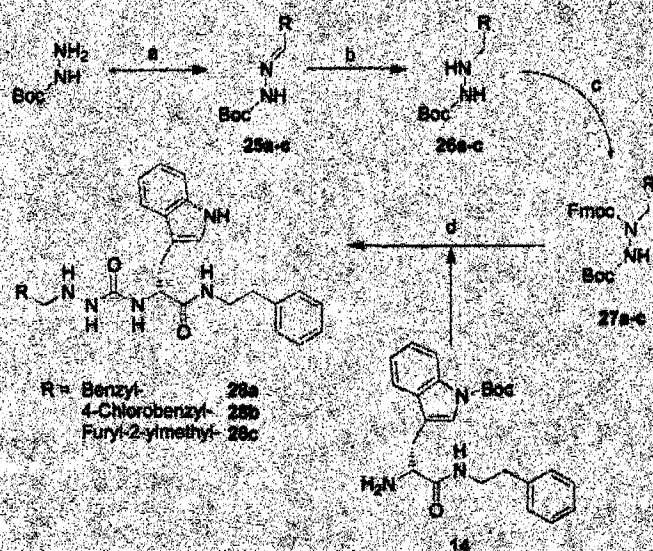
to convert these building blocks into acylating agents selectively at the unsubstituted nitrogen atom of the hydrazine after Boc deprotection in the following steps. Because the site of acylation is dependent on the nature of the substituted hydrazine and the activation reagent and therefore was difficult to predict,^{48,52a,54} Fmoc was introduced at the arylalkylated nitrogen to give building blocks 27a–c. Preliminary attempts to cleave the Boc protecting group were carried out by careful treatment with TFA/CH₂Cl₂/TIPS/H₂O (4:14:1:1) (v/v) at 0 °C and showed approximately 70% deprotection after 1 h as monitored by TLC and HPLC–MS. However, we observed unexpected degradation of the dried deprotected species at room temperature within 24 h. Surprisingly, attempts to remove the Boc with 4 N HCl in dioxane as a solvent at room temperature^{35a} failed because of insufficiently low reaction rates. Therefore, it was decided to cleave the Boc group of 27a–c under more drastic conditions than aforementioned with TFA/CH₂Cl₂/TIPS (20:20:1) (v/v). Without further workup, activated carbazic acid esters were immediately generated using bis(pentafluorophenyl)carbonate and without isolation instantly reacted with the amine 14.^{48,56} This method worked very well and was found to be superior to the use of a solution of phosgene in toluene,^{52a} which seems to require more subtle adjustment of the reaction conditions. Direct cleavage of Boc and Fmoc without intermediate purification finally furnished the desired semicarbazides 28a–c (Scheme 3).

Scaffolds Containing Piperidine/Piperazine (Compounds 35 and 36). Building blocks (4-benzylpiperazin-1-yl)acetic acid (31) and its piperidine ana-

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Scheme 2^a

^a Reagents: (a) 5-(9*H*-fluoren-9-ylmethoxy)-1,3,4-oxadiazol-2(3*H*)-one (19), CH₂Cl₂ (68%, for R¹ = H); (b) 20% piperidine/NMP; (c) carboxylic acid building blocks 7a,b,e-g (compounds 7a,b,e were purchased from commercial sources), TBTU/HOBt/DIEA, NMP; (d) TFA, TIPS, H₂O (18:1:1); (e) (1) 20% piperidine/DMF, (2) arylaldehydes 22a-d (compounds 22a-c were purchased from commercial sources), THF (47-83%); (f) (1) TFA/CH₂Cl₂/TIPS (30:30:1), (2) DMSO/HOAc/H₂O (8:1:1) (56-63%).

Scheme 3^a

^a Reagents: (a) arylaldehydes 22a-c (compounds were purchased from commercial sources), THF (82-96%); (b) (1) NaCNBH₃, THF, AcOH, (2) NaHCO₃, (3) 1*N* NaOH, MeOH (71-93%); (c) Fmoc-Cl, NaHCO₃, dioxane (72-98%); (d) (1) TFA/CH₂Cl₂/TIPS (20:20:1), (2) *tert*-butyl-3-[(2*M*)-2-amino-2-[1-(2-phenylethyl)carbamoyl]ethyl]-1*H*-1-indolecarboxylate (14), bis(pentafluorophenyl)carbonate, DMAP, CH₂Cl₂, (3) TFA/TIPS (40:1), CH₂Cl₂, (4) 20% piperidine/DMF (9-21%).

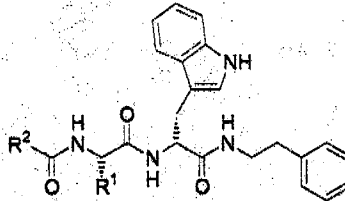
logue 32 were prepared in two steps similar to that described for 8a-d followed by saponification. After coupling to amine 14 using TBTU/HOBt/DIEA activation, Boc deprotection carried out as described above

for the synthesis of 18a-c yielded compounds 35 and 36.

Results and Discussion

The synthesized library is derived from the structure of the recently published BRS-3 agonist 4, which was obtained from the C-terminal optimization of the lead structure H-D-Phe-Gln-D-Trp-Phe-NH₂.²² Functional activity of the compounds was assessed in a FLIPR assay. Therefore, their ability to increase the level of intracellular calcium in CHO cells transfected with the human bombesin receptors NMB-R, GRP-R, and BRS-3 was measured and referenced to the peptide agonist [D-Phe⁶,β-Ala¹¹,Phe¹³,Nle¹⁴]Bn(6-14) (1) for BRS-3, endogenous ligands NMB (2) for NMB-R, and GRP (3) for GRP-R. Further experimental details of this assay have been published earlier²² and are given in the Supporting Information.

One of the most reliable methods in medicinal chemistry to improve in vitro activity is to incorporate properly positioned lipophilic groups or, as could be suggested for our case, to remove "unnecessary" hydrophilic groups. Although C-terminal optimization of the aforementioned lead structure, which resulted in the removal of the amide function, was sufficient to increase functional potency about 15-fold to generate agonist 4 with an EC₅₀ of 710 nM,²² we hoped that further improvement of functional activity could be achieved by exploiting the N-terminal SAR of this molecule. On the other hand, we were aiming at oral availability, which requires solubility. Therefore, regarding Lipinski's "rule of five",⁵⁷ lipophilicity, which can be estimated and

Table 2. Functional Potencies from 4 to 11 Independent Concentration-Response Curves of Modified Analogues of 4 with N-Terminally Deleted Amino Function (15a-d)^a


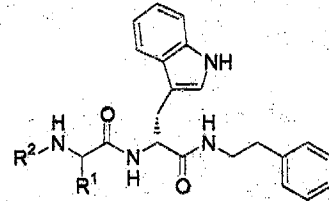
compd	R ¹	R ²	BRS-3		
			EC ₅₀ [nM]	E _{max}	cLogP
15a	(CH ₂) ₂ CONH ₂	benzyl-	1200 (680–2100)	38 ± 0.8	1.81
15b	(CH ₂) ₂ CONH ₂	4-chlorobenzyl-	1400 (990–2000)	62 ± 3.8	2.52
15c	(CH ₂) ₂ CONH ₂	1-[2-(4-chlorophenyl)ethyl]-	230 (170–320)	68 ± 5.9	2.91
15d	(CH ₂) ₂ CONH ₂	1 <i>H</i> -indole-2-ylmethyl-	57 (26–120)	82 ± 4.7	1.8
16a	CH ₃	benzyl-	2.1 (1.2–3.9)	71 ± 4.7	3.62
16b	CH ₃	4-chlorobenzyl-	140 (75–270)	75 ± 8.5	4.33
16c	CH ₃	1-[2-(4-chlorophenyl)ethyl]-	6400 (5800–7200)	30 ± 2.2	4.72
16d	CH ₃	1,3-benzodioxol-5-ylmethyl-	7.8 (2.8–22)	75 ± 7.0	3.18
16e	CH ₃	pyridine-3-ylmethyl-	32 (23–44)	74 ± 1.6	2.12
16f	CH ₃	1,2,3,4-tetrahydroiso-quinoline-1-ylmethyl-	310 (250–390)	69 ± 8.1	3.83
16g	CH ₃	1 <i>H</i> -indole-2-ylmethyl-	79 (50–120)	69 ± 1.2	3.61

^a Additionally, in compounds 16a–g, Gln was replaced by Ala. Functional potencies of the peptides were determined in a previously described FLIPR assay²² (see also Supporting Information) and are given as EC₅₀ ± SEM.⁶³ E_{max} represents the Ca²⁺ signal at 16 μM in percent of maximal response of 1. All compounds tested were inactive on the NMB-R and GRP-R except for 15a, with EC₅₀ = 15.0 μM (13.9–16.3) on NMB-R and EC₅₀ = 19.1 μM (15.5–23.5) on GRP-R, and for 15b, with EC₅₀ = 18.5 μM (16.6–20.7) on NMB-R and EC₅₀ = 12.2 μM (11.1–13.3) on GRP-R. For further experimental details, see Supporting Information.

expressed by the *n*-octanol/water partition coefficient cLogP value, should not exceed the magnitude of five. First, we deleted the N-terminal aminofunction of the agonist 4 (Scheme 1, Figure 1). As a result of this, the cLogP value increased from 1.3 to 1.81 (Table 2), compared to the augmentation of 0.1 of H-D-Phe-Gln-D-Trp-Phe-NH₂ to 1.3 of agonist 4. As shown in Table 2 (compounds 15a–d), the effect of this increase of lipophilicity on functional potency at BRS-3 is low except for compound 15d, with an increase of about 12-fold. Compounds 15a and 15b showed functional potencies on the NMB-R and GRP-R in the micromolar range.

In the second step, a series of compounds, where in addition to the deleted amino function Gln was replaced by Ala and the N-terminal aromatic moiety was differently substituted or replaced by heteroaromatic moieties, were investigated (Table 2). Although the synthetic effort to get from lead structure H-D-Phe-Gln-D-Trp-Phe-NH₂ to compounds 16 was relatively small, the impact on improvement of functional potency was enormous. With compounds 16a and 16d, potencies in the nanomolar range were obtained. Furthermore, all compounds showed excellent selectivity for BRS-3. As seen in the preceding series, for compounds 15a–d, indole-2-yl/3-pyridyl was clearly favored as the N-terminal aromatic moiety over 4-chlorophenyl. On the other hand, these alterations probably do not represent much progress concerning proteolytic stability, since these compounds still comprised three peptide bonds. Therefore, efforts were directed toward a reduction of the peptidic character of the compounds.

In compounds 17a–d the N-terminal peptide bond was removed by incorporation of N-substituted glycine, and in compounds 18a–c it was removed by N-substituted alanine (Table 3). Thus, it is assumed that these modifications introduce a greater flexibility to the backbone accompanied by enhanced metabolic stability.^{28–31} Deletion of the carbonylfunction caused cLogP values to slightly increase by about 0.2–0.7.

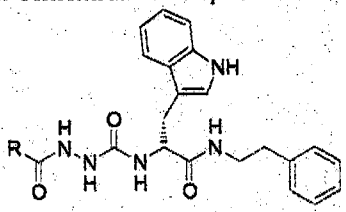
Table 3. Functional Potencies from 7 to 11 Independent Concentration-Response Curves of Modified Analogues of 4 Containing Peptoid Monomer Building Blocks (17a–d) and N-Arylated 2-Aminopropionic Acid Building Blocks (18a–c)^a


compd	R ¹	R ²	BRS-3		
			EC ₅₀ [nM]	E _{max}	cLogP
17a	H	benzyl-	21 (14–31)	87 ± 6.4	3.86
17b	H	4-chlorobenzyl-	2.9 (1.2–6.9)	81 ± 1.7	4.58
17c	H	pyridine-3-ylmethyl-	21 (12–36)	82 ± 8.4	2.37
17d	H	1-(2-phenylethyl)-	17 (8.9–31)	92 ± 16.0	4.08
18a	CH ₃	benzyl-	46 (34–64)	86 ± 12.8	4.17
18b	CH ₃	4-chlorobenzyl-	4.0 (2.3–7.3)	83 ± 12.5	4.89
18c	CH ₃	1-(2-phenylethyl)-	25 (19–34)	82 ± 9.6	4.39

^a All compounds tested were inactive on the NMB-R and GRP-R. For further experimental details, see Table 2 or Supporting Information. E_{max} represents the Ca²⁺ signal at 16 μM in percent of maximal response of 1.

Here, in contrast to the preceding series, the 4-chloro-substituted compounds 17b and 18b showed the highest functional potency with EC₅₀ values around 3–4 nM. The explanation for this shift of high functional potency could probably be that a high lipophilicity is favorable but only at the correct distance from the tryptophan. This distance is obviously too large for compounds 15 and 16. Moreover, a basic functionality located more closely to the tryptophan seems to enhance functional activity. The overall series of compounds 17 and 18 demonstrated that the carbonyl function or the planarity caused by the peptide bond was not contributing to the binding mode of the compound to the receptor and therefore could be omitted.

Moreover, we attempted to exchange C^αH by N^α, which leads to a loss of one chiral center and to a

Table 4. Functional Potencies of Azapeptides **21a–e** from 7 to 14 Independent Concentration–Response Curves^a


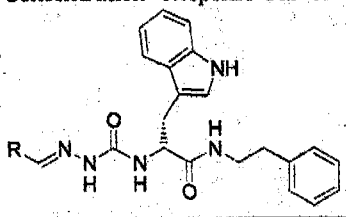
compd	R	EC ₅₀ [nM]	E _{max}	cLogP
21a	benzyl-	3.1 (2.1–4.6)	86 ± 9.5	3.61
21b	4-chlorobenzyl-	0.19 (0.06–0.58)	89 ± 13.5	4.33
21c	pyridine-3-ylmethyl-	1.4 (0.7–2.5)	102 ± 26.1	2.12
21d1	1,2,3,4-tetrahydroiso-	9.3 (7.1–12.3)	95 ± 15.0	3.83
21d2	quinoline-1-ylmethyl-	19 (15–25)	91 ± 15.1	3.83
21e	1 <i>H</i> -indole-2-ylmethyl-	2.2 (0.8–5.9)	84 ± 4.3	3.6

^a All compounds tested were inactive on the NMB-R and GRP-R. For further experimental details, see Table 2 or Supporting Information. E_{max} represents the Ca²⁺ signal at 16 μM in percent of maximal response of 1.

conformational change.⁵⁸ Nevertheless, there are many examples where amino acids have been successfully replaced by their corresponding azaamino acids with retention of biological activity accompanied by an increase of metabolic stability.^{35,49,52} Ab initio calculations showed that diacylhydrazine has relatively easy access to different conformations; however, its flexibility was substantially constrained by N-methylation.^{58a} It is generally believed that a critical amount of rigidity is a prerequisite for high activity and selectivity.⁵⁹ On the other hand, scaffolds bearing azaglycine as their core and structurally dominating unit demonstrated possession of these features.⁵² Obviously the same proves to be true in this case. All azapeptides show excellent activities and selectivities, especially compound **21b** with an EC₅₀ value in the subnanomolar range (Table 4). Although the distance to the lipophilic, 4-chloro-substituted moiety seems to be larger than in compounds **17** and **18** with the removed peptide bond, it is feasible that the azaglycine induces a bentlike structure. Therefore, the actual length of the compound in its bioactive conformation could resemble that of the peptoid–peptide hybrids.

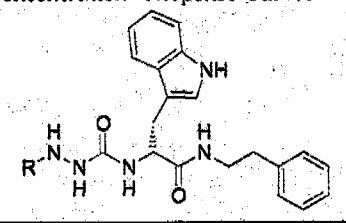
In the next step, we investigated the necessity of the N-terminal peptide bond in the aza compounds. Functional potencies of the semicarbazides **28a–c** were lower than those of the corresponding azapeptides **21a–e** but, as expected, were still excellent (Table 6). As for the peptoid–peptide hybrids, removal of the carbonyl group caused the cLogP values to increase about 0.3 order of magnitude, and the 4-chloro-substituted compound **28b** showed the highest activity. Insertion of a C–N double bond (semicarbazones **24a–d**; Table 5) caused another increase of the cLogP value of about 0.3. This increase was tolerated for 2-furyl as the aromatic moiety but not for too lipophilic residues. In the latter, the drop of functional activity correlated with increasing cLogP value, and because of the high activity of **24c**, it probably was not conformationally related. Taken together, the results confirm those obtained from the peptoid–peptide hybrids **17** and **18**, namely, that the N-terminal peptide bond is not involved in receptor activation.

Finally compounds with incorporated piperazine (**35**) and piperidine (**36**) have been tested (Table 7). Here,

Table 5. Functional Potencies of Semicarbazones **24a–d** from 6 Independent Concentration–Response Curves^a


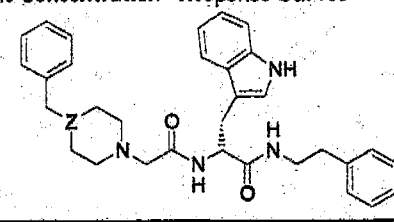
compd	R	EC ₅₀ [nM]	E _{max}	cLogP
24a	phenyl-	240 (110–490)	89 ± 0.4	4.21
24b	4-chlorophenyl-	2700 (1500–5200)	78 ± 0.1	4.92
24c	2-furyl-	1.5 (0.7–3.2)	94 ± 2.6	3.38
24d	4-(2-thienyl)phenyl-	7900 (3800–16200)	58 ± 7.3	5.98

^a All compounds tested were inactive on the NMB-R and GRP-R. For further experimental details see Table 2 or Supporting Information. E_{max} represents the Ca²⁺ signal at 16 μM in percent of maximal response of 1.

Table 6. Functional Potencies of Semicarbazides **28a–c** from 6 Independent Concentration–Response Curves^a


compd	R	EC ₅₀ [nM]	E _{max}	cLogP
28a	benzyl-	15 (8.5–25)	81 ± 0.4	3.9
28b	4-chlorobenzyl-	6.0 (2.3–16)	83 ± 5.3	4.62
28c	furyl-2-ylmethyl-	24 (18–33)	96 ± 14.3	3.08

^a All compounds tested were inactive on the NMB-R and GRP-R. For further experimental details, see Table 2 or Supporting Information. E_{max} represents the Ca²⁺ signal at 16 μM in percent of maximal response of 1.

Table 7. Functional Potencies of Compounds **35** and **36** from 6 Independent Concentration–Response Curves^a


compd	Z	EC ₅₀ [nM]	E _{max}	cLogP
35	N	70 (57–85)	90 ± 1.2	4.98
36	C	30 (4.9–190)	84 ± 5.3	5.85

^a Compounds tested were inactive on the NMB-R and GRP-R. For further experimental details, see Table 2 or Supporting Information. E_{max} represents the Ca²⁺ signal at 16 μM in percent of maximal response of 1.

high activities and selectivities were obtained too; however, the cLogP value clearly exceeded the upper limit of 5.

Additional FLIPR experiments were carried out to assess the selectivity of the new agonists to the BRS-3 receptor over the NMB and the GRP receptors. The ability of compounds **15–18**, **21**, **24**, **28**, **35**, and **36** to inhibit receptor activation of the agonists NMB at NMB-R and GRP at GRP-R was investigated.

As a reference, the known antagonists [D-Phe¹²]Bn, [D-Phe⁶,Leu¹³,*p*-chloro-Phe¹⁴]Bn(6–14), and [D-Phe⁶,Leu-NHET¹³,desMet¹⁴]Bn(6–14) were used.^{60–62} First, a solution of the new agonists and reference antagonists at eight different concentrations were added to CHO cells transfected with the NMB or GRP receptor followed by a solution of the agonist, and calcium mobilization was permanently measured. In all measurements, no reduction of the calcium emission induced by the presence of the aforementioned compounds could be observed. Thus, we conclude that the investigated compounds are not antagonists of the NMB-R and GRP-R.

At present, we are unable to explain the selectivity of the compounds for the BRS-3 receptor.

Conclusions

In summary, we have performed detailed N-terminal structure–activity studies on the known BRS-3 agonist H-D-Phe-Gln-D-Trp-1-(2-phenylethyl)amide (4). On the basis of previous results, which suggested that the side chain of Gln is not essential for functional potency, we developed a library of peptidomimetics with a conserved C-terminal D-Trp-1-(2-phenylethyl)amide moiety and structural variations on the H-D-Phe-Gln unit. It was demonstrated that the N-terminal increase of lipophilicity by simple deletion of the amino function combined with removal of the Gln side chain furnished selective BRS-3 agonists in the nanomolar range. Furthermore, substitution of the H-D-Phe-Gln unit by peptoid monomers showed that the N-terminal peptide bond is not required for receptor activation. Gln can further be replaced by azaglycine, leading to compounds with subnanomolar activities (21b: EC₅₀ = 0.19 nM). For azapeptides, too, a removal of the N-terminal peptide bond is possible, as realized in the semicarbazides 28a–c. However, as shown by the semicarbazones 24a–d, a too lipophilic N-terminus does not seem to be tolerated. The finding that piperidine or piperazine can also be incorporated suggests that a large number of different spacers are able to mimic the function of the former Gln at this position. From the analysis of the substitution and distance pattern for the different series of compounds, it seems very likely that a lipophilicity placed at a certain distance from the tryptophan combined with a basic functionality located between them is favorable for functional potency. A further FLIPR experiment in which the ability of the described peptidomimetics to inhibit activation of the NMB-R and GRP-R by their natural ligands was investigated, showed that these compounds are not antagonists of the NMB-R and GRP-R. This work therefore describes the development of selective tool substances for BRS-3 with improved pharmacokinetic properties, which may prove to be helpful in the understanding of the physiological role of this orphan receptor.

Experimental Section

General. Fmoc-protected amino acids and 2-(4-formyl-3-methoxyphenoxy)ethylpolystyrene (FMPE) resin was purchased from Novabiochem (Darmstadt, Germany). [D-Phe⁶,β-Ala¹¹,Phe¹³,Nle¹⁴]Bn(6–14) (1) was purchased from Polypeptide Laboratories (Wolfenbüttel, Germany). NMB (2), GRP (3), [D-Phe¹²]Bn, [D-Phe⁶,Leu¹³,*p*-chloro-Phe¹⁴]Bn(6–14), and [D-Phe⁶,Leu-NHET¹³,desMet¹⁴]Bn(6–14) were obtained from Bachem (Heidelberg, Germany). HATU and HOAt from

Perseptive Biosystems (Hamburg, Germany), and TBTU and HOBt from Quantum Appligene (Heidelberg, Germany). TFA was obtained from Solvay (Hannover, Germany). Bis(pentafluorophenyl)carbonate was purchased from Senn Chemicals (Dielsdorf, Switzerland) and 3-(4-chlorophenyl)propionic acid from CPS Chemie + Service (Düren, Germany). All other chemicals were purchased from Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), Lancaster (Mühlheim, Germany), or Fluka (Seelze, Germany). Solid-phase synthesis was carried out in PE syringes (2, 5, or 20 mL) from Becton-Dickinson (Fraga, Spain) or Braun (Melsungen, Germany) equipped with PE frits from Roland Vetter Laborbedarf (Ammerbuch, Germany). Air- or moisture-sensitive reactions were carried out in dry glassware and under argon (99.996%) atmosphere. There was no attempt to optimize yields. Thin-layer chromatography was performed on TLC aluminum sheets covered with silica gel 60 F₂₅₄ from Merck (Darmstadt). For flash chromatography, silica gel 60 (230–400 mesh ASTM, Korngrösse 0.040–0.063 mm) from Merck (Darmstadt) was used. Melting points were determined on a Büchi 510 melting point apparatus and are uncorrected. ¹H and ¹³C NMR spectra were recorded in CDCl₃, DMSO-*d*₆, or ACN-*d*₃ as solvent on Bruker AC250 and DMX500 instruments at 300 K. TMS or the solvent peak was used as internal reference. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (*J* values) are given in Hertz (Hz). Purification of compounds 15–18, 21, 23d, 24, 28, 35, and 36, analyses, and analytical purity determination were carried out on RP-HPLC instruments from Amersham Pharmacia Biotech or Beckman (System Gold), equipped with Omicron YMC columns (preparative, ODS-A C₁₈, 250 mm × 30 mm, 10 μm, flow rate of 25 mL/min; semipreparative, ODS-A C₁₈, 250 mm × 20 mm, 5 μm or 10 μm, flow rate of 8 mL/min; analytical, ODS-A C₁₈, 250 mm × 4.6 mm, 5 μm, flow rate of 1 mL/min) or a Macherey-Nagel column (preparative, Nucleosil C₁₈, 250 mm × 40 mm, 7 μm, flow rate of 25 mL/min). Compounds were eluted with linear gradients (30 min) of acetonitrile in water and 0.1% (v/v) trifluoroacetic acid, detected at 220 nm, and lyophilized after purification. ESI mass spectra were obtained on a LCQ Finnigan mass spectrometer, and GC mass spectra were obtained on a Finnigan MAT 8200 instrument. High-resolution mass spectra were recorded on a Finnigan spectrometer using the electrospray ionization time-of-flight (ESI-TOF) technique. cLogP values were calculated using Sybyl 6.8 from Tripos Inc.

Synthesis of N1-(2-Phenylethyl)-(2*R*)-2-[(9*H*-fluoren-9-ylmethoxy)carboxamido]-3-[1-(*tert*-butoxycarbonyl)-3-indolyl]propanamide on FMPE Resin (13).⁶ FMPE resin (2.378 g, theoretical 0.5 mmol/g, 1.19 mmol) was preswollen in DCE (24 mL) for 10 min. Then TMOF (12 mL), 1-(2-phenylethyl)amine (11.89 mmol), and NaBH(OAc)₃ (2.52 g, 11.89 mmol) were added and the mixture was submitted to an ultrasonicator for 10 min and then shaken overnight at room temperature. After the mixture was washed with CH₂Cl₂ (3 × 20 mL) and NMP (3 × 20 mL), Fmoc-D-Trp-OH (2.38 mmol, 2 equiv) was coupled to the resin using [HATU (2 equiv)]/[HOAt (2 equiv)]/[collidine (20 equiv)] activation in NMP (20 mL) for 5 h. Washing with NMP (3 × 20 mL) was followed by a double-coupling step overnight. Finally the resin was washed with NMP (3 × 20 mL) and CH₂Cl₂ (3 × 20 mL) and dried thoroughly in vacuo to give resin-bound N1-(2-phenylethyl)-(2*R*)-2-[(9*H*-fluoren-9-ylmethoxy)carboxamido]-3-[1-(*tert*-butoxycarbonyl)-3-indolyl]propanamide (13).

N1-[(1*R*)-2-(1*H*-3-Indolyl)-1-[1-(2-phenylethyl)carbamoyl]ethyl]-(2*S*)-2-(benzylcarboxamido)pentanediamide (15a). Resin-bound 13 (124 mg, 0.315 mmol/g, 0.039 mmol) was treated two times with 20% piperidine (v/v) in NMP (5 mL) for 15 min and washed after each time with NMP (5 × 5 mL). Then Fmoc-Gln(Trt)-OH (48 mg, 0.078 mmol) dissolved in NMP (2 mL) was coupled two times to the resin for 45 min using [TBTU (2 equiv)]/[HOBt (2 equiv)]/[DIEA (5.7 equiv)] activation. Coupling steps were followed by a washing step with NMP (5 × 5 mL). After repeated Fmoc removal and coupling of 7a (10.6 mg, 0.078 mmol), the resin was washed

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with CH_2Cl_2 (3 × 5 mL) for 30 min and the compound was deprotected and cleaved from the resin with [TFA (18 equiv)]/[TIPS (1 equiv)]/[H_2O (1 equiv)] (3 × 2 mL, 30 min each). The combined filtrates were reduced in vacuo. RP-HPLC purification yielded 11.1 mg of **15a**: colorless powder; mp 226–227 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.77 (s, 1H), 8.30 (d, 1H, $J = 7.1$ Hz), 8.18 (d, 1H, $J = 8.3$ Hz), 7.95 (t, 1H, $J = 5.0$ Hz), 7.55 (d, 1H, $J = 8.0$ Hz), 7.31 (d, 1H, $J = 8.1$ Hz), 7.23–7.27 (m, 6H), 7.16–7.19 (m, 2H), 7.11 (d, 2H, $J = 7.5$ Hz), 7.07 (s, 1H), 7.05 (t, 1H, $J = 7.7$ Hz), 6.97 (t, 1H, $J = 7.2$ Hz), 6.72 (bs, 2H), 4.39–4.44 (m, 1H), 4.15–4.21 (m, 1H), 3.47 (d, 2H, $J = 6.0$ Hz), 3.16–3.21 (m, 2H), 3.11 (dd, 1H, $J = 4.9/14.5$ Hz), 2.86 (dd, 1H, $J = 8.9/14.7$ Hz), 2.57 (t, 2H, $J = 8.0$ Hz), 1.92–2.00 (m, 1H), 1.85–1.92 (m, 1H), 1.69–1.77 (m, 1H), 1.60–1.69 (m, 1H); MS (ESI) m/z 554.2 [$\text{M} + \text{H}$] $^+$.

N1-((1R)-2-(1H-3-Indolyl)-1-[1-(2-phenylethyl)carbamoyl]ethyl)-(2S)-2-[(4-chlorobenzyl)carboxamido]pentanediarnide (15b). Preparation as described for **15a**, using 128 mg of **13** (0.357 mmol/g, 0.046 mmol) and **7b** (15.6 mg, 0.091 mmol), yielded 6.3 mg of **15b**: colorless powder; mp 219–220 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.77 (s, 1H), 8.33 (d, 1H, $J = 7.5$ Hz), 8.19 (d, 1H, $J = 9.0$ Hz), 7.94 (t, 1H, $J = 5.0$ Hz), 7.55 (d, 1H, $J = 7.5$ Hz), 7.29–7.32 (m, 3H), 7.23–7.26 (m, 4H), 7.17 (t, 1H, $J = 7.3$ Hz), 7.11 (d, 2H, $J = 7.6$ Hz), 7.08 (s, 1H), 7.05 (t, 1H, $J = 7.7$ Hz), 6.97 (t, 1H, $J = 8.0$ Hz), 6.73 (bs, 2H), 4.39–4.44 (m, 1H), 4.16–4.20 (m, 1H), 3.47 (d, 2H, $J = 6.0$ Hz), 3.16–3.21 (m, 2H), 3.10 (dd, 1H, $J = 5.0/14.3$ Hz), 2.86 (dd, 1H, $J = 8.5/14.8$ Hz), 2.56 (t, 2H, $J = 7.0$ Hz), 1.92–1.99 (m, 1H), 1.84–1.92 (m, 1H), 1.68–1.76 (m, 1H), 1.60–1.68 (m, 1H); MS (ESI) m/z 588.2 [$\text{M} + \text{H}$] $^+$.

N1-((1R)-2-(1H-3-Indolyl)-1-[1-(2-phenylethyl)carbamoyl]ethyl)-(2S)-2-[1-(2-(4-chlorophenyl)ethyl)carboxamidol]pentanediarnide (15c). Preparation as described for **15a**, using 100 mg of **13** (0.354 mmol/g, 0.035 mmol) and **7c** (13.1 mg, 0.07 mmol), yielded 16.8 mg of **15c**: colorless powder; mp 213–215 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.78 (s, 1H), 8.14 (d, 1H, $J = 8.2$ Hz), 8.08 (d, 1H, $J = 7.4$ Hz), 7.99 (t, 1H, $J = 5.8$ Hz), 7.55 (d, 1H, $J = 8.5$ Hz), 7.28–7.32 (m, 3H), 7.26 (t, 2H, $J = 7.4$ Hz), 7.21 (d, 2H, $J = 8.0$ Hz), 7.18 (t, 1H, $J = 7.3$ Hz), 7.15 (d, 2H, $J = 7.6$ Hz), 7.08 (s, 1H), 7.05 (t, 1H, $J = 7.7$ Hz), 6.97 (t, 1H, $J = 7.7$ Hz), 6.72 (bs, 2H), 4.39–4.45 (m, 1H), 4.15–4.21 (m, 1H), 3.18–3.29 (m, 2H), 3.11 (dd, 1H, $J = 4.7/14.4$ Hz), 2.88 (dd, 1H, $J = 8.8/14.8$ Hz), 2.79 (t, 2H, $J = 7.7$ Hz), 2.64 (t, 2H, $J = 7.5$ Hz), 2.34–2.46 (m, 2H), 1.82–1.98 (m, 2H), 1.65–1.74 (m, 1H), 1.55–1.65 (m, 1H); MS (ESI) m/z 602.2 [$\text{M} + \text{H}$] $^+$.

N1-((1R)-2-(1H-3-Indolyl)-1-[1-(2-phenylethyl)carbamoyl]ethyl)-(2S)-2-[(1H-2-Indolylmethyl)carboxamidol]pentanediarnide (15d). Preparation as described for **15a**, using 100 mg of **13** (0.354 mmol/g, 0.035 mmol) and **7g** (12.3 mg, 0.07 mmol), yielded 6.6 mg of **15d**: colorless powder; mp 195–201 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.86 (s, 1H), 10.76 (s, 1H), 8.24 (d, 1H, $J = 7.5$ Hz), 8.21 (d, 1H, $J = 8.2$ Hz), 7.96 (t, 1H, $J = 5.6$ Hz), 7.55 (d, 1H, $J = 7.8$ Hz), 7.39 (d, 1H, $J = 7.8$ Hz), 7.29 (t, 2H, $J = 8.2$ Hz), 7.22 (t, 2H, $J = 7.4$ Hz), 7.14 (t, 1H, $J = 7.4$ Hz), 7.06–7.08 (m, 3H), 7.04 (t, 1H, $J = 8.0$ Hz), 6.95–6.99 (m, 2H), 6.90 (t, 1H, $J = 7.4$ Hz), 6.72 (bs, 2H), 6.20 (s, 1H), 4.41–4.45 (m, 1H), 4.23–4.27 (m, 1H), 3.63 (d, 2H, $J = 3.4$ Hz), 3.13–3.24 (m, 2H), 3.09 (dd, 1H, $J = 5.4/14.5$ Hz), 2.86 (dd, 1H, $J = 8.7/14.5$ Hz), 2.56 (t, 2H, $J = 7.6$ Hz), 1.87–2.01 (m, 2H), 1.71–1.78 (m, 1H), 1.61–1.69 (m, 1H); MS (ESI) m/z 593.2 (100) [$\text{M} + \text{H}$] $^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1S)-1-(benzylcarboxamidol)ethyl]carboxamidol-3-(1H-3-indolyl)propanamide (16a). Preparation as described for **15a**, using 100 mg of **13** (0.354 mmol/g, 0.035 mmol) and Fmoc-Ala-OH (22 mg, 0.07 mmol), yielded 12.6 mg of **16a**: colorless powder; mp 205–207 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.78 (s, 1H), 8.24 (d, 1H, $J = 6.8$ Hz), 8.18 (d, 1H, $J = 8.4$ Hz), 8.00 (t, 1H, $J = 5.5$ Hz), 7.57 (d, 1H, $J = 7.9$ Hz), 7.31 (d, 1H, $J = 8.1$ Hz), 7.23–7.27 (m, 6H), 7.16–7.19 (m, 2H), 7.14 (d, 2H, $J = 7.5$ Hz), 7.06 (s, 1H), 7.05 (t, 1H, $J = 7.7$ Hz), 6.97 (t, 1H, $J = 7.3$ Hz), 4.38–4.43 (m, 1H), 4.21–4.25 (m, 1H), 3.45 (s, 2H), 3.19–3.24 (m, 2H), 3.11 (dd, 1H, $J = 4.6/14.7$ Hz), 2.84 (dd, 1H, $J =$

9.6/14.6 Hz), 2.61 (t, 2H, $J = 7.6$ Hz), 1.01 (d, 3H, $J = 7.0$ Hz); MS (ESI) m/z 497.2 (80) [$\text{M} + \text{H}$] $^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1S)-1-((4-chlorobenzyl)carboxamidol)ethyl]carboxamidol-3-(1H-3-indolyl)propanamide (16b). Preparation as described for **16a**, using **7b** (12.1 mg, 0.070 mmol), yielded 10.4 mg of **16b**: colorless powder; mp 198–200 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.78 (s, 1H), 8.28 (d, 1H, $J = 6.8$ Hz), 8.18 (d, 1H, $J = 8.4$ Hz), 7.99 (t, 1H, $J = 5.5$ Hz), 7.57 (d, 1H, $J = 7.9$ Hz), 7.29–7.32 (m, 3H), 7.24–7.27 (m, 4H), 7.18 (t, 1H, $J = 7.3$ Hz), 7.13 (d, 2H, $J = 7.5$ Hz), 7.07 (s, 1H), 7.05 (t, 1H, $J = 7.7$ Hz), 6.97 (t, 1H, $J = 7.4$ Hz), 4.37–4.44 (m, 1H), 4.20–4.26 (m, 1H), 3.45 (s, 2H), 3.16–3.26 (m, 2H), 3.11 (dd, 1H, $J = 4.5/14.7$ Hz), 2.84 (dd, 1H, $J = 9.6/14.6$ Hz), 2.60 (t, 2H, $J = 7.6$ Hz), 1.00 (d, 3H, $J = 7.0$ Hz); MS (ESI) m/z 531.1 [$\text{M} + \text{H}$] $^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1S)-1-(1-(2-(4-chlorophenyl)ethyl)carboxamidol)ethyl]carboxamidol-3-(1H-3-indolyl)propanamide (16c). Preparation as described for **16a**, using **7c** (13.1 mg, 0.07 mmol), yielded 10.2 mg of **16c**: colorless powder; mp 214–217 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.78 (s, 1H), 8.14 (d, 1H, $J = 8.5$ Hz), 8.02–8.04 (m, 2H), 7.57 (d, 1H, $J = 7.9$ Hz), 7.24–7.32 (m, 5H), 7.17–7.21 (m, 5H), 7.07 (s, 1H), 7.05 (t, 1H, $J = 7.5$ Hz), 6.97 (t, 1H, $J = 7.4$ Hz), 4.39–4.43 (m, 1H), 4.20–4.26 (m, 1H), 3.22–3.29 (m, 2H), 3.11 (dd, 1H, $J = 4.5/14.6$ Hz), 2.86 (dd, 1H, $J = 9.5/14.6$ Hz), 2.78 (t, 2H, $J = 7.7$ Hz), 2.68 (t, 2H, $J = 7.5$ Hz), 2.36–2.40 (m, 2H), 0.97 (d, 3H, $J = 7.0$ Hz); MS (ESI) m/z 545.1 [$\text{M} + \text{H}$] $^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1S)-1-(1,3-benzodioxol-5-ylmethyl)carboxamidol)ethyl]carboxamidol-3-(1H-3-indolyl)propanamide (16d). Preparation as described for **16a**, using **7d** (12.8 mg, 0.07 mmol), yielded 12.1 mg of **16d**: colorless powder; mp 211 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.78 (s, 1H), 8.17–8.19 (m, 2H), 7.99 (t, 1H, $J = 5.5$ Hz), 7.57 (d, 1H, $J = 7.9$ Hz), 7.31 (d, 1H, $J = 8.1$ Hz), 7.26 (t, 2H, $J = 7.5$ Hz), 7.18 (t, 1H, $J = 7.3$ Hz), 7.13 (d, 2H, $J = 7.5$ Hz), 7.07 (s, 1H), 7.05 (t, 1H, $J = 7.7$ Hz), 6.97 (t, 1H, $J = 7.4$ Hz), 6.81 (s, 1H), 6.78 (d, 1H, $J = 7.9$ Hz), 6.68 (d, 1H, $J = 8.0$ Hz), 5.91 (s, 2H), 4.38–4.43 (m, 1H), 4.19–4.24 (m, 1H), 3.35 (s, 2H), 3.18–3.27 (m, 2H), 3.11 (dd, 1H, $J = 4.5/14.6$ Hz), 2.83 (dd, 1H, $J = 9.7/14.6$ Hz), 2.61 (t, 2H, $J = 7.6$ Hz), 0.99 (d, 3H, $J = 7.0$ Hz); MS (ESI) m/z 541.2 [$\text{M} + \text{H}$] $^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1S)-1-(3-pyridyl)methylcarboxamidol)ethyl]carboxamidol-3-(1H-3-indolyl)propanamide (16e). Preparation as described for **16a**, using **7e** (12.3 mg, 0.07 mmol), yielded 13.2 mg of **16e**: colorless powder; mp 95–97 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.78 (s, 1H), 8.57–8.59 (m, 2H), 8.40 (d, 1H, $J = 7.0$ Hz), 8.20 (d, 1H, $J = 8.4$ Hz), 7.97–8.01 (m, 2H), 7.57 (d, 2H, $J = 7.8$ Hz), 7.31 (d, 1H, $J = 8.1$ Hz), 7.26 (t, 2H, $J = 7.5$ Hz), 7.18 (t, 1H, $J = 7.2$ Hz), 7.13 (d, 2H, $J = 7.5$ Hz), 7.07 (s, 1H), 7.05 (t, 1H, $J = 7.7$ Hz), 6.97 (t, 1H, $J = 7.4$ Hz), 4.04–4.45 (m, 1H), 4.23–4.29 (m, 1H), 3.61 (s, 2H), 3.18–3.28 (m, 2H), 3.09 (dd, 1H, $J = 4.7/14.6$ Hz), 2.84 (dd, 1H, $J = 9.6/14.6$ Hz), 2.60 (t, 2H, $J = 7.5$ Hz), 1.01 (d, 3H, $J = 7.1$ Hz); MS (ESI) m/z 498.2 [$\text{M} + \text{H}$] $^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1S)-1-(1,2,3,4-tetrahydro-1-isoquinolyl)methylcarboxamidol)ethyl]carboxamidol-3-(1H-3-indolyl)propanamide (16f). Preparation as described for **16a**, using **7f** (29 mg, 0.07 mmol), yielded 4.7 mg of **16f**: colorless powder; cis/trans isomeric ratio of 1:1.08; mp 110–113 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.78 (s, 1H), 8.80 and 9.21 (bs, 1H), 8.39 and 8.45 (d, 1H, $J = 7.5$ Hz), 8.21 and 8.23 (d, 1H, $J = 8.5$ Hz), 8.10 (s, 1H), 7.60 (d, 1H, $J = 7.8$ Hz), 7.31 (d, 1H, $J = 8.1$ Hz), 7.15–7.28 (m, 9H), 7.08 (s, 1H), 7.05 (t, 1H, $J = 7.6$ Hz), 6.97 (t, 1H, $J = 7.5$ Hz), 4.77–4.82 (m, 1H), 4.46–4.52 (m, 1H), 4.33–4.44 (m, 1H), 3.40–3.48 (m, 1H), 3.27–3.36 (m, 2H), 3.17–3.24 (m, 1H), 2.81–3.08 (m, 6H), 2.64 (t, 2H, $J = 7.5$ Hz), 0.94 and 1.02 (d, 3H, $J = 7.0$ Hz); MS (ESI) m/z 552.3 [$\text{M} + \text{H}$] $^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1S)-1-(1H-2-indolyl)methylcarboxamidol)ethyl]carboxamidol-3-(1H-3-indolyl)propanamide (16g). Preparation as described for **16a**, using **7g** (12.3 mg, 0.07 mmol), yielded 5.8 mg of **16g**: colorless powder;

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mp 188–193 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.86 (s, 1H), 10.76 (s, 1H), 8.20 (d, 1H, $J = 8.5$ Hz), 8.17 (d, 1H, $J = 7.1$ Hz), 8.01 (t, 1H, $J = 5.5$ Hz), 7.56 (d, 1H, $J = 8.1$ Hz), 7.38 (d, 1H, $J = 7.8$ Hz), 7.29 (t, 2H, $J = 9.1$ Hz), 7.22 (t, 2H, $J = 7.4$ Hz), 7.15 (t, 1H, $J = 7.4$ Hz), 7.11 (d, 2H, $J = 7.6$ Hz), 7.06 (s, 1H), 7.04 (t, 1H, $J = 7.6$ Hz), 6.94–6.99 (m, 2H), 6.90 (t, 1H, $J = 7.0$ Hz), 6.19 (s, 1H), 4.41–4.45 (m, 1H), 4.26–4.31 (m, 1H), 3.60 (d, 2H, $J = 3.5$ Hz), 3.15–3.29 (m, 2H), 3.08 (dd, 1H, $J = 4.8/14.6$ Hz), 2.84 (dd, 1H, $J = 9.5/14.4$ Hz), 2.61 (t, 2H, $J = 7.6$ Hz), 1.00 and 1.01 (s, 3H); MS (ESI) m/z 536.2 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(benzylamino)methylcarboxamido]-3-(1H-3-indolyl)propanamide (17a). Preparation as described for 15a, using 100 mg of 13 (0.354 mmol/g, 0.035 mmol) and 11a (27 mg, 0.07 mmol), yielded 9.8 mg of 17a: colorless powder; mp 107–109 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.82 (s, 1H), 9.11 (bs, 1H), 8.69 (d, 1H, $J = 8.1$ Hz), 8.24 (m, 1H), 7.62 (d, 1H, $J = 7.9$ Hz), 7.38–7.43 (m, 5H), 7.32 (d, 1H, $J = 8.1$ Hz), 7.26 (t, 2H, $J = 7.4$ Hz), 7.18 (t, 1H, $J = 7.2$ Hz), 7.16 (d, 2H, $J = 7.4$ Hz), 7.10 (s, 1H), 7.06 (t, 1H, $J = 7.4$ Hz), 6.98 (t, 1H, $J = 7.5$ Hz), 4.57 (q, 1H, $J = 5.7$ Hz), 4.02 (m, 2H), 3.63–3.66 (m, 1H), 3.53–3.56 (m, 1H), 3.27–3.34 (m, 1H), 3.20–3.26 (m, 1H), 3.04 (dd, 1H, $J = 5.1/14.5$ Hz), 2.86 (dd, 1H, $J = 9.0/14.5$ Hz), 2.64 (t, 2H, $J = 7.5$ Hz); MS (ESI) m/z 455.2 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(4-chlorobenzyl)amino)methylcarboxamido]-3-(1H-3-indolyl)propanamide (17b). Preparation as described for 17a, using 11b (29.8 mg, 0.07 mmol), yielded 11 mg of 17b: colorless powder; mp 138–140 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.82 (s, 1H), 9.13 (bs, 1H), 8.68 (d, 1H, $J = 8.2$ Hz), 8.25 (m, 1H), 7.61 (d, 1H, $J = 7.9$ Hz), 7.49 (d, 2H, $J = 8.2$ Hz), 7.41 (d, 2H, $J = 8.1$ Hz), 7.32 (d, 1H, $J = 8.1$ Hz), 7.26 (t, 2H, $J = 7.4$ Hz), 7.18 (t, 1H, $J = 7.3$ Hz), 7.16 (d, 2H, $J = 7.5$ Hz), 7.10 (s, 1H), 7.06 (t, 1H, $J = 7.3$ Hz), 6.97 (t, 1H, $J = 7.5$ Hz), 4.57 (q, 1H, $J = 5.8$ Hz), 4.03 (m, 2H), 3.62–3.67 (m, 1H), 3.51–3.56 (m, 1H), 3.26–3.34 (m, 1H), 3.19–3.26 (m, 1H), 3.04 (dd, 1H, $J = 5.1/14.5$ Hz), 2.86 (dd, 1H, $J = 9.0/14.5$ Hz), 2.64 (t, 2H, $J = 7.5$ Hz); MS (ESI) m/z 489.2 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(3-pyridyl)methylamino)methylcarboxamido]-3-(1H-3-indolyl)propanamide (17c). Preparation as described for 17a, using the TFA salt of 11c (35.4 mg, 0.07 mmol), yielded 6.6 mg of 17c: colorless powder; mp 89–93 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.82 (s, 1H), 9.20 (bs, 1H), 8.71 (d, 1H, $J = 8.0$ Hz), 8.59–8.62 (m, 2H), 8.26 (t, 1H, $J = 5.5$ Hz), 7.83 (d, 1H, $J = 8.0$ Hz), 7.62 (d, 1H, $J = 8.0$ Hz), 7.46 (dd, 1H, $J = 4.7/7.9$ Hz), 7.32 (d, 1H, $J = 8.0$ Hz), 7.26 (d, 2H, $J = 7.4$ Hz), 7.18 (t, 1H, $J = 7.2$ Hz), 7.16 (d, 2H, $J = 7.5$ Hz), 7.11 (s, 1H), 7.06 (t, 1H, $J = 7.0$ Hz), 6.98 (t, 1H, $J = 7.0$ Hz), 4.57 (q, 1H, $J = 6.0$ Hz), 4.09 (m, 2H), 3.69–3.75 (m, 1H), 3.57–3.64 (m, 1H), 3.27–3.35 (m, 1H), 3.19–3.27 (m, 1H), 3.05 (dd, 1H, $J = 5.2/14.9$ Hz), 2.87 (dd, 1H, $J = 8.8/14.6$ Hz), 2.64 (t, 2H, $J = 7.1$ Hz); MS (ESI) m/z 456.2 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1-(2-phenylethyl)amino)methylcarboxamido]-3-(1H-3-indolyl)propanamide (17d). Preparation as described for 17a, using 11d (28.3 mg, 0.07 mmol), yielded 2.7 mg of 17d: colorless powder; mp 197–199 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.82 (s, 1H), 8.79 (bs, 1H), 8.74 (d, 1H, $J = 8.5$ Hz), 8.26 (t, 1H, $J = 5.5$ Hz), 7.62 (d, 1H, $J = 8.0$ Hz), 7.31–7.34 (m, 3H), 7.25–7.28 (m, 3H), 7.19–7.21 (m, 3H), 7.16 (d, 2H, $J = 7.6$ Hz), 7.12 (s, 1H), 7.05 (t, 1H, $J = 7.5$ Hz), 6.98 (t, 1H, $J = 7.5$ Hz), 4.58 (q, 1H, $J = 5.5$ Hz), 3.73–3.79 (m, 1H), 3.62–3.67 (m, 1H), 3.29–3.34 (m, 1H), 3.20–3.25 (m, 1H), 3.04–3.08 (m, 3H), 2.84–2.92 (m, 3H), 2.64 (t, 2H, $J = 7.4$ Hz); MS (ESI) m/z 469.2 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1-(benzylamino)ethyl)carboxamido]-3-(1H-3-indolyl)propanamide (18a). Compound 14 (0.30 g, 0.736 mmol) and 12a (0.295 g, 0.735 mmol) were coupled in DMF (7 mL) at room temperature within 1 h using [HATU (1.5 equiv)]/[HOAt (1.5 equiv)]/[collidine (15 equiv)] activation. The solvent was removed in vacuo, the residue was chromatographed (ethyl acetate/hexane, 1:1) and subsequently dissolved in CH_2Cl_2 (2.5 mL), and trisopropylsilane (0.25 mL)

was added. After the mixture was cooled to 0 °C, TFA (2.5 mL) was added dropwise over 5 min and the solution was stirred for 1 h. Then the solvent was removed in vacuo and the residue was treated with a mixture of DMSO (20 mL), water (2.5 mL), and acetic acid (2.5 mL) and stirred at room temperature overnight. Evaporation to dryness was followed by treatment with 20% (v/v) piperidine in DMF (10 mL) and stirring for 30 min at room temperature. Finally the solvent was removed in vacuo, and chromatography (ethyl acetate) yielded 0.25 g (73%) of 18a: colorless powder; the ratio of the two isomers was determined from the NMR integrals of the CH_3 group as 1:1.52; mp 100–105 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.83 and 10.85 (s, 1H), 9.1 (m, 1H), 8.74–8.78 (m, 1H), 8.27 and 8.34 (t, 1H, $J = 5.6$ Hz), 7.63 and 7.68 (d, 1H, $J = 7.8$ Hz), 7.17–7.21 and 7.24–7.33 and 7.33–7.37 and 7.40–7.43 (m, 11H), 7.09 and 7.14 (s, 1H), 6.96–7.07 (m, 2H), 4.62–4.68 (m, 1H), 3.98–4.01 (m, 1H), 3.76–3.78 (m, 1H), 3.61–3.67 and 3.41–3.43 (m, 1H), 3.34–3.39 (m, 1H), 3.26–3.30 (m, 1H), 3.05–3.09 (m, 1H), 2.84–2.93 (m, 1H), 2.67–2.72 (m, 2H), 1.11 and 1.34 (d, 3H, $J = 6.9$ Hz); MS (ESI) m/z 469.2 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1-(4-chlorobenzyl)amino)ethyl]carboxamido]-3-(1H-3-indolyl)propanamide (18b). Preparation as described for 18a, using 12b (0.32 g, 0.734 mmol), yielded 320 mg (87%) of 18b: colorless powder; the ratio of the two isomers was determined from the NMR integrals of the CH_3 group as 1:1.24; mp 107–110 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.85 and 10.83 (s, 1H), 9.2 (m, 1H), 8.72 and 8.77 (d, 1H, $J = 8.5$ Hz), 8.28 and 8.34 (t, 1H, $J = 5.5$ Hz), 7.68 and 7.63 (d, 1H, $J = 7.7$ Hz), 7.17–7.20 and 7.24–7.33 and 7.40–7.50 (m, 10H), 7.09 and 7.15 (s, 1H), 6.96–7.07 (m, 2H), 4.61–4.69 (m, 1H), 3.98–4.02 (m, 1H), 3.75 (m, 1H), 3.60–3.67 and 3.39–3.43 (m, 1H), 3.34–3.38 (m, 1H), 3.24–3.29 (m, 1H), 3.05–3.08 (m, 1H), 2.85–2.92 (m, 1H), 2.67–2.71 (m, 2H), 1.33 and 1.10 (d, 3H, $J = 6.9$ Hz); MS (ESI) m/z 503.2 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(1-(2-phenylethyl)amino)ethyl]carboxamido]-3-(1H-3-indolyl)propanamide (18c). Preparation as described for 18a, using 12c (0.305 g, 0.737 mmol), yielded 230 mg (65%) of 18c: colorless powder; the ratio of the two isomers was determined from the NMR integrals of the CH_3 group as 1:1.59; mp 89–91 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.83 and 10.79 (s, 1H), 8.88 (m, 1H), 8.77 (m, 1H), 8.24 and 8.32 (t, 1H, $J = 5.4$ Hz), 7.63 (d, 1H, $J = 7.8$ Hz), 7.15–7.18 and 7.21–7.28 and 7.29–7.33 (m, 11H), 7.04–7.12 (m, 2H), 6.94–7.00 (m, 1H), 4.59–4.64 (m, 1H), 3.82–3.86 (m, 1H), 3.29–3.35 (m, 1H), 3.22–3.30 (m, 1H), 2.75–3.09 (m, 6H), 2.66 (t, 2H, $J = 7.2$ Hz), 1.35 and 1.12 (d, 3H, $J = 6.9$ Hz); MS (ESI) m/z 483.3 $[\text{M} + \text{H}]^+$.

N1-(2-Phenylethyl)-(2R)-2-[(N-(benzoyl)hydrazino)carboxamido]-3-(1H-3-indolyl)propanamide (21a). Resin-bound 13 (100 mg, 0.354 mmol/g, 0.035 mmol) was treated two times with 20% piperidine (v/v) in NMP (5 mL) for 15 min, washed after each time with NMP (5×5 mL), finally washed with dry CH_2Cl_2 (5×5 mL), and subsequently left in dry CH_2Cl_2 (5 mL) for 30 min. Then a solution of 19 (30.5 mg, 0.108 mmol) in dry CH_2Cl_2 (1 mL) was added and the resin was shaken for 90 min at room temperature and washed with CH_2Cl_2 (5×5 mL) and NMP (5×5 mL). After repeated Fmoc cleavage, 7a (9.5 mg, 0.07 mmol) was coupled two times to the resin for 45 min using [TBTU (2 equiv)]/[HOBT (2 equiv)]/[DIEA (5.7 equiv)] activation. Coupling steps were followed by a washing step with NMP (5×5 mL). Finally the resin was washed with CH_2Cl_2 (3×5 mL) for 30 min and the compound was deprotected and cleaved from the resin with [TFA (18 equiv)]/[TIPS (1 equiv)]/[H_2O (1 equiv)] (3×2 mL, 30 min each). The combined filtrates were reduced in vacuo. RP-HPLC purification yielded 2.6 mg of 21a: colorless powder; mp 109–114 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ 10.79 (s, 1H), 9.80 (s, 1H), 7.95–8.00 (m, 2H), 7.51 (d, 1H, $J = 7.9$ Hz), 7.30 (d, 1H, $J = 8.2$ Hz), 7.23–7.28 (m, 6H), 7.19–7.22 (m, 1H), 7.16 (t, 1H, $J = 7.1$ Hz), 7.13 (d, 2H, $J = 7.5$ Hz), 7.02–7.04 (m, 2H), 6.95 (t, 1H, $J = 7.5$ Hz), 6.36 (d, 1H, $J = 8.0$ Hz), 4.36 (q, 1H, $J = 6.6$ Hz), 3.41 (s, 2H), 3.14–3.29 (m, 2H).

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3.01 (dd, 1H, $J = 5.7/14.6$ Hz), 2.91 (dd, 1H, $J = 7.5/14.5$ Hz), 2.58 (t, 2H, $J = 7.1$ Hz); MS (ESI) m/z 484.3 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(4-chlorobenzoyl)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (21b). Preparation as described for 21a, using 7b (12 mg, 0.07 mmol), yielded 3.0 mg of 21b: colorless powder; mp 195/196 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.79 (s, 1H), 9.81 (s, 1H), 8.00 (s, 1H), 7.94 (bs, 1H), 7.51 (d, 1H, $J = 7.9$ Hz), 7.23–7.34 (m, 7H), 7.17 (t, 1H, $J = 7.3$ Hz), 7.13 (d, 2H, $J = 7.5$ Hz), 7.02–7.05 (m, 2H), 6.95 (t, 1H, $J = 7.5$ Hz), 6.38 (d, 1H, $J = 8.1$ Hz), 4.36 (q, 1H, $J = 6.7$ Hz), 3.42 (s, 2H), 3.16–3.24 (m, 2H), 3.01 (dd, 1H, $J = 5.6/14.9$ Hz), 2.91 (dd, 1H, $J = 7.5/14.5$ Hz), 2.58 (t, 2H, $J = 7.5$ Hz); MS (ESI) m/z 518.2 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(2-(3-pyridyl)ethanoyl)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (21c). Preparation as described for 21a, using 7e (12 mg, 0.07 mmol), yielded 4.5 mg of 21c: colorless powder; mp 116–120 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.79 (s, 1H), 9.89 (s, 1H), 8.63 (s, 1H), 8.61 (d, 1H, $J = 5.0$ Hz), 8.02–8.04 (m, 2H), 7.96 (bs, 1H), 7.63 (dd, 1H, $J = 5.8/7.3$ Hz), 7.51 (d, 1H, $J = 7.9$ Hz), 7.30 (d, 1H, $J = 8.2$ Hz), 7.25 (t, 2H, $J = 7.6$ Hz), 7.17 (t, 1H, $J = 7.4$ Hz), 7.13 (d, 2H, $J = 7.5$ Hz), 7.02–7.04 (m, 2H), 6.95 (t, 1H, $J = 8.0$ Hz), 6.44 (d, 1H, $J = 8.1$ Hz), 4.32–4.36 (m, 1H), 3.59 (s, 2H), 3.22–3.26 (m, 1H), 3.15–3.19 (m, 1H), 3.01 (dd, 1H, $J = 5.6/14.4$ Hz), 2.91 (dd, 1H, $J = 7.4/14.6$ Hz), 2.58 (t, 2H, $J = 7.5$ Hz); MS (ESI) m/z 485.3 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(2-(1,2,3,4-tetrahydro-1-isoquinolyl)ethanoyl)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (21d). Prepared as described for 21a, using 7f (29 mg, 0.07 mmol). The two isomers were separated by RP-HPLC and obtained as colorless powders. **21d1:** 1.6 mg; mp 105–110 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.79 (s, 1H), 9.94 (s, 1H), 8.75 and 9.25 (bs, 1H), 8.12 (s, 1H), 8.04 (t, 1H, $J = 5.5$ Hz), 7.54 (d, 1H, $J = 7.5$ Hz), 7.31 (d, 1H, $J = 8.0$ Hz), 7.17–7.26 (m, 7H), 7.14 (d, 2H, $J = 7.4$ Hz), 7.05 (s, 1H), 7.04 (t, 1H, $J = 7.7$ Hz), 6.96 (t, 1H, $J = 7.0$ Hz), 6.49 (d, 1H, $J = 8.0$ Hz), 4.75–4.84 (m, 1H), 4.35–4.39 (m, 1H), 3.31–3.48 (m, 2H), 3.24–3.28 (m, 1H), 3.16–3.20 (m, 1H), 2.87–3.01 (m, 6H), 2.59 (t, 2H, $J = 7.5$ Hz); MS (ESI) m/z 539.3 $[M + H]^+$. **21d2:** 0.9 mg; mp 120–123 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.79 (s, 1H), 9.95 (s, 1H), 8.78 and 9.26 (bs, 1H), 8.12 (s, 1H), 8.03 (t, 1H, $J = 5.8$ Hz), 7.54 (d, 1H, $J = 7.7$ Hz), 7.31 (d, 1H, $J = 8.1$ Hz), 7.17–7.26 (m, 7H), 7.14 (d, 2H, $J = 7.3$ Hz), 7.03–7.05 (m, 2H), 6.95 (t, 1H, $J = 7.0$ Hz), 6.49 (d, 1H, $J = 8.2$ Hz), 4.80–4.86 (m, 1H), 4.37 (q, 1H, $J = 3.6$ Hz), 3.31–3.48 (m, 2H), 3.22–3.28 (m, 1H), 3.15–3.20 (m, 1H), 2.87–3.05 (m, 6H), 2.59 (t, 2H, $J = 7.5$ Hz); MS (ESI) m/z 539.3 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(2-(1H-2-indolyl)ethanoyl)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (21e). Preparation as described for 21a, using 7g (12.3 mg, 0.07 mmol), yielded 5.7 mg of 21e: colorless powder; mp 109–115 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.92 (s, 1H), 10.79 (s, 1H), 9.80 (s, 1H), 8.04 (s, 1H), 7.97 (bs, 1H), 7.52 (d, 1H, $J = 7.9$ Hz), 7.40 (d, 1H, $J = 7.7$ Hz), 7.30 (d, 2H, $J = 8.0$ Hz), 7.24 (t, 2H, $J = 7.4$ Hz), 7.16 (t, 1H, $J = 7.3$ Hz), 7.12 (d, 2H, $J = 7.5$ Hz), 6.90–7.05 (m, 5H), 6.45 (d, 1H, $J = 8.0$ Hz), 6.23 (s, 1H), 4.36–4.40 (m, 1H), 3.60 (s, 2H), 3.15–3.26 (m, 2H), 3.02 (dd, 1H, $J = 5.7/14.5$ Hz), 2.91 (dd, 1H, $J = 7.4/14.6$ Hz), 2.58 (t, 2H, $J = 7.4$ Hz); MS (ESI) m/z 523.2 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(phenylmethylene)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (24a). Compound 23a (120 mg, 0.217 mmol) was dissolved in CH_2Cl_2 (3 mL), triisopropylsilane (0.1 mL) was added, and the solution was cooled to 0 °C. Then TFA (3 mL) was added dropwise over 5 min and the solution was stirred for 30 min. The solvent was removed in vacuo and the residue was treated with DMSO (8 mL), water (1 mL), and acetic acid (1 mL) and stirred overnight at room temperature. Then the solution was evaporated to dryness. RP-HPLC purification yielded 62 mg (63%) of 24a: colorless powder; mp 183 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.85 (s, 1H), 10.43 (s, 1H), 8.06 (t, 1H, $J = 5.5$ Hz), 7.81 (s, 1H), 7.60 (d, 1H, $J = 7.9$ Hz), 7.54 (d, 2H, $J = 7.4$ Hz), 7.36–7.41 (m, 3H), 7.32 (d, 1H, $J = 8.1$ Hz), 7.25 (t, 2H,

$J = 7.5$ Hz), 7.14–7.19 (m, 3H), 7.11 (s, 1H), 7.05 (t, 1H, $J = 7.4$ Hz), 6.94 (t, 1H, $J = 7.5$ Hz), 6.72 (d, 1H, $J = 8.0$ Hz), 4.44 (q, 1H, $J = 7.4$ Hz), 3.27–3.35 (m, 1H), 3.19–3.27 (m, 1H), 3.11 (d, 2H, $J = 6.4$ Hz), 2.66 (t, 2H, $J = 7.4$ Hz); MS (ESI) m/z 454.2 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(4-chlorophenylmethylene)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (24b). Preparation from 23b (120 mg, 0.204 mmol) as described for 24a yielded 56 mg (56%) of 24b: colorless powder; mp 173–175 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.85 (s, 1H), 10.51 (s, 1H), 8.05 (t, 1H, $J = 5.5$ Hz), 7.79 (s, 1H), 7.59 (d, 1H, $J = 8.0$ Hz), 7.56 (d, 2H, $J = 8.4$ Hz), 7.46 (d, 2H, $J = 8.3$ Hz), 7.33 (d, 1H, $J = 8.1$ Hz), 7.25 (t, 2H, $J = 7.5$ Hz), 7.15–7.18 (m, 3H), 7.11 (s, 1H), 7.05 (t, 1H, $J = 7.4$ Hz), 6.94 (t, 1H, $J = 7.5$ Hz), 6.73 (d, 1H, $J = 8.0$ Hz), 4.43 (q, 1H, $J = 7.5$ Hz), 3.27–3.35 (m, 1H), 3.19–3.27 (m, 1H), 3.11 (d, 2H, $J = 6.2$ Hz), 2.66 (t, 2H, $J = 7.5$ Hz); MS (ESI) m/z 488.2 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(furan-2-ylmethylene)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (24c). Preparation from 23c (100 mg, 0.184 mmol) as described for 24a yielded 46 mg (57%) of 24c: colorless powder; mp 100/101 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.82 (s, 1H), 10.41 (s, 1H), 8.09 (t, 1H, $J = 5.5$ Hz), 7.76 (s, 1H), 7.73 (s, 1H), 7.55 (d, 1H, $J = 7.9$ Hz), 7.30 (d, 1H, $J = 8.1$ Hz), 7.25 (t, 2H, $J = 7.4$ Hz), 7.14–7.17 (m, 3H), 7.07 (s, 1H), 7.03 (t, 1H, $J = 7.4$ Hz), 6.93 (t, 1H, $J = 7.5$ Hz), 6.74 (d, 1H, $J = 3.2$ Hz), 6.58–6.60 (m, 2H), 4.45 (q, 1H, $J = 6.8$ Hz), 3.24–3.31 (m, 1H), 3.17–3.24 (m, 1H), 3.02–3.10 (m, 2H), 2.62 (t, 2H, $J = 7.4$ Hz); MS (ESI) m/z 444.2 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(4-(2-thienyl)phenylmethylene)-hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (24d). Preparation from 23d (40 mg, 0.063 mmol) as described for 24a yielded 25 mg (74%) of 24d: colorless powder; mp 219–221 °C; ^1H NMR (500 MHz, DMSO- d_6) δ 10.87 (s, 1H), 10.48 (s, 1H), 8.06 (t, 1H, $J = 5.5$ Hz), 7.81 (s, 1H), 7.69 (d, 2H, $J = 8.2$ Hz), 7.57–7.61 (m, 5H), 7.33 (d, 1H, $J = 8.1$ Hz), 7.25 (t, 2H, $J = 7.5$ Hz), 7.16–7.18 (m, 4H), 7.13 (s, 1H), 7.06 (t, 1H, $J = 7.7$ Hz), 6.95 (t, 1H, $J = 7.5$ Hz), 6.74 (d, 1H, $J = 7.9$ Hz), 4.43 (q, 1H, $J = 7.7$ Hz), 3.27–3.34 (m, 1H), 3.20–3.27 (m, 1H), 3.12 (d, 2H, $J = 6.4$ Hz), 2.66 (t, 2H, $J = 7.4$ Hz); MS (ESI) m/z 536.1 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(benzyl)hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (28a). Compound 27a (0.24 g, 0.54 mmol) was dissolved in CH_2Cl_2 (2 mL), triisopropylsilane (0.1 mL) was added, and the solution was cooled to 0 °C. Then TFA (2 mL) was added dropwise over 5 min and the solution was stirred for 30 min. The solvent was removed in vacuo, and the residue was redissolved in a solution of dry CH_2Cl_2 (8 mL) and DMAP (66 mg, 0.54 mmol). This solution was added dropwise and under stirring to a solution of bis(pentafluorophenyl)carbonate (213 mg, 0.54 mmol) in dry CH_2Cl_2 (20 mL) over a period of 20 min. Upon completion of the addition, a solution of 14 (0.22 g, 0.54 mmol), DMAP (66 mg, 0.54 mmol), and dry CH_2Cl_2 (8 mL) was added. The resulting mixture was stirred for 30 min at room temperature. Then the solvent was removed in vacuo, the residue was redissolved in CH_2Cl_2 (4 mL) and triisopropylsilane (0.1 mL), and the solution was cooled to 0 °C. TFA (4 mL) was added dropwise over 5 min, and the solution was stirred for 30 min. The solution was evaporated to dryness and then treated with a solution of 20% (v/v) piperidine in DMF (10 mL) for 30 min at room temperature. Finally the solvent was removed in vacuo and RP-HPLC purification yielded 34.2 mg (14%) of 28a: colorless powder; mp 81–84 °C; ^1H NMR (500 MHz, CDCl_3) δ 8.13 (s, 1H), 7.56 (d, 1H, $J = 7.9$ Hz), 7.34 (d, 1H, $J = 8.1$ Hz), 7.16–7.29 (m, 9H), 7.11 (t, 1H, $J = 7.1$ Hz), 6.94–6.95 (m, 3H), 6.88 (bs, 1H), 5.99 (bs, 1H), 4.43 (q, 1H, $J = 6.5$ Hz), 3.91 (bs, 2H), 3.34–3.42 (m, 1H), 3.16–3.24 (m, 1H), 3.14 (dd, 1H, $J = 5.5/14.0$ Hz), 3.07 (dd, 1H, $J = 7.4/14.3$ Hz), 2.45–2.59 (m, 2H); MS (ESI) m/z 456.1 $[M + H]^+$.

N1-(2-Phenylethyl)-(2R)-2-([N-(4-chlorobenzyl)hydrazino]carboxamido)-3-(1H-3-indolyl)propanamide (28b). Preparation from 27b (0.30 g, 0.63 mmol) and 14 (0.26 g, 0.63

mmol) as described for **28a** yielded 26.8 mg (9%) of **28b**: colorless powder; mp 75–80 °C; ¹H NMR (500 MHz, ACN-*d*₃) δ 9.74 (bs, 1H), 7.91 (d, 1H, *J* = 7.7 Hz), 7.98 (d, 1H, *J* = 8.1 Hz), 7.74–7.83 (m, 5H), 7.71 (t, 1H, *J* = 7.4 Hz), 7.66–7.69 (m, 4H), 7.62 (t, 1H, *J* = 7.5 Hz), 7.58 (s, 1H), 6.99 (bs, 1H), 6.89 (bs, 1H), 4.87 (q, 1H, *J* = 6.9 Hz), 4.34 (bs, 2H), 3.87–3.94 (m, 1H), 3.76–3.82 (m, 1H), 3.66 (d, 2H, *J* = 6.2 Hz), 3.17 (t, 2H, *J* = 7.3 Hz); MS (ESI) *m/z* 490.1 [M + H]⁺.

N1-(2-Phenylethyl)-(2*R*)-2-[(*N*-(2-furymethyl)hydrazino)carboxamido]-3-(1*H*-3-indolyl)propanamide (28c). Preparation from **27c** (0.30 g, 0.69 mmol) and **14** (0.28 g, 0.69 mmol) as described for **28a** yielded 65.1 mg (21%) of **28c**: colorless powder; mp 59–62 °C; ¹H NMR (500 MHz, ACN-*d*₃) δ 9.72 (s, 1H), 8.14 (d, 1H, *J* = 7.9 Hz), 7.97 (d, 1H, *J* = 8.3 Hz), 7.95 (s, 1H), 7.82 (t, 2H, *J* = 7.5 Hz), 7.76 (t, 1H, *J* = 7.2 Hz), 7.70 (t, 1H, *J* = 7.4 Hz), 7.67 (d, 2H, *J* = 7.5 Hz), 7.62 (t, 1H, *J* = 7.6 Hz), 7.60 (s, 1H), 7.05 (bs, 2H), 6.89 (s, 1H), 6.80 (s, 1H), 4.89 (q, 1H, *J* = 6.8 Hz), 4.41 (bs, 2H), 3.82–3.92 (m, 2H), 3.68 (dd, 1H, *J* = 5.9/14.5 Hz), 3.64 (dd, 1H, *J* = 7.7/14.0 Hz), 3.17 (t, 2H, *J* = 7.0 Hz); MS (ESI) *m/z* 446.2 [M + H]⁺.

N1-(2-Phenylethyl)-(2*R*)-2-[(4-benzylpiperazino)methyl]carboxamido]-3-(1*H*-3-indolyl)propanamide (35). Compound **33** (170 mg, 0.273 mmol) was dissolved in CH₂Cl₂ (3 mL), and trisopropylsilane (0.1 mL) was added. After the mixture was cooled to 0 °C, TFA (3 mL) was added dropwise over 5 min and the solution was stirred for 1 h. Then the solvent was removed in vacuo and the residue was treated with DMSO (8 mL), water (1 mL), and acetic acid (1 mL). The mixture was stirred overnight at room temperature. Evaporation to dryness and RP-HPLC purification yielded 97 mg (68%) of **35**: colorless powder; mp 67–70 °C; ¹H NMR (500 MHz, CDCl₃) δ 8.40 (s, 1H), 7.60 (d, 1H, *J* = 7.9 Hz), 7.55 (d, 1H, *J* = 7.5 Hz), 7.43–7.49 (m, 3H), 7.34–7.37 (m, 3H), 7.23–7.27 (m, 2H), 7.19 (t, 2H, *J* = 7.2 Hz), 7.13 (t, 1H, *J* = 7.5 Hz), 7.05 (d, 2H, *J* = 7.4 Hz), 6.96 (s, 1H), 6.26 (s, 1H), 4.68 (q, 1H, *J* = 7.5 Hz), 4.03 (dd, 2H, *J* = 12.9/23.8 Hz), 3.48–3.55 (m, 1H), 3.38–3.45 (m, 1H), 3.23 (dd, 1H, *J* = 7.4/14.8 Hz), 3.14 (dd, 1H, *J* = 7.2/14.8 Hz), 3.08 (m, 2H), 2.70 (t, 2H, *J* = 6.9 Hz), 2.61–2.75 (m, 8H); MS (ESI) *m/z* 524.4 [M + H]⁺.

N1-(2-Phenylethyl)-(2*R*)-2-[(4-benzylpiperidino)methyl]carboxamido]-3-(1*H*-3-indolyl)propanamide (36). Preparation from **34** (180 mg, 0.289 mmol) as described for **35** yielded 109 mg (72%) of **36**: colorless powder; mp 73–75 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ 10.84 (s, 1H), 8.81 (d, 1H, *J* = 8.3 Hz), 8.25 (t, 1H, *J* = 5.2 Hz), 7.61 (d, 1H, *J* = 7.7 Hz), 7.14–7.31 (m, 11H), 7.09 (s, 1H), 7.02 (t, 1H, *J* = 7.3 Hz), 6.96 (t, 1H, *J* = 6.9 Hz), 4.60 (q, 1H, *J* = 6.9 Hz), 3.81 (d, 1H, *J* = 15.2 Hz), 3.67 (d, 1H, *J* = 13.1 Hz), 3.21–3.34 (m, 3H), 3.06 (dd, 1H, *J* = 4.9/14.4 Hz), 2.97–2.93 (m, 3H), 2.59–2.67 (m, 3H), 2.46–2.48 (m, 2H), 1.57–1.70 (m, 3H), 1.32–1.46 (m, 2H); MS (ESI) *m/z* 523.3 [M + H]⁺.

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Appendix

Abbreviations. BLP, bombesin-like peptide; Bn, bombesin; Boc, *tert*-butoxycarbonyl; BRS-3, bombesin receptor subtype 3; [Ca²⁺]_i, intracellular calcium; CART, constitutively activating receptor technology; CHO, Chinese hamster ovary; DCE, dichloroethane; DIEA,

diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EC, effect concentration; ESI, electrospray ionization; FLIPR, fluorometric imaging plate reader; Fmoc, 9-fluorenylmethoxycarbonyl; FMPE, 2-(4-formyl-3-methoxyphenoxy)ethyl; GPCR, G-protein-coupled receptor; GRP, gastrin-releasing peptide; GRP-R, gastrin-releasing peptide receptor; HATU, 2-(1*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAc, acetic acid; HOAt, *N*-hydroxy-9-azabenzotriazole; HOBt, *N*-hydroxybenzotriazole; mRNA, messenger ribonucleic acid; MS, mass spectrometry; NMB, neuromedin B; NMB-R, neuromedin B receptor; NMP, *N*-methylpyrrolidinone; PE, polyethylene; propylamide is *n*-propylamide; RP-HPLC, reverse-phase high-pressure performance chromatography; SAR, structure–activity relationship; SCLC, small-cell lung cancer; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TIPS, triisopropylsilane; TLC, thin-layer chromatography; TMS, trimethylsilane; TMOF, trimethyl orthoformate; TOF, time of flight.

Supporting Information Available: Detailed descriptions of molecular cloning, transfection, cell culture, the FLIPR assay, and the FLIPR antagonist experiment, synthesis procedures of compounds **7f**, **7g**, **8**–**12**, **14**, **20**, **22d**, **23**, **25**–**27**, **29**–**34**, and tables of analytical data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
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Patente de Invención ☒ Patente de Modelo de Utilidad ☐
Vía Nacional ☐
Vía PCT (Fase Nacional) ☒ Capítulo I ☒ Capítulo II ☐
No. Solicitud Internacional PCT/JP2005/012021
Fecha de Presentación Internacional 23/Junio/2005

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 comunica:

1. Documentos estudiados

Descripción:	Folios 189 a 669	22/Diciembre/2006
Reivindicaciones:	Folios 670 a 701	22/Diciembre/2006
	Folios 767 a 800	01/Diciembre/2008
Dibujos	Folios 703 a 724	22/Diciembre/2006
Listado de Secuencias:	Folios 725 a 738	22/Diciembre/2006
Prioridad:	JP 2004-187671	25/Junio/2004
	JP 2004-363311	15/Diciembre/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 la prioridad reivindicada.

3. Modificaciones aceptadas

Se acepta el nuevo capítulo reivindicatorio porque no se amplía el objeto de la Solicitud.

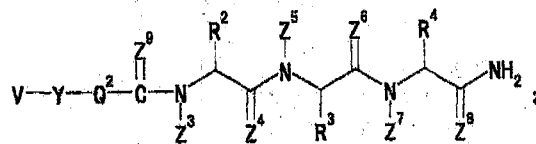
4. Objeto de la invención

Título de la solicitud: "Derivados de metastina y utilización de los mismos".

Expediente 06-128571 1er Art 45

El problema planteado en esta solicitud consiste en suministrar derivados de metastina para el tratamiento del cáncer, control de la función placentaria, entre otras aplicaciones.

Para resolver este problema técnico la solicitud en estudio proporciona derivados de metastina (II) de la fórmula:



El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): C07K 14/47.

5. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 15 – 23, 41 – 44 y 72 – 78 no es patentable de acuerdo con el Art citado.

6. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

El uso de las reivindicaciones 24 – 32 y 45 – 48 no es patentable, de acuerdo con el Art 14.

7. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no es conciso porque contiene un excesivo número de reivindicaciones independientes, lo cual dificulta definir cual es el objeto que se busca proteger. Las reivindicaciones 51 – 71 tienen el mismo alcance de la reivindicación 50.

Las reivindicaciones 1 – 5 y 33 – 37 y 49 no son concisas porque presentan un número demasiado alto de alternativas posibles, lo cual dificulta determinar la extensión del objeto que se busca proteger.

La reivindicación 1 utiliza los términos "opcionalmente sustituido", "opcionalmente posee un sustituyente adicional", "grupo aralquilo", "grupo cíclico aromático", lo cual dificulta determinar la extensión del objeto que se busca proteger. Por lo tanto se invita al solicitante para realizar una delimitación correcta de la invención, basándose en los ejemplos de la descripción, haciendo una generalización razonable de estos y de acuerdo a las definiciones establecidas.

En la reivindicación 4 si bien es cierto que para la persona del oficio versada en la material el término prodroga es conocido, no se evidencia en la descripción que éste pueda llegar a formarse. Por lo tanto se requiere eliminar este término del alcance del capítulo reivindicatorio.

Las reivindicaciones 6 – 14 y 38 – 40 indican el uso que puede darse al producto reivindicado, el cual no es una característica técnica que defina a la invención. Se requiere eliminar estas reivindicaciones del alcance del capítulo reivindicatorio.

La reivindicación 4 utiliza el término "prodroga", el cual no es una traducción apropiada del término "produg". Se sugiere al solicitante reemplazarlo por el término "profármaco".

Las reivindicaciones 5 y 6 utilizan el término "compuesto farmacéutico", para referirse a una formulación farmacéutica. Se sugiere hacer la aclaración respectiva.

En el capítulo reivindicatorio se encuentra subrayado el término "caracterizado". Se sugiere no subrayar este término.

Descripción (Art 28 D 486)

En el Folio 326 no se encuentra la fórmula a la que se hace referencia.

Se requiere al Solicitante hacer las aclaraciones necesarias.

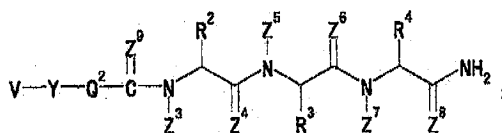
8. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a 2 Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características **esenciales**:

1° Derivado de metastina de la fórmula [Pya (4) 10] MS que corresponde al compuesto preferido No. 17: reivindicaciones 1 – 71 parcialmente (divulgados en D1 y D3).

2° Derivados de metastina de la siguiente fórmula, que corresponden a los compuestos preferidos 18 – 515 (Tablas 1 a 11): reivindicaciones 1 – 71, parcialmente (divulgados en D1 y D2):



No se guarda un concepto común inventivo porque los Grupos 1 y 2 están dirigidos a derivados de metastina definidos por una fórmula Markush de las reivindicaciones 1, 33 y 49 que presentan un número demasiado alto de alternativas posibles que no permite a esta Superintendencia distinguir una característica técnica común. Igualmente derivados de metastina dentro del alcance de la fórmula 1 ya habían sido divulgados en D1 y D2. En vista de esto, el examen de patentabilidad corresponderá al derivado de metastina que corresponde al compuesto preferido 17, como primera invención.

Se sugiere presentar las Solicitudes Divisionales que considere necesarias, ó limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

9. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Junio 15 de 2004 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación
D1	<u>Biochimica et Biophysica Acta, Vol. 1678, pág 102-110</u>	Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta	19/Marzo/2004
D2	<u>Biochemical and Biophysical Research Communications, Vol. 315, pág.85-92</u>	Metastin and its variant forms suppress migration of pancreatic cancer cells	27/Febrero/2004
D3	EP0162575	GnRH antagonists VII	27/Noviembre/1985

D1 <http://www.sciencedirect.com/science/article/pii/S0167478104000557> divulga el derivado de metastina [Tyr54] (45-54), de fórmula YNWSFGLRY-amida (Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr-amida) que activa el receptor de metastina OT7T175 (página 106, columna derecha, líneas 14-22).

D2 <http://www.sciencedirect.com/science/article/pii/S0006291X0400049X> divulga los derivados de metastina FM053a (Gu-Amb-Phe-Gly-Leu-Arg-Trp-NH₂), FM059a (Ac-Trp-Asn-Arg-Phe-Gly-Leu-Arg-Trp-NH₂) y FM052a (Bis(Py)-Amb- Phe-Gly-Leu-Arg-Trp-NH₂) con actividad frente a la supresión de la migración de células de cáncer pancreático (abstract y página 86, columna derecha, líneas 6-8).

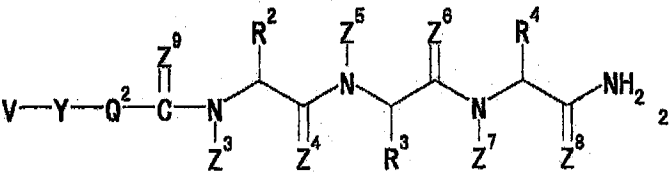
D3http://ep.espacenet.com/publicationDetails/biblio?DB=ep.espacenet.com&ll=0&ND=3&adjacent=true&locale=en_GB&FT=D&date=19851127&CC=EP&NR=0162575A2&KC=A2 divulga decapeptidos sintéticos que inhiben la secreción de gonadotropinas, en los cuales la sustitución de residuos por 4-piridilalanina permite la obtención de decapeptidos análogos con propiedades mejoradas (página 3, líneas 1-11 y página 4, líneas 8-10)

10.Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

Grupo Inventivo 2

La reivindicación 1 consiste en un derivado de metastina (II) caracterizado porque está representado por la fórmula:



sustituyente seleccionado de 1) un hidrocarburo aromático C ₆₋₁₂ opcionalmente sustituido, 2) un grupo heterocíclico aromático;	
Q ² es 1) CH ₂ opcionalmente sustituido con alquilo C ₁₋₄ opcionalmente sustituido, 2) NH opcionalmente sustituido con alquilo C ₁₋₄ , 3) O	
Y es un grupo de fórmula: -CONH-, entre otros, que puede estar opcionalmente sustituido por un alquilo C ₁₋₆ ;	
P y P' iguales o diferentes, cada uno puede formar un anillo combinado P y P' ó P y Q ¹ juntos representan: 1) H; 2) un residuo de aminoácido opcional limitado de manera continua o discontinua del C terminal de la secuencia de aminoácido 1-48 en la secuencia de aminoácido representada por SEQ ID NO: 1; 3) un grupo representado por la fórmula: J ¹ -J ² -C(J ³)(Q ³)Y ¹ C(J ⁴)(Q ⁴)Y ² C(J ⁵)(Q ⁵)Y ³ C(J ⁶)(Q ⁶)C(=Z ¹⁰)-	
Z ¹ , Z ³ , Z ⁶ y Z ⁷ es H o alquilo C ₁₋₃ ;	
Z ² , Z ⁸ y Z ⁹ es H, O ó S;	
R ² es un grupo alquilo C ₁₋₈ opcionalmente sustituido	
R ³ es un grupo alquilo C ₁₋₈ que posee un grupo básico opcionalmente sustituido;	
R ⁴ es un grupo alquilo C ₁₋₄ , opcionalmente sustituido con un hidrocarburo aromático C ₆₋₁₂ opcionalmente sustituido;	
Z ⁹ es H, O ó S;	

Las características **esenciales** del derivado de metastina (II) de la reivindicación 1 habían sido divulgadas previamente en D1 y en D2:

D1 divulga el derivado de metastina [Tyr54] (45-54), de fórmula YNWN SFGLRY-amida (Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr-amida) que activa el receptor de metastina OT7T175 (página 106, columna derecha, líneas 14-22).
D2 divulga los derivados de metastina FM053a (Gu-Amb-Phe-Gly-Leu-Arg-Trp-NH₂), FM059a (Ac-Trp-Asn-Arg-Phe-Gly-Leu-Arg-Trp-NH₂) y FM052a (Bis(Py)-Amb-Phe-Gly-Leu-Arg-Trp-NH₂) con actividad frente a la supresión de la migración de células de cáncer pancreático (abstract y página 86, columna derecha, líneas 6-8).

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en los documentos D1 o D2.

Las reivindicaciones 2 – 5 y 33 – 37 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel Inventivo (Art18 D486)

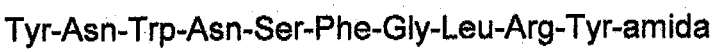
El Grupo Inventivo 1 consiste en un derivado de metastina de la fórmula [Pya (4) 10] MS:



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

Características esenciales	D1	D3
Un derivado de metastina de la fórmula [Pya (4) 10] MS: Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Pya(4)-NH ₂ ,	Derivado de metastina de la fórmula: Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr-amida	La sustitución de residuos por 4-piridilalanina permite la síntesis de decapeptidos análogos con propiedades mejoradas.

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulga derivados de metastina de la siguiente fórmula que activa el receptor de metastina OT7T175 (página 106, columna derecha, líneas 14-22):



La diferencia entre la invención, definida en el Grupo Inventivo 1 y el derivado de metastina de D1 consiste en el aminoácido de la posición 10, el cual representa 4-piridilalanina, Pya(4), mientras que en D1 corresponde a Tyr.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a los derivados de metastina reclamados en esta solicitud porque no parecen presentar una mayor actividad anticancerígena, en el control de la función placentaria, entre otras aplicaciones, según lo establecido en la descripción.

El Problema Técnico Objetivo de esta solicitud consiste en la necesidad de derivados de metastina para el tratamiento del cáncer, control de la función placentaria, entre otras aplicaciones, alternativos a los ya conocidos en D1.

Teniendo en cuenta que D1 ya divulgaba derivados de metastina como moduladores del receptor OT7T175, involucrado en la formación placentaria (abstract), que comprende la secuencia Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Tyr-amida y que en la síntesis de péptidos con actividad hormonal, la introducción de modificaciones químicas o sustituciones con aminoácidos no naturales es una práctica común, como se divulga en D3, el cual muestra que la sustitución de residuos por 4-piridilalanina permite la síntesis de decapeptidos análogos con propiedades mejoradas (página 3, líneas 1-11 y página 4, líneas 8-10) y que la modificación del aminoácido Gly por AzaGly es una práctica común en síntesis de péptidos para mejorar su estabilidad¹ la persona del oficio medianamente versada en la materia habría inferido sin mucho esfuerzo de su parte, sustituir por 4-piridilalanina un residuo del decapeptido derivado de metastina divulgado en D1 o sustituir el residuo glicina por azaglicina, para obtener más compuestos con la misma actividad .

De manera que la solución propuesta por esta solicitud en la reivindicación 1 y sus dependientes 2 – 5, la reivindicación 33 y sus dependientes 34 – 37, y la reivindicación 49 y sus dependientes 50 – 71 son obvias y no tienen Nivel Inventivo.

11. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	<u>Biochimica et Biophysica Acta, Vol. 1678, pág 102-110</u>	1 – 71	Novedad y Nivel Inventivo
D2	<u>Biochemical and Biophysical Research Communications, Vol. 315, pág.85-92</u>	1 – 37	Novedad
D3	EP0162575	1 – 71	Nivel Inventivo

1. 1 D. Weber et al. Design of selective peptidomimetic agonists for the human orphan receptor BRS-3. Journal of Medicinal Chemistry, Vol. 46, pág. 1918-1930, Abril 15 de 2003. Ver página 1923, líneas 1-5.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha

121 MAR 2012

Elaborado por: Juan David Moncaleano.
Revisado por: Ana Delia Pinzón