

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO			
RAD:	12-154938- -6	FECHA:	2014-05-26 17:13:23
DEP:	2020 DIRECCION DE NUEVAS CREACIONES	EVE:	378 FASE NACIONAL INCLUIDO CAPITULO I
TRA:	11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS:	13
ACT:	519 PRESENTACION		

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
SILVANA MARIA LÓPEZ DÍAZ

Asunto: Radicación: 12-154938- -6
Trámite: 11
Evento: 378
Actuación: 519
Oficio: 6333
Folios: 13

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2012/023484				
Fecha de Presentación Internacional	01/02/2012				

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:	No. 12-154938-00000-0000	10/Septiembre/2012
Reivindicaciones:	No. 12-154938-00000-0000	10/Septiembre/2012
	No. 12-154938-00005-0000	11/Julio/2013
Dibujos:	No. 12-154938-00000-0000	10/Septiembre/2012
Secuencias:	No. 12-154938-00000-0000	10/Septiembre/2012
Oposición Procaps S.A.	No. 12-154938-00003-0000	14/Marzo/2013
Prioridad:	61/438.780	02/Febrero/2011

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del capítulo primero del Título 10 de la Circular Única para efectos del examen de patentabilidad, se tendrá en

cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En el presente caso se estudió la totalidad del capítulo reivindicatorio No. 12-154938-00000-0000 (reivindicaciones 1-12).

Título de la solicitud: “Métodos para tratar úlceras de pie diabético”.

El problema planteado en esta solicitud consiste en la necesidad de obtener formulaciones para el tratamiento de las úlceras de pie diabético.

Para resolver este problema técnico la solicitud en estudio proporciona formulaciones farmacéuticas que comprenden HEC (hidroxietil-celulosa) en combinación con el péptido Nle3A (1-7) de SEQ ID NO: 1 (Asp-Arg-Nle-Tyr-Ile-His-Pro) mediante el diseño de un hidrogel.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 38/08.

4. De la solicitud de patente

Título

Los métodos de tratamiento no son objeto de patentabilidad por lo cual se sugiere al solicitante hacer la corrección necesaria. Se sugiere modificar el título al siguiente: Formulaciones tipo hidrogel que comprenden el péptido Nle3A (1-7) en combinación con hidroxietilcelulosa.

Reivindicaciones (Art 30 D 486, Art 22 PCT)

- 1- La reivindicación 1 no es clara debido a que el término: “aproximadamente”, es impreciso, por lo cual no permitiendo distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto. El péptido debe ser definido por su estructura, es decir por el número de secuencia, se sugiere incluir la reivindicación 2 en la reivindicación 1. El rango presentado no es conciso, en la descripción está probada la concentración de 0,03% de péptido con 2% de hidroxietil celulosa. Se sugiere restringir la solicitud a las concentraciones del péptido y de hidroxietil celulosa, que fueron probadas y tienen sustento.
- 2- Las reivindicaciones 4-5, 7-8, 10-11 no son claras debido a que el término: “aproximadamente”, es impreciso, por lo cual no permitiendo distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso o un rango concreto. Adicionalmente, no son concisas debido a que en la descripción está probada la concentración de 0,03% de péptido con 2% de hidroxietil celulosa y no están probados los otros rangos. Se sugiere restringir la solicitud a las concentraciones del péptido y de hidroxietil celulosa, que fueron probadas y tienen sustento.
- 3- La reivindicación 3 reclama la misma materia de la reivindicación 2. Se sugiere eliminar dicha reivindicación.
- 4- Las reivindicaciones 4, 5 reclaman materia que ya está reivindicada en la reivindicación 1. Se sugiere eliminar dichas reivindicaciones.

- 5- Las reivindicaciones 10-12 reclaman la misma materia de las reivindicaciones 7-9. Se sugiere eliminar dichas reivindicaciones.

Se requiere al solicitante hacer las aclaraciones necesarias.

5. Análisis de la Oposición

Argumentaciones de Procaps S.A.

Dice el opositor que esta solicitud pretende obtener patente para métodos para el tratamiento de úlceras de pie diabético. Dice el opositor que dichos métodos no son patentables de acuerdo a la D486 Art. 20 literal d.

Adicionalmente, dice el opositor que las reivindicaciones carecen de nivel inventivo de acuerdo a los documentos de Rodgers et al., 2005 (D1); US-NIH, 2010 (D2). El opositor menciona que el documento D1 revela fragmentos Nle3-angiotensina (1-7) que aceleran la curación en modelos dérmicos, y que el documento D2 divulga un ensayo clínico para comprobar la eficacia del péptido en el tratamiento de úlceras de pie diabético. Argumenta el opositor que el objeto de oposición a la luz del documento D1 solo o en combinación con D2 carece de nivel inventivo.

Respuestas del Solicitante

Dice el solicitante que presenta un nuevo capítulo reivindicatorio eliminando las reivindicaciones que mencionan el método de tratamiento. Dice el solicitante que la composición reclamada presenta un incremento en la liberación de péptidos de 10 veces sobre formulaciones similares del estado del arte y que las matrices de hidroxietil celulosa son más biocompatibles que las presentadas en el estado del arte.

Dice el solicitante que de acuerdo a los documentos presentados no sería posible para el experto en la materia modificar las composiciones para mejorar la efectividad de la formulación.

Respuesta del Examinador Técnico

Los documentos presentados en la oposición se considerarán dentro del examen de patentabilidad para el estudio de nivel inventivo, puesto que mencionan el péptido de la formulación farmacéutica y un vehículo como excipiente para el tratamiento de úlcera de pie de diabético.

6. 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: febrero 02 de 2011, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación
D1	Rodgers et al., 2005. Journal of peptide research, Blackwell publishing Ltd. Oxford, Vol. 66 (1):41-47	"Fragments of Nle 3-angiotensin(1-7) accelerate healing in dermal models"	01/12/2005
D2	US-NIH., 2009. NCT00796744.	"Safety and Efficacy Study of DSC127 in Treating Subjects With Diabetic Ulcers"	12/Nov/2009
D3	Matuszewska et al., 1994. Pharm Res. Vol.11 (1):65-71.	"Acidic fibroblast growth factor: evaluation of topical formulations in a diabetic mouse wound healing model."	1994

El documento D1¹divulga fragmentos de Nle3-angiotensina (1-7) que aceleran la curación en los modelos dérmicos y reporta las cualidades curativas de heridas del péptido NLe-A (1-7).

El documento D2²divulga un ensayo clínico para comprobar la seguridad y eficacia del péptido Nle-A (1-7) en el tratamiento de las úlceras del pie de diabético.

El documento D3³divulga que el uso de HEC como excipiente para el tratamiento de úlcera de pie diabético.

7 . Examen de Patentabilidad(Art 14 D486)

Nivel inventivo (Art18 D486)

Nivel inventivo con los documentos D1 y D3

La reivindicación 1 consiste en: *“una formulación farmacéutica que comprende: a) de 0,5% a 4% de HEC (...)”*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D3
Formulación farmacéutica	Formulación farmacéutica (Pág. 42, Col 2).	Formulación farmacéutica (Resumen)
0,5 a 4% de HEC	10% de CMC	HEC (Resumen)
Péptido de 5 aminoácidos contiguos de Nle3 A (1-7), en concentración de 0,03% a 1%	Nle3 A (1-7) 1mg/ml (Pág. 42, Col 2).	No menciona

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque el documento D1 divulga una formulación farmacéutica que comprende un péptido Nle3 A (1-7) y un vehículo (Pág. 42, Col 2). El documento D1 menciona la eficacia de dicho péptido en el control de úlceras y menciona la formación de hidrogeles para el tratamiento del pie diabético (Pág. 42, Col 1, Pág. 46, Col 1).

La diferencia entre la invención, definida en la reivindicación 1 y la formulación de D1 consiste en el vehículo HEC.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la formulación reclamada en esta solicitud. Dentro de la descripción no hay ningún ejemplo que muestre un efecto técnico inesperado al usar como vehículo HEC y no CMC.

El problema técnico objetivo de esta solicitud consiste en la necesidad de una formulación farmacéutica alternativa en la que se usa como vehículo HEC, a la ya conocida en el documento D1.

Sin embargo, el uso de HEC como excipiente, ya está divulgada en el documento D3 que se refiere al uso de HEC como excipiente para el tratamiento de úlcera de pie diabético (Resumen). Adicionalmente, la eficacia del péptido en el control de úlceras y la divulgación de la formulación de hidrogeles para el tratamiento de pie diabético usando el péptido y un vehículo (Pág. 42, Col 1, Pág. 46, Col 1), ya está

1. <http://onlinelibrary.wiley.com/sci-hub.org/doi/10.1111/j.1747-0285.2006.00350.x/pdf>
2. http://clinicaltrials.gov/archive/NCT00796744/2009_11_12
3. <http://www.ncbi.nlm.nih.gov/pubmed/7511240>

divulgada en el documento D1 y en la literatura^{4, 5}. Adicionalmente, tanto CMC como HEC son equivalentes farmacéuticos siendo ambas celulosas formadoras de hidrogeles, por lo que el diseño de la formulación reclamada no resulta inventiva solo por el hecho de intercambiar el formador de gel.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el vehículo HEC del documento D3 en la formulación farmacéuticadivulgada en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Las reivindicaciones dependientes 4-12 no mencionan alguna característica que haga inventiva la formulación farmacéutica.

Nivel inventivo con los documentos D2 y D3

La reivindicación 1 consiste en: *“una formulación farmacéutica que comprende: a) de 0,5% a 4% de HEC (...)”*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2	D3
Formulación farmacéutica	Formulación farmacéutica	Formulación farmacéutica (Resumen)
0,5 a 4% de HEC	Vehículo (no especifica de que tipo)	HEC (Resumen)
Péptido de 5 aminoácidos contiguos de Nle3 A (1-7), en concentración de 0,03% a 1%	NorLeu3-A(1-7) 0,03%	No menciona

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque el documento D2 divulga un estudio clínico para evaluar la eficacia de NorLeu3-A (1-7) a una concentración del 0,03% junto con un vehículo (Resumen)

La diferencia entre la invención, definida en la reivindicación 1 yla formulación de D2 consiste en el vehículo que es HEC.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la formulación reclamada en esta solicitud. Dentro de la descripción no hay ningún ejemplo que muestre un efecto técnico inesperado al usar como vehículo HEC y no CMC. Adicionalmente, el documento D2 no especifica el tipo de vehículo por lo que podría ser el de HEC, en donde la formulación puede ser usada para el tratamiento de la úlcera de pie diabético.

El problema técnico objetivo de esta solicitud consiste en la necesidad de una formulación farmacéutica alternativa en la que se usa como vehículo HEC, a la ya conocida en el documento D2.

Sin embargo, el uso de HEC como excipiente, ya está divulgada en el documento D3 que se refiere al uso de HEC como excipiente para el tratamiento de úlcera de pie diabético (Resumen). Adicionalmente, la eficacia del péptido en el control de úlceras y la divulgación de la formulación de hidrogeles para el tratamiento de pie diabético usando el péptido y un vehículo (Pág. 42, Col 1, Pág. 46, Col 1), ya está divulgada^{4, 5}. Adicionalmente, tanto CMC como HEC son equivalentes farmacéuticos siendo ambas celulosas formadoras de hidrogeles, por lo que el

4. <http://www.ncbi.nlm.nih.gov/pubmed/14714558>

5. <http://www.ncbi.nlm.nih.gov/pubmed/15953051>

diseño de la formulación reclamada no resulta inventiva solo por el hecho de intercambiar el formador de gel.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el vehículo HEC del documento D3 en la formulación farmacéutica divulgada en el documento D2 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Las reivindicaciones dependientes 4-12 no mencionan alguna característica que haga inventiva la formulación farmacéutica.

La reivindicación 2 consiste en: *“una formulación farmacéutica que comprende: a) de 0,5% a 4% de HEC (...) caracterizada porque el péptido comprende Asp-Arg-Nle-Tyr-Ile-His-Pro (SEQ ID NO: 1)”*

La reivindicación 3 consiste en: *“una formulación farmacéutica que comprende: a) de 0,5% a 4% de HEC (...) caracterizada porque el péptido consiste de Asp-Arg-Nle-Tyr-Ile-His-Pro (SEQ ID NO: 1)”*

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D3
Formulación farmacéutica	Formulación farmacéutica (Pág. 42, Col 2).	Formulación farmacéutica (Resumen)
0,5 a 4% de HEC	10% de CMC	HEC (Resumen)
Péptido de 5 aminoácidos contiguos de Nle3 A (1-7), en concentración de 0,03% a 1%	Nle3 A (1-7) 1mg/ml (Pág. 42, Col 2).	No menciona
Nle3A (1-7) Asp-Arg-Nle-Tyr-Ile-His-Pro (SEQ ID NO: 1	NorLeu3A (1-7)	

El documento D1 se consideran el estado de la técnica cercano a la invención definida en las reivindicaciones 2 y 3 porque el documento D1 divulga una formulación farmacéutica que comprende un péptido Nle3 A (1-7) y un vehículo (Pág. 42, Col 2).

La diferencia entre la invención, definida en la reivindicación 1 y la formulación de D1 consiste en el vehículo HEC y en la SEQ ID NO: 1.

Esta diferencia no parece conceder un efecto técnico inesperado o una ventaja a la formulación reclamada en esta solicitud.

El problema técnico objetivo de esta solicitud consiste en la necesidad de una formulación farmacéutica alternativa en la que se usa como vehículo HEC y la SEQ ID NO: 1, a la ya conocida en el documento D1.

Sin embargo, el uso de HEC como excipiente, ya está divulgada en el documento D3 que se refiere al uso de HEC como excipiente para el tratamiento de úlcera de pie diabético (Resumen). La eficacia del péptido en el control de úlceras y la divulgación de la formulación de hidrogeles para el tratamiento de pie diabético usando el péptido y un vehículo (Pág. 42, Col 1, Pág. 46, Col 1), ya está divulgada en el documento D1. Adicionalmente, tanto CMC como HEC son equivalentes farmacéuticos siendo ambas celulosas formadoras de hidrogeles, por lo que el diseño de la formulación reclamada no resulta inventiva solo por el hecho de intercambiar el formador de gel. Por otra parte, el documento D1 aunque no divulga la SEQ ID NO: 1 si divulga la nomenclatura NorLeu3-A (1-7), en la literatura es conocido que la estructura de NorLeu3A (1-7) corresponde a la misma

de la SEQ ID NO: 1⁶.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el vehículo HEC del documento D3 en la formulación farmacéutica divulgada en el documento D1 para llegar así al objeto de las reivindicaciones 2 y 3 de la solicitud. Por lo cual se consideran obvias.

8. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	Rodgers et al., 2005. Journal of peptide research, Blackwell publishing Ltd. Oxford, Vol. 66 (1):41-47	1 – 12	Nivel inventivo
D2	US-NIH., 2009. NCT00796744.	1 – 12	Nivel inventivo
D3	Matuszewska et al., 1994. Pharm Res. Vol.11 (1):65-71.	1 – 12	Nivel inventivo

6. <http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=14767505&viewopt=PubChem>

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 del día siguiente 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.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

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 2014-06-03

Elaboró: Lina Maria Lizaraso Trespalacios
Revisó: Consuelo Leguizamon Leguizamon

K.E. Rodgers
D.D. Ellefson
T. Espinoza
H. Maulhardt
N. Roda
S. Maldonado
G.S. diZerega

Fragments of Nle³- angiotensin(1–7) accelerate healing in dermal models

Authors' affiliations:

K.E. Rodgers, H. Maulhardt and G.S. diZerega,
US Biotest, 231 Bonetti Drive, Suite 240, San Luis
Obispo, CA 93401-7310, USA

K.E. Rodgers, D.D. Ellefson, T. Espinoza, N. Roda,
S. Maldonado and G.S. diZerega, USC/KECK
School of Medicine, Department of Obstetrics and
Gynecology, University of Southern California,
Los Angeles, CA, USA

Correspondence to:

K.E. Rodgers
US Biotest
231 Bonetti Drive
Suite 240
San Luis Obispo
CA 93401-7310
USA
Tel.: (805) 595-1300
Fax: (805) 595-1350
E-mail: Kathy.Rodgers@usbiotest.com

Key words: analogue; angiotensin; collagenase; dermal; proteases; stability

Abstract: The renin angiotensin system (RAS) has been shown to be present in dermal tissue and exogenous peptides from the RAS accelerates healing and reduces scarring. An analogue of Angiotensin(1–7) [A(1–7)], Norleucine³-A(1–7) [Nle³-A(1–7)], is the lead compound under development for treatment of dermal injuries. As proteases are prevalent in wounded tissue and at very high levels in chronic wounds, the ability of fragments of Nle³-A(1–7) to accelerate healing and the effect of proteases on the peptide were determined. Daily application of fragments of Nle³-A(1–7) of five or six amino acids accelerated healing in two models of dermal injury. In addition, the peptide was found to be stable (not substantially degraded) after incubation for 4 h in the presence of Cathepsin G, collagenases blend (from clostridium), matrix metalloprotease [MMP] 2, MMP 3, MMP 9, elastase (human leukocytic or porcine pancreatic) or plasmin. Only kallikrein, an enzyme known to cleave peptides of the RAS, cleaved the peptide into two major fragments one of which was identified as NorLeu³-A(1–4). These data support the activity of Nle³-A(1–7) on dermal wounds.

Abbreviations: A II, Angiotensin II; A(1-7), Angiotensin II (1-7); CMC, carboxymethyl cellulose; MAU, milliabsorbance units; MMP, matrix metallo protease; Nle, Norleucine; RAS, renin angiotensin system.

Introduction

A physiological role for the renin angiotensin system (RAS) in multiple systems involved in cellular proliferation and differentiation has been proposed. The skin represents one of the few mammalian tissues with a very high turnover rate (generation time of keratinocytes is approximately 40–65 days) and is continuously undergoing differentiation.

Dates:

Received 20 September 2005
Revised 14 November 2005
Accepted 29 December 2005

To cite this article:

Rodgers, K.E., Ellefson, D.D., Espinoza, T., Maulhardt, H., Roda, N., Maldonado, S. & diZerega, G.S. Fragments of Nle³-angiotensin(1–7) accelerate healing in dermal models. *J. Peptide Res.*, 2006, **66** (Suppl. 1), 41–47.

Copyright Blackwell Munksgaard, 2006

Studies by multiple laboratories have shown the expression of the RAS system in the skin (1–5). Angiotensin receptors are expressed both in rat neonates and adults, with the receptor phenotype changing with maturation. Upon injury, the expression of receptors is upregulated and down regulates as the tissue heals (1–3,5). In human skin, AT₁ and AT₂ receptors were found in the epidermis and in dermal vessel walls (4). The same expression pattern was found for angiotensinogen, the gene product from which active peptides are derived, renin and ACE. All components were also demonstrated at the mRNA level in cultured primary keratinocytes, melanocytes (except AT₂ receptors), dermal fibroblasts, and dermal microvascular endothelial cells. The synthesis of AII by cultured keratinocytes was increased in an in vitro model of injury (4). Most recently, mice deficient for the angiotensin receptor Mas were found to spontaneously develop dermal lesions upon aging (personal communication, T. Walther).

The peptides, angiotensin II (AII), and angiotensin II (1–7) (A(1–7)), of the RAS are highly effective in stimulating wound healing in a variety of in vivo animal models (6–12). Both angiotensin peptides are naturally expressed in the body. A(1–7) was identified and shown to be comparable to AII in facilitating wound healing (9). An analogue of A(1–7), Nle³-A(1–7), that is more potent than A(1–7) in accelerating wound repair, was also identified and is undergoing pre-clinical development as a therapeutic for chronic wounds (10,11). In normal rats and diabetic mice, the peptide, when delivered in a viscoelastic vehicle, was highly effective in accelerating re-epithelialization of full thickness wounds. Full healing was observed in 60% of wounds in mice treated topically or systemically with Nle³-A(1–7). In contrast, Regranex® (human recombinant platelet derived growth factor in carboxymethyl cellulose) did not fully heal any wounds and A(1–7) only healed 20% of the wounds (10). Further, administration of Nle³-A(1–7) reduced fibrosis and scarring in the healed wound (10,12). This action was more pronounced the longer the peptide was given after injury. In fact, if the administration of Nle³-A(1–7) by systemic administration continued during the remodeling phase, the formation of new adnexal structures at the center of full-thickness excision wounds was observed including an increase in the appearance of immature hair follicles at the site of the excision wound (10,11). Histological examination showed that Nle³-A(1–7) accelerated collagen deposition six-fold and increased the number of blood vessels at the wound site three-fold (11). Thus, Nle³-A(1–7) appears to be more active in preclinical models than FDA approved drug for wound healing (Regranex).

The mechanisms by which A(1–7) and Nle³-A(1–7) stimulate wound healing are not entirely understood. A(1–7) has little affinity for AT₂ receptors (13–15) and is believed to act via a unique receptor (16–20). The effects of A(1–7) were not altered by AT₁ or AT₂ receptor antagonists, but were blocked by D-Ala⁷-A(1–7), which is believed to be a selective antagonist of the A(1–7) receptor (21,22). Actions of A(1–7) are not present in Mas knockout mice suggesting that this is the preferred receptor for A(1–7) and dAla⁷-A(1–7) blocks the actions of A(1–7) in cells transfected by Mas (23). Similarly, Nle³-A(1–7) actions on wound healing are blocked by this A(1–7) receptor antagonist (11).

There is extensive evidence that increased levels of proteases in chronic wounds contribute to reduced efficacy of agents developed for acceleration of chronic ulcer healing. In order to understand the possible contribution of increased protease levels to the efficacy of our lead compound, studies were undertaken to evaluate the ability of fragments of Nle³-A(1–7) to accelerate healing in normal rats and diabetic mice. Further, the stability of this peptide was assessed in the presence of enzymes commonly found in wound fluid, cathepsin G, collagenase, MMPs, elastase, and plasmin in vitro. Kallikrein, an enzyme known to cleave angiotensin peptides, was used as a positive control for these in vitro studies.

Materials and Methods

Materials

The viscous vehicle was prepared from carboxymethyl cellulose (CMC sodium salt, low viscosity, Sigma, St. Louis, MO, Lot Number 34H0310) consisting of 10% low viscosity CMC in 0.05 M phosphate buffer, pH 7.2, and sterilized by autoclaving followed by mixing with sterile peptide solutions. The active peptide that is the lead compound, Nle³-A(1–7), was prepared by Bachem under Good Manufacturing Practices. The remaining peptides, Nle³-A(1–4), Nle²-A(2–7), Nle¹-A(3–7), Nle³-A(1–6), and Nle³-A(1–5), were manufactured at the USC Microchemical Core facility.

Enzymes were purchased from Sigma (St. Louis, MO): cathepsin G from human leukocytes, collagenase mixed from *Clostridium*, elastase from human leukocytes, elastase from porcine pancreas, kallikrein from porcine pancreas, matrix metalloproteinase 2 (MMP 2), MMP 3, MMP 9, and plasmin from human plasma.

Rat full-thickness excision model

During the experimental period, Sprague-Dawley rats were housed one per cage and maintained in a central animal care facility with a 12 h light/dark cycle. Water and standard rodent laboratory chow were supplied *ad libitum*. All animals used for this study received humane care as defined by the National Research Council's criteria for humane care. The study protocols were approved by the University of Southern California's Institutional Animal Care and Use Committee before initiation of the studies.

Female Sprague-Dawley rats, 175–200 g, were anesthetized with intramuscular ketamine/rompun. The backs of the rats were shaved with animal clippers, scrubbed with betadine, and washed with 70% alcohol before surgery. Two 2.25 cm² excisions, approximately 1–2 cm apart, extending to the panniculus carnosus were made mid-dorsally. The edges of the wound were traced onto a glass slide immediately after wounding and at each postoperative time point. In the group of animals that received topical medication, immediately after tracing the wound, the various medicaments were administered topically at the wound site. After treatment, the wounds were covered with a semipermeable polyurethane dressing and the covering sealed with benzoin solution. Rats were lightly anesthetized with intramuscular ketamine/rompun on day 2 and 4. After anesthesia, the bandages were removed and the area was cleaned before further administration of peptide in 10% carboxymethyl cellulose.

Wound measurement

Wound areas were measured on days 0, 2, 4, 7, and 9. The animals were either lightly anesthetized with Metofane (if just measuring wounds) or intramuscular ketamine/rompun (if bandages were changed), and the area of wound closure and the area of the wounds covered with granulation tissue were traced. The wounds were considered closed if moist granulation tissue was no longer apparent and the wound appeared covered with epithelium. Wound areas were determined by tracing the wound area onto graph paper and weighing the graph paper. The tracing of the wound on day 0 was used as the original area, and all of the subsequent areas are expressed as a percentage of the original area. For each measurement time, the percentage of the original area from the treated wound area was divided by the percentage of the original area of the vehicle treated wound to determine the percentage of control response. A value less than 100% would be indicative of acceleration in wound repair.

Diabetic mouse surgical model

Male C57Bl/6-db/db diabetic mice, 6–8 weeks old, were used for these studies. All animals were handled and maintained in accordance to the 'Guide for the Animal Care and Use of Laboratory Animals' and were approved by the USC Institutional Animal Care and Use Committee. At day 0, animals were anesthetized with ketamine/xylazine and backs were shaved and cleaned with Betadine and 70% isopropyl alcohol. Afterwards, a 1.3 cm diameter full thickness skin area was excised on the back of each animal. Wound margins were treated with Benzoin Tincture (Western Medical Supply, Arcadia, CA), and 100 mL of the peptide preparation or vehicle was placed in each wound. Wounded areas were covered with Tegaderm transparent dressing that was sealed at the edges with Benzoin Tincture. Daily over 10 days, 100 mL of medicament or vehicle preparation was injected with a 26G needle through the Tegaderm into the wound bed. At day 0 (directly after wounding), and every 3–4 days, animals were anesthetized with isoflurane, and wound margins were traced onto glass slides. The bandages were changed every 2–3 days through day 14.

To evaluate dermal healing, wound areas were measured on days 0, 3, 7, 10, 14, 17, 21, and 24. The animals were either lightly anesthetized with Metofane (if just measuring wounds) or intramuscular ketamine/rompun (if bandages were changed), and the area of the wounds covered with epithelium was traced (7,10,11). The wounds were considered closed if moist granulation tissue was no longer apparent and the wound appears covered with epithelium. The measurements were performed by a trained observer blinded to the treatment the animals received. At necropsy, the dressing was removed and the wound was traced. Wound areas were determined by tracing the wound area onto graph paper (1 mm² square) and weighing the graph paper. The tracing of the wound on day 0 was used as the original area, and all of the subsequent areas were expressed as a percentage of the original area that was still open.

In vitro stability studies

The enzymes were diluted in 2 tubes (duplicate) in 9 mL of phosphate buffered saline, pH 7.2, and warmed to 37 °C at the following concentrations (represents concentration in final 10 mL volume): cathepsin G from human leukocytes 0.5 Units/mL; collagenase, Sigma blend, 1 mg/mL; elastase from human leukocytes 0.01 Units/mL; elastase from

porcine pancreas 0.05 mg/mL; kallikrein from porcine pancreas 10 Units/mL; MMP 2 0.01 µg/mL; MMP 3 0.01 µg/mL; MMP 9 0.01 µg/mL; and plasmin 10 µg/mL.

At time 0, 1 mL of 10 mg/mL Nle³-A(1–7) (final concentration 1 mg/mL) was added to each tube and the solutions mixed. Immediately, 1 mL was removed from each tube (duplicate for each enzyme and time point) and placed in a bath containing dry ice and ethanol for snap freezing. At 1, 5, 15, 30 min, 1, 2, and 4 h, 1 mL samples were removed from the tubes and snap frozen in the appropriate containers. Samples were kept frozen until HPLC testing for degradation products.

During the HPLC testing, the samples were thawed one at a time and kept cold until processing to reduce enzyme activity. Sample tubes were removed from –20 °C and thawed by hand. The thawing process took approximately 5 min. Thawed samples were filtered using a 0.45 µm syringe filter (PALL PN4556T) directly into a glass vial. Injections were made immediately after filtration was complete.

Results

Rat full thickness excision model

The ability of several fragments of Nle³-A(1–7) to accelerate the healing of full thickness excision wounds was initially tested on rats. The data, expressed as a percent of the control wound size (therefore when lines are below 100 there is an acceleration of healing), showed that Nle³ substituted peptides of five or greater amino acids accelerated wound closure (Fig. 1). Fragments of AII of four amino acids were effective at promoting wound repair (data not shown). However, with Nle substitution at position 3 (of AII), peptides with less than five amino acids were not effective at increasing wound repair (Fig. 1). This may indicate that the conformational changes that occur in the shorter peptides with the substitution of Nle reduces biological activity, perhaps by altering receptor interaction.

Diabetic mouse full thickness excision model

In Figs 2 and 3, the ability of fragments of Nle³-A(1–7) to accelerate healing of full thickness wounds in diabetic mice was evaluated. Fragments of Nle³-A(1–7), Nle²-A(2–7), Nle³-A(1–6), Nle¹-A(3–7), and Nle³-A(1–5), accelerated healing comparable to the intact peptide. The ability of

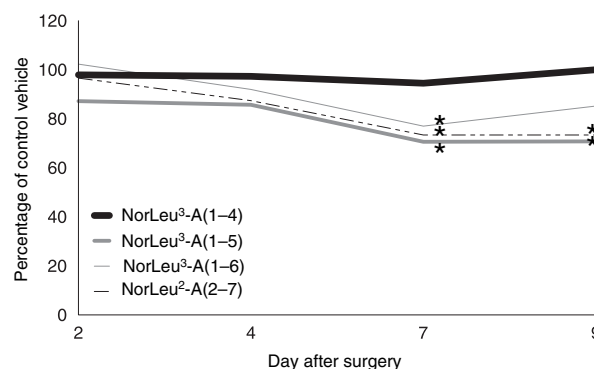


Figure 1. The ability of fragments of Nle³-A(1–7) to accelerate healing was evaluated in a full thickness excision model in rats. Five female Sprague Dawley rats per group received two wounds. Daily, through day 5, the placebo (control) or vehicle containing peptide (100 µL, 1 mg/mL) was applied. The amount of wound not re-epithelialized was measured on days 2, 4, 7, and 9. These data are represented as the percentage of the wound size as a ratio (treated wound size/placebo wound size) × 100. Points marked with (*) were significantly different ($P < 0.05$) from the placebo treated wound.

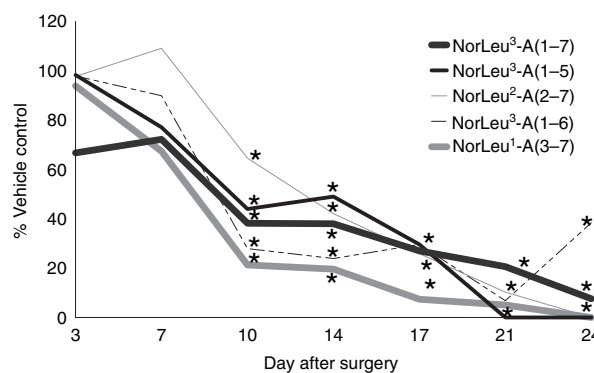


Figure 2. The ability of fragments of Nle³-A(1–7) to accelerate healing was evaluated in a full thickness excision model in diabetic mice. Five males diabetic (db/db) mice per group received one wound. Daily, through day 5, the placebo (control) or vehicle containing peptide (100 µL, 1 mg/mL) was applied. The amount of wound not re-epithelialized was measured on days 3, 7, 10, 14, 17, 21, and 24. These data are represented as the percentage of the wound size as a ratio of (treated wound size/placebo wound size) × 100. Points marked with (*) were significantly different ($P < 0.05$) from the placebo treated wound.

these molecules to accelerate healing in this model is also demonstrated by the percentage of wounds fully healed (Fig. 3). All fragments tested (five and six amino acids in length) accelerated healing in this model.

In vitro stability with enzymes

In order to assess the stability of the peptides in the presence of enzymes found in chronic wound fluid, the peptide Nle³-A(1–7) was incubated in the presence of purified

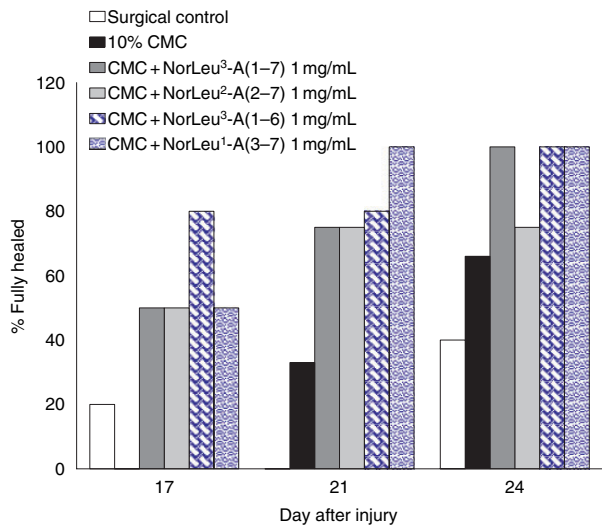


Figure 3. The data from Fig. 2 is represented here as the percentage of wounds fully healed at days 17, 21, and 24 including wounds from surgical and vehicle controls. As can be seen, Nle³-A(1–7) and all fragments tested accelerated the healing of the diabetic wounds.

Table 1. Levels of intact Nle³-A(1–7) after in vitro incubation with purified enzymes. Intact peptide levels (μg/mL)

Intact peptide levels (μg/mL)			
Enzyme	0 min	15 min	4 h
Cathepsin G	–	960	946
Collagenase Blend	–	910	869
Human Elastase	–	988	971
Porcine Elastase	943	921	663
Kallikrein	177	13	0
MMP 2	987	995	982
MMP 3	–	974	1010
MMP 9	–	992	977
Plasmin	–	1009	992

enzymes (Table 1). Only the positive control, kallikrein, an enzyme known to be involved in the RAS, has substantial degradation potential and none of the human enzymes found in wounds have ability to degrade the peptide (Figs 4 and 5). The preferential cleavage site of tissue kallikrein is Arg-/Xaa. This reaction is selectively involved in the release of bradykinin or kallidin from kininogen in which the preferential cleavage is Met-/Xaa or Leu -/Xaa. However, for this peptide cleavage occurred between Tyr and Ile as the major fragment equal (% to the remaining major peak) as identified as Nle³-A(1–4) as the remaining peak is of equal area, it is expected that this peak is A(5–7), the fragment that would be cleaved from Nle³-A(1–7) to generate Nle³-A(1–4). There is slight degradation with two of

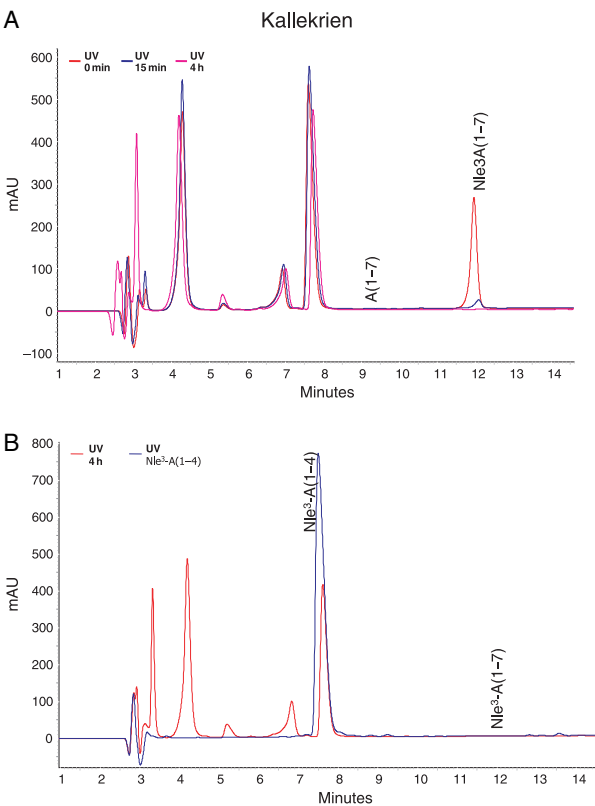


Figure 4. Peptide, one part Nle³-A(1–7) (10 mg/mL), was mixed with nine parts kallikrein enzyme at time = 0 and duplicate tubes were immediately snap frozen. Additional tubes were collected at 15 min and 4 h. The frozen samples were then thawed and the degradation profile analyzed by HPLC (panel a). The Y axis is mAU (milliabsorbance units) at 220 nm sampled every 0.2 s over a 26 min interval. The tracing of the synthetic peptide Nle³-A(1–4) is overlaid on this graph to show the identity of one of the two major peaks formed by exposure to kallikrein (panel b).

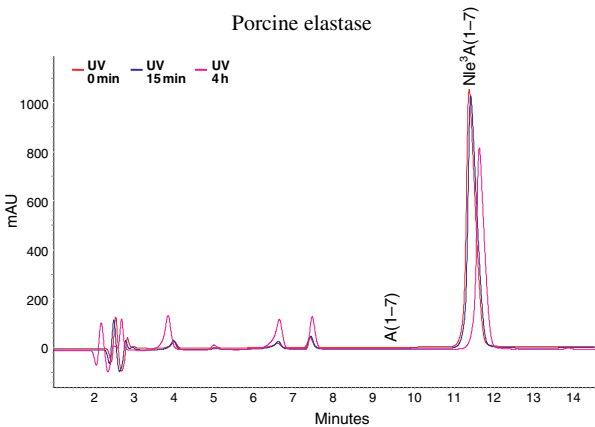


Figure 5. Peptide, one part Nle³-A(1–7) (10 mg/mL), was mixed with nine parts porcine pancreatic elastase at time = 0 and duplicate tubes were immediately snap frozen. Additional tubes were collected at 15 min and 4 h. The frozen samples were then thawed and the degradation profile analyzed by HPLC. The Y axis is mAU (milliabsorbance units) at 220 nm sampled every 0.2 s over a 26 min interval.

the crude enzyme preparations (collagenase blend and porcine pancreatic elastase), but not human elastase from leukocytes or purified collagenases. Within 15 min of exposure to the porcine pancreatic elastase, approximately 8% of the peptide was degraded. By 4 h, 66.4% of the peptide remained intact. The degradation of the peptide with the crude preparation of porcine pancreatic elastase was more pronounced than that with collagenase blend (Table 1).

Discussion

Recent studies have made clear that components of RAS are present in dermal tissue, that receptor profiles change upon injury of dermal tissue and receptor antagonists delay dermal healing. Through structure activity studies, Nle³-A(1–7) was identified in preclinical in vivo and in vitro studies as a therapeutic that accelerates healing better than the native peptide and in a manner superior to Regranex, and reduces scarring (9–12). This peptide is being developed as a therapeutic for the treatment of dermal wounds, including diabetic ulcers and scar reduction.

One aspect of injured tissue, particularly chronic diabetic wounds, is the presence of high levels of proteases. The effect of proteases on the efficacy of a therapeutic is an important consideration for the treatment of chronic ulcers. One means of forestalling proteolytic degradation is through the slow release of the therapeutic over time by use of a slow release vehicle. Release studies with our viscoelastic formulations indicate a slow steady release of the peptide over 24 h. However, if the drug is not stable for a period of time in the presence of proteases, efficacy may still be reduced. In this paper, evidence is provided that five and six amino acid fragments of Nle³-A(1–7) are effective at accelerating the healing of full thickness excision wounds in normal rats and diabetic mice. Further, the peptide was shown not to degrade in the presence of enzymes common to chronic wounds, a group that includes cathepsin G, collagenase, plasmin, elastase, and MMPs.

These data provide additional evidence that Nle³-A(1–7) is an attractive compound to evaluate in human clinical trials.

Acknowledgements: This study was funded by US Biotest and the National Institutes of Health (Grant Number 5R44AR047481-03).

References

1. Abiko, M., Rodgers, K.E., Campeau, J.D., Nakamura, R.M. & di Zerega, G.S. (1996) Alterations in angiotensin II receptor levels in full thickness excisional wounds in rat skin. *Wound Repair Regen* **4**, 363–367.
2. Abiko, M., Rodgers, K.E., Campeau, J.D., Nakamura, R.M. & di Zerega, G.S. (1996) Alterations in angiotensin II receptor levels in sutured wounds in rat skin. *J. Invest. Surg.* **9**, 447–453.
3. Kimura, B., Summers, C. & Phillips, M.I. (1992) Changes in skin angiotensin II receptors in rats during wound healing. *Biochem. Biophys. Res. Commun.* **16**, 1083–1090.
4. Steckelings, U.M., Artuc, M., Paul, M., Stoll, M. & Henz, B.M. (1996) Angiotensin II stimulates proliferation of primary human keratinocytes via a non-AT₁, non-AT₂ angiotensin receptor. *Biochem. Biophys. Res. Commun.* **229**, 329–333.
5. Viswanathan, M. & Saavedra, J.M. (1992) Expression of angiotensin II AT₂ receptors in the rat skin during experimental wound healing. *Peptides* **13**, 783–786.
6. Okuyama, N., Roda, N., Guerrero, A., Dougherty, W., Nguyen, T., di Zerega, G.S. & Rodgers, K.E. (1999) Effect of angiotensin II on the viability, vascularity of random flaps in a rat model. *Ann. Plast. Surg. Res.* **68**, 913–918.
7. Rodgers, K.E., Abiko, M., Girgis, M., St. Amand, K., Campeau, J. & di Zerega, G.S. (1997a) Acceleration of dermal tissue repair by angiotensin II. *Wound Repair Regen* **5**, 175–183.
8. Rodgers, K.E., Decherney, A.H., St., Amand, K.M., Dougherty, W.R., Felix, J.C., Girgis, W. & di Zerega, G.S. (1997b) Histologic alterations in dermal repair after thermal injury: effects of topical angiotensin II. *Burn Care Rehabil* **18**, 381–388.
9. Rodgers, K.E., Xiong, S., Felix, J.C., Roda, R., Espinoza, T., Maldonado, S. & di Zerega, G.S. (2001) Development of angiotensin (1–7) as an agent to accelerate dermal regeneration. *Wound Repair Regen* **9**, 241–250.
10. Rodgers, K.E., Espinoza, T., Felix, J.C., Roda, N., Maldonado, S. & di Zerega, G.S. (2003a) Acceleration of healing, reduction of fibrotic scar and normalization of tissue architecture by angiotensin analog, Nle³-A(1–7) Plast. *Reconstr. Surg* **111**, 1195–1206.
11. Rodgers, K.E., Roda, N., Felix, J.C., Espinoza, T., Maldonado, S. & di Zerega, G.S. (2003b) Histological evaluation of the effects of angiotensin peptides on wound repair in diabetic mice. *Exp. Dermatol.* **12**, 784–790.
12. Rodgers, K.E., Ellefson, D.D., Espinoza, T., Roda, N., Maldonado, S. & di Zerega, G.S. (2005) Effect of Nle³-A(1–7) on scar formation over time after full thickness incision injury in the rat. *Wound Repair Regen* **13**, 309–317.
13. Bernier, S.G., Servant, G., Boudreau, M., Fournier, A. & Guillemette, G. (1995) Characterization of a binding site for angiotensin IV on bovine aortic endothelial cells. *Eur. J. Pharmacol.* **291**, 191–200.
14. Chappell, M.C., Jacobsen, D.W. & Tallant, E.A. (1995) Characterization of angiotensin II receptor subtypes in pancreatic acinar AR42J cells. *Peptides* **16**, 741–747.

15. Tallant, E.A., Diz, D.I., Khosla, M.C. & Ferrario, C.M. (1991) Identification and regulation of angiotensin II receptor subtypes on NG108-15 cells. *Hypertension* **17**, 1135–1143.
16. Benter, I.F., Diz, D.I. & Ferrario, C.M. (1993) Cardiovascular actions of angiotensin-(1–7). *Peptides* **14**, 679–684.
17. Brosnihan, K.B., Li, P. & Ferrario, C.M. (1996) Angiotensin-(1–7) dilates canine coronary arteries through kinins and nitric oxide. *Hypertension* **27**, 523–528.
18. Porsti, I., Bara, A.T., Busse, R. & Hecker, M. (1994) Release of nitric oxide by angiotensin-(1–7) from porcine coronary endothelium: implications for a novel angiotensin receptor. *Br. J. Pharmacol.* **111**, 652–654.
19. Tallant, E., Lu, X., Weiss, R., Chappel, M. & Ferrario, C. (1997) Bovine aortic endothelial cells contain an angiotensin-(1–7) receptor. *Hypertension* **29**, 388–393.
20. Tallant, E.A., Diz, D.I. & Ferrario, C.M. (1999) State-of the-art lecture. antiproliferative actions of angiotensin 1–7 in vascular smooth muscle. *Hypertension* **34**, 950–957.
21. Santos, R.A.S., Campagnole-Santos, M.J., Baracho, N.C.V., Fontes, M.A.P., Silva, L.C.S., Neves, L.A.A., Oliveira, D.R., Caligiome, S.M., Rodrigues, A.R.V., Gropen, C. Jr, Carvalho, W.S., Simoes e Silva, A.C. & Khosla, M.C. (1994) Characterization of a new angiotensin antagonist selective for angiotensin-(1–7): evidence that the actions of angiotensin-(1–7) are mediated by specific angiotensin receptors. *Brain Res. Bull.* **35**, 293–298.
22. Vallon, V., Heyne, N., Richter, K., Khosla, M.C. & Fechter, K. (1998) 7-D-Ala-angiotensin 1–7 blocks renal actions of angiotensin 1–7 in the anesthetized rat. *J. Cardiovasc. Pharmacol.* **32**, 164–167.
23. Santos, R.A., Simoes e Silva, A.C., Maric, C., Silva, D.M., Machado, R.P., de Buhr, I., Heringer-Walther, S., Pinheiro, S.V., Lopes, M.T., Bader, M., Mendes, E.P., Lemos, V.S., Campagnole-Santos, M.J., Schultheiss, H.P., Speth, R. & Walther, T. (2003) Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc. Natl. Acad. Sci. USA* **100**, 8258–8263.