



DIRECCIÓN DE NUEVAS CREACIONES
PRESENTACIÓN DE OPOSICIÓN

1. Oposición a solicitud de:

- ☒ Patente de invención
☐ Registro de Diseño Industrial
☐ Patente de Modelo de Utilidad
☐ Esquema de Trazado de Circuitos Integrados

Número de expediente: 13 301132

Solicitante: OPKO BIOLOGICS LTD.

Apoderado: SILVANA MARIA LOPEZ DIAZ

Título de la Nueva Creación: AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1 DE ACCIÓN PROLONGADA

Gaceta: 698

2. Datos del Opositor

☐ Persona Natural ☒ Persona Jurídica
PROCAPS S.A.

Nombre o denominación /Razón social completa

WALTER TANAKA TATEKAWA

Nombre del representante legal

Documento de identificación: C.C. ☐ C.E. ☐ NIT ☒ Otro ☐ Cuál _____

Número 890106527-5 _____

Dirección. CALLE 80 78B 201 _____ Ciudad BARRANQUILLA
Teléfono 055 3719900 Fax: _____ Correo electrónico _____

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del representante o apoderado:

MORENO BOHORQUEZ ELIANA ISaura	51.975.664	83823
Apellidos y Nombre	Documento de identificación	Tarjeta profesional
Dirección del representante		
CRA 13 119-95 OFICINA 104		
Dirección electrónica negocios@optimum-co.com	No. Fax (571)6121979	Número telefónico (571)2131213

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general:

4. Fundamentos de la oposición

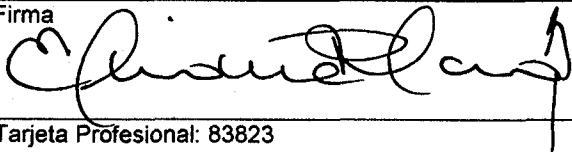
Causal: Art14, 16, 18,20 y 30 de la decisión 486 de la CAN no cumple con los requisitos De patentabilidad.

Justificación: La presente solicitud no cumple con los requisitos de patentabilidad dado que lo revelado, ya se encontraba en el estado de la técnica o es posible derivarlo de la misma tal y como se demostrará en el capítulo "fundamento de hecho".

Prórroga: SI ☐ NO ☐

5. Anexos

- ☒ Comprobante de pago de la tasa.
- ☒ Poder, si fuera el caso.
- ☒ Fundamentos de la oposición. Se anexan 8 folios (obligatorios indicar cuántos).
- ☒ Anexo de fundamentos de la oposición. Se anexan 46 folios (obligatorios indicar cuántos)
- ☒ Copias para traslado.
- ☐ Copia de la solicitud y sus anexos en formato magnético.

6. Firma	
Nombre del Firmante	Firma
ELIANA ISaura MORENO BOHORQUEZ	
CC. 51.975.664	Tarjeta Profesional: 83823

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800.176.089-2

- / -



Industria y Comercio
SUPERINTENDENCIA

RECIBO DE CAJA

No. 14 - 101340

Bogotá D.C., Septiembre 23 de 2014 - 12:59:05

RECIBIDO DE : PROCAPS S.A.

NI 890.106.527

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	633199755	23/09/2014	1.705.000.00

*** Conceptos Pagados ***

CANT.	RENTISTICO	CONCEPTO	Vr. UNIDITARIO	Vr. CONCEPTO
1 50005-01-04	PRESENTACION DE OBSERVACIONES A SOLICITUDES	12 OPOSICION POR CADA CLASE-MARCAS Y LEMAS COMERCIALES	335.000.00	335.000.00
				\$335.000.00

SON: **TRESCIENTOS TREINTA Y CINCO MIL PESOS MONEDA CORRIENTE**

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 13-301132- -00009-0000

Fecha: 2014-09-23 13:10:58 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 400 OPOSIOBSERVTER Folios: 63

Sede Centro: Carrera 13 No. 27 - 00 Pls 3,4,5 y 10 Bogotá, D.C.- Colombia

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Septiembre 23 de 2014 - 12:59:05



Derecho de la Competencia, Propiedad
Industrial y Comercio Exterior

Señor Director,
DIRECCIÓN DE NUEVAS CREACIONES
Superintendencia de Industria y Comercio
E. S. D.

Referencia : Expediente N° 13 - 301132
Asunto : Oposición a la solicitud de patente de Invención titulada
"AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1
DE ACCIÓN PROLONGADA"
Solicitante : OPKO BIOLOGICS LTD..
Opositora : PROCAPS S.A.

Publicada en la Gaceta de la Propiedad Industrial
N° 698 del 27 de Junio de 2014

Yo, **ELIANA ISaura MORENO BOHÓRQUEZ**, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 del Consejo Superior de la Judicatura, en mi condición de apoderada de la sociedad **PROCAPS S.A.** conformada bajo las leyes de la República de Colombia y domiciliada en la ciudad de Barranquilla, Colombia, atentamente manifiesto que estando dentro del término de ley y por orden expresa de mi representada, presento oposición a la concesión del registro de la Patente de Invención titulada "**AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1 DE ACCIÓN PROLONGADA**", cuyo titular **OPKO BIOLOGICS LTD.**, y pido a Usted declarar fundada esta oposición y en consecuencia negar el registro de la patente solicitada.

1. INTERÉS PARA ACTUAR

Conforme a lo dispuesto en el artículo 42 de la Decisión 486 de 2000 de la CAN, estando dentro del término estipulado por la ley, manifiesto el legítimo interés de nuestra representada para oponerse al registro de la patente de la referencia, atendiendo a que la solicitud pretendida no cumple con los requisitos dispuestos en la ley como se analizará más adelante y de ser concedida afectaría directamente la

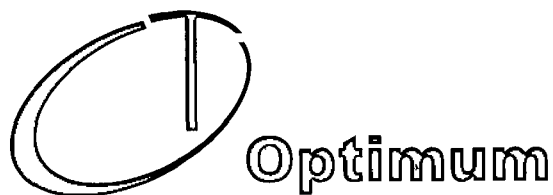
comercialización de algunos productos de mi representada, que contienen, en particular *AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1*. Por lo tanto, solicito respetuosamente a ese Despacho se sirva tener en cuenta la presente oposición dentro del trámite administrativo que se surte en esta Superintendencia.

2. FUNDAMENTOS DE HECHOS

- 2.1. La solicitud objeto de la presente fue presentada el 27 de diciembre de 2013, reivindicando prioridad bajo las solicitudes US201161492448P del 2011-06-02; y, US201261624589P del 2012-04-16; y fue publicada en Colombia, en la Gaceta de la Propiedad Industrial N° 698 del 27 de Junio de 2014, cuya publicación internacional corresponde a la WO2012167251 del 2012-12-06.
- 2.2. La solicitud colombiana 13 - 301132 objeto de la presente oposición se refiere a agonistas del receptor de glucagón/GLP-1 pegilado y pegilado inverso que incluye composiciones farmacéuticas que los comprenden y métodos para su uso.
- 2.3. En primera instancia, se debe advertir que las reivindicaciones se refieren también a métodos de tratamiento y no sólo a la composición farmacológica o a un método de fabricación mediante la cual se obtenga un producto tangible y según la legislación, decisión 486 de la Comunidad Andina de Naciones, Artículo 14.- *“Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento (...)”*, los usos no están contemplados como patentables; y, Artículo 20.- *“No serán patentables (...) d) los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales”*. Solo serán patentables los productos o procedimientos. Por lo tanto a luz de la norma, dicha materia referente métodos de tratamiento no sería patentable.
- 2.4. Por otro lado, es de resaltar que en materia de unidad de invención, la presente comprende varios grupos de invenciones que no conforman una única unidad inventiva, contrario a lo que dicta la Norma. Esto es:

Grupo I: Reivindicaciones 1-6: Composición que consiste en oxintomodulina, un polímero de polietilenglicol (PEG polímero), y 9 fluorenilmetoxycarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS).

Grupo II: Reivindicaciones 7-11: Método para prolongar la vida media biológica de oxintomodulina que consiste en la etapa de conjugación de la



oxintomodulina, un polímero de polietilenglicol (PEG polímero) y 9-fluorenylmethoxycarbonyl (Fmoc) o 2-sulfo-9-fluorenilmetoxicarbonilo (FMS) en una relación molar de aproximadamente 1: 1: 0,5 a 1: 1: 3,5.

Grupo III: Reivindicaciones 12-16: Procedimiento de inducción de tolerancia a la glucosa, control de la glucemia, en tanto en un sujeto en necesidad del mismo, que comprende la etapa de administrar a dicho sujeto una cantidad eficaz de la composición de la reivindicación 1 y un producto farmacéutico aceptable portador.

Grupo IV: Reivindicaciones 17-26: Método para mejorar el área bajo la curva (AUC) de oxintomodulina que incluye un método de reducir la frecuencia de dosificación de oxintomodulina, que consiste en la etapa de conjugado de un polímero de polietilenglicol (PEG polímero) a la amino terminal de dicho oxintomodulina a través de 9 fluorenilmetoxicarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxicarbonilo (FMS).

Grupos V: Reivindicaciones 27-31: Método para reducir la ingesta de alimentos, reducir el peso corporal, o ambas en un sujeto, que comprende la etapa de administrar oxintomodulina conjugado con polímero de polietilenglicol (PEG polímero) a través de un enlazador flexible, en donde dicho flexibles enlazador es 9-fluorenilmetoxicarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxicarbonilo (FMS).

Grupo VI: Reivindicaciones 32-39: Método para aumentar la sensibilidad a la insulina en un sujeto, que comprende la etapa de administrar al sujeto una cantidad eficaz de una composición que comprende oxintomodulina conjugado con polímero de polietilenglicol (PEG; polímero).

Las invenciones que figuran como los Grupos I a VI no se refieren a un único concepto inventivo general conforme la Norma, Decisión 486 de la CAN, artículo 25: *“La solicitud de patente sólo podrá comprender una invención o un grupo de invenciones relacionadas entre sí, de manera que conformen un único concepto inventivo”*. Estos grupos inventivos carecen de las mismas o correspondientes características técnicas especiales para considerarse como un unico concepto inventivo. Por tanto no se ajustan a lo que dicta la Norma en Colombia.

2.5. Ahora bien, con base en el artículo 14 de la Decisión 486 de la CAN consagra lo siguiente: *“Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel*

inventivo y sean susceptibles de aplicación industrial". Así las cosas, las invenciones objeto de patente deben ser nuevas y tener nivel inventivo. Bien, pues antes de la fecha de prioridad de esta solicitud se encuentran los siguientes documentos que afecta la patentabilidad de la presente solicitud:

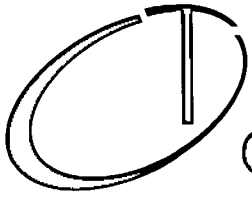
- D1: US2010144617 publicada el 10 de octubre de 2010.
- D2: US20060171920 publicada el 3 de agosto de 2006.

2.6. Ahora bien, el documento D1, se refiere en el párrafo [0001] a derivados de oxintomodulina, su síntesis y su uso para el tratamiento de trastornos metabólicos como la diabetes y la obesidad. Particularmente, en el párrafo [0071] esta anterioridad D1 se refiere a derivados de OXM modificados. Según se informa, los derivados de OXM son desarrollados por PEGilación o conjugación a otros restos o proteínas portadoras para mejorar la estabilidad y la farmacocinética y/o mediante la incorporación de sustituciones de residuos de aminoácidos para hacer que los péptidos resistentes a la escisión DP-IV. Además, según D1, los derivados de OXM estabilizadas no exhiben actividad agonista del receptor de glucagón y de ese modo pueden ofrecer ciertas ventajas en el tratamiento de la hiperglucemia y la obesidad en los sujetos diabéticos o prediabéticos. En el párrafo [0127], D1 contempla el uso de derivados poliméricos multi-funcionales, como se ejemplifica por los derivados de PEG bifuncional N-maleimidilo y multi-arm. Una amplia variedad de (PEG) especies de glicol polietileno puede ser utilizado según D1, para la PEGilación de los nuevos derivados de OXM. Igualmente se afirma que sustancialmente cualquier reactivo de PEG adecuado puede ser utilizado pero no se limitan a las que estén disponibles para la venta. D1 también define que los reactivos de PEG se prefieren a menudo en diversas realizaciones: multi-Arm PEG, mPEG(MAL)2, mPEG2(MAL), cualquiera de los SUNBRIGHT PEG activado (incluyendo pero no limitado a carboxilo-PEG, p-NP-PEG, Tresyl-PEG, PEG aldehído, acetal-PEG, amino-PEG, PEG-tiol, maleimido-PEG, hidroxilo-PEG-amina, amino-PEG-COOH, hidroxilo-PEG-aldehído, anhídrido carboxílico de tipo PEG, PEG funcionalizado-fosfolípido) y otros PEG reactivos similares y/o adecuados como seleccionados. Adicionalmente, en el párrafo [0128] D1 agrega que los péptidos derivados OXM también pueden contener dos restos de PEG que están unidos covalentemente a través de un carbamato o un enlace amida a un resto espaciador, en el que el resto



espaciador está unido covalentemente al enlazador de amida terciaria del péptido. Cada uno de los dos restos de PEG usados en tales formas de realización de D1 puede ser lineal y puede ser unidas entre sí en un solo punto de unión. D1 define cada resto de PEG que tiene un peso molecular de aproximadamente 10 kilodaltons (10K) a aproximadamente 60K. Cada uno de los dos restos de PEG puede tener un peso molecular de aproximadamente 20K a aproximadamente 40K. Un experto en la técnica será capaz de seleccionar el tamaño del polímero deseado basándose en consideraciones tales como la dosificación deseada; tiempo de circulación; resistencia a la proteólisis; efectos, si los hay, sobre la actividad biológica; facilidad de manejo; grado o falta de antigenicidad y otros efectos de la PEG conocida en un péptido terapéutico.

- 2.7. El documento D2, define una composición que consiste en una proteína, un polímero de polietilenglicol (PEG polímero) y 9 fluorenilmetoxycarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS), en el que dicha composición se logra mediante la conjugación de un polímero de polietilenglicol (polímero de PEG) al extremo amino terminal de la proteína a través de un enlazador flexible, tal como 9-fluorenilmetoxycarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS). En fármacos pegilados, el resto de PEG y el residuo de la droga no son vinculadas directamente entre sí, sino más bien ambos residuos están vinculados a diferentes posiciones de la estructura Fmoc o FMS que es altamente sensible a bases y es extraíble en condiciones fisiológicas. Igualmente se define en D2, que los fármacos son preferiblemente fármacos que contienen un grupo amino, lo más preferiblemente péptidos y proteínas de bajo o medio peso en el párrafo [0066] por lo que el resto de PEG y el residuo de drogas no están ligados directamente entre sí, como en los procedimientos de pegilación estándar, sino que ambos residuos están vinculados a diferentes posiciones de una estructura que son muy sensibles a bases y es extraíble en condiciones fisiológicas según los párrafos [0359] a [0360]. Así, basándose en los rendimientos de secuenciación, el grupo PEG.sub.40-FMS está asociado principalmente a la alfa N-terminal .alpha.-amino grupo de PYY.sub.3-36.
- 2.8. Así las cosas, un experto en la técnica encontraría que es obvio el uso de dicho enlazador flexible FMS o Fmoc para modificar el conjugado de PEG oxintomodulina divulgado en D1 de modo que las principales deficiencias de la tecnología de proteínas de pegilación, principalmente la pérdida de potencias biológicas y farmacológicas en los conjugados de PEG in vivo, pueden ser



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superados, como se describe por D2 (párrafo [0015]) y llegar así de manera obvia al objeto de la presente solicitud. Por lo tanto, este documento objeto de oposición a la luz del documento D1 carece evidentemente de ALTURA INVENTIVA.

2.9. En consecuencia y de acuerdo a lo descrito anteriormente, se deriva que la solicitud colombiana 13 - 301132 no puede pretender un monopolio por patente toda vez que lo que pretende no cumple con dos de los tres requisitos necesarios para su protección.

2.10. Que por consiguiente, respecto a la solicitud pretendida se tiene que:

2.8.1 CARENCIA DE ALTURA INVENTIVA. Según la Decisión 486 de la Comunidad Andina de Naciones, Artículo 18.- *"Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica."* A la fecha de prioridad, 2 de junio de 2011, a partir del documento D1, un experto en la materia puede llegar de manera evidente al uso de dicho enlazador flexible FMS o Fmoc para modificar el conjugado de PEG oxintomodulina de modo que las principales deficiencias de la tecnología de proteínas de pegilación, principalmente la pérdida de potencias biológicas y farmacológicas en los conjugados de PEG in vivo, como se describe por D2 (párrafo [0015]) y llegar a una composición que consiste en oxintomodulina, un polímero de polietilenglicol (PEG polímero) y 9 fluorenilmetoxycarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS), sobre lo cual busca protección en el capítulo reivindicatorio.

2.8.2 CARENCIA DE APLICACIÓN INDUSTRIAL La Decisión 486 de la CAN consagra en su artículo 14 lo siguiente: *"Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial"*. La presente solicitud carece de aplicación Industrial ya que como se demostró anteriormente, algunas cláusulas reclaman un método de tratamiento y estos no son aplicables industrialmente porque primero no se obtiene un producto o proceso; y segundo no es

reproducible o repetible a escala industrial. Por lo tanto, el objeto de la presente invención referido a métodos de tratamiento, no cumple con el requisito de la aplicación industrial.

2.9 Dado lo anterior, es posible deducir que la solicitud y lo reivindicado no es objeto de patentabilidad pues es contrario a lo que dicta la Decisión 486 de la Comunidad Andina de Naciones, Artículo 14.- *“Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial.”*. La solicitud pretendida no cumple con dos de los tres requisitos de patentabilidad.

2.10 Por todo lo anterior, atentamente solicito a usted que se sirva declarar fundada ***“AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1 DE ACCIÓN PROLONGADA”***, cuyo titular **OPKO BIOLOGICS LTD.**, solicitud Colombiana que apareció publicada en la Gaceta de la Propiedad Industrial N° 698 del 27 de Junio de 2014.

3. PRUEBAS

De conformidad con lo dispuesto por las normas vigentes, solicito a usted tener como prueba en este asunto los siguientes:

- D1: US2010144617 publicada el 10 de octubre de 2010.
- D2: US20060171920 publicada el 3 de agosto de 2006.

4. FUNDAMENTO DE DERECHO

Fundamento esta observación en las normas legales siguientes:

- 4.1. Artículo 45 de la Decisión 486 de la Comisión del Acuerdo de Cartagena.
- 4.2. En las demás normas concordantes.



Derecho de la Competencia, Propiedad
Industrial y Comercio Exterior

5. ANEXOS

Para los efectos correspondientes anexo al presente escrito los siguientes documentos:

1. Poder para actuar en el presente.
2. Documento que acredita al representante legal de **PROCAPS S.A.**
3. Recibo de caja del respectivo pago de tasa.
4. Copias de los documentos:
 - D1: US2010144617 publicada el 10 de octubre de 2010.
 - D2: US20060171920 publicada el 3 de agosto de 2006.



Derecho de la Competencia, Propiedad
Industrial y Comercio Exterior

6. NOTIFICACIONES

Para efectos de notificaciones que debe hacer su Despacho, informo que mi representada y la suscrita recibiremos notificaciones en la Secretaría de su Despacho o en mis oficinas situadas en la Carrera 13 N° 119-95, Of. 104 de esta ciudad de Bogotá.

Señor director,

ELIANA ISAURA MORENO BOHÓRQUEZ

C.C. 51'975.664 de Bogotá

T.P.A. 83823 del C.S. de la J.

ANEXO 1



US 20100144617A1

(19) **United States**

(12) **Patent Application Publication**
Sinha Roy et al.

(10) **Pub. No.: US 2010/0144617 A1**
(43) **Pub. Date: Jun. 10, 2010**

(54) **OXYNTOMODULIN DERIVATIVES**

(86) PCT No.: **PCT/US2007/004306**

(76) Inventors: **Ranabir Sinha Roy**, Edison, NJ (US); **Ellsabetta Bianchi**, Roma (IT); **Antonello Pessi**, Rome (IT); **Paolo Ingallinella**, Pomezia (Rome) (IT); **Donald J. Marsh**, Hillsborough, NJ (US); **George J. Eiermann**, Marlboro, NJ (US); **Yingjun Mu**, Fanwood, NJ (US); **Yun-Ping Zhou**, East Brunswick, NJ (US)

§ 371 (c)(1),
(2), (4) Date: **Aug. 13, 2008**

Related U.S. Application Data

(60) Provisional application No. 60/775,544, filed on Feb. 22, 2006, provisional application No. 60/834,452, filed on Jul. 31, 2006.

Publication Classification

(51) Int. Cl.
A61K 38/26 (2006.01)
C07K 14/605 (2006.01)
A61P 3/00 (2006.01)
A61P 3/08 (2006.01)

(52) U.S. Cl. **514/12; 530/308**

(57) **ABSTRACT**

Modified oxyntomodulin derivatives. Such derivatives can be used for the treatment of metabolic diseases such as diabetes and obesity.

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RAHWAY, NJ 07065-0907 (US)

(21) Appl. No.: **12/223,942**

(22) PCT Filed: **Feb. 16, 2007**

OXYNTOMODULIN DERIVATIVES

FIELD OF THE INVENTION

[0001] The present invention relates to oxyntomodulin derivatives, their synthesis, and their use for the treatment of metabolic disorders such as diabetes and obesity.

BACKGROUND OF THE INVENTION

[0002] The hormone oxyntomodulin (OXM, glucagon-37) is a posttranslational product of preproglucagon processing in the intestine and central nervous system (CNS) and is secreted from L-cells in the gut in response to food intake. Discovered in 1983, OXM has been implicated in the regulation of food intake and energy expenditure. Central or peripheral administration of OXM in rats causes a decrease in short term food intake with minimal effects on gastric emptying (Dakin et al. *Endocrinology*, 142:4244-4250 (2001), Dakin et al. *Endocrinology*, 145:2687-2695 (2004)). Repeated intracerebroventricular administration of OXM in rats results in elevated core temperatures and reduced weight gain compared to pair-fed animals, suggesting effects on both caloric intake and energy expenditure (Dakin et al. *Am. J. Physiol. Endocrinol. Metab.*, 283:E1173-E1177 (2002)).

[0003] OXM is a 37-amino acid peptide. It has been reported that the effects of OXM in inhibiting gastric acid secretion can be mimicked by the 8-residue C-terminal fragment Oxm(30-37), known as SP-1 (Caries-Bonnet et al., *Peptides*, 1996, 17:557-561. In humans, a single 90 min intravenous infusion of OXM in normal weight healthy subjects reduced hunger scores and food intake at a buffet meal by ~19%. Cumulative 12 hour caloric intake was reduced by ~11% with no reports of nausea or changes in food palatability (Cohen et al., *J. Clin. Endocrinol. Metab.*, 88:4696-4701 (2003)). More recently, pre-prandial injections of OXM over a 4 week period in obese healthy volunteers (BMI ~33) led to a significant reduction of caloric intake on the first day of treatment (~25%) that was maintained over the course of the study (35% reduction after 4 weeks) (Wynne et al., *Diabetes* 54:2390-2395 (2005)). Robust weight loss was observed at the end of the study in treated subjects (1.9%, placebo-corrected). Plasma levels of OXM were similar to that observed in the infusion study (peak concentration ~950 μ M). The absence of any tachyphylaxis and a low incidence of mild and transient nausea (~3%) despite the relatively high doses necessitated by the poor in vivo stability of OXM (plasma $t_{1/2}$ < 12 min) renders this hormone one of the few obesity targets with both human validation and an attractive tolerability profile.

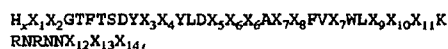
[0004] OXM has a very short half-life and is rapidly inactivated by the cell surface dipeptidyl peptidase IV (hereafter DP-IV). However, DP-IV inhibitors are weight-neutral in the clinic, suggesting that supraphysiological levels of OXM (900-1000 pM) may be required to achieve weight loss in humans.

[0005] Oxyntomodulin therefore shows potential as a treatment for metabolic disorders such as diabetes and obesity. However, because of the poor in vivo stability of OXM, there exists a need to develop OXM derivatives that can be safely and efficaciously administered for the treatment of metabolic diseases, such as diabetes and obesity. It would be further desirable if analogs or derivatives were developed that were modified by conjugation to moieties that would improve stability and pharmacokinetics, more particularly modifications

that confer resistance to DP-IV cleavage. The instant invention provides OXM polypeptide derivatives and methods for the treatment or prevention of metabolic disorders such as obesity and diabetes by administering the derivatives described herein.

SUMMARY OF THE INVENTION

[0006] The present invention provides a polypeptide comprising:



[0007] wherein H_x is selected from the group consisting of His; imidazole-lactic acid (ImiH); desamino-His (ΔNH_2 -H); acetyl His; pyroglutamyl His (PyrH); N-methyl-His (Me-H); N,N-dimethyl-His (Me_2 -H); Benzoyl His (Bz-H); Benzyl His (Bzl-H); and Phe;

[0008] X_1 is selected from the group consisting of Ser; Gly; Ala; Arg; Asn; Asp; Glu; Gln; His; Ile; Lys; Met; Phe; Pro; Thr; Trp; Tyr; Val; D-Ala; D-Ser; and α -aminoisobutyric acid;

[0009] X_2 is Gln, Asp, Glu, Pro, Leu or L-norleucine;

[0010] X_3 is Ser, Ala, Cys, Cys(mPEG), or Cys(cholesteryl);

[0011] X_4 is Lys, Cys, Cys(mPEG), or Cys(cholesteryl);

[0012] X_5 is Ser or Ala;

[0013] X_6 is any amino acid;

[0014] X_7 is Gln, Cys, Cys(mPEG), or Cys(cholesteryl);

[0015] X_8 is Asp, Cys, Cys(mPEG), or Cys(cholesteryl);

[0016] X_9 is Met, Met(O), Val, norleucine, alanine, α -aminoisobutyric acid or O-methyl-homoserine;

[0017] X_{10} is Asn, Cys, Cys(mPEG), or Cys(cholesteryl);

[0018] X_{11} is Thr, Cys, Cys(mPEG), or Cys(cholesteryl);

[0019] X_{12} is Ile, Cys, Cys(mPEG), or Cys(cholesteryl);

[0020] X_{13} is Ala, Cys, Cys(mPEG), or Cys(cholesteryl); and

[0021] X_{14} is amide, carboxylate, secondary amide, Ala, K(palmitoyl), Cys, Cys(mPEG), Cys(cholesteryl) or any linker to which mPEG or cholesterol is linked with a chemical bond. Pharmaceutically acceptable salts thereof are contemplated as well.

[0022] Additionally, any one or two of X_3 , X_4 , X_6 - X_8 , and X_{10} - X_{14} may be Cys(mPEG) Cys(cholesteryl); Cys(mPEG) teine may also be C_1 , C_2 , C_3 or C_6 , wherein C_1 =Cys(mPEG)₅ kDa, C_2 =Cys(mPEG)₂₀ kDa, C_3 =Cys(mPEG)₄₀ kDa, C_6 =Cys(mPEG)₆₀ kDa and each corresponds to a cysteine residue PEGylated via the side chain thiol with linear methoxyPEG (mPEG) or branched mPEG₂ of the indicated molecular weight.

[0023] The present invention relates to OXM polypeptide derivatives of the formula:



wherein C_3 =Cys[(mPEG)₂40 kDa], each corresponding to an amidated cysteine residue PEGylated via the side-chain thiol with a branched mPEG [(mPEG)₂] of the indicated MW; α is α -amino isobutyric acid (aib); and m=methionine sulfoxide (Met(O)).

[0024] In another embodiment of the present invention, there is provided a polypeptide comprising:

H_x SQGTFTSDYSKYLDSSRAQDFVQWLMNTRNRNNIA,

wherein H_x is selected from the group consisting of His, H_1 =Imidazole-lactic acid (ImiH); desamino-His (ΔNH_2 —H), acetyl His, pyroglutamyl His, N-methyl-His (Me-H), N,N-dimethyl-His (Me₂-H); Benzoyl His (Bz-H), Benzyl His (Bzl-H), and Phe.

[0025] In yet another embodiment of the present invention, there is provided a polypeptide comprising:

HX₁QGTFTSDYSKYLDSSRAQDFVQWLMNTRNRNNIA

[0026] wherein X_1 is selected from the group consisting of Ser, Gly, Ala, Arg; Asn, Asp, Glu, Gln, His, Ile, Lys, Met, Phe, Pro, Thr, Trp, Tyr, Val, D-Ala, D-Ser, and α -aminoisobutyric acid.

[0027] The present invention further provides for a polypeptide comprising:

HSX₂GTFTSDYSKYLDSSRAQDFVQWLMNTRNRNNIA

[0028] X_2 is selected from the group consisting of Gln, Asp, Glu, Pro, Leu, and L-norleucine.

[0029] In another embodiment of the present invention, there is provided a polypeptide comprising:

HSQGTFTSDYX₃X₄YLDX₅X₆AX₇X₈FVX₉WLMX₁₀X₁₁KRNRN
NX₁₂X₁₃X₁₄

[0030] wherein X_3 is Ser, Ala, Cys(mPEG), or Cys(cholesteryl);

[0031] X_4 is Lys, Cys(mPEG), or Cys(cholesteryl);

[0032] X_6 is any one of Arg, Cys(mPEG), or Cys(cholesteryl);

[0033] X_7 is any one of Gln, Cys(mPEG), or Cys(cholesteryl);

[0034] X_8 is Asp, Cys(mPEG), or Cys(cholesteryl);

[0035] X_{10} is Asn, Cys(mPEG), or Cys(cholesteryl);

[0036] X_{11} is Thr, Cys(mPEG), or Cys(cholesteryl);

[0037] X_{12} is Ile, Cys(mPEG), or Cys(cholesteryl);

[0038] X_{13} is Ala, Cys(mPEG), or Cys(cholesteryl); and

[0039] X_{14} is amide, carboxylate, secondary amide, Ala, K(palmitoyl), Cys(mPEG), Cys(cholesteryl), or any linker to which mPEG or cholesterol is linked with a chemical bond.

[0040] wherein one or two of X_3 , X_4 , X_6 - X_8 , and X_{10} - X_{14} is Cys(mPEG) or Cys(cholesteryl).

[0041] In an embodiment of the present invention, there is provided a polypeptide comprising:

HxX₁₅GTFTSDYSKYLDSSZAX₁₆DFVQWLX₁₇NTX₁₈

[0042] wherein X_{15} is D or Q;

[0043] Z is any amino acid;

[0044] X_{16} is C₈, Cys(N-ethylmaleimidyl), Q or C;

[0045] X_{17} is m or M;

[0046] X_{18} is an amidated k or K.

[0047] In another embodiment of the present invention, there is provided a polypeptide comprising:

HaDGTFTSDYSKYDSZZAQDFVQWLMNTRNRNNIA₁₉,

[0048] wherein X_{19} is C or C₈, Cys(N-ethylmaleimidyl).

[0049] In yet another embodiment of the present invention, there is provided a polypeptide of the formula:

HaDGTFTSDYSKYLDs-TtdsEC-COMH₂

[0050] In an embodiment of the present invention, there is provided a polypeptide of the formula:

HaDGTFTSDYSKYLDSSRAQDFVQWLMNTRNRNNIA-Ttds-EEEEEC-COOH,

wherein Ttds is 1-amino-4,7,10-trioxa-13-tridccanamine succinimic acid.

[0051] In another embodiment of the present invention there is provided a method for the treatment of a metabolic disease in a subject comprising administering to the subject a polypeptide as described above. The metabolic disease may be selected from the group consisting of diabetes, metabolic syndrome, hyperglycemia, and obesity and may be administered via a route peripheral to the brain, such as an oral, mucosal, buccal, sublingual, nasal rectal, subcutaneous, transdermal intravenous, intramuscular or intraperitoneal route.

[0052] In yet another embodiment of the present invention, there is provided a pharmaceutical composition comprising a polypeptide as described above and a pharmaceutically suitable carrier.

[0053] The present invention further relates to the use of the polypeptides of the present invention in the preparation of a medicament useful for the treatment or prevention of metabolic disorders such as diabetes or obesity in a subject in need thereof by administering the polypeptides and pharmaceutical compositions of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0054] FIG. 1 depicts activation of a mutant form of the human GLP-1 receptor by (a) native porcine OXM and (b) PEGylated OXM2 and loss of potency due to preincubation of the peptides with DP-IV.

[0055] FIG. 2 shows incretin activity of porcine oxyntomodulin in the lean mouse intraperitoneal glucose tolerance test (IPGTT). % inhibition of glucose excursion is indicated for each group. "ctrl"=vehicle-treated saline-challenged mice, "veh"=vehicle-treated dextrose-challenged mice.

[0056] FIG. 3 illustrates the efficacy of the polypeptides denoted by sequences OXM2 and OXM3 in reducing overnight food intake and body weight gain in lean mice. * $p \leq 0.05$ relative to the vehicle group.

[0057] FIG. 4 depicts the effects of GLP-1 and OXM on glucose-stimulated insulin secretion (GSIS) in islets and MIN6 cells.

[0058] FIG. 5 shows the effects of GLP-1R deletion and receptor antagonism on OXM, GCG and GLP-1 mediated GSIS in islets.

[0059] FIG. 6 illustrates the effect of OXM on GSIS in glucagon receptor -/- islets.

[0060] FIG. 7 shows the effects of OXM and exendin-4 on blood glucose and insulin levels during IPGTT in wild-type and GLP-1 R -/- mice.

[0061] FIG. 8 shows the acute glucose-lowering effects of OXM99 in the lean mouse IPGTT.

[0062] FIG. 9 depicts the effect of OXM99 in reducing blood glucose in lean mice.

[0063] FIG. 10 illustrates the glucose-lowering effects of OXM117 in the lean mouse IPGTT.

[0064] FIG. 11 shows the duration of action of the OXM analogs OXM117 and OXM99 compared with exendin-4.

[0065] FIG. 12 depicts the pharmacokinetics for OXM117 using subcutaneous dosing in the rat.

[0066] FIG. 13 shows the results of studies demonstrating that the OXM117 peptide shows no glucagon-like activity in vitro.

[0067] FIG. 14 summarizes the in vitro activity data at the GLP1 and GCG receptors in tabular form.

[0068] FIG. 15 shows in vivo activity of (mPEG)₂40 kDa conjugates on food intake and body weight loss in the DIO mouse model.

[0069] FIG. 16 illustrates in vitro potency data for the C-terminal truncated analogs acting at the GLP1 and GCG receptors.

[0070] FIG. 17 presents in vitro potency data for select PEGylated OXM analogs acting at the GLP1 and GCG receptors.

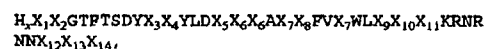
DETAILED DESCRIPTION OF THE INVENTION

[0071] The invention relates to modified OXM derivatives. The OXM derivatives are developed by PEGylation or conjugation to other moieties or carrier proteins to improve stability and pharmacokinetics, and/or by incorporation of substitutions of amino acid residues to render the peptides resistant to DP-IV cleavage. In addition, the stabilized OXM derivatives do not exhibit glucagon receptor agonist activity, and may thereby offer certain advantages in the treatment of hyperglycemia and obesity in diabetic or prediabetic subjects. For those subjects, up-regulation of glucagon receptor signaling should be avoided, since it may result in elevated blood glucose levels.

[0072] Unless otherwise specified, the terms "polypeptide," "protein," and "peptide" is understood by the skilled artisan to also encompass various modified and/or stabilized forms. Such modified forms may be chemically modified forms, including, without limitation, PEGylated forms, palmitoylated forms, cholesterol-modified forms, etc. Modifications also include intra-molecular crosslinking and covalent attachment to various moieties such as lipids, flavin, biotin, polyethylene glycol derivatives, etc. In addition, modifications may also include cyclization, branching and cross-linking. Further, amino acids other than the conventional twenty amino acids encoded by genes may also be included in a polypeptide.

The Structures of the OXM Derivatives

[0073] The present invention provides modified OXM derivatives. In particular, the present invention relates to novel stabilized modified OXM polypeptide derivatives of the formula:



[0074] wherein H_x is selected from the group consisting of His; imidazole-lactic acid (ImiH);

[0075] desamino-His (ΔNH_2-H); acetyl His; pyroglutamyl His (PyrH); N-methyl-His (Me-H); N,N-dimethyl-His (Me_2-H); Benzoyl His (Bz-H); Benzyl His (Bzl-H); and Phe;

[0076] X_1 is selected from the group consisting of Ser; Gly; Ala; Arg; Asn; Asp; Glu; Gln; His; Ile; Lys; Met; Phe; Pro; Thr; Trp; Tyr; Val; D-Ala; D-Ser; and α -aminoisobutyric acid;

[0077] X_2 is Gln, Asp, Glu, Pro, Leu or L-norleucine;

[0078] X_3 is Ser, Ala, Cys, Cys(mPEG), or Cys(cholesteryl);

[0079] X_4 is Lys, Cys, Cys(mPEG), or Cys(cholesteryl);

[0080] X_5 is Ser or Ala;

[0081] X_6 is any amino acid;

[0082] X_7 is Gln, Cys, Cys(mPEG), or Cys(cholesteryl);

[0083] X_8 is Asp, Cys, Cys(mPEG), or Cys(cholesteryl);

[0084] X_9 is Met, Met(O), Val, norleucine, alanine, α -aminoisobutyric acid or O-methyl-homoserine;

[0085] X_{10} is Asn, Cys, Cys(mPEG), or Cys(cholesteryl);

[0086] X_{11} is Thr, Cys, Cys(mPEG), or Cys(cholesteryl);

[0087] X_{12} is Ile, Cys, Cys(mPEG), or Cys(cholesteryl);

[0088] X_{13} is Ala, Cys, Cys(mPEG), or Cys(cholesteryl); and

[0089] X_{14} is amide, carboxylate, secondary amide, Ala, K(palmitoyl), Cys, Cys(mPEG), Cys(cholesteryl) or any linker to which mPEG or cholesterol is linked with a chemical bond.

[0090] Additionally, any one or two of X_3 , X_4 , X_6 , X_8 , and X_{10} - X_{14} may be Cys(mPEG) or Cys(cholesteryl); Cys(mPEG)teine may also be C_1 ; C_2 ; C_3 or C_6 , wherein C_1 =Cys(mPEG)5 kDa, C_2 =Cys(mPEG)20 kDa, C_3 =Cys(mPEG)240 kDa, C_6 =Cys(mPEG)260 kDa and each corresponds to a cysteine residue PEGylated via the side chain thiol with linear methoxyPEG (mPEG) or branched mPEG₂ of the indicated MW.

[0091] The present invention further provides an OXM polypeptide of the formula:



wherein C_3 =Cys[(mPEG)₂40 kDa], each corresponding to an amidated cysteine residue PEGylated via the side-chain thiol with a branched mPEG [(mPEG)₂] of the indicated MW; α is α -amino isobutyric acid (aib); and m=methionine sulfoxide [Met(O)].

[0092] In an embodiment of the present invention, there is provided a polypeptide comprising:



[0093] wherein X_1 is selected from the group consisting of Ser, Gly, Ala, Arg; Asn, Asp, Glu, Gln, His, Ile, Lys, Met, Phe, Pro, Thr, Trp, Tyr, Val, D-Ala, D-Ser, and α -aminoisobutyric acid.

[0094] The present invention further provides for a polypeptide comprising:



[0095] X_2 is selected from the group consisting of Gln, Asp, Glu, Pro, Leu, and L-norleucine.

[0096] In another embodiment of the present invention, there is provided a polypeptide comprising:

HSQGTFTSDYX₃X₄YLDX₆X₆AX₇X₈PVX₇WLMX₁₀X₁₁KRNRRN
X₁₂X₁₃X₁₄

- [0097] wherein X₃ is Ser, Ala, Cys(mPEG), or Cys(cholesteryl);
[0098] X₄ is Lys, Cys(mPEG), or Cys(cholesteryl);
[0099] X₆ is Arg, Cys(mPEG), or Cys(cholesteryl);
[0100] X₇ is Gln, Cys(mPEG), or Cys(cholesteryl);
[0101] X₈ is Asp, Cys(mPEG), or Cys(cholesteryl);
[0102] X₁₀ is Asn, Cys(mPEG), or Cys(cholesteryl);
[0103] X₁₁ is Thr, Cys(mPEG), or Cys(cholesteryl);
[0104] X₁₂ is He, Cys(mPEG), or Cys(cholesteryl);
[0105] X₁₃ is Ala, Cys(mPEG), or Cys(cholesteryl); and
[0106] X₁₄ is amide, carboxylate, secondary amide, Ala, K(palmitoyl), Cys(mPEG), Cys(cholesteryl), or any linker to which mPEG or cholesterol is linked with a chemical bond.
[0107] wherein one or two of X₃, X₄, X₆-X₈, and X₁₀-X₁₄ is Cys(mPEG) or Cys(cholesteryl).
[0108] In an embodiment of the present invention, there is provided a polypeptide comprising:

HQX₁₅GTFTSDYSKYLDSSZAX₁₆DFVQWLX₁₇NTX₁₈

- [0109] wherein X₁₅ is D or Q;
[0110] Z is any amino acid;
[0111] X₁₆ is C8, Q or C;
[0112] X₁₇ is m or M;
[0113] X₁₈ is amidated k or K.
[0114] In another embodiment of the present invention, there is provided a polypeptide comprising:

HaDGTFTSDYSKYDSZZAQDFVQWLmNTRNRNNIAx₁₉,

- [0115] wherein X₁₉ is C or C₈.
[0116] In yet another embodiment of the present invention, there is provided a polypeptide of the formula:

HaDGTFTSDYSKYLDS-TtdeEC-CONH₂

- [0117] In an embodiment of the present invention, there is provided a polypeptide of the formula:

HaDGTFTSDYSKYLDSRRAQDFVQWLmNTRNRNNIA-Ttde-EEEE
EC-COOH.

[0118] As used herein, the abbreviations of amino acid residues are shown as follows:

Amino Acids	Three-Letter Abbreviations	One-Letter Abbreviations
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Histidine	His	H
Isoleucine	Ile	I
Glutamine	Gln	Q
Glutamate	Glu	E
Glycine	Gly	G
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

[0119] Unless specifically designated otherwise, all the amino acid residues are in the L-form.

[0120] In comparison to the wild-type OXM, the OXM derivatives of the present invention contain several amino acid substitutions, and/or can be PEGylated or otherwise modified (e.g. with cholesterol moieties). Analogs may be double conjugated, e.g., with to both cholesterol and PEG. Such OXM derivatives are resistant to cleavage and inactivation by dipeptidyl peptidase IV (DP-IV).

[0121] By "receptor agonist" is meant any endogenous or exogenous (drug) substance or compound that can interact with a receptor, for example, the GLP-1R or the glucagon receptor, and thereby initiate a pharmacological or biochemical response characteristic of receptor activation. Typically, the OXM derivatives of the instant invention are characterized by their affinity to the human GLP-1R and display an EC50 for this receptor in the range of 0.1 pM to 1 μM. The OXM derivatives of the instant invention also are characterized by their affinity to the GcgR, displaying an EC50>1 μM.

[0122] The OXM derivatives of the present invention may be useful in the reduction of food intake and body weight and may mediate glucose-stimulated insulin secretion (GSIS) from pancreatic islets, thereby providing a treatment option for individuals afflicted with a metabolic disorder such as obesity, diabetes, metabolic syndrome X, hyperglycemia, impaired fasting glucose, and other prediabetic states.

TABLE 1

OXM Derivatives		
SEQ ID NO	Peptides	Sequences
1	OXM1	HQGTFTSDYSKYLDSRRAQDFVQWLmNTRNRNNIAA-COOH
2	OXM2	HQGTFTSDYSKYLDSRRAQDFVQWLmNTRNRNNIAC ₂ -COOH

TABLE 1-continued

OXM Derivatives		
SEQ ID NO	Peptides	Sequences
3	OXM3	HQGGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIAC ₃ -COOH
4	OXM-NH ₂	HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
5	Ac-OXM-NH ₂	Ac-HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
6	Ac-OXM	Ac-HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
7	OXM4	HqQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
8	OXM5	HVQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
9	OXM6	HaQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
10	OXM7	HsQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
11	OXM8	HSEGTFSTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
12	OXM9	HSDGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
13	OXM10	HSLGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
14	OXM11	HSnGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
15	OXM12	HGEGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
16	OXM13	FSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
17	OXM14	Pyr-HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
18	OXM15	HSPGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
19	OXM16	H ₁ SQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
20	OXM17	Me-HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
21	OXM18	H ₂ SQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
22	OXM19	Me ₂ -HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
23	OXM20	Br-HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
24	OXM21	Ba1-HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
25	OXM23	HARGTFTSDVSSYLEGQAAKEPIAWLMNTKRNRRNNIA-CONH ₂
26	OXM24	HSQGTFTSDYAKYLDARRAQDFVQWLMNTKRNRRNNIA-CONH ₂
27	OXM25	HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNI-CONH ₂
28	OXM26	HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNN-CONH ₂
29	OXM27	HSQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRN-CONH ₂
30	OXM28	HqQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
31	OXM29	HqQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIAC-CONH ₂
32	OXM30	HqQGTFTSDYSKYLSRRAQDFVQWLMCTKRNRRNNIA-CONH ₂
33	OXM31	HsQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-COOH
34	OXM32	HsQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIA-CONH ₂
35	OXM33-36 precursor	HsQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIAC-CONH ₂
36	OXM33	HsQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIAC ₁ -CONH ₂
37	OXM34	HsQGTFTSDYSKYLSRRAQDFVQWLMNTKRNRRNNIAC ₂ -CONH ₂

TABLE 1-continued

OXM Derivatives		
SEQ ID NO	Peptides	Sequences
145	OXM143	H _α QGTFTSDYSKYLSRRAC ₈ DFVQWLmNTK-CONH ₂
146	OXM144	H _α DGTFTSDYSKYLSRRAC ₈ DFVQWLmNTK-CONH ₂
147	OXM147	H _α QGTFTSDYSKYLSRRACDFVQWLmNTK-CONH ₂
148	OXM148	H _α QGTFTSDYSKYLSRRAC ₈ DFVQWLmNTK-CONH ₂
149	OXM149	H _α DGTFTSDYSKYLSRRACDFVQWLmNTK-CONH ₂
150	OXM150	H _α DGTFTSDYSKYLSRRAC ₈ DFVQWLmNTK-CONH ₂
151	OXM151	H _α DGTFTSDYSKYLS ₈ EAQDFVQWLmNTKRNRNNIAC-CONH ₂
152	OXM152	H _α DGTFTSDYSKYLS-TtdeEC-CONH ₂
153	OXM153	H _α DGTFTSDYSKYLS ₈ EAQDFVQWLmNTKRNRNNIAC ₈ -CONH ₂
154	OXM154	H _α DGTFTSDYSKYLS ₈ EAQDFVQWLmNTKRNRNNIAC ₃ -CONH ₂
155	OXM155	H _α DGTFTSDYSKYLSRRACDFVQWLmNTKRNRNNIA-Ttde-EEEEEC-COOH
156	OXM174	H _α QGTFTSDYSKYLSRRAC ₃ DFVQWLANTKRNRNNIA-CONH ₂
157	OXM175	H _α QGTFTSDYSKYLSRRAC ₃ DFVQWLmNTKRNRNNIA-CONH ₂
158	OXM176	H _α QGTFTSDYSKYLSRRAC ₃ DFVQWLmNTKRNRNNIA-CONH ₂
159	OXM199	H _α QGTFTSDYSKYLSRRAC ₃ DFVQWLmNTKRNRNNIA-CONH ₂

α = α-aminoisobutyric acid (Aib);

a = D-Ala;

s = D-Ser;

n = L-norleucine (Nle);

X = O-methyl-homoserine;

C₁ = Cys (mPEG) 5 kDa;

C₂ = Cys (mPEG) 20 kDa;

C₃ = Cys (mPEG)₂ 40 kDa, each corresponding to a cysteine residue PEGylated

via the side-chain thiol with linear methoxyPEG (mPEG) or branched mPEG

[(mPEG)₂] of the indicated MW;

C₄ = Cys (Cholesteryl), corresponding to a cysteine residue linked to cholesterol via the side-chain thiol;

C₅ = Cys(CH₂CONH₂), corresponding to a cysteine residue in which the side-

chain thiol was reacted with iodoacetamide;

C₆ = Cys (mPEG)₂ 60 kDa, each corresponding to a cysteine residue PEGylated

via the side-chain thiol with linear methoxyPEG (mPEG) or branched mPEG₂mPEG

[(mPEG)₂] of the indicated MW;

H₁ = Imidazole-lactic acid (ImH);

H₂ = desamino-His (ΔNH₂-H)¹

Ac = Acetyl;

Pyr = pyroglutamyl;

Me-H = N-methyl-His;

Me₂-H = N,N-dimethyl-His;

Bz = Benzoyl (C₇H₅O);

Bzl = Benzyl (C₇H₇);

m = methionine sulfoxide.

C₇ = (Cys)₂ (mPEG) 2-40 kDa, each corresponding to two cysteine residues PEGy-

lated via the side chain thiol to the same one linear methoxyPEG (mPEG) or

one branched mPEG [(mPEG)₂];

C₈ = Cys (N-ethylmaleimidyl); Ttde, 1-amino-4,7,10-trioxa-13-tridecanamine succinimic acid; k, D-Lysine.

1.1. Amino Acid Substitutions and Modifications

[0123] Substitution at X₁ (position 2 of OXM) is designed to improve the resistance of the OXM derivatives to proteolysis by DP-W, which plays a key role in the degradation of many peptides, including OXM and GLP-1. It has been reported that substitution of Ser at position 2 with Gly in GLP-1 improves resistance to DP-IV cleavage (Lotte, B. K., *J. Med. Chem.*, 47:4128-4134 (2004)). In spite of the high

degree of sequence homology between OXM and GLP-1, the Ser→Gly substitution at position 2 was not found to confer a similar effect on the modified OXM. However, the substitution of Ser at position 2 with Val, Ile, Asp, Glu, Met, Trp, Asn, D-Ala, D-Ser, or α-aminoisobutyric acid rendered the corresponding OXM derivative more resistant to DP-IV than the wild-type OXM, as discussed *infra* in the Examples. Peptides with a substitution at X₁ (position 2 of OXM) include:

OXM4-7, 12, 23, 28-59, and the precursors of OXM133-36 and OXM55-59.

[0124] The substitutions at X_2 (position 3 of OXM) are designed to create OXM derivatives that are selective agonists of GLP-1R with minimal or no activation of GcgR. Such OXM derivatives may be advantageous when treating obese diabetics. Peptides with a substitution at X_2 (position 3 of OXM) include: OXM8-12, 15, 23, 53, 95, 96 and 97.

[0125] Similarly, the substitutions to Ala at X_3 and X_5 (positions 11 and 16) are designed to create OXM derivatives that are selective for GLP-1R and have no activity against GcgR. One such example of the substitutions to Ala at X_3 and X_5 (positions 11 and 16) is OXM24.

[0126] The substitutions to cysteine at any one or more of positions X_3 , X_4 , X_6 - X_8 , and X_{10} - X_{14} allow for the PEGylation or cholesterylation of the OXM derivative at specific sites. Other substitutions or modifications are known in the art and include those which physically adhere to the surface of the active agent but do not chemically bond to or interact with the active agent. Two or more such modifications can be employed and may be selected from known organic and inorganic pharmaceutical excipients, including various polymers, low molecular weight oligomers, natural products, and surfactants.

1.2. PEGylation and/or Cholesterylation

[0127] The invention contemplates the use of multi-functional polymer derivatives, as exemplified by bifunctional and multi-arm N-maleimidyl PEG derivatives. A wide variety of polyethylene glycol (PEG) species may be used for PEGylation of the novel OXM derivatives of the present invention. Substantially any suitable reactive PEG reagent can be used and suitable species include, but are not limited to, those which are available for sale in the Drug Delivery Systems catalog of NOF Corporation (Yebisu Garden Place Tower, 20-3 Ebisu 4-chome, Shibuya-ku, Tokyo 150-6019) and, for exemplary purposes, of the Molecular Engineering catalog of Nelstar Therapeutics (490 Discovery Drive, Huntsville, Ala. 35806). By way of example and not limitation, the following PEG reagents are often preferred in various embodiments: multi-Arm PEG, mPEG(MAL)₂, mPEG2(MAL), any of the SUNBRIGHT activated PEGs (including but not limited to carboxyl-PEGs, p-NP-PEGs, Tresyl-PEGs, aldehyde PEGs, acetal-PEGs, amino-PEGs, thiol-PEGs, maleimido-PEGs, hydroxyl-PEG-amine, amino-PEG-COOH, hydroxyl-PEG-aldehyde, carboxylic anhydride type-PEG, functionalized PEG-phospholipid), and other similar and/or suitable reactive PEGs as selected by those skilled in the art for their particular application and usage.

[0128] The novel OXM derivative peptides of the present invention can also contain two PEG moieties that are covalently attached via a carbamate or an amide linkage to a spacer moiety, wherein the spacer moiety is covalently bonded to the tertiary amide linker of the peptide. Each of the two PEG moieties used in such embodiments of the present invention may be linear and may be linked together at a single point of attachment. In one embodiment of the invention, each PEG moiety has a molecular weight of about 10 kilodaltons (10K) to about 60K (the term "about" indicating that in preparations of PEG, some molecules will weigh more, and some less, than the stated molecular weight). Each of the two PEG moieties may have a molecular weight of about 20K to about 40K. One skilled in the art will be able to select the desired polymer size based on such considerations as the desired dosage; circulation time; resistance to proteolysis;

effects, if any, on biological activity; ease in handling; degree or lack of antigenicity; and other known effects of PEG on a therapeutic peptide.

[0129] In an embodiment of the present invention, the polymer backbone of the N-maleimidyl polymer derivative is a poly(alkylene glycol), copolymer thereof, terpolymer thereof, or mixture thereof. Examples include poly(ethylene glycol), poly(propylene glycol), and copolymers of ethylene glycol and propylene glycol. As explained in greater detail below, more preferred embodiments of the invention utilize PEG polymers, such as bifunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG, and PEG with degradable linkages therein. However, it should be understood that other related polymers are also suitable for use in the practice of this invention and that the use of the term PEG or poly(ethylene glycol) is intended to be inclusive and not exclusive in this respect. The term PEG includes poly(ethylene glycol) in any of its forms, including bifunctional PEG, multiarmed PEG, forked PEG, branched PEG, pendent PEG (i.e. PEG or related polymers having one or more functional groups pendent to the polymer backbone), or PEG with degradable linkages therein.

[0130] The polymer backbone can be linear or branched. PEG is commonly used in branched forms that can be prepared by addition of ethylene oxide to various polyols, such as glycerol, glycerol oligomers, pentaerythritol and sorbitol. The central branch moiety can also be derived from several amino acids, such as lysine. The branched poly(ethylene glycol) can be represented in general form as R-(PEG-OH)_m in which R is derived from a core moiety, such as glycerol, glycerol oligomers, or pentaerythritol, and m represents the number of arms. Multi-armed PEG molecules, such as those described in U.S. Pat. No. 5,932,462, which is incorporated by reference herein in its entirety, can also be used as the polymer backbone.

[0131] Those of ordinary skill in the art will recognize that the foregoing list for substantially water soluble and non-peptidic polymer backbones is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated.

[0132] The sites of PEGylation on the OXM derivatives of the present invention are chosen taking into account the structure of OXM and its interactions with glucagon and GLP-1 receptors. Hence, the PEGylation is preferably site-specific. PEGylation at the thiol side-chain of cysteine has been widely reported (see, e.g., Caliceti & Veronese, 2003). If there is no Cys residue in the peptide, it can be introduced through substitution. The OXM derivatives of the present invention may be PEGylated through the side chains of cysteine. The OXM derivatives may contain Cys(mPEG)teine. The mPEG in Cys(mPEG)teine can have various molecular weights. The range of the molecular weight is preferably 5 kDa to 200 kDa, more 5 kDa to 100 kDa, and further preferably 20 kDa to 60 kDa. The mPEG can be linear or branched. For instance, the Cys(mPEG)teine of present invention may be C₁, C₂, C₃ or C₆. As exemplified herein, C₁ is Cys(mPEG)teine with a linear mPEG with a molecular weight of 5 kDa (Cys(mPEG)₅ kDa) (e.g., MPEG-MAL-5000, NEKTAR 2F2MOH01); C₂ is Cys(mPEG)teine with a linear mPEG with a molecular weight of 20 kDa (Cys(mPEG)₂₀ kDa) (e.g., MPEG-MAL-20K, NEKTAR 2F2MOP01); C₃ is Cys(mPEG)teine with a branched mPEG with a molecular weight of 40 kDa (Cys(mPEG)₂40 kDa) (e.g., MPEG2-MAL-40K, NEKTAR 2D3Y0T01 or Y Shape PEG Maleimide, MW40K (JenKem Technology, item

number Y-MAL-40K or SUNBRIGHT GL2-400MA Maleimide, (NOF Corporation) and C₆ is Cys(mPEG)teine with a branched mPEG with a molecular weight of 60 kDa (Cys (mPEG)₂60 kDa) (e.g., MPEG2-MAL-60K, NEKTAR 2D3Y0V01).

[0133] Alternatively, the cysteine residues in the OXM derivatives can also be derivatized with cholesterol via the side-chain thiol. Examples of cholesteryl OXM derivatives include: OXM36, OXM59, OXM65, OXM70, OXM76, OXM82, OXM88, and OXM101.

1.3. Other Modifications

[0134] The N-terminal Histidine H_x can be mutated with derivatives from the group consisting of His, H₁=Imidazole-lactic acid (ImiH); desamino-His (ANH₂-H), acetyl His, pyroglutamyl His (PyrH, N-methyl-His (Me-H), N,N-dimethyl-His (Me₂-H); Benzoyl His (Bz-H), Benzyl His (Bzl-H) and Phe. Acetylation and other modification and N-terminal capping groups at the N-terminus may stabilize OXM against DP-IV cleavage, while the amidation of C-terminus may prevent potential degradation in vivo by carboxypeptidases. OXM derivatives with N-terminal modifications include OXM14, and OXM16-22.

[0135] As illustrated in the examples, equimolar doses of OXM2 and OXM3 were effective in reducing overnight body weight gain in mice fed ad libitum, whereas similar doses of OXM1 and wild type or native (wt) Oxm were not efficacious in this model. OXM3 had the highest in vivo efficacy in dose-dependently reducing overnight body weight gain, likely reflecting its higher potency against GLP-1R compared to OXM2, which has a bulkier PEG moiety that likely interferes with receptor binding. For the PEGylated OXM derivatives, increased in vivo efficacy relative to wt OXM suggests that some stabilization against proteolysis, and for renal clearance, is induced by PEGylation alone, since both OXM2 and OXM3 are significantly less potent than native OXM against GLP-1R in vitro.

[0136] Additionally, a blood component may be utilized to stabilize the peptide. Preferred blood components comprise proteins such as immunoglobulins, serum albumin, ferritin, steroid binding proteins, transferrin, thyroxin binding protein, alpha-2-macroglobulin, haptoglobin and the like.

2. Synthesis of the OXM Derivatives

2.1. The Synthesis of Peptides

[0137] The following general procedure was used to synthesize some of the OXM derivatives. Solid phase peptide synthesis was performed using Fmoc chemistry under batch or continuous flow conditions (see, for example, Pennington and Dunn, *Peptide Synthesis Protocols* (1994), vol. 35) using PEG-polystyrene resins. Peptides were cleaved from the resin and deprotected using trifluoroacetic acid (TFA), and cation scavengers such as phenol triisopropylsilane, and water. Peptides were precipitated with cold methyl-t-butyl ether and the precipitated peptide was washed twice with cold ether prior to lyophilization. Peptide identity was confirmed by reversed-phase HPLC on a C4 column using water/acetonitrile with 0.1% TFA as typical mobile phases, and by electrospray mass spectrometry. Peptides were purified to >95% by reverse phase HPLC.

2.2 PEGylation of Peptides

[0138] Peptides are first synthesized and are then PEGylated at the thiol side-chain of cysteine. The following general procedure was used for PEGylation of peptides.

[0139] PEGylation reactions were run between a thiolated peptide precursor and a maleimide-mPEG to form a thioether bond. The reaction was run at a pH 7.3 and the maleimide-mPEG amount ranged from 0.5 to 10-fold molar excess with respect to the thiolated peptide. The PEGylated OXM peptide was then isolated using reverse-phase HPLC or ion-exchange chromatography followed by size exclusion chromatography. Finally, PEGylated peptides were characterized using analytical RP-HPLC, and MALDI tof mass spectrometry.

3. Implications of OXM-Based Therapy

[0140] OXM-based therapy has the potential to favorably impact both obesity and diabetes. Weight loss efficacy and reduction in food intake upon peripheral administration of OXM has been well validated in humans. Studies by the present inventors have shown that peripherally administered porcine OXM is sufficient to reduce short term food intake and overnight body weight gain in mice. Although the incretin (antihyperglycemic) activity of OXM has not been well investigated to date, it has been demonstrated for the first time that the glucose lowering activity of OXM is comparable to that of GLP-1 in a mouse intraperitoneal glucose tolerance test (IPGTT). Like GLP-1, OXM induces robust glucose stimulated insulin secretion (GSIS) from static isolated murine islets and perfused rat pancreata (Jarrousse et al., *Endocrinology*, 115:102-105 (1984)), suggesting a low risk of hypoglycemia compared to conventional insulin secretagogues. In rats, negligible effects of OXM on gastric emptying have been reported (Dakin et al., *Endocrinology*, 145: 2687-2695 (2004)). In mice, OXM reduces gastric emptying by ~25% at a maximally efficacious dose for glucose lowering, which is less than that produced by a maximally efficacious dose of the GLP-1 receptor agonist exendin 4 (47% reduction). Potentially benign effects of OXM on gastric emptying in humans may therefore play a role in the enhanced tolerability of this peptide hormone compared to current GLP-1 mimetics.

[0141] It is suggested that the polypeptides of the present invention may be useful for the treatment of obesity and/or diabetes. Secondary indications are metabolic syndrome, hyperglycemia, impaired fasting glucose, and other prediabetic states. Alternate indications for the polypeptides of the present invention include any and all indications for GLP-1 such as irritable bowel syndrome and other absorptive diseases of the gut, ischemia, stroke, and neurological disorders including anxiety, impaired cognition, and Alzheimer's disease.

[0142] The peptidyl nature of OXM precludes oral therapy with the native hormone. By contrast, the OXM derivatives presented herein may be administered as a pharmaceutical composition comprising one of the polypeptides of the present invention in combination with a pharmaceutically acceptable carrier which is suitable for administration by a variety of routes, including but not limited to oral, intranasal, sublingual, intraduodenal, subcutaneous, buccal, intracolonic, rectal, vaginal, mucosal, pulmonary, transdermal, intradermal, parenteral, intravenous, intramuscular and intraocular at a dosage range of 0.001 mg/kg to 10 mg/kg, more preferably from 1 µg/kg to 200 mg/kg with a dosing frequency ranging from twice daily to once per week or longer. The peptide pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. Suitable unit dosage forms, include, but are not limited to powders, tablets, pills,

capsules, lozenges, suppositories, patches, nasal sprays, injectables, implantable sustained-release formulations, lipid complexes, etc. The peptides are typically combined with a pharmaceutically acceptable carrier or excipient which can contain one or more physiologically acceptable compound(s) that may act to stabilize the composition or to increase or decrease the absorption of the active agent(s). Physiologically acceptable compounds can include, for example, carbohydrates, such as glucose, sucrose, or dextran, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins, protection and uptake enhancers such as lipids, compositions that reduce the clearance or hydrolysis of the active agents, or excipients or other stabilizers and/or buffers. Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives that are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, for example, phenol and ascorbic acid. One skilled in the art would appreciate that the choice of pharmaceutically acceptable carrier(s), including a physiologically acceptable compound depends, for example, on the route of administration of the active agent(s) and on the particular physio-chemical characteristics of the active agent(s).

[0143] The peptides can be administered in the native form or, if desired, in the form of salts provided the salts are pharmaceutically acceptable. Salts of the active agents may be prepared using standard procedures known to those skilled in the art of synthetic organic chemistry. Polypeptides of the present invention, as detailed in the Examples, were prepared as acetate salts.

[0144] An OXM polypeptide of the instant invention can be used in combination with other agents used in the treatment or prevention of diseases implicating the GLP1-R. Specific compounds of use in combination with a polypeptide of the present invention include: simvastatin, mevastatin, ezetimibe, atorvastatin, sitagliptin, metformin, sibutramine, orlistat, Qnexa, topiramate, naltrexone, bupropion, phentermine, and losartan, losartan with hydrochlorothiazide. Specific CB1 antagonists/inverse agonists of use in combination with a polypeptide of the present invention include: those described in WO03/077847, including: N43-(4-chlorophenyl)-2(S)-phenyl-[(S)-methylpropyl]-2-(4-trifluoromethyl-2-pyrimidyl)-2-methylpropanamide, N-[3-(4-chlorophenyl)-2-(3-cyanophenyl)-1-methylpropyl]-2-(5-trifluoromethyl-2-pyridyl)-2-methylpropanamide, N-[3-(4-chlorophenyl)-2-(5-chloro-3-pyridyl)-1-methylpropyl]-2-(5-trifluoromethyl-2-pyridyl)-2-methylpropanamide, and pharmaceutically acceptable salts thereof; as well as those in WO05/000809, which includes the following: 3-{1-[bis(4-chlorophenyl)methyl]azetidin-3-ylidene}-3-(3,5-difluorophenyl)-2,2-dimethylpropanenitrile, 1-{1-[1-(4-chlorophenyl)pentyl]azetidin-3-yl}-1-(3,5-difluorophenyl)-2-methylpropan-2-ol, 3-[(S)-(4-chlorophenyl){3-[(1S)-1-(3,5-difluorophenyl)-2-hydroxy-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(S)-(4-chlorophenyl){3-[(1S)-1-(3,5-difluorophenyl)-2-fluoro-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(S)-(4-chlorophenyl){3-[(1S)-1-(3,5-difluorophenyl)-2,2-dimethylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(1S)-1-{1-[(S)-(3-cyanophenyl)(4-cyanophenyl)methyl]azetidin-3-yl}-2-fluoro-2-methylpropyl)-5-fluorobenzonitrile, 3-[(S)-(4-chlorophenyl){3-[(1S)-2-fluoro-1-[3-fluoro-5-(4H-1,2,4-triazol-4-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile,

and 5-[(4-chlorophenyl){3-[(1S)-1-(3,5-difluorophenyl)-2-fluoro-2-methylpropyl]azetidin-1-yl)methyl}thiophene-3-carbonitrile, and pharmaceutically acceptable salts thereof; as well as: 3-[(S)-(4-chlorophenyl){3-[(1S)-2-fluoro-1-[3-fluoro-5-(5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(S)-(4-chlorophenyl){3-[(1S)-2-fluoro-1,3-fluoro-5-(1,3,4-oxadiazol-2-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(S)-{3-[(1S)-1-[3-(5-amino-1,3,4-oxadiazol-2-yl)-5-fluorophenyl]-2-fluoro-2-methylpropyl]azetidin-1-yl}(4-chlorophenyl)methyl}benzonitrile, 3-[(S)-(4-cyanophenyl){3-[(1S)-2-fluoro-1-[3-fluoro-5-(5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(S)-(3-[(1S)-1-[3-(5-amino-1,3,4-oxadiazol-2-yl)-5-fluorophenyl]-2-fluoro-2-methylpropyl]azetidin-1-yl)(4-cyanophenyl)methyl}benzonitrile, 3-[(S)-(4-cyanophenyl){3-[(1S)-2-fluoro-1-[3-fluoro-5-(1,3,4-oxadiazol-2-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(1S)-1-(1-{(S)-(4-cyanophenyl){3-(1,2,4-oxadiazol-3-yl)phenyl]-methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 5-(3-{1-[1-(diphenylmethyl)azetidin-3-yl]-2-fluoro-2-methylpropyl]-5-fluorophenyl)-1H-tetrazole, 5-(3-{1-[1-(diphenylmethyl)azetidin-3-yl]-2-fluoro-2-methylpropyl]-5-fluorophenyl)-1-methyl-1H-tetrazole, 5-(3-{1-[1-(diphenylmethyl)azetidin-3-yl]-2-fluoro-2-methylpropyl]-5-fluorophenyl)-2-methyl-2H-tetrazole, 3-[(4-chlorophenyl){3-[(2-fluoro-1-[3-fluoro-5-(2-methyl-2H-tetrazol-5-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(4-chlorophenyl){3-[(2-fluoro-1-[3-fluoro-5-(1-methyl-1H-tetrazol-5-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(4-cyanophenyl){3-[(2-fluoro-1-[3-fluoro-5-(1-methyl-1H-tetrazol-5-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 3-[(4-cyanophenyl){3-[(2-fluoro-1-[3-fluoro-5-(2-methyl-2H-tetrazol-5-yl)phenyl]-2-methylpropyl]azetidin-1-yl)methyl}benzonitrile, 5-{3-[(S)-{3-[(1S)-1-(3-bromo-5-fluorophenyl)-2-fluoro-2-methylpropyl]azetidin-1-yl}(4-chlorophenyl)methyl}phenyl]-1,3,4-oxadiazol-2(3H)-one, 3-[(1S)-1-(1-{(S)-(4-chlorophenyl){3-(5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)phenyl]methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 3-[(1S)-1-(1-{(S)-(4-cyanophenyl){3-(5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)phenyl]methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 3-[(1S)-1-(1-{(S)-(4-chlorophenyl){3-(1,3,4-oxadiazol-2-yl)phenyl]methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 3-[(1S)-1-(1-{(S)-[3-(5-amino-1,3,4-oxadiazol-2-yl)phenyl](4-chlorophenyl)methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 3-[(1S)-1-(1-{(S)-[3-(5-amino-1,3,4-oxadiazol-2-yl)phenyl](4-cyanophenyl)methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 3-[(1S)-1-(1-{(S)-(4-cyanophenyl){3-(1,2,4-oxadiazol-3-yl)phenyl]methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 3-[(1S)-1-(1-{(S)-(4-chlorophenyl){3-(1,2,4-oxadiazol-3-yl)phenyl]methyl}azetidin-3-yl)-2-fluoro-2-methylpropyl]-5-fluorobenzonitrile, 5-{3-[(S)-(4-chlorophenyl){3-[(1S)-1-(3,5-difluorophenyl)-2-fluoro-2-

methylpropyl]azetidin-1-yl)methyl]phenyl]-1,3,4-oxadiazol-2(1H)-one, 5-[3-((S)-(4-chlorophenyl){3-[(1S)-1-(3,5-difluorophenyl)-2-fluoro-2-methylpropyl]azetidin-1-yl)methyl]phenyl]-1,3,4-oxadiazol-2(3H)-one, 4-[(8)-{3-[(1S)-1-(3,5-difluorophenyl)-2-fluoro-2-methylpropyl]azetidin-1-yl}]-3-(5-oxo-4,5-dihydro-1,3,4-oxadiazol-2-yl)phenyl)methyl]-benzonitrile, and pharmaceutically acceptable salts thereof.

[0145] Specific NPY5 antagonists of use in combination with a polypeptide of the present invention include: 3-oxo-N-(5-phenyl-2-pyrazinyl)-spiro[isobenzofuran-1(3H), 4'-piperidine]-1'-carboxamide, 3-oxo-N-(7-trifluoromethylpyrido[3,2-b]pyridin-2-yl)spiro[isobenzofuran-1(3H), 4'-piperidine]-1'-carboxamide, N-[5-(3-fluorophenyl)-2-pyrimidinyl]-3-oxospiro[isobenzofuran-1(3H), 4'-piperidine]-1'-carboxamide, trans-3-oxo-N-(5-phenyl-2-pyrimidinyl)spiro[cyclohexane-1,1'(3'H)-isobenzofuran]-4-carboxamide, trans-3'-oxo-N41-(3-quinolyl)-4-imidazolyl]spiro[cyclohexane-1,1'(3'H)-isobenzofuran]-4-carboxamide, trans-3-oxo-N-(5-phenyl-2-pyrazinyl)spiro[4-azaisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, trans-N15-(3-fluorophenyl)-2-pyrimidinyl]-3-oxospiro[5-azaisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, trans-N45-(2-fluorophenyl)-2-pyrimidinyl]-3-oxospiro[5-azaisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, trans-N-[1-(3,5-difluorophenyl)-4-imidazolyl]-3-oxospiro[7-azaisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, trans-3-oxo-N-(1-phenyl-4-pyrazolyl)spiro[4-azaisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, trans-N41-(2-fluorophenyl)-3-pyrazolyl]-3-oxospiro[6-azaisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, trans-3-oxo-N-(1-phenyl-3-pyrazolyl)spiro[6-aisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, trans-3-oxo-N-(2-phenyl-1,2,3-triazol-4-yl)spiro[6-azaisobenzofuran-1(3H), 1'-cyclohexane]-4'-carboxamide, and pharmaceutically acceptable salts and esters thereof.

[0146] Specific ACC-1/2 inhibitors of use in combination with a polypeptide of the present invention include: 1'-[(4,8-dimethoxyquinolin-2-yl)carbonyl]-6-(1H-tetrazol-5-yl)spiro[chroman-2,4'-piperidin]-4-one, (5-{1'-[4,8-dimethoxyquinolin-2-yl)carbonyl]-4-oxospiro[chroman-2,4'-piperidin]-6-yl]-2H-tetrazol-2-yl)methyl pivalate, 5-{1'-[(8-cyclopropyl-4-methoxyquinolin-2-yl)carbonyl]-4-oxospiro[chroman-2,4'-piperidin]-6-yl}nicotinic acid, 1'-(8-methoxy-4-morpholin-4-yl-2-naphthoyl)-6-(1H-tetrazol-5-yl)spiro[chroman-2,4'-piperidin]-4-one, and 1'-[(4-ethoxy-8-ethylquinolin-2-yl)carbonyl]-6-(1H-tetrazol-5-yl)spiro[chroman-2,4'-piperidin]-4-one, and pharmaceutically acceptable salts thereof.

[0147] Specific MCH1R antagonist compounds of use in combination with a polypeptide of the present invention include: 1-[4-[(1-ethylazetidin-3-yl)oxy]phenyl]-4-[(4-fluorobenzyl)oxy]pyridin-2(1H)-one, 4-[(4-fluorobenzyl)oxy]-1-[4-[(1-isopropylazetidin-3-yl)oxy]phenyl]pyridin-2(1H)-one, 144-(azetidin-3-yloxy)phenyl]-4-[(5-chloropyridin-2-yl)methoxy]pyridin-2(1H)-one, 4-[(5-chloropyridin-2-yl)methoxy]-1-[4-[(1-ethylazetidin-3-yl)oxy]phenyl]pyridin-2(1H)-one, 4-[(5-chloropyridin-2-yl)methoxy]-1-[4-[(1-propylazetidin-3-yl)oxy]phenyl]pyridin-2(1H)-one, and 4-[(5-chloropyridin-2-yl)methoxy]-1-[4-[(2S)-1-ethylazetidin-2-yl]methoxy]phenyl]pyridin-2(1H)-one, or a pharmaceutically acceptable salt thereof.

[0148] Specific DP-IV inhibitors of use in combination with a polypeptide of the present invention are selected from 7-[(3R)-3-amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-a]pyrazine. In particular, the compound of formula I is favorably combined with 7-[(3R)-3-amino-4-(2,4,5-trifluorophenyl)butanoyl]-3-(trifluoromethyl)-5,6,7,8-tetrahydro-1,2,4-triazolo[4,3-a]pyrazine, and pharmaceutically acceptable salts thereof.

[0149] Additionally, other peptide analogs and mimetics of the incretin hormone glucagon-like peptide 1 (GLP-1), may also be of use in combination with a polypeptide of the present invention.

[0150] Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

EXAMPLES

Example 1

Synthesis of Oxyntomodulin (OXM) Analogs

[0151] The peptide OXM analogs (see Table 1) were synthesized by solid phase using Fmoc/tBu chemistry on a peptide multisynthesizer APEX 396 (Advanced Chemtech) using a 40-well reaction block. Each peptide was synthesized in a single well. For peptide amides 0.1 g of a resin Fmoc-Linker AM-Champion, 1% cross-linked (Biosearch Technologies, Inc.) and a PEG-PS based resin derivatized with a modified Rink linker p-[(R,S)- α -(9H-Fluoren-9-yl-methoxyformamido)-2,4-dimethoxybenzyl]-phenoxyacetic acid (Rink, H., 1987, *Tetrahedron Lett.* 28:3787-3789; Bernatowicz, M. S. et al., 1989, *Tetrahedron Lett.* 30:4645-4667) was used. For peptide acids, 0.1 g of Champion resin, 1% cross-linked (Biosearch Technologies, Inc.) was used, which was previously derivatized with a hydroxymethylphenoxyethyl handle. All the amino acids were dissolved at a 0.5 M concentration in a solution of 0.5M HOBt (Hydroxybenzotriazole) in DMF. The acylation reactions were performed for 60 min with 6-fold excess of activated amino acid over the resin free amino groups. The amino acids were activated with equimolar amounts of HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and a 2-fold molar excess of DIEA (N,N-diisopropylethylamine).

[0152] Alternatively, the peptides were synthesized by solid phase using Fmoc/t-Bu chemistry on a Pioneer Peptide Synthesizer (Applied Biosystems). In this case, all the acylation reactions were performed for 60 minutes with a 4-fold excess of activated amino acid over the resin free amino groups following the end of peptide assembly on the synthesizer. The side chain protecting groups were: tert-butyl for Asp, Glu, Ser, Thr and Tyr; trityl for Asn, Cys, Gln and His; tert-butoxy-carbonyl for Lys, Trp; and, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg. For the OXM2 and OXM3 peptides, the acetylation reaction was performed at the end of the peptide assembly by reaction with a 10-fold excess of acetic anhydride in DMF.

[0153] For OXM14, L-pyroglutamic acid was acylated by reaction with equimolar amounts of DIPC (diisopropylcarbo-

diimide) and HOBt (N-hydroxybenzotriazole) with a 4-fold excess of activated acylant over the resin free amino groups.

[0154] For OXM16, imidazole-lactic acid (Imi-H) was acylated by reaction with equimolar amounts of PyBOP (Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate), HOBt and a 2-fold molar excess of DIEA (N,N-diisopropylethylamine) with a 4-fold excess of activated acylant over the resin free amino groups.

[0155] For OXM17, N-methyl-His (Me-H) was acylated by reaction with equimolar amounts of HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and a 2-fold molar excess of DMA. The acylation reaction was performed for 180 min with a 3-fold excess of activated acylant over the resin free amino groups.

[0156] For OXM18, desamino-His (ANH₂-H) was acylated by reaction with equimolar amounts of HBTU and a 2-fold molar excess of DIEA. The acylation reaction was performed for 180 min with a 3-fold excess of activated acylant over the resin free amino groups.

[0157] For OXM19, N,N-dimethyl-His (Me₂-H) was acylated by reaction with equimolar amounts of HBTU and a 2-fold molar excess of DMA. The acylation reaction was performed overnight with a 3-fold excess of activated acylant over the resin free amino groups.

[0158] For OXM20, benzoyl-His (Bz-H) was acylated by reaction with equimolar amounts of HBTU and a 2-fold molar excess of DIEA. The acylation reaction was performed for 240 min with a 3-fold excess of activated acylant over the resin free amino groups.

[0159] For OXM21, Benzyl-His (Bzl-H) was acylated by reaction with equimolar amounts of HBTU and a 2-fold molar excess of DIEA. The acylation reaction was performed overnight with a 3-fold excess of activated acylant over the resin free amino groups.

[0160] At the end of the synthesis, the dry peptide-resins were individually treated with 20 mL of the cleavage mixture, 88% TFA, 5% phenol, 2% triisopropylsilane and 5% water (Sole, N. A. and G. Barany, 1992, *J. Org. Chem.* 57:5399-5403) for 1.5 hours at room temperature. Each resin was filtered and the solution was added to cold methyl-t-butyl ether in order to precipitate the peptide. After centrifugation, the peptide pellets were washed with fresh cold methyl-t-butyl ether to remove the organic scavengers. The process was repeated twice. Final pellets were dried, resuspended in H₂O, 20% acetonitrile, and lyophilized.

[0161] The synthesis of peptide OXM54 was performed by dissolving the thiol containing OXM peptide precursor (SEQ ID NO: 41) in TrisHCl 0.1M pH 8, guanidinium chloride 6M. A 10 molar excess of iodoacetamide was added. After 1 hour incubation, the peptide solution was purified by HPLC.

[0162] The synthesis of peptide OXM55 was performed by dissolving the thiol containing OXM peptide precursor (SEQ ID NO: 64) in TrisHCl 0.1M pH 8, guanidinium chloride 6M. A 10 molar excess of iodoacetamide was added to this solution. After 1 hour incubation, the peptide solution was purified by HPLC.

[0163] The crude peptides were purified by reverse-phase HPLC using semi-preparative Waters RCM Delta-Pak™ C₄ cartridges (40x200 mm, 15 μm) and using as eluents (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. The following gradient of eluent B was used: 20%-20% over 5 min and 20%-35% over 20 min, flow rate 80 mL/min. Analytical HPLC was performed on a Phenomenex, Jupiter C₄ column (150x4.6 mm, 5 μm) with the following gradient of eluent B:

20%-40% B (in 20 min)-80% (in 3 min), flow rate 1 mL/min. The purified peptide was characterized by electrospray mass spectrometry on a Micromass LCZ platform.

Example 2

PEGylation of Oxyntomodulin (OXM) Analogs

[0164] PEGylation reactions were run under conditions permitting thioester bond formation. The PEGylated OXM peptide was then isolated using reverse-phase HPLC or ion exchange chromatography and size exclusion chromatography (SEC). PEGylated OXM analogs were characterized using RP-HPLC, HPLC-SEC and MALDI-T of Mass Spectrometry.

[0165] OXM33, 34, 35, 36 and 54 peptides were synthesized from the thiol-containing OXM peptide precursor (SEQ ID NO: 41) to produce derivatives with PEG covalently attached via a thioether bond.

Synthesis of OXM433

[0166] 10 mg of peptide precursor (2.2 μmoles) were dissolved in 0.2 mL of HEPES 0.1M pH 7.3, Guanidinium Chloride 6M, 2 mM EDTA. 22 mg of MPEG-MAL-5000 (NEKTAR 2F2MOH01) (4.4 μmoles) dissolved in 0.4 mL HEPES 0.1M, pH 7.3 (1:2 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide was purified by RP-HPLC and characterized by MALDI-T of.

Synthesis of OXM34

[0167] 10 mg of peptide precursor (2.2 μmoles) were dissolved in 0.2 mL of HEPES 0.1M pH 7.3, Guanidinium Chloride 6M, 2 mM EDTA. 80 mg of MPEG-MAL-20K (NEKTAR 2F2MOP01) (4.0 μmoles) dissolved in 0.5 mL HEPES 0.1M, pH 7.3 (1:1.8 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide was purified by RP-HPLC and characterized by MALDI-T of.

Synthesis of OXM35

[0168] 10 mg of peptide precursor (0.92 μmoles) were dissolved in 0.4 mL of HEPES 0.1M pH 7.3, Guanidinium Chloride 6M, 2 mM EDTA. 70 mg of MPEG2-MAL-40K (NEKTAR 2D3Y0T01) (1.7 μmoles) dissolved in 0.8 mL HEPES 0.1M, pH 7.3 in a 1:1.8 mole/mole ratio of peptide to PEG was added to this solution. After 1 hour incubation, the PEGylated peptide was purified by RP-HPLC and characterized by MALDI-T of.

[0169] The control peptide OXM54, was prepared by incubating the thiol containing peptide precursor with 10 eq. of iodoacetamide in 0.1 M TrisHCl pH 7.5, 6M guanidinium chloride. After 30 minutes incubation the peptide was purified by RP-HPLC and characterized by electrospray mass spectrometry.

[0170] The peptides OXM 56, 57, 58 were synthesized from the thiol containing OXM peptide precursor (SEQ ID NO: 64) to produce derivatives with PEG covalently attached via a thioether bond.

Synthesis of OXM56

[0171] 5 mg of peptide precursor (1.1 μmoles) were dissolved in 0.2 mL of HEPES 0.1M pH 7.3. 57 mg of MPEG-MAL-5000 (NEKTAR 2F2MOH01) (11.4 μmoles) dissolved

in 0.4 mL HEPES 0.1M, pH 7.3 (1:10 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide solution was acidified to 1% acetic acid and purified by cation exchange chromatography (DCC) on fractogel TSK CM-650S with a linear gradient of NaCl in sodium acetate 50 mM pH 4.8. The DCC purified PEGylated-peptide was further purified by size-exclusion chromatography (SEC) and characterized by MALDI-T of.

Synthesis of OXM57

[0172] 10 mg of peptide precursor (2.2 μ moles) were dissolved in 0.2 mL of DMSO. 50 mg of MPEG-MAL-20K (NEKTAR 2F2MOP01) (2.5 μ moles) dissolved in 0.6 mL HEPES 0.1M pH 7.3, 0.3M TRIS(2-carboxy-ethyl)phosphine with a 1:1.13 mole/mole ratio of peptide to PEG was added to this solution. After 1 hour incubation, the PEGylated peptide was purified by RP-HPLC and characterized by MALDI-T of.

Synthesis of OXM58

[0173] 10 mg of peptide precursor (0.92 μ moles) were dissolved in 0.4 mL of HEPES 0.1M pH 7.3, Guanidinium Chloride 6M, 2 mM EDTA. 70 mg of MPEG2-MAL-40K (NEKTAR 2D3Y0T01) (1.7 μ moles) dissolved in 0.8 mL HEPES 0.1M, pH 7.3 (1:1.8 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide was purified by RP-HPLC and characterized by MALDI-T of.

[0174] The control peptides OXM102, OXM112, and OXM116 were prepared by incubating the thiol-containing peptide precursor with 10 eq. of iodoacetamide in 0.1 M TrisHCl pH 7.5, 6M guanidinium chloride. After 30 minutes incubation the peptide was purified by RP-HPLC and characterized by electrospray mass spectrometry.

Synthesis of OXM103, OXM105, OXM107, OXM113

[0175] 10 mg of the corresponding peptide precursors (2.26 μ moles) were dissolved in 2 mL of urea 8M, HEPES 0.1M pH 7.3, 2 mM EDTA. 109 mg of MPEG2-MAL-40K (NEKTAR 2D3Y0T01) (2.71 μ moles) dissolved in H₂O (1:1.2 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide solution was acidified to 1% acetic acid and purified by cation exchange chromatography (IXC) on TSK CM-650S with a linear gradient of NaCl in sodium acetate 50 mM pH 4.8. The DCC purified PEGylated-peptide was further purified by SEC and characterized by MALDI-T of.

Synthesis of OXM109

[0176] 10 mg of the corresponding peptide precursors (2.25 μ moles) were dissolved in 2 mL urea 8M, HEPES 0.1M pH 7.3, 2 mM EDTA. 108 mg of MPEG2-MAL-40K (NEKTAR 2D3Y0T01) (2.7 μ moles) dissolved in 2 mL H₂O (1:1.2 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide solution was acidified to 1% acetic acid and purified by cation exchange chromatography (DCC) on TSK CM-650S with a linear gradient of NaCl in sodium acetate 50 mM pH 4.8. The IXC purified PEGylated-peptide was further purified by SEC and characterized by MALDI-T of.

Synthesis of OXM117

[0177] 10 mg of the corresponding peptide precursors (2.19 μ moles) were dissolved in 2 mL of urea 8M, HEPES 0.2M pH

6.5, 2 mM EDTA. 105 mg of MPEG₂-MAL-40K (NEKTAR 2D3Y0T01) (2.63 μ moles) dissolved in H₂O (1:1.2 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide solution was acidified to 1% acetic acid and purified by cation exchange chromatography (DCC) on TSK CM-650S with a linear gradient of NaCl in sodium acetate 50 mM pH 4.8. The DCC purified PEGylated-peptide was further purified by SEC and characterized by MALDI-T of.

Synthesis of OXM125

[0178] 10 mg of the corresponding peptide precursors (2.26 μ moles) were dissolved in 2 mL of Urea 8M, HEPES 0.25M pH 6.5, 2 mM EDTA. 105 mg of MPEG₂-MAL-40K (NEKTAR 2D3Y0T01) (2.71 μ moles) dissolved in H₂O (1:1.2 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide solution was acidified to 0.2% formic acid pH 2.8 and purified by cation exchange chromatography (IXC) on TSK SP-5PW with a linear gradient of NaCl in formic acid 0.2%. The DCC purified PEGylated-peptide was further purified by SEC and characterized by MALDI-T of.

Synthesis of OXM129

[0179] 10 mg of the corresponding peptide precursors (2.26 μ moles) were dissolved in 2 mL of Urea 8M, HEPES 0.25M pH 6.5, 2 mM EDTA. 109 mg of MPEG₂-MAL-40K (NEKTAR 2D3Y0T01) (2.72 μ moles) dissolved in H₂O (1:1.2 mole/mole ratio of peptide to PEG) was added to this solution. After 1 hour incubation, the PEGylated peptide solution was acidified to 0.2% formic acid and purified by cation exchange chromatography (DCC) on TSK SP-5PW with a linear gradient of NaCl in formic acid 0.2%. The DCC purified PEGylated-peptide was further purified by SEC and characterized by MALDI-T of.

Example 3

Design and Synthesis of Peptide Sequences

[0180] The biological activity of different PEG sizes (mPEG)₅ kDa, (mPEG)₂₀ kDa, (mPEG)₄₀ kDa and (mPEG)₆₀ kDa was compared in a series of experiments which demonstrated that the optimal PEG size to confer the maximal and durable activity in the mice is generally 40 kDa.

[0181] The peptide OXM103 (Aib (α) at position 2) was designed to explore position 20 within the oxyntomodulin sequence as the site for conjugation with (mPEG)₂40 kDa. OXM102 is a control peptide (CH₂CONH₂), in which the side-chain thiol (of cysteine at position 20) was reacted with iodoacetamide.

[0182] The peptide OXM105 (Aib (α) at position 2) was designed to explore position 21 within the oxyntomodulin sequence, as the site for conjugation with (mPEG)₂40 kDa. OXM104 is a control peptide (CH₂CONH₂), in which the side-chain thiol (of cysteine at position 21) was reacted with iodoacetamide.

[0183] The peptide OXM107 (Aib (α) at position 2, Met(O) at position 27) was designed to explore position 24 within the oxyntomodulin sequence, as the site for conjugation with (mPEG)₂40 kDa. OXM106 is a control peptide (CH₂CONH₂), in which the side-chain thiol (of cysteine at position 24) was reacted with iodoacetamide.

[0184] The peptide OXM109 (Aib (α) at position 2,) was designed to explore position 28 within the oxyntomodulin sequence, as the site for conjugation with (mPEG)₂40 kDa, OXM108 is a control peptide (CH₂CONH₂), in which the side-chain thiol (of cysteine at position 28) was reacted with iodoacetamide.

[0185] The peptide designated OXM141 has a Gln to Asp mutation at position 3, which confers specific selectivity to the GLP1-receptor. The peptide OXM141, which has a Gln to Asp mutation at position 3, Met(O) at position 27, and two conjugation sites at position 20 and 38, was designed to explore the potential of having a peptide conjugated with both a cholesterol group at C38 and PEG at position C20.

[0186] The peptide OXM142 (Aib (cc), Met(O) at position 27, and two conjugation sites at position 20 and 38) was designed to explore the potential of having a peptide conjugated with both a cholesterol group at C38 and PEG at position C20.

[0187] FIG. 14 summarizes the in vitro activity data for GLP1R and GCG receptors (also showing the GLP-1R and GLGR specificities) in tabular form. The (mPEG)₂40 kDa conjugate at position C20 retains activity on both receptors, therefore OXM103 as is referred to as a +/-analog, while all the other (mPEG)₂40 kDa conjugates lose potency at the GCG receptor. In particular there is a between 2-3 orders of magnitude selectivity towards the GLP-1 receptor over the Gcg receptor for the (mPEG)₂40 kDa conjugates at position 38, 24 and 28. Therefore, the analogs OXM107 and 109 are referred to as +/- analogs.

[0188] FIG. 15 shows the in vivo activity of the (mPEG)₂40 kDa conjugates on food intake and body weight loss on DIO mice. Ad libitum fed, DIO (~51 g each), male C57BL/6 mice were dosed i.p. with either vehicle (water) or Oxm analogs 99, 103, 105, 107, and 109~30 min prior to the onset of the dark phase. Food intake measured ~2 h and 18 h (overnight) later on day 1 and 26 and 42 h (overnight) later on day 2. *P<0.05 vs. vehicle, n=5-6 per group).

[0189] The peptide OXM110 (D-Ser (s) at position 2,) was designed to explore position 38 within the oxyntomodulin sequence, as the site for conjugation with a lipid such as a palmitoyl group. The palmitoyl group was acylated to the ϵ -amino group of a lysine added at the C-terminus of the oxyntomodulin sequence.

[0190] The peptide OXM113 (desamino-His (ANH₂—H) at position 1, Aib (α) at position 2, Met(O) at position 27, conjugation site 38) was designed to explore the potential of substituting the wild type (wt) His with a desamino-His at position 1 for protection from DPPIV proteolysis. OXM113 is the (mPEG)₂40 kDa conjugate, OXM114 is the cholesterol conjugate and OXM112 is a control peptide (CH₂CONH₂), in which the side-chain thiol (of cysteine at position 38) was reacted with iodoacetamide). (OXM111 is the thiolated peptide precursor).

[0191] The peptides OXM117 and OXM118 (Aib (α) at position 2, Gln to Asp mutation at position 3, Met(O) at position 27, conjugation site 38) were designed to explore the potential of substituting the wild type Gln with an Asp at position 3. As mentioned above, this mutation confers specific selectivity towards the GLP-1R. OXM117 is the (mPEG)₂40 kDa conjugate and the OXM118 is the cholesterol conjugate. (OXM116 is the thiolated peptide precursor).

[0192] The peptide OXM121 (Aib (α) at position 2, Met(O) at position 27, conjugation site 11) was designed to explore position 11 within the oxyntomodulin sequence as the site for

the conjugation. OXM121 is the (mPEG)₂40 kDa conjugate, OXM119 is the thiolated peptide precursor and OXM120 is a control peptide (CH₂CONH₂), in which the side-chain thiol (of cysteine at position 1.1) was reacted with iodoacetamide.

[0193] The peptide OXM124 (Aib (α) at position 2, Met(O) at position 27, conjugation site 12) was designed to explore position 12 within the oxyntomodulin sequence as the site for conjugation. OXM124 is the (mPEG)₂40 kDa conjugate, OXM122 is the thiolated peptide precursor and OXM123 is a control peptide (CH₂CONH₂), in which the side-chain thiol (of cysteine at position 12) was reacted with iodoacetamide.

[0194] The peptide OXM125 (Aib (α) at position 2, Gln to Asp mutation at position 3, Met(O) at position 27, conjugation site 20) was designed to explore position 20 within the oxyntomodulin sequence as the site for conjugation of (mPEG)₂40 kDa as well as the Gln to Asp substitution at position 3 that confers selectivity towards the GLP-1R.

[0195] The peptide OXM127 (Aib (α) at position 2, Met(O) at position 27, conjugation site 22) was designed to explore position 22 within the oxyntomodulin sequence as the site for conjugation for cholesterol. OXM126 is the thiolated peptide precursor).

[0196] The peptide OXM129 (desamino-His (ANH₂—H) at position 1, Aib (α) at position 2, Gln to Asp mutation at position 3, Met(O) at position 27, conjugation site 20) was designed to explore the potential of the following combinations: substituting the wt His with desamino-His at position 1 for protection from DPPIV proteolysis, the Gln to Asp substitution at position 3 that confers selectivity towards the GLP-1R, and conjugation at position 20.

[0197] The peptide OXM134 (Aib (α) at position 2, Met(O) at position 27, conjugation site 20) is a (mPEG)₂40 kDa conjugate similar to the potent +/-OXM103 analogue that only differs for the methionine substitution to methionine sulfoxide. This peptide is conjugated at the position 20 therefore displaying a +/-pattern for GLP1R/GcgR selectivity.

Example 4

C-Terminal Truncated Analogs or GcgK Analogs

C-Terminal Truncated Analogs

[0198] A series of peptide OXM C-terminal truncated analogs were designed. An analysis of in vitro activity on both the GLP1 receptor (GLP1R) and the Gcg receptor (GcgR) demonstrated that the OXM sequence can be truncated at the C-terminus to have only one extra lysine residue with respect to wt glucagon, yielding a peptide such as OXM93 that is extremely potent on both the GLP1R and GcgR.

[0199] The potency of OXM93 is at least one order (and possibly two) of magnitude higher than that of wt OXM. Because there is only one extra Lys residue with respect to Gcg, this new class is referred to as GcgK analogs. FIG. 16 illustrates in vitro potency data for the C-terminal truncated analogs acting at the GLP1 and GCG receptors in tabular form.

C-terminal truncated analogs which are selective GcgK Analogs

[0200] Peptides OXM130 and OXM131 were designed to confirm the in vitro analysis and introduce other mutations that are known to confer stability and suitable properties. OXM130 and OXM131 are C-terminal truncated analogues of the same length as OXM93. OXM130 has Aib (α) at

position 2, while OXM131 has Aib (α) at position 2 and a Gln to Asp mutation at position 3 to confer selectivity for the GLP1R.

[0201] The following PEGylated analogs were designed based on these truncated sequences:

[0202] The peptide OXM136 (Aib (α) at position 2, Met(O) at position 27, conjugation site 20) is the (mPEG)₂40 kDa conjugate. From prior studies (see above) it is known that the choice of PEG conjugation at position 20 allows for activity on both receptors GLP1R and GcgR, therefore OXM136 would be defined as the prototype Gcg +/- analogue. (OXM135 is the thiolated peptide precursor).

[0203] The peptide OXM138 (Aib (α) at position 2, Gln to Asp mutation at position 3, Met(O) at position 27, conjugation site 20) was designed to explore the potential of the following combinations: the Gln to Asp substitution at position 3 that confers selectivity towards the GLP-1R; conjugation

at position 20 and mutating the wt Gln to Asp at position 3. Therefore OXM137 would be defined as the prototype GcgK +/-0 analog (OXM137 is the thiolated peptide precursor).

[0204] FIG. 17 provides in vitro potency data at the GLP1 and GCG receptors for select PEGylated OXM analogs.

Example 5

Peptides with Alternative PEG Moieties

[0205] The peptide OXM145 (Aib (cc) at position 2, Gln to Asp mutation at position 3, Met(O) at position 27, conjugation site 38 with Y Shape PEG Maleimide from JenKem Therapeutics) was produced to explore the potential of using alternative branched 40 kDa PEG moieties. This is the conjugate obtained using a (mPEG)₂40 kDa from JenKem Therapeutics (Y-Shaped PEG Maleimide, MW40K, item number Y-MAL-40K and its structure is shown below:

ANEXO 2



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536/28.1; 530/351; 424/85.1(73) Assignee: **Yeda Research and Development Co., Ltd.**, Rehovot (IL)(21) Appl. No.: **11/244,402**(22) Filed: **Oct. 6, 2005****Related U.S. Application Data**

(63) Continuation-in-part of application No. PCT/IL04/00321, filed on Apr. 8, 2004.

(60) Provisional application No. 60/460,816, filed on Apr. 8, 2003.

(57)

ABSTRACT

Reversible pegylated drugs are provided by derivatization of free functional groups of the drug selected from amino, hydroxyl, mercapto, phosphate and/or carboxyl with groups sensitive to mild basic conditions such as 9-fluorenylmethoxycarbonyl (Fmoc) or 2-sulfo-9-fluorenylmethoxycarbonyl (FMS), to which group a PEG moiety is attached. In these pegylated drugs, the PEG moiety and the drug residue are not linked directly to each other, but rather both residues are linked to different positions of the scaffold Fmoc or FMS structure that is highly sensitive to bases and is removable under physiological conditions. The drugs are preferably drugs containing an amino group, most preferably peptides and proteins of low or medium molecular weight. Similar molecules are provided wherein a protein carrier or another polymer carrier replaces the PEG moiety.

REVERSIBLE PEGYLATED DRUGS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application is a continuation-in-part application of PCT application No. PCT/IL 2004/000701, filed Apr. 8, 2004, in which the US is designated, and claims the benefit of U.S. Provisional Patent Application No. 60/460,816, filed Apr. 08, 2003, the entire contents of each and both these applications being hereby incorporated by reference herein in their entirety as if fully disclosed herein.

FIELD OF THE INVENTION

[0002] The present invention relates to reversible pegylation of drugs and to pegylated drugs that are slowly converted to the drugs in physiological conditions.

[0003] Abbreviations: ANP, atrial natriuretic peptide; t-Boc, tert-butyloxycarbonyl; BSA, bovine serum albumin; DCC, N,N'-dicyclohexylcarbodiimide; DCU, N,N'-dicyclohexylurea; DMF, N,N'-dimethylformamide; DMB, 5,5-dimethyl-2-methyl-2-oxobenzene-1,3-dithiolane-2-carboxylic acid; EIMS, electrospray ionization mass spectra; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, Fmoc-N-hydroxysuccinimide ester; FMS, 2-sulfo-9-fluorenylmethoxycarbonyl; GSII, reduced glutathione; hGH, human growth hormone; HOSu, N-hydroxysuccinimide; HPLC, high-performance liquid chromatography; HSA, human serum albumin; HSA-Fmoc-insulin, a conjugate of human serum albumin and insulin; IDDM, insulin dependent diabetes mellitus; hTN- α 2, human interleukin- α 2; hTN- β 2-EC, extracellular part of hTN- α 2 receptor; MAI-FMS-NHS-N-[2-(maleimido-propionyl)amino-7-sulfo-fluorenyl-methoxy-carbonyloxy]succinimide (Precursor 8); MAI-FMS-OSu, MAI-FMS-NHS, MID-NHS, maleimido benzoic N-hydroxysuccinimide ester; NHS, N-hydroxysuccinimide; PBS, phosphate-buffered saline; PEG, polyethylene glycol; PEG₅₀₀₀, 5,000 Da-PEG; PEG₁₀₀₀₀, 10,000 Da-branched PEG; PEG₁₀₀₀₀-SH, a 40 kDa branched PEG containing a sulfhydryl moiety; PEG₁₀₀₀₀-OSu, PEG₁₀₀₀₀-N-hydroxysuccinimide ester; SC, subcutaneous; STZ, streptozocin; TCA, trichloroacetic acid; TEA, triethylamine; THF, tetrahydrofuran; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

BACKGROUND OF THE INVENTION

[0004] Most peptide and protein drugs are short lived and have often a short circulatory half-life in vivo. This is particularly valid for nonglycosylated proteins of a molecular mass less than 20 kDa. The short lifetime of proteins in vivo is attributed to several factors, including glomerular filtration in the kidney and proteolysis. Considering that peptide and protein drugs are not absorbed orally, prolonged maintenance of therapeutically active drugs in the circulation is a desirable feature of obvious clinical importance. Proteins with molecular masses above ~60 kDa largely avoid glomerular filtration and are not, for the most part, filtered in the kidney. Therefore they remain in the circulation longer than smaller proteins.

[0005] An attractive strategy for improving clinical properties of small protein drugs has come to be known as PEGylation (or pegylation, as used hereinafter). By this strategy several hydrophilic chains of polyethylene glycol (PEG) are covalently linked to the protein in order to

increase its effective molecular mass. Important clinical advantages are gained by pegylation. For example, life-time in vivo can be prolonged in some instances from minutes to hours, owing to the steric interference that protects conjugates from proteolysis in vivo and the increase in molecular mass, which precludes filtration by the kidney. Protein pegylation also decreases immunogenicity, presumably by protecting conjugates from being recognized as foreign antigens by the immune system.

[0006] In spite of the profound advantages often gained by pegylating therapeutic proteins, this technology suffers from a principal drawback. On the one hand, covalently attaching PEG chains to proteins prolongs their lifetime in vivo, protecting the conjugates from proteolysis and shielding them from the immune system. On the other hand, the steric interference of the PEG chains often leads to a drastic loss or even abolish the biological and the pharmacological potencies of the proteins in the conjugates (Fertges and Abuchowski, 1990; Katre, 1993; Bailon and Berthold, 1998; Nucci et al., 1991; Delgado et al., 1992; Fung et al., 1997; Reddy, 2000; Veronese, 2001). In principle, this deficiency can be overcome by introducing the PEG chains via a chemical bond that is sensitive to hydrolysis, or can be cleaved enzymatically by serum proteases or esterases. Clearly, a consistent rate of hydrolysis is crucial. A prerequisite condition is therefore that the hydrolysis of the PEG chains from the conjugate is to take place at a slow rate, and in a homogenous fashion in vivo.

[0007] It would be highly desirable to design PEG derivatives of proteins or peptides or small drug molecules from which PEG can be released by hydrolysis. An appropriate reversible PEG conjugate would have to be hydrolyzed slowly and spontaneously in physiological conditions and would permit time-dependent reactivation of inactive pegylated proteins and peptides.

[0008] Several methods for reversible pegylation were proposed (Greenwald et al., 1999, 2000; Lee et al., 2001; Garmay and Kafindjian, 1987; Zalipsky et al., 1999). They suffer, however, from major potential drawbacks. For example, reliance on enzymatic detachment as a rate-determining step (Greenwald et al., 1999, 2000; Lee et al., 2001) of PEGs from conjugates by serum proteases and/or esterases might not yield desirable pharmacokinetic profiles in situ. Moreover, it is dependent on enzymes availability. Disulfide-bonded conjugate is not to be cleaved in the non-reducing environment of the body fluids (Zalipsky et al., 1999). A reversibly pegylated conjugate which still retain an active moiety capable of reacting with free SH functions may result in complex undesired cross linking (Garmay and Kafindjian, 1987). It would be very desirable to design a version of reversible pegylation that would overcome these deficiencies.

[0009] International PCT Publication No. WO 98/05361 or the present applicants describes a novel conceptual approach for prolonging the half-life of drugs by derivatizing a drug having at least one free amino, carboxyl, hydroxyl and/or mercapto groups with a moiety that is highly sensitive to bases and is removable under mild basic conditions. The prodrug obtained is inactive but undergoes transformation into the active drug under physiological conditions in the body. Examples of said moieties are the radicals 9-fluorenylmethoxycarbonyl (Fmoc) and 2-sulfo-9-fluorenyl-

methoxycarbonyl (Fmoc). According to this concept, Fmoc and FMS derivatives of peptidic drugs such as insulin and human growth hormone as well as of non-peptidic drugs such as propranolol, cephalixin and piperacillin have been described in said WO 98/05361. Later on, FMS derivatives of cytokines have been disclosed in WO 02/36057, and FMS derivatives of enkephalin, doxorubicin, amphotericin B, gentamicin and gonadotropin releasing hormone (GnRH) were disclosed in WO 02/7859.

[0010] U.S. Pat. No. 6,433,438 discloses a pegylated derivative of an analogue of luteinizing hormone releasing hormone (LHRH or GnRH) in which the PEG moiety is covalently bound to a serine residue of said LHRH analogue. In the process of preparation of said PEG-LHRH analogue by solid phase peptide synthesis, a pegylated serine residue such as Fmoc-Ser(PEG)-OH or t-Boc-Ser(PEG)-OH is introduced into the LHRH analogue, and the produced PEG-LHRH analogue is recovered (without the protective group Fmoc or t-Boc).

[0011] JP Patent Application JP 5148798 describes PEG-peptide conjugates, e.g., PEG-GnRH conjugate, obtained by reacting the guanidino group of an arginine residue with PEG, while protecting the amine groups present in the molecules.

[0012] Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicants at the time of filing and does not constitute an admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

[0013] It has been found, in accordance with the present invention, that drugs with a prolonged circulating half-life can be obtained by combining the technology of derivatization of the drug with Fmoc or FMS or similar moieties removable under mild basic conditions with the technology of attaching a suitable natural or synthetic carrier to the thus derivatized drug molecule, such carrier serving for delivery of the drug and providing further benefits.

[0014] The carrier may be a protein such as albumin or a protein containing a globin-like domain or a polymeric carrier consisting of a biocompatible and biodegradable polymer containing functional groups. The polymeric carrier is preferably in the form of nanoparticles or it is attached to liposomes.

[0015] In one preferred embodiment, the polymeric carrier is PEG. It has been found, in accordance with the present invention, that by combination of the protein-pegylation technology with the technology of derivatization with Fmoc or FMS or similar moieties removable under mild basic conditions, major deficiencies of the protein-pegylation technology, mainly the loss of biological and pharmacological potencies in the PEG conjugates in vivo, may be overcome.

[0016] In one embodiment of the present invention, PEG-protein conjugates are provided from which PEG can be released by hydrolysis under physiological conditions in the body.

[0017] In another embodiment, reversible PEG-protein conjugates are provided that are inactive when administered and permit time-dependent reactivation of the inactivated pegylated protein under physiological conditions in the body.

[0018] The present invention thus relates, in one aspect, to a compound of the formula:



[0019] wherein

[0020] Y is a moiety of a drug bearing at least one functional group selected from free amino, carboxyl, phosphate, hydroxyl and/or mercapto; and

[0021] X is a radical that is highly sensitive to bases and is removable under mild basic conditions, said radical carrying a protein or a polymeric carrier moiety.

[0022] n is an integer of at least one; and

[0023] pharmaceutically acceptable salts thereof.

[0024] The prodrug obtained is inactive but undergoes transformation into the active drug Y under physiological conditions in the body.

[0025] In preferred embodiments of the invention, the radical X is Fmoc or 2-sulfo Fmoc (herein "FMS"), Y is a peptide or protein drug linked to Y through an amino group, n is 1 or 2, the protein carrier is albumin and the polymeric carrier is a linear or branched PEG moiety having a molecular weight of 5,000-40,000 Da.

[0026] In another aspect, the present invention provides novel methods and intermediates and precursors for the preparation of the conjugates of the invention.

[0027] In a further aspect, the present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and a prodrug of the invention.

BRIEF DESCRIPTION OF THE FIGURES

[0028] FIG. 1 shows the stability of the maleimide functional moiety in MAL-FMS-NHS in aqueous solutions having different pH values. MAL-FMS-NHS (1 mM) was incubated at room temperature in H₂O (pH 6.0), in 0.09 M acetic acid (pH 4.0), in 0.1 M phosphate buffer (pH 7.4), and in 0.1 M NaHCO₃ (pH 8.5). At the indicated time points, aliquots were allowed to react with a slight excess of GSH (15 mM at pH 7.2) and the concentration of unreacted GSH was determined with 5,5'-dithiobis(2-nitrobenzene acid) (DTNB).

[0029] FIG. 2 shows the degree of incorporation of MAL-FMS-NHS at pH 7.2 into α -lactalbumin (α -LA) as a function of the amount of added reagent. To samples of α -LA (1.0 ml of 1 mg/ml in 0.1M phosphate buffer, pH 7.2), MAL-FMS-NHS was added at concentrations ranging from 1 equivalent up to 13 molar equivalents of MAL-FMS-NHS. For each treatment the amount incorporated into the protein was determined by the absorbance at 280 nm, after dialysis, and by quantitating the amount of unmodified amino side chain moieties with trinitrobenzene sulfonic acid (TNBS).

[0030] FIGS. 3A-3B show the time course of reactivation of (PEG₄₀₀₀-Fmoc)-pentamycin and (PEG₄₀₀₀-Fmoc)-gentamicin conjugates, respectively. After incubation at pH 8.5, 37°C, aliquots were withdrawn at the indicated time points

and analyzed for their potency to arrest *E. coli* replication. The IC_{50} for each aliquot was determined. Native penicillin inhibited *E. coli* replication with IC_{50} value $= 0.22 \pm 0.02$ μ M.

[0031] FIGS. 4A-4B show progressive modification of the amino acid moieties of human insulin with PEG_{1000} -Fmoc-OSu and loss of biological potency as a function of PEG_{1000} . Insulin incorporated into insulin, respectively. (4A) Insulin (17.21 μ moles in 0.2 ml 0.01 M $NaHCO_3$) reacted with increasing concentrations of PEG_{1000} -Fmoc-OSu at a molar excess over the protein as indicated in the figure for 2 hours at $25^\circ C$. The number of free amino groups that remained unmodified were quantified with TNBS. (4B) Aliquots containing 0.4, 0.7, 1.1, 1.5 and 2.2 μ moles PEG_{1000} -Fmoc covalently attached per mole insulin, were assayed for their lipogenic potency in rat adipocytes. Under the assay conditions, human insulin stimulates lipogenesis, 4-6 times above basal levels with ED_{50} value of 0.2 ± 0.02 ng/ml. An insulin derivative exhibiting ED_{50} of 2.0 ± 0.2 ng/ml in this assay is considered as having 10% the lipogenic potency of native insulin.

[0032] FIG. 5 shows the rate of reactivation of PEG_{1000} -Fmoc-insulin conjugates upon incubation at pH 8.5, $37^\circ C$. PEG_{1000} -Fmoc-insulin conjugates containing one and two moles of PEG_{1000} -Fmoc/mole insulin were incubated at a concentration of 0.172 μ M in 0.1 M $NaHCO_3$ -0.5% bovine serum albumin and 1 mM NaN_3 at $37^\circ C$. At the indicated time points aliquots were analyzed (in several concentrations for each aliquot) for their lipogenic potencies in rat adipocytes.

[0033] FIG. 6 shows prolonged glucose-lowering effect after a single subcutaneous (SC) administration of $(PEG_{1000}-Fmoc)_1$ insulin in mice. Mice received SC, either native insulin (Zn^{2+} -free, 1.72 μ moles/mouse in 0.2 ml PBS buffer) or $(PEG_{1000}-Fmoc)_1$ -insulin (17.2 μ moles/mouse, in 0.2 ml PBS buffer). Blood glucose levels were determined at the indicated time points. Each point is the arithmetic mean $\pm$ SEM of blood glucose of five mice.

[0034] FIG. 7 shows glucose-lowering pattern in mice following single intraperitoneal (IP) administration of $(PEG_{1000}-Fmoc)_1$ insulin. Groups of mice received IP either insulin (Zn^{2+} -free, 0.345 μ moles/mouse, in 0.2 ml PBS buffer) or $(PEG_{1000}-Fmoc)_1$ -insulin (3.45 μ moles/mouse, in 0.2 ml PBS buffer). Blood glucose levels were determined at the indicated time points. Each point in the figure is the arithmetic mean $\pm$ SEM of five mice.

[0035] FIG. 8 shows the rate of release of exendin-4 from PEG_{1000} -FMS-exendin-4 conjugate, upon incubation at pH 8.5, $37^\circ C$. At the indicated time points, aliquots (50 μ l) were loaded on HPLC and run under conditions resolving well exendin-4 from the conjugate. Results are expressed as percent of maximal peak area of released exendin-4, as a function of time. Exendin-4 (50 μ g) was assigned at 100% peak area.

[0036] FIG. 9 shows the rate of hydrolysis of PEG_{1000} -FMS conjugates, upon incubation at pH 8.5, $37^\circ C$. Solutions of PEG_{1000} -FMS-exendin-4 (circles) and PEG_{1000} -FMS-4-nitro-phenethyl amine (squares) were incubated in PBS at pH 8.5, $37^\circ C$. At the indicated time points, aliquots (50 μ l) were analyzed using HPLC on a RP-4 column. Results are expressed as percent of the maximal peak area of released exendin-4 and 4-nitrophenethylamine, as a function of time.

[0037] FIGS. 10A-10B show glucose-lowering patterns of native exendin-4 and PEG_{1000} -FMS-exendin-4 following a single subcutaneous administration to CD1-mice. (10A) CD1-mice were SC-administered with either native exendin-4 (10 μ g/mouse) or with PEG_{1000} -FMS-exendin-4 (10 μ g/mouse of exendin-4 equiv). At the indicated time points, circulating glucose levels were determined. Each experimental group consisted of five mice. Data are presented as mean $\pm$ SE. (10B) Three groups of CD1 mice (n=5 per group) underwent one subcutaneous administration of saline, native exendin-4 (4 μ g/mouse) or PEG_{1000} -FMS-exendin-4 (4 μ g peptide/mouse). Circulating glucose levels were then monitored. Results are expressed as percent decrease in plasma glucose concentration in the groups treated with exendin-4 or PEG_{1000} -FMS-exendin-4 relative to that found in the saline-treated group measured at the same time-point during the day.

[0038] FIGS. 11A-11C show release of active I-I-Nr2 upon incubation of PEG_{1000} -FMS-I-I-Nr2 at pH 8.5, $37^\circ C$. PEG_{1000} -FMS-I-I-Nr2 (0.3 mg protein/ml) was incubated in 0.1 M phosphate buffer with 2 mM NaN_3 and 6 mg/ml BSA (pH 8.5, $37^\circ C$). At the indicated time points, aliquots were withdrawn. (11A) Analysis of I-I-Nr2 discharge from the conjugate by SDS-PAGE: the amounts of I-I-Nr2 discharge were quantified relative to an I-I-Nr2 reference of known concentration and intensity (the time increments and the percentages are indicated). (11B) Aliquots withdrawn at the indicated time points were analyzed for their I-I-Nr2 binding capacity on BIAcore. (11C) Fitted BIAcore profile of native I-I-Nr2 discharge from PEG_{1000} -FMS-I-I-Nr2.

[0039] FIG. 12 shows the results of SC administration of native I-I-Nr2 and PEG_{1000} -FMS-I-I-Nr2. Rats were SC injected with the indicated concentrations of native I-I-Nr2 (100 μ g/rat) or the PEG_{1000} -FMS-I-I-Nr2 conjugate (12, 60, 120 μ g/rat) (0.2 ml/rat, dissolved in PBS). Blood aliquots were withdrawn at the indicated time points. Circulating antiviral activities in the aliquots were determined in human WISH cells with 5 fold serial dilutions of each aliquot.

[0040] FIG. 13 shows the result of intravenous administration of PEG_{1000} -FMS-I-I-Nr2 to rats. Rats were intravenously injected with the indicated concentrations of native I-I-Nr2 (30 μ g/rat) or the PEG_{1000} -FMS-I-I-Nr2 conjugate (30 μ g/rat) (0.2 ml/rat, dissolved in PBS). Blood aliquots were withdrawn at the indicated time points. Circulating antiviral activities in the aliquots were determined in human WISH cells with 5 fold serial dilutions of each aliquot.

[0041] FIGS. 14A-14B show experimental vs. simulated behavior of I-I-Nr2. (14A) following SC administration with initial concentrations of 50 nM and 1.5 nM of PEG_{1000} -FMS-I-I-Nr2 and native I-I-Nr2, respectively; (14B) following intravenous administration to rats with initial concentrations of 20 nM of PEG_{1000} -FMS-I-I-Nr2, 1.5 nM of native I-I-Nr2 in the SC volume and no conjugate in circulation. The inserts are the experimental curves.

[0042] FIG. 15 shows dose response of PYY_{1-36} in food intake in mice. Male C57BL/6J mice (10 per group), were deprived of food for 24 h. At time 23 h, the mice received a SC injection of either saline or the indicated doses of PYY_{1-36} . At time 24 h, the mice were allowed to consume an excess of pre-weighed chow for 2 h. Drinking water was provided at all times. The amount of food consumed per 10 mice during 2 h is shown as a function of PYY_{1-36} dose.

[0043] FIG. 16 shows time-dependent reduction in food intake in mice by PYY₁₋₃₆. Male C57BL/6J mice (10 per group) were deprived of food as described in FIG. 15 and PYY₁₋₃₆ (5 nmol/mouse) was administered at the indicated times prior to start of the re-feeding period. Results are average of four identical experiments.

[0044] FIG. 17 shows the effects of irreversible pegylation on the biological activity of PYY₁₋₃₆. Native PYY₁₋₃₆ was allowed to react with PEG₄₀₀-OSn. Groups of 10 mice were injected SC with saline, PYY₁₋₃₆ or PEG₄₀₀-PYY₁₋₃₆ (5 nmol/mouse) at 1 h prior to start of the re-feeding period. Results are the average of two identical experiments.

[0045] FIG. 18 shows that PEG₄₀₀-FMS is linked to the α-amino group of PYY₁₋₃₆. PEG₄₀₀-FMS-PYY₁₋₃₆ (100 μg) was acetylated by a 500 molar excess of acetic anhydride at pH 7.0, hydrolyzed, and incubated for 3 days at pH 8.5, 37° C. to quantitatively remove the PEG₄₀₀-FMS moiety. The resulting acetylated PYY₁₋₃₆ was then subjected to three cycles of N-terminal protein sequence analysis. The sequence obtained was Ile-Ile-Arg-Tyr-Phe. Sequence analysis of the native peptide yielded Ile, Tyr, and Phe on cycles 1, 2, 3, respectively (not shown).

[0046] FIG. 19 shows the kinetics of PYY₁₋₃₆ release from PEG₄₀₀-FMS-PYY₁₋₃₆. PEG₄₀₀-FMS-PYY₁₋₃₆ (750 μM in 0.1 M phosphate buffer pH 8.5, 2 mM Na₂S₂O₃) was incubated at 37° C. Aliquots (100 μl) were withdrawn at the indicated times and free PYY₁₋₃₆ was measured by HPLC. The cumulative amount of PYY₁₋₃₆ released is shown as a function of time. The amount of PYY₁₋₃₆ in the initial conjugate was determined by acid hydrolysis of a 20 μl aliquot, followed by amino acid analysis.

[0047] FIG. 20 shows the rate of PEG₄₀₀-FMS-PYY₁₋₃₆ hydrolysis in normal mouse serum. PEG₄₀₀-FMS-PYY₁₋₃₆ (0.5 μM) in normal mouse serum was incubated at 37° C. Aliquots were withdrawn at the indicated times and the amount of 2-PEG₄₀₀-9-sulfonate released from PEG₄₀₀-FMS-PYY₁₋₃₆ was determined by HPLC and used for calculating the rate of PEG₄₀₀-FMS-PYY₁₋₃₆ hydrolysis. The insert shows that PYY₁₋₃₆ degrades rapidly in normal mouse serum at 37° C. PYY₁₋₃₆ (50 μM) in normal mouse serum was incubated at 37° C. At the indicated times, aliquots (0.1 ml) were removed, de-proteinized by 3 volumes of ethanol and the quantity of PYY₁₋₃₆ was determined in the supernatants by HPLC.

[0048] FIG. 21 shows that PEG₄₀₀-FMS-PYY₁₋₃₆ elicits prolonged satiety. The protocol described in FIG. 15 was repeated, except that the mice received SC either saline or PEG₄₀₀-FMS-PYY₁₋₃₆ (5 nmol/mouse) at the indicated times prior to re-feeding. Results are average of three identical experiments, normalized according to the saline control.

[0049] FIG. 22 shows the time course of reactivation of PEG₄₀₀₀-FMS-hGII upon incubation at pH 8.5, 37° C. PEG₄₀₀₀-FMS-hGII (1 mg/ml) was incubated in 0.1 M phosphate buffer, 0.6% BSA and 2 mM Na₂S₂O₃ at 37° C. Aliquots were withdrawn at the indicated time points, and analyzed for their potencies to displace [¹²⁵I]-hGII from enriched hGII receptor preparation extracted from rabbit liver plasma membranes. Native hGII displaces [¹²⁵I]-hGII in this assay half maximally at a concentration of 0.3±0.03 nM. An hGII derivative exhibiting half maximal displacement in this assay at a concentration of 3.0±0.3 nM is considered to

have 10% of the native receptor's binding potency. The insert shows the rate of release of hGII from PEG₄₀₀₀-FMS-hGII upon incubation at pH 8.5, 37° C. PEG₄₀₀₀-FMS-hGII (1 mg protein) was incubated as described above. At the indicated time points, 0.1 ml aliquots withdrawn and subjected to analytical HPLC analysis.

[0050] FIG. 23 shows the rate of hydrolysis of PEG-FMS conjugates upon incubation at pH 8.5, 37° C. The concentration of PEG₄₀₀₀-FMS-exendin-4 and PEG₄₀₀₀-FMS-hGII was determined for each time point by HPLC. The linear plot obtained indicates that the rate of hydrolysis is of first order reaction. The half-life time of the conjugates was calculated from $t_{1/2} = \ln 2/\lambda$, when λ is the slope of the linear plot (d^{-1}).

[0051] FIG. 24 shows the pharmacokinetic profiles of subcutaneously administered radiolabeled insulin and human serum albumin in rats. At t=0, groups of Wistar rats (170±5 g each, n=5 per group) were injected subcutaneously with 0.3 ml PBS (pH 7.4) and 0.5% BSA containing either 10 μg [¹²⁵I]-insulin (specific activity 2800 cpm/μg) or 10 μg [¹²⁵I]-BSA (specific activity 1400 cpm/μg). Blood aliquots obtained from the tail vein at the indicated time points were spotted onto Whatman #3 paper and weighed immediately. Each paper was washed with 10 ml 10% TCA and measured for its radioactive content. Results are expressed as ng of TCA precipitable protein (insulin or BSA) per ml blood. Each point in the figure represents the arithmetic mean of 5 rats±SE. Arrows indicate the times at which peak values were attained and the species $t_{1/2}$ values.

[0052] FIGS. 25A-25B show the HPLC analysis of purified HSA-Fmoc-insulin before and after the release of insulin by hydrolysis. HPLC was conducted with a linear gradient from 0 to 100% of solution A (0.1% TFA) to solution B (acetonitrile-H₂O, 75:25 in 0.1% TFA) over 10 minutes and then 4 minutes in solution B, using a Chromolith RP 18e (100×4 mm) column at a rate of 3 ml/minutes. The effluent was monitored at 220 nm. (25A) Purified HSA-Fmoc-insulin (100 μg loaded). (25B) Purified HSA-Fmoc-insulin following 4 hrs hydrolysis through incubation at pH 10.3, 25° C. Under the same experimental conditions, insulin elutes with Rt=6.91 min and has a surface area of 187,000±9,000 m²/μg insulin.

[0053] FIG. 26 shows the dosage-dependent stimulation of lipogenesis in rat adipocytes. Lipogenesis was carried out for 2 h at 37° C. in plastic vials containing 0.5 ml of fat cell suspension (1.5×10⁵ cells) and 0.2 mM [¹⁴C]-glucose in the presence or absence of the indicated concentrations of native insulin or HSA-insulin conjugates. Results are expressed as a percentage of maximal stimulation. Insulin (100 ng/ml) stimulated lipogenesis four to five-fold above basal levels. HSA-Fmoc-insulin (1 μg/ml) was taken as containing 24±3 μg insulin per mg HSA in this assay (see Table 6). The ED₅₀ values for native insulin (0.3 μg/ml) for HSA-Fmoc-insulin (2.4 μg/ml) and for HSA-Benz-insulin (130 ng/ml) are indicated with arrows on the figure.

[0054] FIGS. 27A-27B show the rate of insulin release from HSA-Fmoc-insulin and reactivation of the conjugate upon incubation at pH 8.5, 37° C. Activation of HSA-Fmoc-insulin (1 μg/ml) was incubated in 0.1 M phosphate buffer at pH 8.5, 37° C. At the indicated time points, aliquots (100 nl) were analyzed by HPLC for the amount of released insulin (27A) and for biological potency in rat adipocytes

(2714). Results are expressed as the amount of insulin released per μg HSA-Fmoc-insulin. An aliquot of HSA-Fmoc-insulin exhibiting ED_{50} value=3.0 $\mu\text{g}/\text{ml}$ in a lipogenic assay was considered to have 10% the native biological potency.

[0055] FIGS. 28A-28B show the circulating glucose levels in mice following a single subcutaneous or intraperitoneal administration of HSA-Fmoc-insulin. Mice were injected intraperitoneally (28A) or subcutaneously (28B) with Zn^{2+} free insulin (3 $\mu\text{g}/\text{mouse}$ in 0.2 ml saline) or HSA-Fmoc-insulin (0.4 $\mu\text{g}/\text{mouse}$). Blood glucose levels were determined at the indicated time points. Food was removed during the experiment. Each point is the arithmetic mean of $n=5$ mice $\pm$ SE.

[0056] FIG. 29 shows the effect of a single subcutaneous administration of HSA-Fmoc-insulin on blood glucose levels in STZ-rats. STZ-rats received saline solution (control group), Zn^{2+} free insulin (20 $\mu\text{g}/\text{STZ rat}$) or HSA-Fmoc-insulin (7 $\mu\text{g}/\text{STZ rat}$). Blood glucose levels were determined at the time points indicated in the Figure. Each point represents the arithmetic mean of the blood glucose levels of $n=5$ rats $\pm$ SE.

DETAILED DESCRIPTION OF THE INVENTION

[0057] The present invention provides a new conceptual approach for delivery of drugs, particularly peptides and proteins of low or medium molecular weight, by natural or synthetic carriers, whereby the carrier moiety and the drug residue are not linked directly to each other or the drug molecule is not encapsulated within the carrier, as in standard drug delivery using such carriers, but rather both residues are linked to different positions of a scaffold structure that is highly sensitive to bases and is removable under physiological conditions.

[0058] The carrier used in the present invention may be a protein such as albumin or a modified albumin, e.g., cationized bovine serum albumin (CBSA) or cationized human serum albumin (CHSA), or a protein containing globin-like domains having long half-life in circulation, e.g., a hemoglobin-like protein such as hemoglobin A or S.

[0059] In one embodiment, the protein carrier is albumin, namely, human serum albumin (HSA). In another embodiment, the protein carrier is cationized albumin. Cationized albumin (pI greater than 8), unlike native albumin (pI approximately 4), enters cerebrospinal fluid (CSF) rapidly from blood. This suggests that a specific uptake mechanism for cationized albumin may exist at the brain capillary wall, i.e. the blood brain barrier, and thus it may be used for brain targeting. Cationized albumins may be obtained, for example, by substituting anionic carboxyl groups with cationic aminoethyl-amine groups. The cationized albumin may be linked to the drug through the scaffold containing the structure that is highly sensitive to bases and is removable under physiological conditions or it may be conjugated with a polymer such as poly(ethylene glycol)-poly(lactide) (PEG-PLA) nanoparticles (CBSA-NP), designed for brain drug delivery. The CBSA is covalently conjugated with the maleimide function group at the distal of PEG surrounding the nanoparticles. The cationized albumin may also be coupled to sterically stabilized liposomes.

[0060] In another embodiment, the carrier is a polymer carrier moiety such as, but not limited to, linear or branched poly(ethylene glycol) (PEG) and block copolymers thereof, poly(lactic acid) and copolymers thereof, polyesters having suitable functional groups based on polylactide (PLA), polyglycolide (PGA), polycaprolactone (PCL), and their copolymers, and polyamides based on polymethacrylamide and their copolymers. All these polymers should have suitable functional groups for linking to a scaffold structure of the formula (I)-(V) that is highly sensitive to bases and is removable under physiological conditions, preferably through a spacer. Examples of the polymer carriers include PLG, poly(lactic acid)-block-poly(ethylene glycol), N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer with suitable functional groups or poly-D,L-lactide-co-glycolide (PLGA) nanoparticles. The functional groups may be hydroxy, amino, carboxyl, mercapto, sulfonic acid group, and the like.

[0061] The polymer may also be a block polymer as disclosed in U.S. Pat. No. 5,929,177, herein incorporated by reference in its entirety as if fully disclosed herein. These block polymers have functional groups, e.g., amino group, carboxyl group or mercapto group on α -terminal, and hydroxy group, carboxyl group, aldehyde group or vinyl group on ω -terminal, and comprise hydrophilic/hydrophobic segments. Hydrophilic segment comprises poly(ethylene oxide), while hydrophobic segment is derived from lactide, lactone or (meth)acrylic acid ester. These block polymers form polymeric micelles which are usable as bio-compatible materials.

[0062] The invention also encompasses as polymer carriers liposomes containing phospholipids with covalently attached poly(ethylene glycol) (PEG-lipids).

[0063] Polymer carriers have several advantages over other delivery methods such as liposomes and antibodies. Because liposomes—spherical vesicles made of phospholipids—are particles, they get taken up by macrophages. High levels can be found in the liver and spleen, even when the liposomes are given “stealth” characteristics by coating them with PEG. In contrast, water-soluble polymers flow working with a single molecule rather than a large particle. It is possible to choose a material which doesn't go to the liver and the spleen. It is in effect a “macro-molecular prodrug”. To avoid the liver and spleen, one can use uncharged hydrophilic polymers, such as PEG and N-(2-hydroxypropyl)-methacrylamide. When these polymers are hydrated, they can circulate in the blood for periods of up to about 24 hours.

[0064] In one preferred embodiment, the polymeric carrier is in the form of nanoparticles. Nanoparticle drug delivery, utilizing degradable and absorbable polymers, provides a more efficient, less risky solution to many drug delivery challenges. Nanoparticles are generally defined as particles between 10 nanometers (nm) and 1000 nm in size, and can be either spherical or vesicular.

[0065] The advantages of using polymeric nanoparticles (PNPs) in drug delivery are many, the most important being that they generally increase the stability of any volatile pharmaceutical agents and that they are easily and cheaply fabricated in large quantities by a multitude of methods. Additionally, the use of absorbable or degradable polymers, such as polyesters, provides a high degree of biocompat-

ability for PNP delivery systems. Among the adaptations that can be made are surface modifications of the polymer, use of different fabrication methods, selection of a variety of pre-existing polymers or copolymers. In one embodiment, the polymer carrier are nanoparticles of organically modified silica.

[0066] In one preferred embodiment of the invention, the polymer carrier is PEG. The present invention thus provides a new conceptual approach for reversible pegylation of drugs, particularly peptides and proteins of low to medium molecular weight, whereby the PEG moiety and the drug residue are not linked directly to each other, as in standard pegylation procedures, but rather both residues are linked to different positions of a scaffold structure that is highly sensitive to bases and is removable under physiological conditions.

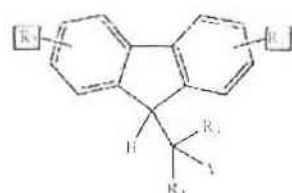
[0067] In one aspect, the present invention provides a compound of the formula:



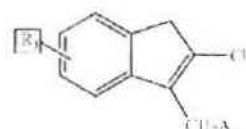
[0068] wherein

[0069] Y is a moiety of a drug bearing at least one functional group selected from free amino, carboxyl, phosphate, hydroxyl and/or mercapto; and

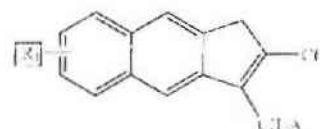
[0070] X is a radical selected from the group of radicals consisting of the formulas (i) to (iv).



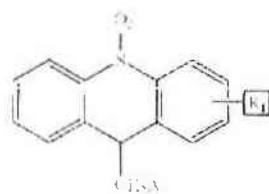
(i)



(ii)



(iii)



(iv)

[0071] wherein

[0072] R₁ is a radical containing a protein or polymer carrier moiety; polyethylene glycol (PEG) moiety;

[0073] R₂ is selected from the group consisting of hydrogen, alkyl, alkoxy, alkoxyalkyl, aryl, alkaryl, aralkyl, halogen, nitro, SO₂H, SO₂NHR, amino, ammonium, carboxyl, PO₃H₂, and OPO₃H₂;

[0074] R is selected from the group consisting of hydrogen, alkyl and aryl;

[0075] R₃ and R₄, the same or different, are each selected from the group consisting of hydrogen, alkyl and aryl;

[0076] A is a covalent bond when the radical is linked to a carboxyl, phosphate or mercapto group of the drug Y, or A is O=C-O- when the radical is linked to an amino or hydroxyl group of the drug Y;

[0077] n is an integer of at least one;

[0078] and pharmaceutically acceptable salts thereof;

[0079] The terms "alkyl", "alkoxy", "alkoxyalkyl", "aryl", "alkaryl" and "aralkyl" in the definitions of R₁, R₂, R₃ and R₄ herein are used to denote alkyl radicals of 1-8, preferably 1-4 carbon atoms, e.g. methyl, ethyl, propyl, isopropyl and butyl, and aryl radicals of 6-10 carbon atoms, e.g. phenyl and naphthyl. The term "halogen" includes bromo, fluoro, chloro and iodo.

[0080] In one preferred embodiment of the invention, X is a radical of the formula (i), more preferably a radical of formula (i) wherein R₁, R₃ and R₄ are each hydrogen and A is -O=C-O-, namely the 9-fluorenylmethoxycarbonyl radical (hereinafter "Fmoc"), or most preferably, a radical of formula (i) wherein R₁ is -SO₂H at position 2 of the fluorene ring, R₃ and R₄ are each hydrogen, and A is -O=C-O-, namely the 2-sulfo-9-fluorenylmethoxycarbonyl radical (hereinafter "TMS").

[0081] In another embodiment of the invention, the functional group is the radical (ii), wherein R₁, R₃ and R₄ are hydrogen and A is a covalent bond, i.e. the 9-fluorenylmethyl (fm) group, which is applicable for reversible masking of free mercapto groups, of carboxylic functions of aspartic and glutamic acid moieties, and of C-terminal carboxyl functions of the cyclic molecules. The resulting 9-fluorenylmethyl esters (fm esters) generate the parent free carboxylic functions following a β-elimination reaction pathway upon mild basic treatment, and thus can be similarly employed for reversible masking of carboxylic functions of drugs. The Fmoc-group is of further potential similar use in the reversible protection of hydroxyl groups of tyrosine, serine and threonine.

[0082] The halogenated Fmoc radicals (i) wherein R₁ is halogen in the 2 or 7 position, preferably Cl or Br, the 2-chloro-1-indenylmethoxycarbonyl (CLIMOC) radical (ii), the 1-benzofluorenylmethoxycarbonyl methane (BIMOC) radical (iii), the methane sulfone radical (iv) and corresponding radicals (i) to (iv) wherein A is a covalent bond, can be used similarly to Fmoc and fm for substitution of free amino, carboxyl, hydroxyl and mercapto functions of drugs, thus providing a wide range of sensitivity toward removal of such groups under basic, e.g. physiological, conditions. In fact, the above radicals (i) to (iv) belong to a general family of rare chemical entities that undergo hydrolysis at neutral or slightly alkaline pH and mild conditions, and can therefore be used for temporary reversible protection of α- and ε-amino groups, for example in peptide synthesis, and even

be removed from the amino function by a β -elimination reaction, under mild basic conditions.

[0083] According to the invention, a radical (R) to PEG, preferably Fmoc or FMS covalently linked to amine and/or hydroxyl moieties or Fm covalently linked to carboxyl and/or mercapto moieties, undergoes hydrolysis (via β -elimination) back to the free amine, hydroxy, mercapto or carboxyl functions, under physiological conditions in the body fluid, namely at pH 7.4 and 37° C.

[0084] In one embodiment, R1 contains a protein carrier, preferably albumin, linked through a spacer to the ring. In another embodiment, R1 contains a polymer carrier moiety, linked through a spacer to the ring. Preferably, the polymer carrier is a polyethylene glycol (PEG) moiety.

[0085] In one embodiment of the invention, R1 is a radical of the formula:

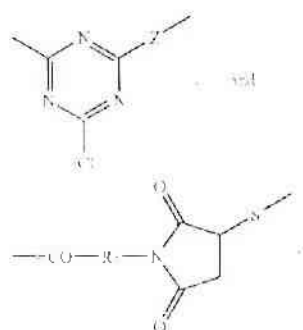


[0086] wherein:

[0087] R_2 is selected from the group consisting of $-NH-$, $-S-$, $-CO-$, $-C(=O)-$, $-CH_2-$, $-SO_2-$, $-SO_2-$, $-PO_2-$, and $-PO_2-$, and

[0088] R_2 is a bond or a radical by which the PEG moiety is covalently attached to R_2 .

[0089] In a more preferred embodiment, R_2 is $-NH-$ and R_2 is selected from the group consisting of $-CO-$, $-C(=O)-$, $-CH_2-$, $-CH_2CH_2-$, $-CO-NH-$, $-CS-$, $-NH-CO-$, $-CH_2-NH-CO-$, $-CO-CH_2CH_2-$, $-NH-CO-$, $-CO-CH_2-NH-CO-NH-$.

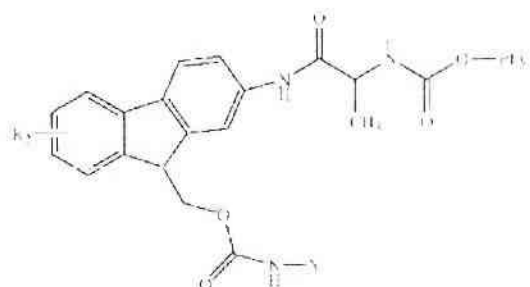


[0090] Z is O, S or NH, and

[0091] R_1 is selected from the group consisting of C1-C18 straight or branched alkylene, phenylene, an oxyalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, and a residue of a saccharide containing 1-10 monosaccharide residues.

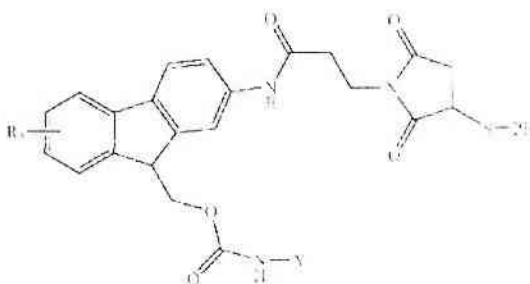
[0092] In the 4-chloro-6-Z-triazine-2-yl radical above, the 6-Z group is linked to the PEG moiety while the 2 position is linked to R_1 , which is $-NH-$ in this case. In the $-CO-CH_2-NH-CO-$ succinimide radical above, the thio $-S-$ group at position 3 is linked to the PEG moiety while the $-CO-$ is linked to R_2 , which is $-NH-$ in this case.

[0093] In one preferred embodiment, the pegylated drug compound of the invention is a conjugate of the formula



[0094] wherein R_1 is H or $-SO_2H$ at position 2 of the fluorene ring, and Y is preferably a peptide or protein drug. When R_2 is H, a herein designated PEG-Fmoc-drug Y conjugate is obtained. In a most preferred embodiment, R_2 is $-SO_2H$ at position 2 of the fluorene ring, and a herein designated PEG-FMS-drug Y conjugate is obtained.

[0095] In a more preferred embodiment, the pegylated drug of the invention is a compound of the formula:



[0096] wherein R_1 is H or $-SO_2H$.

[0097] In a most preferred embodiment, the pegylated drug of the invention is a compound of the formula above, wherein R_2 is $-SO_2H$ at position 2 of the fluorene ring, and the PEG moiety is a 40 kDa branched PEG. These conjugates are herein identified as $(PEG)_n$ -FMS₄-peptide/protein, wherein n is 1 to 3, preferably 1 or 2, most preferably 1.

[0098] The above compounds of the invention wherein R_1 is $-NH-$ can be prepared from N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) or N-(2-sulfo-9-fluorenylmethoxycarbonyloxy)succinimide (FMS-OSu) substituted by $-NH-$ in the fluorene ring (depicted in Scheme 7, page a, first row, first column), by reacting the amino group with the activated PEG-OH (e.g. PEG-CO-CO-Cl) or an activated derivatized PEG such as: PEG-carboxylate (PEG-COOH, e.g. via PEG-CO-Cl), PEG-aldehyde (PEG-CHO), PEG-isocyanate (PEG-N=C=O), PEG-isothiocyanate (PEG-N=C=S), 2,4-dichloro-6-S-PEG-1,3,5-triazine, 2,4-dichloro-6-NH-PEG-1,3,5-triazine, or 2,4-dichloro-6-O-PEG-1,3,5-triazine (Scheme 7, page a, right column) in order to obtain the derivatives wherein $-R_2$ PEG is as presented in Scheme 7 (page a, middle column).

[0099] In one preferred embodiment of the invention, the conjugate of the invention is a (PEG-Fmoc)_n-peptide/protein, and the starting compound for their preparation is the maleimide derivative of Fmoc-OSu, herein designated Precursor 7 or MAL-Fmoc-NHS or MAL-Fmoc-OSu, of the formula depicted in Scheme 3.

[0100] In a most preferred embodiment of the invention, the conjugate of the invention is a (PEG-FMS)_n-peptide/protein, and the starting compound for their preparation is the maleimide derivative of FMS-OSu, herein designated Precursor 8 or MAL-FMS-NHS or MAL-FMS-OSu, of the formula depicted in Scheme 3.

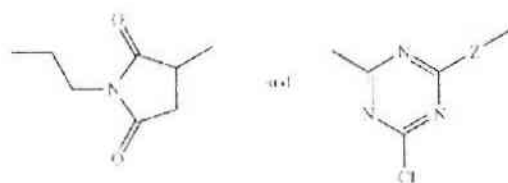
[0101] Two possible pathways for the pegylation of target peptides/proteins and preparation of the (PEG-Fmoc)_n-peptide/protein or (PEG-FMS)_n-peptide/protein conjugates are provided by the invention, as depicted in Scheme 6. Both pathways are two-step procedures.

[0102] According to one pathway, MAL-FMS-NHS or MAL-Fmoc-NHS is first attached to the amine component of the target peptide/protein, thus obtaining a MAL-FMS-peptide/protein or MAL-Fmoc-peptide/protein conjugate, and then substituting PEG-Su for the maleimide moiety, producing the (PEG-FMS)_n-peptide/protein or (PEG-Fmoc)_n-peptide/protein conjugate, respectively.

[0103] In the second pathway, MAL-FMS-NHS or MAL-Fmoc-NHS is first reacted with PEG-Su, thus forming a PEG-FMS-NHS or PEG-Fmoc-NHS conjugate, and then reacting it with the amine component of the target peptide or protein resulting in the desired (PEG-FMS)_n-peptide/protein or (PEG-Fmoc)_n-peptide/protein conjugate, respectively. This pathway is suitable for sulfhydryl- or disulfide-containing peptides and proteins.

[0104] The compounds wherein R₁ is —NH— and R₂ is —CO—NH— or —CS—NH— can be prepared from Fmoc-OSu or FMS-OSu substituted by —N=C(=O) or —N=C=S in the fluorene ring (depicted in Scheme 7, page 6, first row and last rows, first column), respectively, by reaction with PEG-NH₂. No activation is necessary because these Fmoc/FMS species are already activated.

[0105] In a further embodiment of the invention, R₁ is —S—; and R₂ is selected from the group consisting of

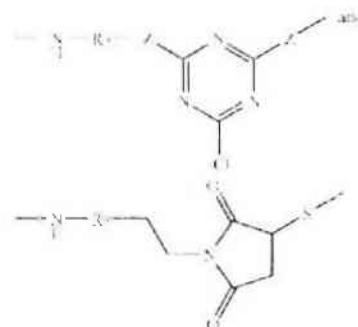


[0106] wherein Z is O, S or NH.

[0107] The above compounds of the invention wherein R₁ is —S— can be prepared from Fmoc-OSu or FMS-OSu substituted by —SH in the fluorene ring (depicted in Scheme 7, page 6, second row, first column), by reaction with PEG-maleimide of the formula shown in Scheme 7, page 6, second row, right column), thus obtaining a pegylated compound of the invention wherein the PEG moiety is linked to the fluorene ring through a residue as depicted in Scheme 7,

page 6, second row, middle column, or by reaction with 2,4-dichloro-6-S-PEG-1,3,5-triazine, 2,4-dichloro-6-NH-PEG-1,3,5-triazine, or 2,4-dichloro-6-O-PEG-1,3,5-triazine.

[0108] In yet another embodiment, in the pegylated drug of the invention, R₁ is —CO—; R₂ is selected from the group consisting of —O—, —NH—, —NH—R—CO—, —NH—R—, —NH—NH—R—CO—NH—,



[0109] Z is O, S or NH; and

[0110] R₁ is selected from the group consisting of C1-C18 straight or branched alkylene, phenylene, or oxalkylene radical having 3-18 carbon atoms in the backbone, a residue of a peptide containing 2-10 amino acid residues, and a residue of a saccharide containing 1-10 monosaccharide residues.

[0111] The above compounds of the invention wherein R₁ is —CO— can be prepared from Fmoc-OSu or FMS-OSu substituted by —COOH in the fluorene ring (depicted in Scheme 7, page 6, third row, first column). When R₁ is —O— or —NH—, the reaction will occur with PEG (PEG-OH) or PEG-amine (PEG-NH₂), respectively, thus obtaining a pegylated compound wherein the PEG moiety is linked to the fluorene ring through a residue —CO—O— or —CO—NH—, respectively, as depicted in Scheme 7, page 6, middle column (3rd and 4th rows).

[0112] The compounds of the invention wherein R₁ is —CO— and R₂ is NH—R₂—COO— can be prepared from Fmoc-OSu or FMS-OSu substituted by —COOH in the fluorene ring, by reaction with H₂N—R₂—COO— using a coupling reagent (e.g. DCC/HOBt, or PyBOP/benzotriazol-1-yloxy)tripyrroldiophosphonium hexafluorophosphate/triethylamine), removal of the tBu protecting group under acidic conditions (e.g. trifluoroacetic acid or HCl in dioxane), activation of the free carboxyl group by triphosgen and reaction of the —NH—R₂—COCl formed with PEG-OH to obtain the —CO—NH—R₂—CO—O-PEG derivative.

[0113] The compounds of the invention wherein R₁ is —CO— and R₂ is NH—R₂—NH— can be prepared from Fmoc-OSu or FMS-OSu substituted by —COOH in the fluorene ring, by reaction with H₂N—R₂—NH-tBu using a coupling reagent and removing the tBu protecting group as described above, reacting the free amino group with PEG-OSu to obtain the —CO—NH—R₂—NH-PEG derivative.

[0114] The compounds of the invention wherein R₁ is —CO— and R₂ is NH—R₂—CO—NH— can be prepared

from Fmoc-OSu or FMS-OSu substituted by $-\text{COOH}$ in the fluorene ring, by reaction with $\text{H}_2\text{N}-\text{R}_1-\text{CO}-\text{NH}-\text{tBu}$ using a coupling reagent and removing the tBu protecting group as described above, activating the free carboxyl group with DCC/NHS and reacting of the $-\text{NH}-\text{R}_1-\text{CO}-\text{N}$ -hydroxysuccinimide ester formed with PEG-NH₂ to obtain the $-\text{CO}-\text{NH}-\text{R}_1-\text{CO}-\text{NH}$ -PEG derivative.

[0115] The compounds of the invention wherein R_1 is $-\text{O}-$ and R_2 is $-\text{NH}-\text{R}_2-\text{NH}-$ 7-(4-chloro-6-*Z*-PEG-1,3,5-triazin-2-yl) can be prepared from Fmoc-OSu or FMS-OSu substituted by $-\text{COOH}$ in the fluorene ring, by reaction with $\text{H}_2\text{N}-\text{R}_2-\text{NH}-\text{tBoc}$ or $\text{H}_2\text{N}-\text{R}_2-\text{NH}-\text{tBu}$ using a coupling reagent and removing the tBoc, tBu or triyl protecting group as described above, reacting the free NH₂, OH or SH group with 2,4,6-trichloro-1,3,5-triazine, and further reacting the $-\text{NH}-\text{R}_2-\text{NH}[\text{O}$ or $\text{S}]-$ 4-chloro-6-NH[O or S]-1,3,5-triazin-2-yl thus formed with PEG-NH₂, PEG-OH or PEG-SH to obtain the corresponding $-\text{CO}-\text{NH}-\text{R}_2-\text{NH}[\text{O}$ or $\text{S}]-$ 4-chloro-6-NH[O or S]-PEG-1,3,5-triazine derivative.

[0116] The compounds of the invention wherein R_1 is $-\text{CO}-$ and R_2 is $-\text{NH}-\text{R}_2$ -ethylene-succinimido can be prepared from Fmoc-OSu or FMS-OSu substituted by $-\text{COOH}$ in the fluorene ring, by reaction with $\text{H}_2\text{N}-\text{R}_2$ -ethylene-maleimide using a coupling reagent (e.g. DCC, EDCB, PyBOP, PyBt, HBTU) followed by reaction of the maleimide moiety with PEG-SH (1, 6 or 8) to obtain the $-\text{CO}-\text{NH}-\text{R}_2$ -ethylene-succinimido-S-PEG derivative.

[0117] The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the one-step procedure described in Example 5 hereinafter.

[0118] In yet a further embodiment of the invention, R_1 is $-(\text{CH}_2)_n-$ and R_2 is $-(\text{CH}_2)_m-\text{S}-$ or $-(\text{CH}_2)_m-\text{NH}-$, wherein n is 0 to 18, preferably 1.

[0119] The above compounds of the invention wherein R_1 is $-\text{CH}_2-$ and R_2 is $-(\text{CH}_2)_n-\text{S}-$ or R_2 is $-(\text{CH}_2)_n-\text{NH}-$ can be prepared from Fmoc-OSu or FMS-OSu substituted by $-\text{COOH}$ in the fluorene ring (depicted in Scheme 7, page c, 4th row, first column), by reaction with PEG-NH₂ or PEG-SH, thus obtaining a pegylated compound of the invention wherein the PEG moiety is linked to the fluorene ring through a residue as depicted in Scheme 7, page d, middle column, 3rd and 4th rows, respectively.

[0120] The above compounds of the invention wherein R_1 is $-\text{CH}_2-$ and R_2 is $-(\text{CH}_2)_n-\text{S}-$ or R_2 is $-(\text{CH}_2)_n-\text{NH}-$ can be prepared from Fmoc-OSu or FMS-OSu substituted by $-(\text{CH}_2)_n-\text{Hal}$ in the fluorene ring, wherein Hal is I , Cl , Br or F (depicted in Scheme 7, page d, 3rd row, first column), by reaction with PEG-NH₂ or PEG-SH, thus obtaining a pegylated compound of the invention wherein the PEG moiety is linked to the fluorene ring through a residue as depicted in Scheme 7, page d, middle column, 3rd and 4th rows, respectively.

[0121] The pegylated-Fmoc/FMS drugs of the invention are then prepared from the above intermediates by the one-step procedure described in Example 5 hereinafter.

[0122] In still another embodiment, R_1 is $-\text{SO}_2-$ and R_2 is $-\text{O}-$, $-\text{NH}-$ or $-(\text{CH}_2)_n-\text{CH}_2-\text{S}-$. The compounds wherein R_1 is $-\text{O}-$ or $-\text{NH}-$ can be prepared from Fmoc-OSu or FMS-OSu substituted by $-\text{SO}_2\text{Cl}$ in the fluorene ring (depicted in Scheme 7, page c, first column, 3rd row), by reaction with PEG-OH or PEG-NH₂, respectively.

The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the one-step procedure described in Example 5 hereinafter.

[0123] The compounds wherein R_1 is $-\text{CH}_2-$, $-\text{CH}_2-\text{S}-$, can be prepared from Fmoc-OSu or FMS-OSu substituted by $-\text{SO}_2\text{CH}_2-\text{CH}_2-$ in the fluorene ring (depicted in Scheme 7, page c, first column, 2nd row), by reaction with PEG-SH. The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the two-step procedure described in Examples 16 or 17 hereinafter.

[0124] In yet still another embodiment, R_1 is $-\text{PO}_3-$ and R_2 is $-\text{O}-$ or $-\text{NH}-$. These compounds can be prepared from Fmoc-OSu or FMS-OSu substituted by $-\text{PO}_3\text{Cl}$ in the fluorene ring (depicted in Scheme 7, page d, first column, first row), by reaction with PEG-OH or PEG-NH₂, respectively. The pegylated-Fmoc/FMS-drugs of the invention are then prepared from these intermediates by the one-step procedure described in Example 5 hereinafter.

[0125] According to the present invention, Y is a moiety of a drug bearing at least one functional group selected from free amino, carboxyl, hydroxyl, phosphate and/or mercapto. In a more preferred embodiment of the invention, the drug contains at least one free amino group and is a peptide or a protein drug or a non-peptide drug.

[0126] In one embodiment of the invention, the drug is a non-peptide drug that contains at least one amino group and the invention relates to PEG-Fmoc and PEG-FMS conjugates thereof. Non-peptide drugs that are amenable to the technology of the invention include antibiotic aminoglycosides such as gentamicin and amphotericin B, and anticancer drugs such as aminolevulinic acid, daunorubicin and doxorubicin.

[0127] In a more preferred embodiment of the invention, the drug containing at least one amino group is a peptide or protein drug, most preferably a peptide or protein of low or medium molecular weight, that can be used as a drug for human or veterinary use.

[0128] Thus, further provided by the invention are pegylated drug conjugates PEG-FMS-Y and PEG-Fmoc-Y herein identified by the formulas:



[0129] wherein Y is a moiety of a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the FMS or Fmoc radical through at least one amino group. In the most preferred embodiment the conjugate is $(\text{PEG-FMS})_n\text{-Y}$ and the PEG moiety is PEG₁₀₋₁₀₀.

[0130] Examples of peptides and proteins Y that can be pegylated according to the invention include, but are not limited to: insulin, an interferon such as IFN- α_2 , a PYY agonist such as the peptide PYY_{1-36}}, an exendin such as exendin 3 and exendin 4 and exendin analogues and agonists, atrial natriuretic peptide (ANP), human growth hormone (hGH), erythropoietin, TNF- α , calcitonin, gonadotropin releasing hormone (GnRH) or an analogue thereof such as leuprolide and D-Lys⁶-GnRH, hirudin, glucagon, and a monoclonal antibody fragment such as anti-TNF- α monoclonal antibody fragment.

[0131] In one preferred embodiment of the invention, the peptide drug is insulin and the invention provides PEG-

Exenatide-insulin and PEG-FMS-insulin conjugates and pharmaceutical compositions comprising them for treatment of diabetes mellitus and hyperglycemia. Examples of such conjugates are the (PEG)₂₀₋₄₀-P(moc)₂-insulin, (PEG)₂₀₋₄₀-Exenatide-insulin, and (PEG)₂₀₋₄₀-FMS-insulin conjugates.

[0132] In another preferred embodiment of the invention, the drug is an exendin or an exendin agonist.

[0133] In a more preferred embodiment, the drug is exendin-1 of the sequence represented by SEQ. ID. NO.1:

[0134] HGEGETTSDI SKQMPTFAWR LLEWLKNGG PSSGAPPS-NH₂.

[0135] In another preferred embodiment, the drug is exendin-3 of the sequence represented by SEQ. ID. NO.2:

[0136] HSDGTFITSDI SKQMPTFAWR LLEWLKNGG PSSGAPPS-NH₂.

[0137] In a further preferred embodiment, the drug is an exendin agonist defined herein as a compound that mimics the activities of exendin-3 or exendin-4 by binding to the receptor(s) at which exendin-3 or exendin-4 exerts its actions which are beneficial as insulinotropic and in the treatment of diabetes mellitus or by mimicking the effects of exendin on increasing urine flow, increasing urinary sodium excretion and/or decreasing urinary potassium concentration, by binding to the receptor(s) where exendins cause these effects. Preferably, the exendin agonist is selected from the group of insulinotropic exendin-4 fragments and analogues consisting of exendin agonists represented by SEQ. ID. NO.3 to SEQ. ID. NO. 10:

exendin-4 (1-11) [SEQ. ID. NO.3]

HGEGETTSDI SKQMPTFAWR LLEWLKNGG

Y¹⁰ exendin-4 (1-11)

HGEGETTSDI SKQMPTFAWR LLEWLKNGG

exendin-4 (1-10)

HGEGETTSDI SKQMPTFAWR LLEWLKNGG

exendin-4 (1-11) amide

HGEGETTSDI SKQMPTFAWR LLEWLKNGG NH₂

exendin-4 (1-18) amide

HGEGETTSDI SKQMPTFAWR LLEWLKNGG

L¹⁴, L¹⁵ exendin-4 amide

HGEGETTSDI SKQMPTFAWR LLEWLKNGG PSSGAPPS-NH₂

L¹⁰, P¹⁰ exendin-4 (1-20) amide

HGEGETTSDI SKQMPTFAWR LLEWLKNGG

and

L¹⁴, R¹⁴, P¹⁰ exendin-4 (1-20) amide

HGEGETTSDI SKQMPTFAWR LLEWLKNGG

[0138] According to this embodiment, the invention provides PEG-Fmoc-exendin/exendin agonist and PEG-FMS-exendin/exendin agonist conjugates and pharmaceutical compositions comprising them for prevention of hypergly-

cemia and for treatment of diabetes mellitus selected from the group consisting of non-insulin dependent diabetes mellitus, insulin-dependent diabetes mellitus, and gestational diabetes mellitus. In a most preferred embodiment, the pegylated exendin conjugate of the invention is (PEG)₂₀₋₄₀-FMS-exendin-4.

[0139] In a further preferred embodiment of the invention, the peptide drug is an interferon, preferably IFN-α, and the invention provides PEG-Fmoc-IFN-α and PEG-FMS-IFN-α conjugates and pharmaceutical compositions comprising them for treatment of diseases treatable by IFN-α, particularly viral diseases, more particularly hepatitis B or C, both as sole therapy or in conjunction with an antiviral agent such as ribavirin, or for treatment of cancer, e.g. transitional cell carcinoma, the most common type of bladder cancer, ovarian cancer, pancreatic cancer melanoma, non-Hodgkin's lymphoma, hairy cell leukemia, and AIDS-related Kaposi's sarcoma, both as sole therapy or in conjunction with a cytotoxic agent such as carboplatin and/or cyclophosphamide. In a more preferred embodiment, the conjugate is (PEG)₂₀₋₄₀-FMS₂-IFNα2 or, most preferably, (PEG)₂₀-FMS-IFNα2.

[0140] In still another preferred embodiment of the invention, the peptide drug is a PYY agonist, herein defined as a molecule that has a PYY- or PYY[3-36]-like biological activity such as reducing food intake in mammals, and acts by a mechanism similar to that of PYY and PYY[3-36], for example by binding to the Y2 receptor. The PYY agonist is preferably an agonist specific for the Y2 receptor and is preferably a peptide containing, at a minimum, the sequence of amino acids 25-36 of PYY, most preferably the sequence 3-36 of PYY.

[0141] In one embodiment of the invention, the PYY agonist is the 36-mer peptide PYY of the sequence represented by SEQ. ID. NO. 11:

[0142] YPIKPEAPGEDASPELNRYYYASIRIYINIAITRQRY-NH₂.

[0143] In a more preferred embodiment of the invention, the PYY agonist is the peptide PYY[3-36] of the sequence represented by SEQ. ID. NO. 12:

[0144] IKPEAPGEDASPELNRYYYASIRIYINIAITRQRY-NH₂.

[0145] According to this embodiment, the invention provides PEG-Fmoc-PYY agonist and PEG-FMS-PYY agonist conjugates and pharmaceutical compositions comprising them for particularly for reduction of food intake, for inducing weight loss and for the treatment of diseases or disorders which can be alleviated by reduction of food intake such as obesity, hypertension, dyslipidemia, cardiovascular risk, insulin-resistance, and diabetes mellitus (particularly type II diabetes). In a preferred embodiment, the pegylated PYY agonist of the invention is (PEG)₂₀₋₄₀-FMS-PYY₃₋₃₆.

[0146] In another preferred embodiment, the peptide drug is human growth hormone (hGH) and the invention provides PEG-Fmoc-hGH and PEG-FMS-hGH conjugates and pharmaceutical compositions comprising them for treatment of conditions and disorders treatable by hGH, particularly for treatment of children of pathologically short stature and as

[0163] Precursor 8 is the compound N-[2-(maleimido-propionyl)-amino-7-sulfo-fluoren-9-yl-methoxycarbonyloxy]-succinimide [or 9-hydroxy-methyl-2-amino-3-maleimidepropionate-7-sulfo-fluorene-N-hydroxysuccinimide] and is also herein identified as MAL-FMS-OSu.

[0164] MAL-FMS-NHS is a water-soluble hetero-bifunctional reagent, consisting of a sulfonated fluorenyloxycarbonyl N-hydroxysuccinimide ester that reacts with peptide and protein amine groups. A maleimide group was attached to the fluorenyl backbone to enable coupling to sulfhydryl-containing PEG, most preferably PEG₄₀₀₀-SH.

[0165] Thus, further provided by the invention are precursor drug conjugates herein identified by the formula:



[0166] wherein Y is a moiety of a drug, more preferably a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the FMS or Fmoc radical through an amine group.

[0167] The present invention also provides a method for the preparation of a conjugate (PEG-Fmoc)_n-Y, wherein Y is a moiety of a drug, more preferably a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the Fmoc radical through an amine group, which comprises:

[0168] (i) reacting a drug Y, e.g. a peptide or protein drug, with at least one equivalent of Precursor 7, thus obtaining a conjugate (MAL-Fmoc)_n-Y; and

[0169] (ii) reacting the conjugate (MAL-Fmoc)_n-Y with PEG-SH.

[0170] In a most preferred embodiment, the invention relates to a method for preparation of a conjugate (PEG-FMS)_n-Y, wherein Y is a moiety of a drug, more preferably a peptide or protein drug, n is an integer of at least one, preferably 1 or 2, and Y is linked to the FMS radical through an amine group, which comprises:

[0171] (i) reacting a drug Y, e.g. a peptide or protein drug, with at least one equivalent of MAL-FMS-NHS, thus obtaining a conjugate (MAL-FMS)_n-Y; and

[0172] (ii) reacting the conjugate (MAL-FMS)_n-Y with PEG-SH, thus obtaining the conjugate (PEG-FMS)_n-Y.

[0173] In another most preferred embodiment, the invention relates to a method for preparation of a conjugate (PEG-FMS)_n-Y, which comprises:

[0174] (i) reacting MAL-FMS-NHS with PEG-SH, thus obtaining a conjugate PEG-FMS-NHS; and

[0175] (ii) reacting the drug Y, e.g. a peptide or protein drug, with at least one equivalent of the conjugate PEG-FMS-NHS, thus obtaining the conjugate (PEG-FMS)_n-Y.

[0176] The PEG-SH reagent is preferably PEG₄₀₀₀-SH, wherein the PEG moiety is a branched PEG moiety of molecular weight 10,000 Da.

[0177] As mentioned in the Background section herein, the pegylation technique has been extensively used for modifying molecules, in particular peptide and protein drugs, in an attempt to improve some of its characteristics such as improved stability and solubility, reduced immunogenicity, reduced proteolysis, reduced toxicity, reduced

clearance by the kidneys, improved bioavailability, and extended circulating life thus less frequent dosing being required. However, one of the main problems of pegylation is that covalent bonding between the PEG moiety and the drug most often causes loss of biological activity or drastic decrease of pharmacological potency of the drug. For this reason, pegylation is used more for high-molecular weight proteins, that are less likely to be inactivated by the reaction with PEG, but is less frequent for peptides and low-molecular weight proteins.

[0178] Theoretically, decreased bioactivity of peptides and proteins by pegylation can be overcome by linking the PEG chains via a chemical bond sensitive to mild alkaline and/or acid hydrolysis, or enzymatically cleavable by serum proteases or esterases. Obviously, an inconsistent rate of hydrolysis would render such an approach impractical. A prerequisite condition is therefore that the hydrolysis of the PEG-chains from the conjugate take place at a slow rate and in a homogeneous fashion under the strictly homeostatic pH and temperature conditions of the mammalian circulatory system.

[0179] Previously, we have prepared 2-sulfo-9-fluorenylmethoxycarbonyl N-hydroxysuccinimide (FMS-OSu) (Uerskov et al., 1999, 2000; Shechter et al., 2001, 2001a) as a reversible protein modifier. Protein-linked FMS moieties undergo slow and spontaneous hydrolysis under physiological conditions generating the unmodified parent molecule. Hydrolysis of FMS protein conjugates at 37°C in normal human serum, in aqueous buffers of pH 8.5, or in the circulatory system in vivo, takes place with a t_{1/2} of 5-7 hrs. Using the FMS moiety as the scaffold for the suggested reversible pegylation technology enables the hydrolysis rates of various PEG-FMS-protein conjugates to be predicted. The t_{1/2} for the hydrolysis of modified small molecules and PEG-conjugated polypeptides and proteins falls within a relatively narrow range of 8-11 h. The constant hydrolysis rate for PEG-FMS-protein conjugates is due to the β-elimination reaction, which occurs at position 9 of the fluorenyl moiety, being solely dependent on the pK_a of the surrounding medium. Thus, in contrast to what occurs in approaches based on enzymatic hydrolytic bond cleavage, similar PEG-FMS hydrolysis rates are expected from all conjugates, regardless of the identity of the protein-peptide moiety conjugated. Using the present approach one can further control the hydrolysis rate by substitution of the fluorenyl moiety with electron-withdrawing or electron-inducing groups that increase or decrease, respectively, the hydrolysis rate. The number of PEG-FMS chains attached to the drug should also affect the rate at which the active drug is released.

[0180] The sulfonated fluorene moiety is not toxic, as previously shown (Shechter et al., 2001). High molecular-weight PEG is known to be safe in terms of toxicity and immunogenicity and is widely used in the food, cosmetic and pharmaceutical industries (Working et al., 1997; Roberts et al., 2002).

[0181] Thus, the present invention provides a procedure herein designated "reversible pegylation". In this new conceptual approach, that was implemented according to the invention with low molecular weight polypeptides and proteins, the PEG moiety is not attached directly to the drug, as in the standard pegylation procedure, but rather the PEG

located fluorenylmethoxycarbonyl N-hydroxy-succinimide ester that reacts with peptide and protein amino groups. A maleimide group was attached to the fluorenyl backbone to enable coupling to sulfhydryl-containing PEG_{400} -SH.

[0250] MAL-FMS-NHS is a cleavable reagent capable of reacting covalently with the amino side chains of peptides, proteins and aminoglycosides. According to the present invention, it enables PEG chains to be linked to peptides and proteins through a slowly hydrolyzable chemical bond. PEG-FMS peptide/protein conjugates thus formed undergo spontaneous hydrolysis at a slow rate upon incubation at pH 8.5, 37° C., with a $t_{1/2}$ value of 8-14±2 h, generating the unmodified parent molecule.

[0251] Chemical features of MAL-FMS-NHS. Table 1 summarizes several of the characteristic features of MAL-FMS-NHS. It is a water- and DMF-soluble compound. Mass spectrum analysis has yielded a calculated mass of 585 Da. The compound absorbs in the UV region with a molar extinction coefficient $\epsilon_{255}=21,000\pm200$ mol⁻¹cm⁻¹. The curve shows a maximum at 250 nm and a shoulder extending up to 330 nm. At 320 nm, where peptides or proteins absorb negligibly, MAL-FMS-NHS, either free or covalently bound to proteins, absorbs with a molar extinction coefficient $\epsilon_{320}=16,100\pm150$ mol⁻¹cm⁻¹ (Table 1).

[0252] Stability of the MAL-functional moiety in aqueous solutions. In order to test the stability of the maleimide function of MAL-FMS-NHS in aqueous solutions at different pH values, MAL-FMS-NHS (1 mM) was incubated at room temperature in water (pH 6.9), in 0.007M acetic acid (pH 4.0), in 0.1M phosphate buffer (pH 7.4), and in 0.1M NaHCO₃ (pH 8.5). At several time points, aliquots were allowed to react with a slight excess of reduced glutathione (GSH) (12 min at pH 7.2) and the concentration of unreacted OSu was determined with DTNB (5,5'-dithio-bis-2-nitrobenzoic acid, Ellman's reagent), by measuring the absorbance of the produced yellow colored 5-thio-2-nitrobenzoic acid (TNB) at 405 or 412 nm. FIG. 1 demonstrates the potency of the reaction of MAL-FMS-NHS with GSH as a function of time, at different pH values. MAL-FMS-NHS was found fully stable at pH 4 to 6 over a period of a day or two and at pH 7.2 it is stable over a period of several hours. At pH 8.5, the maleimide moiety was destroyed at an accelerated rate ($t_{1/2}$ value=2.7±0.3 hr, FIG. 1).

Example 14

Degree of Incorporation of MAL-FMS-NHS at pH 7.2 into α -lactalbumin as a Function of the Amount of Added Reagent

[0253] To evaluate the degree of incorporation of the maleimide moiety into peptides/proteins as a function of the reagent, α -lactalbumin was used as a model. Thus, to samples of α -lactalbumin (α -LA, 1.0 ml of 1 mg/ml), MAL-FMS-NHS was added in 0.1M PBS, pH 7.2, at concentrations ranging from 1 equivalent up to 14 molar equivalents of MAL-FMS-NHS, for 30 min at 25° C., that is, under experimental conditions where the maleimide-alkylating capacity of the spacer remains unmodified. For each reaction, the amount incorporated into the protein was determined by UV absorbance at 280 nm, after dialysis, and by quantitating the amount of unmodified amino side-chain moieties with TNBS. As shown in FIG. 2, about one mole

of MAL-FMS was incorporated into α -LA per 2.1 mole of reagent added, up to a fourteen molar excess over the protein.

Example 15

Synthesis of PEG_{400} -SH

[0254] PEG_{400} -SH was prepared either by reaction of 2-tritylthioethylamine and PEG_{400} -OSu as described below in 15(a), or from PEG_{400} -OSu and cystamine [2,2'-dithio-bis-(triethylamine)]dihydrochloride, as described below in 15(b).

15(a). Synthesis of PEG_{400} -SH from 2-tritylthioethylamine and PEG_{400} -OSu

[0255] Synthesis of 2-tritylthioethylamine. 2-Amino-ethanol (3 g, 26.5 mmol) and triphenylmethyl alcohol (7 g, 26.9 mmol) were dissolved in THF (20 ml) and stirred for 30 min at room temperature. The THF was removed by evaporation and the remaining oil residue was dissolved in ether (400 ml) and stored at -20° C. The product isolated was washed with cooled ether and water. A concentrated ammonia solution (25%, 30 ml) was then added, and the aqueous phase was washed several times with ether. The ether was removed by vacuum and the product was desiccated. Yield, 78% (6.6 g, 20.7 mmol), R_f=5.85 min.

[0256] 2-Tritylthioethylamine and PEG_{400} -OSu (0.25 g, 6.25 μ mol) were dissolved in 4 ml THF/acetonitrile (1:2, v/v). After one hour, the product was precipitated with cooled ether and washed (x3). The dry pellet was dissolved in THF:triethylsilane:water (95:2.5:2.5, 6 ml) and, after one hour, PEG_{400} -SH was precipitated by cooled ether (100 ml) and desiccated.

15(b). Synthesis of PEG_{400} -SH from cystamine and PEG_{400} -OSu

[0257] PEG_{400} -OSu was dissolved at a concentration of 40 mg/ml in an aqueous solution of cystamine di HCl (1M) and brought to pH 8.5 with NaHCO₃. Reaction was carried out for 2 h at 25° C. The product thus obtained was dialyzed overnight against 0.1 M NaHCO₃, treated with 30 mM dithiothreitol (25° C., 1 h) and re-dialyzed against 0.01 M HCl containing 10 mM ascorbic acid. PEG_{400} -SH was obtained with a 93% yield. It contained 1 mole sulfhydryl moiety per mole PEG_{400} , as determined with DTNB in the presence of ascorbate. PEG_{400} -SH was kept frozen until used.

Example 16

Preparation of PEG-FMS-Drug Conjugates from MAL-FMS-NHS (Procedure I)

[0258] For the preparation of PEG-FMS-drug conjugates according to the invention, a "two-step" Procedure I can be used as depicted in Scheme 6 whereby MAL-FMS-NHS is first reacted with an amino-containing drug or with the amino group of a peptide or protein drug and the resulting MAL-FMS-drug conjugate is further reacted with PEG -SH, as follows:

16(a). Preparation of MAL-FMS-Drug Conjugates

[0259] In the first step, a MAL-FMS-drug conjugate is prepared by adding one to seven molar excess of MAL-FMS-NHS, Precursor 8, to a stirred solution of the drug (1

mg/ml) in 0.1 M PBS buffer, pH 7.2. The reaction is carried out for 30 min at 25° C., and then dialyzed against water at 7° C. over a period of 24 h to remove excess MAL-FMS-NHS. The amount of FMS-MAL residues incorporated into the drug, e.g., a peptide or protein drug, is determined both by the absorption at 320 nm using molar extinction coefficient of $\epsilon_{320}=15,100$, and by quantitating the incorporated MAL function into the drug. This is performed by adding a measured aliquot into a GSH solution (0.1 mM in phosphate buffer, pH 7.2). The concentration of unreacted (5%) remained is determined with DTNB.

16(b) Preparation of PEG-FMS-Drug Conjugates

[0260] In the second step, a stoichiometric amount of solid PEG₁₀₀₀-SH is added to the MAL-FMS-drug conjugate of step 16(a), and the reaction proceeds for additional 60 min in 0.1 M phosphate buffer, pH 7.2, containing 2 mM ascorbic acid. The PEG-FMS drug conjugate thus obtained is further purified by HPLC-procedures using C4 or C18 reverse phase columns under conditions resolving the conjugate from the drug, preferably a peptide or protein drug (or protein-FMS-MAL) that has not been covalently linked to PEG₁₀₀₀-SH.

Example 17

Preparation of PEG-FMS-Drug Conjugates From MAL-FMS-NHS (Procedure II)

[0261] PEG-FMS-drug conjugates according to the invention may be also prepared by an alternative "two-step" Procedure II depicted in Scheme 6 whereby MAL-FMS-NHS is first reacted with PEG-SH and the resulting PEG-FMS-NHS conjugate is further reacted with an amino-containing drug or with the amino group of a peptide or protein drug as follows:

17(a) Preparation of PEG-FMS-NHS Conjugate

[0262] In the first step, a PEG-FMS-NHS conjugate is prepared by MAL-FMS-NHS to a stirred solution of PEG-SH in PBS buffer, pH 7.4. The reaction is carried out for 30 min at 25° C., and the product is then purified by HPLC and lyophilized.

17(b) Preparation of PEG-FMS-Drug Conjugates

[0263] In the second step, a stoichiometric amount of the MAL-FMS-NHS conjugate of step 17(a) is added to the amino containing drug, and the reaction proceeds under stirring for 2 hours. The product is then purified by HPLC and lyophilized.

Example 18

Synthesis of (PEG₁₀₀₀-FMS)₄-Insulin

[0264] (PEG₁₀₀₀-FMS)₄-Insulin, was prepared by the two-step Procedure I described in Example 16. Thus, Mal-FMS-NHS (6 g, 10 μ mol) was dissolved in water (0.25 ml) and added to an insulin (Zn²⁺-free, 25 mg, 4.16 μ mol) solution in PBS (0.5 ml, pH=7.4). The pH was adjusted to 7-8 with NaHCO₃. The reaction was stopped after 30 min with diluted hydrochloric solution (pH=6). The product was isolated using preparative HPLC (C18 column, 10-80% acetonitrile/water, 75:25, v/v, 50 min, 10 ml/min) and identified as monosubstituted insulin derivative Mal-FMS-insu-

lin using electrospray mass spectrometry (ESMS (ca. 6280), M-1: 6278.83, M+1: 6280.73). Analytical HPLC (C18-melith column), Rt=4.88 min.

[0265] PEG₁₀₀₀-SH (20 mg, 0.5 μ mol) was added to a solution of the Mal-FMS insulin intermediate (2 mg, 0.337 μ mol) in PBS (0.3 ml, pH=7.4), the pH was adjusted to 7-8 with NaHCO₃, and the reaction was stopped after 30 min with diluted hydrochloric solution (pH=6). The title product, PEG₁₀₀₀-FMS-insulin, was subjected to analytical HPLC (C18-melith column), Rt=6.6 min.

Example 19

Synthesis of PEG₁₀₀₀-FMS-exendin-4

[0266] PEG₁₀₀₀-FMS-exendin-4 was prepared by the two-step Procedure I described in Example 16. Thus, 280 mg of MAL-FMS-NHS (28 μ l from a fresh solution of MAL-FMS-NHS in DMF, 10 mg/ml, 2.0 molar excess over the peptide) was added to a stirred solution of exendin-4 (1 mg in 1.0 ml 0.1 M phosphate buffer, 2 mM ascorbate, pH 7.2, 0.239 mM). After 7 min, solid PEG₁₀₀₀₀-SH was added to a final concentration of 0.5 mM (2.1 molar excess over the peptide). The reaction was carried out for 1 h, then, dialyzed overnight against water and then further filtered through Centricon with a molecular weight cutoff of 50 kDa to remove any residual exendin-4 or MAL-FMS-exendin-4 that had not linked to PEG₁₀₀₀₀-SH. The concentration of the conjugate was determined by acid hydrolysis of a 20 μ l aliquot followed by amino acid analysis, according to a specific acid (2 residues) alanine (2 residues) and valine (1 residue) and absorbance at 280 nm was monitored. The calculated extinction coefficient at 280 nm is 26940=100 $\times$ value that is the additive absorbance of exendin-4 ($\epsilon_{280}=5740$) and PEG₁₀₀₀₀-FMS ($\epsilon_{280}=21200$). A solution of 20 μ M PEG₁₀₀₀₀-FMS-exendin had OD₂₈₀=0.53. Thus the conjugate had a 1:1 PEG₁₀₀₀₀-FMS-exendin stoichiometry (as determined from the UV absorption of the conjugate following purification and acid hydrolysis followed by amino acid analysis as described above).

Example 20

Synthesis of PEG₁₀₀₀-FMS-exendin-4

[0267] PEG₁₀₀₀₀-FMS-exendin-4 was prepared by the two-step Procedure II described in Example 17. Thus, MAL-FMS-NHS (1.8 mg, 3 μ mol) was added to a solution of PEG₁₀₀₀₀-SH (15 mg, 3 μ mol) in PBS (0.5 ml, pH=7.4) and the reaction solution was stirred for 30 min. The product, PEG₁₀₀₀₀-FMS-NHS, was purified by HPLC on a RP-4 column and lyophilized.

[0268] PEG₁₀₀₀₀-FMS-NHS (2.3 mg, 4.02 μ mol) was then added to a solution of exendin-4 (4.6 mg, 0.4 μ mol) in PBS (0.5 ml, pH=8) and the reaction solution was stirred for 2 h. The product PEG₁₀₀₀₀-FMS-exendin-4 was purified by HPLC on a RP-4 column and lyophilized.

Example 21

Synthesis and Characterization of PEG₁₀₀₀-FMS-H Nc2

[0269] The attachment of a single PEG-chain of 40 kDa to DNa2 appears sufficient to grossly arrest kidney filtration of

the conjugate (Bailon et al., 2001). We therefore envisioned that the linkage of a single PEG₄₀-FMS chain to IFN α 2 would suffice to obtain a prolonged-acting conjugate that releases IFN α 2, with a desirable pharmacokinetic profile. In the procedure found most optimal for introducing one mole PEG₄₀-FMS/mol protein, IFN α 2 was allowed to react first with 3 equivalents of MAL-FMS-NHS, at pH 7.2, for 7 min, followed by the addition of 3 equivalents of PEG₄₀-SH, at pH 7.2, for 1 hour. The NHS function of MAL-FMS-NHS is relatively unstable at prolonged aqueous neutral conditions, whereas the MAL function of the spacer preserves its alkylating capacity for several hours at pH 7.2 (not shown). We therefore preferred to use Procedure 1 of Example 16 and to react MAL-FMS-NHS first with IFN α 2 and to subsequently link PEG₄₀-SH to the IFN α 2-FMS-MAL conjugate.

[0270] To a stirred solution of IFN α 2 (1 mg/1.0 ml) in phosphate buffer, pH 7.2 (52 mM), 91 μ g of MAL-FMS-NHS was added (9.1 μ l from a fresh solution of MAL-FMS-NHS, (10 mg/ml) in DMF, (5.0 molar excess over the protein). After 7 min, PEG₄₀-SH was added to obtain a final concentration of 156 μ M (three molar excess over the protein). The reaction was carried out for 1 h, and then dialyzed overnight against H₂O to remove residual DMF and phosphate buffer. The conjugate thus obtained was characterized by MALDI-TOF as PEG₄₀-FMS-IFN α 2.

[0271] Table 2 summarizes several characteristic features of the conjugate thus obtained. MALDI-TOF mass spectra analysis shows a 1:1 PEG₄₀-FMS:IFN α 2 stoichiometry. The experimental mass obtained, 63540 Da, corresponds to the additive masses found for PEG₄₀-SH (43818 Da), IFN α 2 (19278 Da) and of the spacer molecule following conjugation (473 Da). PEG₄₀-FMS-IFN α 2 migrates on analytical HPLC as a wide peak with R_t value=13 min. The conjugate is highly soluble in aqueous solutions. It has a molar extinction coefficient ϵ_{280} =39270 $\pm$ 100, corresponding to the absorption of the native cytokine and of FMS (ϵ_{280} =21,200) (Gershonov et al., 2000).

Example 22

Synthesis of (PEG₄₀-FMS)₂-IFN α 2

[0272] (PEG₄₀-FMS)₂-IFN α 2 was prepared as described in Example 21 above for PEG₄₀-FMS-IFN α 2, but using 6 eq. (16 μ g, 44.5 μ mol, in 10 μ l DMF) of MAL-FMS-NHS.

Example 23

Synthesis of PEG₄₀-FMS-PYY₁₋₃₆

[0273] In the procedure found most optimal for coupling about one mole of PEG₄₀-SH per mol of MAL-FMS-PYY₁₋₃₆, PYY₁₋₃₆ was allowed to react first with 2 equivalents of MAL-FMS-NHS, at pH 7.2, for 7 min, followed by the addition of 2.1 equivalents of PEG₄₀-SH, at pH 7.2, for 1 hour.

[0274] To a stirred solution of PYY₁₋₃₆ (1 mg, in 1.0 ml phosphate buffer, pH 7.2, 10 mM Na ascorbate (0.247 μ M), 288 μ g of MAL-FMS-NHS was added (28.8 μ l, from a fresh 10 mg/ml solution of MAL-FMS-NHS in DMF; twofold molar excess over the peptide). After 7 min, PEG₄₀-SH was added to obtain a final concentration of 0.52 mM (2.1 molar excess over the peptide). The reaction was carried out for 1

h and the mixture was then dialyzed overnight against water. The resulting PEG₄₀-FMS-PYY₁₋₃₆ was characterized by MALDI-TOF MS.

Example 24

Synthesis of (PEG₄₀-FMS)₂-hGH

[0275] (PEG₄₀-FMS)₂-hGH was prepared by the two-step procedure described in Example 16, as follows: To a stirred solution of human growth hormone (4.4 mg, 0.22 μ mol) in phosphate buffer (1 ml, 0.1 M, pH 7.2), MAL-FMS-NHS (4 eq, 0.47 mg, 0.9 μ mol) was added. The reaction was carried out for 30 min at 25° C., and then dialyzed against H₂O (pH 6) at 4° C., for 24 h, to remove excess MAL-FMS-NHS. Solid PEG₄₀-SH (7 eq, 65 mg, 1.5 μ mol) was added, and the reaction proceeded for 2 h in phosphate buffer (1 ml, 0.1 M, pH 7.2, containing 2 mM ascorbic acid). The thus obtained title compound was purified by RP-HPLC using a C4 column, and characterized by SDS gel electrophoresis (12.5%), indicating the presence of (PEG₄₀-FMS)₂-hGH.

Example 25

Synthesis of PEG₄₀-FMS-hGH

[0276] MAL-FMS-NHS (280 μ g) was added to a solution of hGH (4.5 mg, 0.225 μ mol) in 0.5 ml PBS, pH=7.4. After 40 min, PEG₄₀-SH (30 mg) was added and the reaction mixture was stirred for 1 h and dialyzed overnight against water. The product, PEG₄₀-FMS-hGH, was purified by RP-HPLC.

Example 26

Synthesis of PEG₄₀-FMS-ANP

[0277] To a stirred solution of atrial natriuretic peptide (ANP) (0.32 μ mol, 1 mg) in phosphate buffer (1 ml, 0.1 M, pH 7.2), MAL-FMS-NHS (2 eq, 0.2 mg, 0.7 μ mol) was added. The reaction was carried out for 10 min at 25° C. Solid PEG₄₀-SH (2.2 eq, 30 mg, 0.7 μ mol) was added, and the reaction proceeded for 2 h in phosphate buffer (1 ml, 0.1 M, pH 7.2, containing 2 mM ascorbic acid). The product was further purified by HPLC.

II. Biological Section

[0278] In this section, the biological activity of the PEG-Fmoc and PEG-FMS conjugates with gentamicin, peptides and proteins prepared in the examples above, was tested.

Example 27

Inactive PEG-Fmoc-gentamicin Conjugates Undergo Renaturation

[0279] For assaying the antibacterial potency of gentamicin and its derivatives, a suspension of *Escherichia coli* (ATCC strain N-4156-WJ, 1% v/v in LB medium) was divided into plastic tubes (0.5 ml per tube) and incubated in a shaking water bath at 37° C., in either the absence or the presence of increasing concentrations of gentamicin (0.02 to 2 μ M), and increasing concentrations of PEG₄₀-gentamicin derivatives (0.02-50 μ M). *E. coli* replication was evaluated by measuring the absorbance at 600 nm. Incubation was terminated when O.D.₆₀₀ nm in the tubes containing no gentamicin reached a value of 0.6 $\pm$ 0.1. Under our assay

conditions, native gentamicin inhibited half-maximally *E. coli* replication at a concentration of $0.22 \pm 0.02 \mu\text{M}$ ($0.1 \mu\text{g/ml}$). A PEG-gentamicin derivative showing an IC_{50} value of $2.2 \pm 0.2 \mu\text{M}$ in this assay is considered having 10% the antibacterial potency of native gentamicin.

[0280] $(\text{PEG}_{1000}\text{-Fmoc})_1\text{-gentamicin}$ and $(\text{PEG}_{1000}\text{-Fmoc})_2\text{-gentamicin}$, containing one or two moles of $\text{PEG}_{1000}\text{-Fmoc}$, respectively, were prepared as a model in order to assess the reversibility of the PEG-Fmoc moieties. Both derivatives (0.2 mM of each) were incubated in 0.1 M NaHCO_3 (pH 8.5) at 37°C , and aliquots were withdrawn at different times and then analyzed for their potency to arrest *E. coli* replication (see method (xi) above). The IC_{50} for each aliquot was determined. Native gentamicin inhibited *E. coli* replication with IC_{50} value of $0.22 \pm 0.02 \mu\text{M}$. $(\text{PEG}_{1000}\text{-Fmoc})_1$ - and $(\text{PEG}_{1000}\text{-Fmoc})_2$ -gentamicin had 0.01 and $2.1 \pm 0.1\%$ of the antibacterial potency of gentamicin, respectively.

[0281] The results are shown in FIGS. 3A-3B. $(\text{PEG}_{1000}\text{-Fmoc})_1$ -gentamicin showed a prolonged lag period (about 18 hours) prior to noticeable reactivation (FIG. 3A). A sharp elevation in antibacterial potency then occurred, reaching 50 or 100% of the native potency after 30 ± 2 or 60 ± 3 hours of incubation, respectively (FIG. 3A). No lag period was observed upon incubation of $(\text{PEG}_{1000}\text{-Fmoc})_2$ -gentamicin. Reactivation proceeded continuously with half-maximal reactivation at 6 ± 0.5 hours, and full activation (100%) after 30 hours of incubation (FIG. 3B).

Example 28

Biological Activity of PEG-Fmoc-Insulin Conjugates

Materials and Methods

[0282] (i) Materials. Recombinant human insulin was from Biotechnology General (Rehovot, Israel). $\text{D-[1,2-}^{14}\text{C]}$ Glucose (1.7 mCi/mole) was obtained from Du Pont NEN (Boston, Mass., USA). Collagenase type 1 (134 U/mg) was purchased from Worthington (Freehold, N.J., USA).

[0283] (ii) Rat adipocytes were prepared from fat pads of male Wistar rats ($100\text{--}200 \text{ g}$) by collagenase digestion as described (Rodbell, 1964). The fat pads were cut into small pieces with scissors and suspended in 3 ml of KRB buffer containing NaCl , 110 mM ; NaHCO_3 , 25 mM ; KCl , 5 mM ; KH_2PO_4 , 1.2 mM ; CaCl_2 , 1.3 mM ; MgSO_4 , 1.3 mM ; and 0.7% BSA (pH 7.4). Digestion was performed with collagenase type 1 (1 U/ml) in a 25 ml flexible plastic bottle under an atmosphere of carbogen ($95\% \text{ O}_2$, $5\% \text{ CO}_2$) for 40 min at 37°C with vigorous shaking. Five ml of buffer was then added, and the cells were passed through a mesh screen. The cells were then allowed to stand for several minutes in a 25 ml plastic test tube at room temperature, floating, and the buffer underneath was removed. This procedure (suspension, floating, and removal of buffer underneath) was repeated three times.

[0284] (iii) Lipogenesis (incorporation of $[U\text{-}^{14}\text{C}]$ glucose into the lipids of intact adipocytes). The incorporation of $[U\text{-}^{14}\text{C}]$ glucose into adipose tissue in rat adipocytes was performed as described (Moody et al., 1974). Adipocyte suspensions (3×10^6 cells/ml) were divided into plastic vials (0.5 ml per vial) and incubated for 60 min at 37°C under

an atmosphere of carbogen with $0.2 \text{ mM } [U\text{-}^{14}\text{C}]$ glucose, in either the absence or presence of insulin. Lipogenesis was terminated by adding toluene-based scintillation fluid (1.0 ml per vial) and the radioactivity in extracted lipids was counted. In a typical experiment, insulin-stimulated lipogenesis was 4-5 fold higher than basal (basal: 2000 cpm per 3×10^6 cells; V_{max} : 8,000-10,000 cpm per 3×10^6 cells). In this assay, insulin stimulates lipogenesis with ED_{50} value = $0.15 \pm 0.03 \text{ ng/ml}$. An insulin analog exhibiting ED_{50} value = 15 ng/ml is considered to have $\sim 1\%$ of the biological potency of the native hormone.

[0285] (iv) Glucose-lowering potency of insulin and its derivatives was determined in mice following administration under the conditions specified in each experiment. Blood samples were taken from the tail vein at different time points after administration, and blood glucose levels were determined with glucose analyzer (Beckman Instrument, Brea, Calif., USA) by the glucose oxidase method. The level of glucose in normal healthy CD1-mice was $140 \pm 7 \text{ mg/dl}$ (7.77 mM). Each group consisted of 4-5 mice. Data are presented as mean $\pm$ SEM.

28 (i). Progressive Modification of Amino Acid Moieties of Insulin with $(\text{PEG}_{1000}\text{-Fmoc})_n\text{-OSn}$

[0286] Human insulin was modified with increasing concentrations of Precursor 1 and loss of biological potency as a function of $(\text{PEG}_{1000}\text{-Fmoc})_n$ incorporated into insulin was determined.

[0287] Insulin (17.24 nmole in $0.2 \text{ ml } 0.01 \text{ M NaHCO}_3$) reacted with increasing concentrations of $(\text{PEG}_{1000}\text{-Fmoc})_n\text{-OSn}$ at a molar excess over the protein as indicated in FIG. 4A for 2 hours at 25°C . The degree of derivatization was quantitated by determining the number of free amino groups that remained unmodified and were available for reaction with NHS. Theoretically, insulin can incorporate 5 moles of $(\text{PEG}_{1000}\text{-Fmoc})_n$ on the amino side chains of Lys B29, PheB1 and GlyA1. As shown in FIG. 4A, upon reacting insulin with 0.6, 1.3, 1.9, 2.5 and 5.0 molar excess of $(\text{PEG}_{1000}\text{-Fmoc})_n\text{-OSn}$, 0.3 ± 0.03 , 0.59 ± 0.05 , 1.1 ± 0.1 , 1.5 ± 0.2 and 2.2 ± 0.2 moles of $(\text{PEG}_{1000}\text{-Fmoc})_n$ were incorporated into insulin, respectively, indicating that two (of the three) amino side chains of insulin are readily accessible for derivatization.

[0288] Aliquots containing 0.4, 0.7, 1.1, 1.5 and $2.2 \text{ moles } (\text{PEG}_{1000}\text{-Fmoc})_n$ covalently attached per mole insulin, were assayed for their biological potencies in a lipogenic assay in rat adipocytes. Under the assay conditions, human insulin stimulates lipogenesis, 4-6 times above basal levels with ED_{50} value of $0.2 \pm 0.02 \text{ ng/ml}$. An insulin derivative exhibiting ED_{50} of $2.0 \pm 0.2 \text{ ng/ml}$ in this assay is considered as having 10% the lipogenic potency of native insulin. The results in FIG. 4B show that the respective biological potencies were 87 ± 4 , 60 ± 3 , 8 ± 1 , 4 ± 0.3 and less than 1% when 0.3, 0.6, 1.1, 1.5 and $2.2 \text{ mol } (\text{PEG}_{1000}\text{-Fmoc})_n$ moles of insulin has been incorporated.

28 (ii). $(\text{PEG}_{1000}\text{-Fmoc})_n$ -Insulins Undergo Time-Dependent Reactivation

[0289] Insulin conjugates containing one and two moles of $(\text{PEG}_{1000}\text{-Fmoc})_n$ moles of insulin were incubated for different times at a concentration of $0.172 \mu\text{M}$ in 0.1 M NaHCO_3 , 0.5% bovine serum albumin (BSA) and $1 \text{ mM NaH}_2\text{PO}_4$ (pH

8.5) at 37° C. The results are shown in FIG. 5. At the indicated time points, aliquots were analyzed (in several concentrations for each aliquot) for their lipogenic potencies in rat adipocytes. With (PEG₃₀₀₀-Emoc)₂-insulin, no reactivation could be observed in the first 10 hours of incubation. Lipogenic activity then increased, reaching 45±3 and 90% of the native insulin potency at 30±2 and 80±4 hours, respectively.

[0290] With (PEG₃₀₀₀-Emoc)₂-insulin, no such lag period was observed. Activity was regenerated slowly but continuously yielding 16, 24, 30, 36 and 95% of the native potency following 2, 4, 10, 17 and 80 hours of incubation, respectively. Insulin containing about one mole of PEG₃₀₀₀-Emoc₂-insulin (PEG₃₀₀₀-Emoc)₂-insulin was therefore selected for further studies. Mass-spectrum and analytic HPLC analyses revealed that this preparation contains predominantly monomodified derivatives of insulin (MW=11,066 kDa), the remainder being unmodified insulin (about 5%, MW=5,813 kDa) and small quantities of bis (MW=16,587 kDa) and tris (MW=22,661 kDa) modified derivatives.

[0291] As shown in FIG. 4B, (PEG₃₀₀₀-Emoc)₂-insulin has ±1% the biological potency of the native hormone prior to undergoing PEG-Emoc hydrolysis. Based on these findings *in vitro*, (PEG₃₀₀₀-Emoc)₂-insulin was administered *in vivo* at ten times higher concentrations than the native hormone, in order to obtain the same glucose-lowering effect (see below).

28(ii). (PEG₃₀₀₀-Emoc)₂-Insulin Facilitates Prolonged Glucose-Lowering Action *in vivo*

[0292] Native insulin (Zn²⁺-free, 1/2 mU/mouse) or (PEG₃₀₀₀-Emoc)₂-insulin (1/2 mU/mouse) were administered subcutaneously to groups of mice (n=5 in each group), and the glucose lowering profiles were determined. The results are shown in FIG. 6. Native insulin reduced blood glucose level maximally at 30 min. Circulating glucose levels then returned to normal values with t_{1/2}=1.8±0.2 hours. Following subcutaneous administration of (PEG₃₀₀₀-Emoc)₂-insulin, circulating glucose levels were lowered maximally at 4 hours, and were then maintained at the low glucose level for an additional 4 hours, before returning to the normal values with a t_{1/2}=12±1 hours (FIG. 6).

28(iii). (PEG₃₀₀₀-Emoc)₂-Insulin Manifests Prolonged Glucose Lowering Action Also After Intraperitoneal Administration

[0293] The prolonged action of subcutaneously administered (PEG₃₀₀₀-Emoc)₂-insulin can be attributed, theoretically, to a slower absorption rate from the subcutaneous compartment to the circulation as well as to the low fraction of the administered material exposed to receptor-mediated degradation and hydrolysis of the inactive to the active species. In order to differentiate between these factors, the subcutaneous compartment was bypassed by administering native insulin (Zn²⁺-free, 0.345 mU/mouse, in 0.2 ml PBS buffer) or (PEG₃₀₀₀-Emoc)₂-insulin (0.45 mU/mouse, in 0.2 ml PBS buffer) intraperitoneally. The glucose lowering capacities were then monitored. Blood glucose levels were determined at different time points.

[0294] The results are shown in FIG. 7. Following administration of insulin, circulating glucose levels fell maximally at 15 min and returned to normal level with a t_{1/2} value of

1.0±0.1 hours. In the case of intraperitoneally administered (PEG₃₀₀₀-Emoc)₂-insulin, circulating glucose level declined more gradually, reaching a maximal fall at 1.0 hour. Levels were then gradually elevated, showing a t_{1/2} value of 3.1±0.1 hours, and returning to normal values only 6 hours after administration (FIG. 7).

Example 29

Biological Activity of PEG₃₀₀₀-PMS-exendin-4 Conjugates

[0295] Exendin-4 is an insulinotropic glucagon-like peptide-1 (GLP-1) agonist associated with the β-pancreatic cells, elevates endogenous cAMP levels, enhances secretion of insulin, and lowers circulating glucose levels (Ing et al., 1992; Goke et al., 1993; Schepp et al., 1994; Polmann et al., 1999). A profound pharmacological advantage of this GLP-1 agonist is that, following administration at any dosage, the circulating blood glucose level (BG) never falls below a threshold glucose level that, in non-diabetic healthy CD1-mice, is 74±7 mg/dl (Shechter et al., 2003).

[0296] According to the present invention, the conjugate PEG₃₀₀₀-PMS-exendin-4 was prepared (Example 19). Exendin-4 contains one His Nε amino function and two Lys Nε amino groups, enabling modification at these three positions. Indeed, N-terminal amino acid sequencing revealed that, although the PEG₃₀₀₀-PMS-exendin-4 product eluted as a single peak on HPLC, it was actually a mixture containing primarily the Nε-modified hormone. However, in view of the pegylation reaction's reversibility according to the present invention, regeneration the native peptide and protein hormones in physiological environment, this point deserves only minor consideration.

Materials and Methods

[0297] (i) Materials. Exendin-4 and a non-lysine-containing synthetic irrelevant peptide of 27 amino acids (S1-Q10 NO: 14) were synthesized by the solid phase method using a multiple-peptide synthesizer, AMS 4771 (Abimed Analyser Technik GmbH, Langenfeld, Germany). An Emoc-strategy was employed throughout the peptide chain assembly. 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH) and 2-nitrobenzoic acid (TNBS) were purchased from Sigma Chemical Co., (St. Louis, Mo., USA). All other materials used in this study were of analytical grade.

[0298] PEG₃₀₀₀-PMS-exendin-4 and PEG₃₀₀₀-PMS-exendin-4 were prepared as described in Examples 18 and 19, respectively.

[0299] For the preparation of PEG₃₀₀₀-exendin-4, exendin-4 (0.3 mg, 75 nmol) was dissolved in PBS (pH=7.5) and reacted with PEG₃₀₀₀-OSu (20 mg, 470 nmol) for 3 h, filtered (>7) through Centricon (cut off=50 kDa) and characterized by MALDI-TOF mass spectrometry (found 48074 Da).

[0300] PEG₃₀₀₀-PMS-Peptide 27 was prepared as described for PEG₃₀₀₀-PMS-exendin-4.

[0301] A Centricon-50 ultrafiltration device for aqueous solutions was purchased from Millipore S.A. (France).

[0302] Ultraviolet spectra were obtained by Beckman DU 7500 spectrophotometer in 1 cm path length UV cuvettes.

Mass spectra were determined using MALDI-TOF and ESMS techniques (Bruker-Reflex-Reflectron model, Germany, and VG-platform-II electrospray single quadrupole mass spectrometer, Micro Mass, UK, respectively).

[0303] (ii) HPLC analyses were performed using a Spectra-Physics SP8800 liquid chromatography system equipped with an Applied Biosystems 757 variable wavelength absorbance detector, and a Spectra-SYSTEM P2000 liquid chromatography system equipped with a Spectra-SYSTEM AS100 auto-sampler and a Spectra-SYSTEM UV1000, all controlled by a ThermoQuest chromatography data system (ThermoQuest Inc., San Jose, Calif., USA). The column effluents were monitored by UV absorbance at 220 nm. Analytical RP-HPLC was performed using a pre-packed Chromolith[®] Performance RP-18e (4.6×100mm, Merck, Darmstadt, Germany). The column was eluted with a binary gradient of 10–100% solution B over 10 min with a flow rate of 3 ml/min (solution A was 0.1% TFA in H₂O and solution B was 0.1% TFA in acetonitrile [H₂O, 3:1, v/v]). PEGylated compounds were analyzed using a RP-4 column (250×4 mm, 5 µm bead size, VYDAC, Hesperia, Calif., USA) with a binary gradient of 10–100% solution B in 50 min at a flow rate of 1 ml/min.

[0304] (iii) Preparative separations were performed with pre-packed VYDAC[®] RP-18 or RP-4 columns (250×22 mm; Hesperia, Calif.). The column was eluted with 10–100% solution B over 60 min (12 ml/min).

[0305] (iv) Glucose-lowering assay. Three groups of CD1 mice (n=6 per group) were subcutaneously administered with saline, native exendin-4 (1 µg/mouse) or PEG₄₀₀₀-FMS-exendin-4 (4 µg peptide/mouse). Circulating glucose levels were measured with a glucose analyzer (Beckman Instrument, Fullerton, Calif., USA) by the glucose oxidase method. Blood samples for the blood glucose analyses were taken from the tail veins. The level of glucose in normal healthy CD1-mice was 140±7 mg/dl (7.77 mM).

29(i) PEG-FMS-Exendin-4 Releases Exendin-4 Upon Incubation

[0306] A solution of PEG₄₀₀₀-FMS-exendin-4 (0.25 mM, 1 ml/ml in terms of exendin-4 in 0.1 M NaHCO₃, pH 8.5) was incubated at 37° C. At different time points, aliquots (50 µl) were withdrawn and analyzed for the release of exendin-4 from the conjugate, using HPLC on a RP-4 column. As shown in FIG. 8, exendin-4 was released from the conjugate [PEG₄₀₀₀-FMS]-exendin-4 in a slow, homogeneous fashion, with a t_{1/2} of 16±2 h. Exendin-4 was fully released from the conjugate after 48 h of incubation.

29(ii) The Hydrolysis Rates and Reaction Orders of PEG₄₀₀₀-FMS-exendin-4, PEG₄₀₀₀-FMS-Exendin-4 and PEG₄₀₀₀-FMS-4-nitro-phenethylamine

[0307] The hydrolysis rates and reaction orders of the PEG-FMS conjugates PEG₄₀₀₀-FMS-4-nitro-phenethylamine, PEG₄₀₀₀-FMS-exendin-4 and PEG₄₀₀₀-FMS-exendin-4, and one MAI-FMS conjugate, N-(MAI-FMS)-Peptide 27, were evaluated at pH =8.5, 37° C. The structures and half-lives of the conjugates are presented in Table 3. PEG₄₀₀₀-FMS-4-nitro-phenethylamine was prepared as described in Example 20. The conjugate MAI-FMS-Peptide 27 was prepared by reaction of MAI-FMS-NHS with the non-relevant peptide 27 (SEQ ID NO.14).

[0308] FIG. 9 shows the hydrolysis of PEG₄₀₀₀-FMS-exendin-4 (circles) and of PEG₄₀₀₀-FMS-4-nitro-phenethylamine (squares) after incubation in PBS at pH 8.5, 37° C. Results in FIGS. 8 and 9 are expressed as percent of the maximal peak area of released exendin-4 and 4-nitrophenethylamine, as a function of time.

[0309] The hydrolysis rate at pH 8.5 is equivalent to the hydrolysis rate in serum. The release of 4-nitro-phenethylamine and exendin-4 was monitored by RP-HPLC and was determined to be a first order reaction (Table 3 and FIGS. 8, 9). As shown (FIGS. 8-9, Table 3), the peptides and protein were released from the conjugates in a slow homogeneous fashion, with t_{1/2} of 9.4 h (PEG₄₀₀₀-FMS-4-nitro-phenethylamine), 13.8 h (PEG₄₀₀₀-FMS-exendin-4), and 11.9 h (PEG₄₀₀₀-FMS-exendin-4). Exendin-4 was fully released from the PEG₄₀₀₀ conjugate after 48 h of incubation.

[0310] The conjugate MAI-FMS-Peptide 27 is 7-NHS negative as the sole α-amino moiety of Peptide 27 was derivatized. A solution of MAI-FMS-Peptide 27 (0.5 mM in 0.1 M NaHCO₃, pH 8.5) was incubated at 37° C. Aliquots (0.2 ml) were withdrawn at different time points and analyzed for the appearance of the free α-amino group using TNBS. The FMS-MAI (N-ethyl maleimide) moiety was hydrolyzed in a slow and nearly homogeneous fashion from the α-amino moiety of the peptide, with a t_{1/2} value of 8.4 h. Hydrolysis was complete after 32 hrs of incubation.

29(iii) PEG₄₀₀₀-FMS-Exendin-4 Facilitates Prolonged Glucose Lowering Action in Mice

[0311] FIGS. 10A-10B show the glucose-lowering profile of subcutaneously administered native exendin-4 and of PEG₄₀₀₀-FMS-exendin-4, both at a dose of 10 µg (FIG. 10A) or 4 µg (FIG. 10B) peptide/mouse relative to a saline-treated group of mice.

[0312] FIG. 10A shows the glucose lowering profile of subcutaneously administered native-exendin-4 and of PEG₄₀₀₀-FMS-exendin-4, both at a dose of 10 µg/mouse. With the native peptide, blood glucose levels declined from 139±10 to 96±7 within 0.5 h reaching a maximal fall in 2-4 hours after administration (74 mg/dl). The return to initial glucose level was then proceeded with a t_{1/2} value of 14.2±1 h. Following the subcutaneous administration of PEG₄₀₀₀-FMS-exendin-4, little decrease in blood glucose level is seen at 0.5 h after administration. Circulating glucose level then fell gradually, with the lowest glucose concentration reached at 6-8 hours after administration (80-90 mg/dl). Stable, low circulating glucose concentrations then maintained over nearly 20 hours, prior to the return to initial glucose levels with a t_{1/2} value of 70±3 hrs.

[0313] FIG. 10B shows the glucose lowering profile of subcutaneously administered native exendin-4 and of PEG₄₀₀₀-FMS-exendin-4, both at a dose of 4 µg/mouse. With the native peptide, blood glucose levels declined by 26-28% (from 140 mg/dl to 102-101 mg/dl), with the largest percent change in blood glucose levels occurring 0.5 h after administration. Glucose concentrations then returned to their initial levels with a t_{1/2} value of 3.7±0.3 h. Following the subcutaneous administration of PEG₄₀₀₀-FMS-exendin-4, the decrease in blood glucose level took place at a more moderate rate. Circulating glucose reached its lowest concentration 8-12 hours after administration (92 mg/dl, 33%). Stable, low circulating glucose concentrations were then maintained

for a further 12 hours. Return to initial glucose levels took place with a $t_{1/2}$ of 30 ± 2 h, being 7.5 times longer than that obtained by the same dose of the native hormone.

[0314] Calculations based on the hydrolysis rates of FMS-exendin-4 (described in U.S. patent application Ser. No. 10/408,262) and PEG₁₀₀₀-FMS-exendin-4 at 37° C. in normal sera and PBS (pH 8.5), respectively, revealed that ~2% of exendin-4 is released from the conjugate each hour in vivo (Shechter et al., 2001). We further hypothesized that this release rate, if combined with prolonged maintenance of the conjugate in the circulatory system, prior to exendin-4 hydrolysis, should yield a long-lasting glucose-lowering signal in mice, as indeed is found following a single subcutaneous administration of PEG₁₀₀₀-FMS-exendin-4 conjugate (FIG. 10B). Irreversibly conjugated PEG₁₀₀₀-exendin-4 has only 4.1% of the activity of native exendin-4 ($IC_{50} = 250 \pm 30$ pmol/mouse versus $IC_{50} = 2.5 \pm 0.24$ pmol/mouse for the native exendin-4, not shown).

Example 30

Biological Activity of PEG₁₀₀₀-FMS-IFN α 2

[0315] Type I interferons (IFNs) are proteins that initiate antiviral and antiproliferative responses. Interferons are clinically important, and several subtypes of IFN α 2 were approved as drugs for the treatment of Hepatitis B and C as well as for cancers such as chronic myelogenous leukemia and hairy cell leukemia. Interferons regulate signals through the Janus tyrosine kinase (JAK/STAT proteins), and by reducing phosphorylation and activation of MEK1 and ERK1/2 through a Ras-Raf independent pathways (Romero et al., 2000). Human type I interferons induce differential cellular effects, but act through a common cell surface receptor complex comprised of the two subunits, Ifnar1 and Ifnar2. Human Ifnar2 binds all type I IFNs, but with a lower affinity and specificity than the Ifnar1 complex. Human Ifnar1 has a low intrinsic binding affinity towards human IFNs, but modulates specificity and affinity of other ligands of the Ifnar complex (Cutrone and Langer, 2001).

[0316] IFN α 2 may be administered intramuscularly, subcutaneously or intravenously, resulting in different pharmacokinetic profiles. In any mode, the administered cytokine is rapidly inactivated by body fluids and tissues (O'Kelly et al., 1985), and cleared from the blood plasma several hours following administration (Rosting et al., 1998). The major routes of IFN α 2 elimination from the circulatory system are through proteolysis, receptor mediated endocytosis and kidney filtration (Goodman and Geman, 2001).

[0317] Prolonging the maintenance dose of IFN α 2 in circulation is a desirable clinical task. A non-reversible, 12 kDa-PEG-IFN α 2 conjugate, has been therapeutically approved in 2001. It is administered once a week to hepatitis C patients, and facilitates a sustained anti-viral response rate of 24%, as opposed to a 12% response rate obtained by the native cytokine (Schering Plough Corporation, 2001 press release; Baker, 2003). However, while the covalent attachment of PEG chains to proteins prolongs their lifetime in vivo, it often results in a dramatic reduction or even loss of biological and pharmacological activities (Pierous and Abuchowski, 1990; Katre, 1993; Bailon and Berthold, 1995; Nacer et al., 1991; Delgado et al., 1992; Fung et al., 1997; Reddy, 2000; Vecenese, 2001). The pegylated formulation of

IFN α 2 currently in use has 7% the activity of the native cytokine, calling for higher doses to be administered (Bailon et al., 2001). Furthermore, PEG-IFN does not readily penetrate all tissues, while 12 kDa PEG-IFN α 2b is widely distributed, 40 kDa -PEG-IFN α 2a is restricted to the blood and the interstitial fluid (Gline et al., 2000; Reddy et al., 2002). This major drawback can be overcome by designing a PEG-IFN α 2 conjugate capable of generating native IFN α 2 at a slow rate under physiological conditions.

[0318] These problems of the prior art can be overcome by the mono- and bis-PEG₁₀₀₀-FMS-IFN α 2 conjugates of the invention, in which IFN- α 2 is linked to the PEG moiety through the FMS moiety that provides the slowly hydrolysable bond. These novel reversibly pegylated-conjugates and their prolonged anti-viral activity in vivo are discussed here in detail.

Materials and Methods

[0319] (i) Materials. Non-glycosylated human IFN- α 2 was prepared as described in WO 02/36067 as previously described by Piehler and Schreiber (1999). Preparation of PEG₁₀₀₀-FMS-IFN α 2 and (PEG₁₀₀₀-FMS)₂-IFN α 2 is described in Examples 21 and 22, respectively.

[0320] (ii) Receptor binding affinities were evaluated by BiAcore (SPR Detection) measurements. The BiAcore 2000 system, sensor chips CM5, HBS (10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% surfactant P20, pH 7.4) and the amine coupling kit were from BiAcore (Sweden). Chip immobilization by Ifnar2 and the BiAcore measurements were carried out according to Piehler and Schreiber (2001). In short, Ifnar2-LC was immobilized to the surface using the non-neutralizing anti Ifnar2-FC (Ifnar2 extra cell. human mAb 4610, followed by cross-linking with a second mAb (117.7) (kindly provided by D. Nowick, Weizmann Institute of Science, Rehovot, Israel). The binding curves were evaluated with the BiAevaluation software (BiAcore AB, Sweden) using a simple one to one kinetic model. Increase in RU (resonance unit) after specific binding to the receptor corresponds to the amount of protein bound to the sensor surface. To estimate the increase in RU resulting from the nonspecific effect of the protein on the bulk refractive index, binding of the protein to a control surface with no immobilized ligand was also measured and subtracted. For the determination of the active interferon concentration the equilibrium response was plotted against the estimated initial concentration. The data were fitted using Kaleidagraph (version 3.0.4, Abelbeck Software) using the equation:

$$R = \frac{1 \times 10^6 \times m1 \times m2}{1 \times 10^6 \times m1 + m2 + 1}$$

[0321] whereby a K_D of 1×10^6 was determined independently for IFN α 2 binding and fixed for all samples, m1 is $R_{\text{un}}/R_{\text{max}}$ (the percent of active interferon measured in the sample), m2 is R_{max} , and m0 is the observed RU.

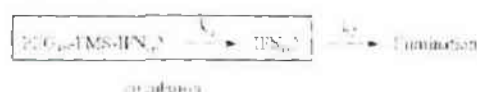
[0322] (iii) In vivo experiments were performed using male Wistar rats (150-170 g). Rats were injected either subcutaneously or intravenously (0.2 ml/rat).

[0323] (iv) Antiviral activity of IFN α 2 and its derivatives was determined by the capacity of the cytokine to protect

human amnion WISH cells against Vesicular Stomatitis Virus (VSV)-induced cytopathic effects (Rubinstein et al., 1981).

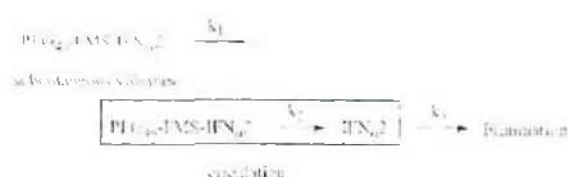
[0324] (v) Simulations of experimental data were performed using Pro-Kineticist 1st, a 2nd Order Global Kinetic Analysis software (Applied Photophysics Ltd., England).

[0325] For the i.v. administration, the following model was considered:



[0326] Whereby, $k_1=0.01 \text{ hr}^{-1}$ and $k_2=0.63 \text{ hr}^{-1}$ (determined experimentally by antiviral assay).

[0327] For the s.c. administration, the following model was considered:



[0328] Whereby, $k_1=0.02 \text{ hr}^{-1}$, $k_2=0.01 \text{ hr}^{-1}$, and $k_3=0.63 \text{ hr}^{-1}$.

[0329] k_3 , which could not be determined directly, was estimated to be 0.63 hr^{-1} from the fit of the simulation to the experimental data.

30(ii) PEG₁₀-FMS-IFN α_2 Has Modified Receptor-Binding Capacity

[0330] The binding capacity of the modified IFN α_2 of the invention toward the immobilized recombinant extracellular part of IFN α_2 receptor (Ifnar2-EC) was monitored under flow-through conditions by an optical probe called reflectometric interference spectroscopy (RIFS). This method detects biomolecular interactions of ligands to transducer-bound proteins as a shift in the interference spectrum caused by change of the apparent optical thickness of the transducer chip. A shift of 1 pm corresponds to approximately 1 pg/mm² protein on the surface. The transducer surface was modified with a dextran layer and carboxylated by reaction with molten glutaric anhydride (Sigma) at 75° for 2-8 h. On such surfaces, electrostatic pre-concentration and covalent immobilization of proteins were carried out by standard BiAcore protocols. After this procedure, Ifnar2-EC was immobilized into a carboxylated dextran layer. All measurements were carried out in 50 mM HEPES (pH 7.4) containing 150 mM NaCl and 0.01% Triton X-100. A sample of 0.8 ml was injected for 80 s with a data acquisition rate of 1 Hz. Flow rates of 50 $\mu\text{L}/\text{min}$ were applied. Under these conditions, the samples in the flow cell were exchanged within one second, allowing the analysis of processes within 5 seconds.

[0331] The results are summarized in Table 1. Monomodified and bis-modified conjugates of PEG₁₀-FMS-IFN α_2 had

9 ± 1 and $0.4 \pm 0.05\%$ the receptor-binding capacity of IFN α_2 , respectively. These conjugated derivatives, however, underwent time-dependent reactivation upon incubation at 37° C. in phosphate-buffer pH 8.5 or in normal human serum (not shown). Reactivation proceeded with a $t_{1/2}$ value of 9.4 and 24.5 hrs for the mono and bis-polylated derivatives, respectively reaching nearly full reactivation following 50 hours of incubation (Table 4).

30(iii) PEG₁₀-FMS-IFN α_2 Releases Native-Active IFN α_2 Upon Incubation at a Rate Constant of 0.01 hr⁻¹

[0332] In the set of experiments summarized in FIG. 11A-C, PEG₁₀-FMS-IFN α_2 was incubated in 0.1 M phosphate buffer in the presence or absence of 0.6% BSA and 2 mM sodium azide (NaN₃, pH 8.5, 37° C.). At this pH value, the rate of FMS hydrolysis from FMS-proteins is nearly identical to that obtained in normal human serum, in vitro or in the circulatory system in vivo (Gierschkov et al., 2000; Saechter et al., 2001 and 2002). Aliquots were drawn at different time points and analyzed for the release of IFN α_2 from the conjugate by SDS-PAGE (FIG. 11A) and by BiAcore, measuring the active concentration of Ifnar2 according to the law of mass action (FIG. 11B). The interferon-binding curve on the Ifnar2 surface resembles that of a homogeneous population of native interferon, suggesting that PEG₁₀-FMS-IFN α_2 does not bind Ifnar2. For the SDS-PAGE analysis, the amounts of IFN α_2 discharged were quantified relative to an IFN α_2 reference of known concentration and intensity. In both cases the discharge profiles are in good agreement. The 10% active interferon observed at time zero in the BiAcore profile is due to native interferon present in the sample. The rate of discharge was determined by fitting the quantity of active interferon to a single exponential equation (FIG. 11C). Accordingly, IFN α_2 is released from the conjugate with a rate constant of 0.01 hr⁻¹ (FIG. 11). Upon 66 hours of incubation, 50% of the IFN α_2 in the conjugate is discharged and is fully active. From the extrapolation of the curve in obtained, it is assumed that nearly all of the interferon will eventually be released and regain full activity. No BiAcore data was collected at very long time points as keeping proteins for weeks at 37° C. is not advisable.

30(iii) Subcutaneous Administration of PEG₁₀-FMS-IFN α_2 Dramatically Increased its Half-life in vivo

[0333] Next, we determined the half-life and activity of PEG₁₀-FMS-IFN α_2 in rats. Human IFN α_2 is not active in rats, albeit its concentration can be determined from the antiviral potency in rat serum by measuring the VSV-induced cytopathic effects in WISH cells (see Methods above, antiviral activity assay). Native IFN α_2 or PEG₁₀-FMS-IFN α_2 were administered subcutaneously to rats. Blood aliquots were drawn at various time points, and analyzed for their antiviral potency. Following the administration of the native unmodified IFN α_2 (100 $\mu\text{g}/\text{rat}$), circulating antiviral activity declined with a $t_{1/2}$ value of ~1 hr reaching a level lower than 20 pM IFN α_2 12 h after administration (FIG. 12).

[0334] The circulatory behavior of PEG₁₀-FMS-IFN α_2 following subcutaneous administration to rats (at doses of 12 $\mu\text{g}/\text{rat}$, 60 $\mu\text{g}/\text{rat}$, or 120 $\mu\text{g}/\text{rat}$) shows a clearly visible

dose-dependent behavior (FIG. 12). Administration of 12 $\mu\text{g}/\text{rat}$ of the conjugated IFN $\alpha 2$ yielded maintenance levels of 70 ± 10 pM IFN $\alpha 2$ which were maintained 56 hours following administration. When a 40-fold increase in PEG $_{40}$ -FMS-IFN $\alpha 2$ was administered, IFN $\alpha 2$ was continuously present in the serum 56 hours at a molar concentration of 450 pM. Administration of 50 $\mu\text{g}/\text{rat}$ of the conjugate resulted in interferon levels of 225 pM at 56 hours, and 25 pM at 200 hours. Native IFN $\alpha 2$ present in the administered sample (approximately 10% as determined by HPLC) contributed to the initially high levels of IFN $\alpha 2$ observed in the rats' serum. These values display a clearance curve similar to that of native IFN $\alpha 2$. The remaining 90% of the IFN $\alpha 2$ were slowly discharged from the conjugate.

30(iv) Intravenous Administration of PEG $_{40}$ -FMS-IFN $\alpha 2$ to Rats

[0335] To eliminate the contribution of the subcutaneous exchange, both the conjugate and the native cytokine were administered to rats (30 $\mu\text{g}/\text{rat}$) intravenously. For native interferon, the same half-life was measured, indicating that it readily penetrates the circulatory system following subcutaneous administration (FIG. 13). For PEG $_{40}$ -FMS-IFN $\alpha 2$, antiviral activity was still detected 150 hours following intravenous administration, demonstrating the prolonged effects of the pegylated cytokine. Discharged IFN $\alpha 2$ level of 10 pM still remained 150 hours post administration, while native IFN $\alpha 2$ was eliminated within 30 hours post-administration. It should be noted that the large shoulder observed following subcutaneous administration of the conjugate (FIG. 12) is not observed when PEG $_{40}$ -FMS-IFN $\alpha 2$ was administered intravenously (FIG. 13).

30(v) Simulations of Experimental Data

[0336] Using the rate constants obtained from both BIAcore data ($k=0.01 \text{ hr}^{-1}$ for the discharge of IFN $\alpha 2$ from the PEG conjugate) and the antiviral activity assay of native IFN $\alpha 2$ ($k=0.65 \text{ hr}^{-1}$ for the elimination of interferon), both the subcutaneous and intravenous administration modes of PEG $_{40}$ -FMS-IFN $\alpha 2$ to rats were simulated (FIGS. 14A and 14B, respectively). In both cases, the simulated data and the experimental results were in good agreement. As became evident from the simulated data, the passage of the conjugate, but not of the native interferon, from the subcutaneous volume to the bloodstream, proceeds at a slow rate and is in the order of the discharge of interferon from the conjugate. This explains the shoulder observed between 10-70 hours in the active protein concentration. As expected, this shoulder is not found when PEG $_{40}$ -FMS-IFN $\alpha 2$ is administered intravenously.

Discussion

[0337] As mentioned before, the pegylating technology applied to therapeutic proteins often leads to a drastic loss in the biological and the pharmacological potencies of the conjugates. In principle, this deficiency can be overcome by introducing the PEG chains via a chemical bond that is either sensitive to hydrolysis or can be cleaved enzymatically by serum proteases or esterases. A prerequisite condition for efficient pegylation is that the hydrolysis of the PEG chains from the conjugate is to take place at a slow rate, and in a homogeneous fashion in vivo.

[0338] Two basic irreversible PEG-IFN $\alpha 2$ conjugates are in therapeutic use at present. The first, a 12 kDa-PEG-

IFN $\alpha 2$, which satisfactorily permeates into tissues. This preparation, however, is a relatively short-lived in vivo, since its low molecular mass (calculated mass=32 kDa) is insufficient to markedly arrest kidney filtration. The second formulation, a 40 kDa-PEG-IFN $\alpha 2$, is an extremely long-lived species in vivo. This conjugate, however, has poor permeability into tissues. Following administration, the conjugate is distributed only in the blood and in the intestinal fluids (Barton et al., 2001). We have, therefore, anticipated that the two prerequisite features for an optimal PEG-IFN $\alpha 2$ conjugate, namely, a prolonged maintenance in vivo combined with free access to peripheral tissues, can be obtained by linking a slowly hydrolyzable PEG $_{40}$ -chain to IFN $\alpha 2$.

[0339] We have previously found that upon linkage of FMS to proteins, the FMS-protein conjugate undergoes hydrolysis at physiological conditions with a desirable pharmacokinetic pattern (Gershonov et al., 2000; Schechter et al., 2001, 2002, 2003). The rate of FMS hydrolysis is dictated exclusively by the pH and the nucleophilicity of the serum, both of which are maintained in mammals under strict homeostasis (Schechter et al., 2001). We therefore based our new development on the FMS principle. In neutral, aqueous solutions, FMS moieties undergo slow, spontaneous hydrolysis, resulting in the regeneration of the native proteins (Schechter et al., 2001). For this purpose, NHS-FMS-MAI was synthesized, enabling us to link salhydroly-containing PEG chains to the amino groups of peptides and proteins via the hydrolyzable FMS function. The working hypothesis was that an inactive PEG-interferon conjugate could regenerate the native protein in its active form in a continuous fashion over a long period of time. The principal monomodified PEG $_{40}$ -FMS-IFN $\alpha 2$ conjugate obtained (Table 2) is devoid of the cytokine binding potency and can therefore be referred to as a "prodrug". Upon incubation, the native cytokine is released by hydrolysis, and the binding potency of IFN $\alpha 2$ to IFN $\alpha 2$ is regenerated with a rate constant of 0.01 hr^{-1} .

[0340] A single subcutaneous administration of PEG $_{40}$ -FMS-IFN $\alpha 2$ significantly prolonged the levels of IFN $\alpha 2$ in the serum of rats. While IFN $\alpha 2$ was short-lived in vivo, having a half-life of ~1 hr, the PEG $_{40}$ -FMS-IFN $\alpha 2$ conjugate exerted its antiviral activity over a period of 200 hours. Furthermore, there is a dose-dependent ratio between the quantity administered and the active interferon levels over a prolonged duration in vivo. This observation is beneficial for optimization of dosing regimens in future clinical use.

[0341] It should be noted that the IFN $\alpha 2$ molecule contains 13 amino functions theoretically available for PEG $_{40}$ -FMS attachment. The exact site of pegylation was not determined. In view however, of its reversibility and the generation of the native protein, it seems that this point deserves a rather minor consideration.

[0342] In summary, following the new conceptual approach for reversible-pegylation of the invention, whereby a pharmacologically "silent" conjugate is "trapped" in the circulatory system and releases the parent protein with a desirable pharmacokinetic profile, we have succeeded in combining prolonged maintenance of IFN $\alpha 2$ in vivo with the release of active-native IFN $\alpha 2$ to ensure access to peripheral tissues.

Example 31

Biological Activity of the PEG_{40} -FMS-PYY $_{1-36}$ Conjugate

[0343] The hypothalamic family of neuropeptide Y (NPY) receptors plays a major role in regulating satiety and food intake (Schwartz, 2000). The putative inhibitory Y2 presynaptic receptor (Y2R) is expressed in the arcuate nucleus, which is accessible to local and peripheral agonists of the NPY family (Broberger et al., 1997; Kalra et al., 1999). One such Y2R agonist is peptide YY $_{1-36}$ (PYY $_{1-36}$), which is released from the gastrointestinal tract post-prandially in proportion to the caloric content of a meal (Pedersen-Bjergaard et al., 1996; Adrian et al., 1985; Grandt et al., 1994). Recently, it was demonstrated that peripheral administration of PYY $_{1-36}$ inhibits food intake in humans, mice and rats and reduces weight gain in rats [Fittner et al., 2002; Batterham et al., 2002, 2003]. Thus, infusion of PYY $_{1-36}$ to reach the normal post-prandial circulatory concentrations of this peptide lead to a peak in serum PYY $_{1-36}$ within 15 min, followed by a rapid decline to normal levels within 30 min. Despite this rapid clearance, administration of PYY $_{1-36}$ to fasting individuals decreases their appetite and reduces food intake by 33% within a 12 h period following PYY $_{1-36}$ administration. Furthermore, no compensatory food intake occurs over the next 12 h (Batterham et al., 2002). Therefore, PYY $_{1-36}$ may find a clinical use in treatment of obesity and its associated disorders, including type II diabetes mellitus and cardiovascular diseases (Schwartz and Morton, 2002).

[0344] The short circulatory half-life of PYY $_{1-36}$ and its potential in management of obesity prompted us to develop a longer-acting form of PYY $_{1-36}$. Indeed, in our hands the satiety induced by PYY $_{1-36}$ in mice lasted for only 2-4 h following subcutaneous (sc) injection. As mentioned before, covalent attachment of PEG to proteins and peptides prolong their half-life in vivo but often leads to a drastic loss of their biological or pharmacological activity. We found that PYY $_{1-36}$, pegylated using standard chemistry, i.e. through formation of a stable bond, indeed lost its biological activity. We have then prepared and test here the biological activity of the PEG_{40} -FMS-PYY $_{1-36}$ pro-drug of the invention.

Materials and Methods

[0345] (i) Animals. C57BL/6J male mice (Harlan Labs) aged 9-11 week (30-35 g body weight) were used. Mice were kept under controlled temperature (21-23° C) and light conditions (light on 6:00-18:00) at the Weizmann Institute of Science (Rehovot, Israel) animal facility. The mice were acclimated for at least one week prior to the initiation of the study. Mice had free access to drinking water at all times during the experiments. All experimental protocols were in accordance with the Israeli regulations of laboratory animal welfare and were approved by the Institutional Animal Committee for Animal Welfare.

[0346] (ii) Reagents. Peptide YY $_{1-36}$ was synthesized by the solid phase method, using a multiple-peptide synthesizer AMS 422 (Abimed Analyser Technik GmbH, Langenfeld, Germany). The resulting peptide was HPLC purified and characterized by MALDI-TOF mass spectrometry (MS) and N-terminal protein micro sequencing. All other reagents were of analytical grade and were purchased from Sigma Chemical Co. (Ness Ziona, Israel).

[0347] (iii) Analytical Procedures. Mass spectra were determined using MALDI-TOF and Electro Spray (ES-MS) techniques (Bruker-Reflex-Reflection model, Germany, and VG-platform-II electrospray single quadrupole mass spectrometer, Micro Mass, UK, respectively). PYY $_{1-36}$, PEG_{40} -FMS-PYY $_{1-36}$ and PEG_{40} were resolved by analytical HPLC (Lichrosorb RP-4 column, 4x250 mm, Merck). Buffer A was 0.1% aq. trifluoroacetic acid (TFA) and Buffer B was 0.1% TFA in 75% aq. Acetonitrile. A gradient of 10-100% Buffer B was used over 30 min. at a flow rate of 1 ml/min. The retention times (Rt) of PYY $_{1-36}$, PEG_{40} -FMS-PYY $_{1-36}$ and PEG_{40} -9-sulfate heptene were 21.53, 35.03 and 44.97 min., respectively. Amino acid analyses were performed following 6N HCl hydrolysis at 110° C. for 24 h using a Dionex Automatic amino acid analyzer EEP1090 (Palo Alto, Calif., USA). N-terminal sequence analyses were performed with a Model 491A Protein Sequencer (Applied Biosystems, Foster City, Calif., USA).

[0348] (iv) Synthetic Procedures

[0349] PEG_{40} -FMS-PYY $_{1-36}$ was prepared as in Example 23.

[0350] PEG_{40} -FMS-Glycine ethyl ester (PEG_{40} -FMS-GEE), to a stirred solution of PEG_{40} -SH (0.25 mM in 0.1M phosphate buffer pH 7.2-10 mM Na ascorbate), 292 μ g MAL-FMS-NHS was added (two-fold molar excess over PEG_{40} -SH). After 7 min, 0.2 ml from a solution of 0.8M glycine ethyl ester was added. The reaction was carried out for 1 h, and the mixture was then dialyzed overnight against water. The resulting PEG_{40} -FMS-glycine ethyl ester was characterized and quantitated by its absorbance at 280 nm (ϵ_{280} =21,200) and by the amount of glycine in the preparation following acid hydrolysis of a 20 μ l aliquot and amino acid analysis.

[0351] PEG_{40} -PYY $_{1-36}$, irreversibly pegylated PYY $_{1-36}$ was prepared by reacting PYY $_{1-36}$ (1 μ g/ml in 0.1 M phosphate buffer pH 7.2) with four equivalents of solid PEG_{40} -OSu (43 μ g). The reaction was carried out for 1 h and the mixture was then dialyzed overnight against water. The conjugate thus obtained contains one PEG_{40} residue per PYY $_{1-36}$, as determined by MALDI-TOF MS (PEG_{40} -OSu, 43,626 D; PYY $_{1-36}$, 4,047 D; conjugate, 47,712 D).

[0352] (v) Food intake measurements: Groups of 10 mice were deprived of food for 24 h and then given excess pre-weighed standard chow for a period of 2 h. Drinking water was provided at all times. The mice received sc injections of either saline (0.1 ml/mouse), native PYY $_{1-36}$, or PYY $_{1-36}$ derivatives at the indicated doses and times within 24 h prior to the start of the 2 h re-feeding period. A minimum interval of 1 h was introduced between injection and re-feeding to avoid a stress-dependent decrease in food intake. The amount of food consumed per group was determined at the end of the feeding period. Remaining chow was weighed after the 2 h re-feeding period and the cumulative food intake per 10 mice was calculated. Values of food intake in replicate experiments were normalized according to the saline controls. Results are expressed as food intake per 10 mice $\pm$ SD from 2-5 replicate experiments.

[0353] (vi) Statistical analysis. The significance of differences in food consumption was determined by the Student's t-test, using the total weight of food consumed by each group of 10 mice as a single value. A saline-injected group

of 10 mice was included in each experiment as a control. A two-tailed, paired t-test was performed between the control and treated groups.

31(b) The effect of PYY₁₋₃₆ on Food Intake in Mice

[0354] To evaluate the duration and magnitude of the effect of PYY₁₋₃₆ and its derivatives on food intake, we employed the mouse re-feeding model of 24 h starvation followed by a re-feeding period of 2 h. Initially we repeated previous studies, where PYY₁₋₃₆ was administered immediately prior to the re-feeding period. However we found that the stress of mere handling the mice had a profound and inconsistent effect on food intake, thereby reducing the difference between saline and PYY₁₋₃₆. We then injected the mice 1 h prior to start of the re-feeding period and obtained a much more consistent difference between PYY₁₋₃₆ and saline. FIG. 15 shows that PYY₁₋₃₆ inhibited food intake in a dose-dependent manner. Mice receiving saline or PYY₁₋₃₆ at a dose of 5 nmol/mouse consumed 10.7±1.26 and 5.26±1.47 g chow per 10 mice, respectively ($P<0.001$, $N=8$). These figures correspond to a 50% decrease in food intake by the PYY₁₋₃₆ groups as compared with the control saline groups. Inhibition was statistically significant at all doses used, including the lowest dose of 0.2 nmol/mouse (0.8 µg/mouse, $P<0.05$, $N=3$). The dose-response corresponded to a half-maximal effect of PYY₁₋₃₆ at about 0.5 nmol/mouse (FIG. 15).

[0355] We then determined the duration of the satiety induced by sc administration of PYY₁₋₃₆ (5 nmol/mouse) at different times prior to the re-feeding period. The satiety induced by PYY₁₋₃₆ was maximal when given 1 h prior to re-feeding and rapidly decreased when PYY₁₋₃₆ was administered at earlier times. The half-life of its biological response was about 3 h and no effect was seen with 5 nmol PYY₁₋₃₆ when administered nearly 10 h prior to the re-feeding period (FIG. 16).

[0356] Intraperitoneal (rather than subcutaneous) administration of PYY₁₋₃₆ to mice has induced a considerably shorter-lived satiety effect. For example, an ip dose of 5 nmol PYY₁₋₃₆/mouse was fully effective when administered 30 min before re-feeding, but had no effect when administered 2 h before meal (not shown).

[0357] We then attempted to extend the biological activity of PYY₁₋₃₆ by conventional pegylation. Thus, mono-pegylated PYY₁₋₃₆ was prepared (see Materials and Methods above) and tested for its biological activity by sc administration (5 nmol/mouse) 1 h prior to the re-feeding period. No significant reduction in food intake was obtained as compared with the saline control, indicating that conventional pegylation, which results in a stable pegylated peptide, abolished the biological activity of PYY₁₋₃₆ (FIG. 17).

[0358] We then tested if the inactivation of PYY₁₋₃₆ was due to the bulkiness of the PEG group or due to blocking of the amine residues of PYY₁₋₃₆. Acetylation of the two amine groups of PYY₁₋₃₆ largely abolished the effect of PYY₁₋₃₆ in inducing satiety *in vivo*. Thus, mice injected with 20 µg NaAc diacetyl PYY₁₋₃₆ per mouse ate 10.54±0.08 g chow per 10 mice, as compared with 10.6±1.26 g in the saline group and 5.26±1.47 g in the PYY₁₋₃₆ group. Hence, even attachment of a small acetyl group was sufficient to disrupt the biological activity of PYY₁₋₃₆.

31(c) Preparation and Characterization of PEG₄₀-FMS-PYY₁₋₃₆

[0359] The lack of biological activity following pegylation or even acetylation of PYY₁₋₃₆ prompted us to prepare the PEG₄₀-FMS-PYY₁₋₃₆ conjugate described in Example 23 hereinabove. This product was analyzed by mass spectrometry to determine the ratio of PEG₄₀ to PYY₁₋₃₆ by MALDI-TOF MS analyses. PYY₁₋₃₆ exhibited a molecular mass of 4047.51 D (calc. 4049.6 D). PEG₄₀-SH had an average mass of 43.626 D, and the conjugate yielded a major peak corresponding to a molecular mass of 47.712.6 D (not shown). The calculated mass of the 1:1 conjugate of PEG₄₀-SH and MAL-FMS-PYY₁₋₃₆ is 48.087 D. Thus the main product corresponds to such a 1:1 conjugate.

31(d) PEG₄₀-FMS is Linked to the ϵ -Amino Group of PYY₁₋₃₆

[0360] Peptide YY₁₋₃₆ contains the N-terminal α -amino group of isoleucine and one ϵ -amino group of lysine at position 2, both of which are potentially available for acylation by MAL-FMS-OSu. To determine the site(s) of acylation, PEG₄₀-FMS-PYY₁₋₃₆ was reacted with a 500 molar excess of acetic anhydride at pH 7.0 for 1 h, followed by dialysis against water overnight. The PEG₄₀-FMS group was then removed by incubating the acetylated PEG₄₀-FMS-PYY₁₋₃₆ for 4 days at pH 8.5. The product was then subjected to N-terminal protein sequence analysis. As shown in FIG. 18, cycles 1, 2 and 3 yielded the expected amounts of isoleucine, ϵ -acetyl lysine and proline, respectively. No free lysine was found in cycle 2. Thus, the conjugate consists of PEG₄₀-FMS and PYY₁₋₃₆ at a 1:1 ratio. Furthermore, based on the sequencing yields, the PEG₄₀-FMS group is primarily linked to the N-terminal ϵ -amino group of PYY₁₋₃₆. Nevertheless, this analysis could not entirely exclude the presence of a PYY₁₋₃₆ mono-substituted at its ϵ -lysine side chain. However, in view of the complete regeneration of the native peptide, this point is of a rather minor significance.

31(e) PEG₄₀-FMS-PYY₁₋₃₆ hydrolyses to Yield PYY₁₋₃₆ Under Physiological Conditions

[0361] To evaluate the rate of PYY₁₋₃₆ release from PEG₄₀-FMS-PYY₁₋₃₆, we incubated the conjugate in phosphate buffer (pH 8.5, 0.1 M, 37°C). Aliquots were withdrawn at different times and subjected to analytical HPLC using eluting conditions, which resolve PEG₄₀-FMS-PYY₁₋₃₆ from PYY₁₋₃₆. At pH 8.5, the rate of FMS hydrolysis from FMS-peptides or proteins is nearly identical to that obtained in normal human serum (Shechter et al., 2001). As shown in FIG. 19, PYY₁₋₃₆ was released from the conjugate in a slow, nearly homogeneous fashion with a half-life of 5.3 h. After 40 h, the cumulative amount of free PYY₁₋₃₆ reached 76% of the input PEG₄₀-FMS-PYY₁₋₃₆.

31(f) Hydrolysis of PEG₄₀-FMS-PYY₁₋₃₆ in Normal Mouse Serum

[0362] We found that PYY₁₋₃₆ undergoes rapid proteolysis in normal mouse serum at 37°C, with a half-life of 3±0.7 min. (FIG. 20, insert). Therefore, the rate of PEG₄₀-FMS-PYY₁₋₃₆ hydrolysis was evaluated by the amount of 2-PLA₁₀₀-9-amino-fluorene released during hydrolysis. PEG₄₀-FMS-PYY₁₋₃₆ was incubated in normal mouse serum at 37°C. Aliquots were withdrawn at different times, ethanol



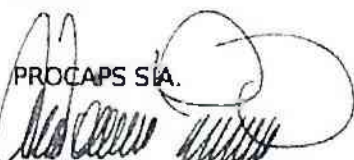
PODER ESPECIAL

Yo, **WALTER TANAKA TATEKAWA**, mayor de edad y vecino de la ciudad de Barranquilla (Colombia), identificado como aparece al pie de mi firma, quien en el presente documento actúa en calidad de Representante Legal de la sociedad **PROCAPS S.A.** sociedad legalmente constituida y domiciliada en la ciudad de Barranquilla (Colombia), por el presente documento otorgo a la doctora **ELIANA ISAURA MORENO BOHORQUEZ**, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 del C.S. de la J., poder especial, amplio y suficiente, para formular oposiciones u observaciones a la solicitud de patente No. 13 - 301132, titulada "AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1 DE ACCIÓN PROLONGADA", cuyo titular es **PROLOR BIOTECH INC.** Publicada en la gaceta No. 699 del 26 de junio de 2014.

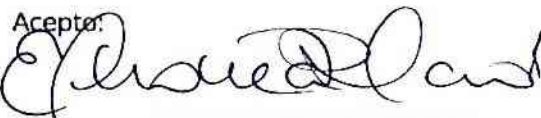
Nuestro apoderado queda facultado, de manera expresa, para realizar todo tipo de actos necesarios para llevar a cabo el trámite de la oposición, incluyendo, sin limitación, preparar y presentar solicitudes, hacer declaraciones, pagar tasas, contribuciones e impuestos, recabar los títulos o certificados. Asimismo quedan autorizados para impugnar administrativamente todo lo que fuere resuelto en el expediente a que se hace expresa referencia en el presente poder, con todas las facultades generales y específicas que fuera requerido para ella.

De la misma manera, además de las facultades que le confiere la naturaleza del presente mandato, se encuentran facultados para recibir, desistir, transigir, sustituir, y reasumir el presente poder, incoar acciones, interponer recursos y demás facultades conferidas por la ley.

Dado y Firmado en Barranquilla (Colombia) a los 19 días del mes de Septiembre de 2014.


PROCAPS S.A.

et
C.C. N° 16'251.923
NIT: 890.106.527-5
Representante Legal

Acepto:


ELIANA ISAURA MORENO BOHORQUEZ
C.C. 51.975.664
T.P. 83823 del C.S.J

Señor Director,
 DIRECCIÓN DE NUEVAS CREACIONES
 Superintendencia de Industria y Comercio
 E. S. D.

Referencia : Expediente N° 13 - 301132
 Asunto : Oposición a la solicitud de patente de Invención titulada
 "AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1
 DE ACCIÓN PROLONGADA"
 Solicitante : OPKO BIOLOGICS LTD.,
 Opositora : PROCAPS S.A.

Publicada en la Gaceta de la Propiedad Industrial
 N° 698 del 27 de Junio de 2014

Yo, *ELIANA ISAURA MORENO BOHÓRQUEZ*, abogada en ejercicio, identificada como aparece al pie de su firma, con tarjeta profesional No. 83823 del Consejo Superior de la Judicatura, en mi condición de apoderada de la sociedad PROCAPS S.A. conformada bajo las leyes de la República de Colombia y domiciliada en la ciudad de Barranquilla, Colombia, atentamente manifiesto que estando dentro del término de ley y por orden expresa de mi representada, presento oposición a la concesión del registro de la Patente de Invención titulada "AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1 DE ACCIÓN PROLONGADA", cuyo titular OPKO BIOLOGICS LTD., y pido a Usted declarar fundada esta oposición y en consecuencia negar el registro de la patente solicitada.

1. INTERÉS PARA ACTUAR

Conforme a lo dispuesto en el artículo 42 de la Decisión 486 de 2000 de la CAN, estando dentro del término estipulado por la ley, manifiesto el legítimo interés de nuestra representada para oponerse al registro de la patente de la referencia, atendiendo a que la solicitud pretendida no cumple con los requisitos dispuestos en la ley como se analizará más adelante y de ser concedida afectaría directamente la

comercialización de algunos productos de mi representada, que contienen, en particular *AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1*. Por lo tanto, solicito respetuosamente a ese Despacho se sirva tener en cuenta la presente oposición dentro del trámite administrativo que se surte en esta Superintendencia.

2. FUNDAMENTOS DE HECHOS

- 2.1. La solicitud objeto de la presente fue presentada el 27 de diciembre de 2013, reivindicando prioridad bajo las solicitudes US201101492448P del 2011-06-02; y, US201261624589P del 2012-04-16; y fue publicada en Colombia, en la Gaceta de la Propiedad Industrial N° 698 del 27 de Junio de 2014, cuya publicación internacional corresponde a la WO2012167251 del 2012-12-06.
- 2.2. La solicitud colombiana 13 - 301132 objeto de la presente oposición se refiere a agonistas del receptor de glucagón/GLP-1 pegilado y pegilado inverso que incluye composiciones farmacéuticas que los comprenden y métodos para su uso.
- 2.3. En primera instancia, se debe advertir que las reivindicaciones se refieren también a métodos de tratamiento y no sólo a la composición farmacológica o a un método de fabricación mediante la cual se obtenga un producto tangible y según la legislación, decisión 486 de la Comunidad Andina de Naciones, Artículo 14: “Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento (...)”, los usos no están contemplados como patentables; y, Artículo 20: “No serán patentables (...) d) los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales”. Solo serán patentables los productos o procedimientos. Por lo tanto a luz de la norma, dicha materia referente métodos de tratamiento no sería patentable.
- 2.4. Por otro lado, es de resaltar que en materia de unidad de invención, la presente comprende varios grupos de invenciones que no conforman una única unidad inventiva, contrario a lo que dicta la Norma. Esto es:

Grupo I: Reivindicaciones 1-6: Composición que consiste en oxintomodulina, un polímero de polietilenglicol (PEG polímero), y 9 fluorenilmetoxicarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxicarbonilo (FMS).

Grupo II: Reivindicaciones 7-11: Método para prolongar la vida media biológica de oxintomodulina que consiste en la etapa de conjugación de la

oxintomodulina, un polímero de polietilenglicol (PEG polímero) y 9-fluorenylmethoxycarbonyl (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS) en una relación molar de aproximadamente 1:1:0,5 a 1:1:3,5.

Grupo III: Reivindicaciones 12-16: Procedimiento de inducción de tolerancia a la glucosa, control de la glucemia, en tanto en un sujeto en necesidad del mismo, que comprende la etapa de administrar a dicho sujeto una cantidad eficaz de la composición de la reivindicación 1 y un producto farmacéutico aceptable portador.

Grupo IV: Reivindicaciones 17-26: Método para mejorar el área bajo la curva (AUC) de oxintomodulina que incluye un método de reducir la frecuencia de dosificación de oxintomodulina, que consiste en la etapa de conjugado de un polímero de polietilenglicol (PEG polímero) a la amino terminal de dicho oxintomodulina a través de 9-fluorenilmetoxycarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS).

Grupos V: Reivindicaciones 27-31: Método para reducir la ingesta de alimentos, reducir el peso corporal, o ambas en un sujeto, que comprende la etapa de administrar oxintomodulina conjugado con polímero de polietilenglicol (PEG polímero) a través de un enlazador flexible, en donde dicho flexible enlazador es 9-fluorenilmetoxycarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS).

Grupo VI: Reivindicaciones 32-39: Método para aumentar la sensibilidad a la insulina en un sujeto, que comprende la etapa de administrar al sujeto una cantidad eficaz de una composición que comprende oxintomodulina conjugado con polímero de polietilenglicol (PEG polímero).

Las invenciones que figuran como los Grupos I a VI no se refieren a un único concepto inventivo general conforme la Norma, Decisión 486 de la CAN, artículo 25: *“La solicitud de patente sólo podrá comprender una invención o un grupo de invenciones relacionadas entre sí, de manera que conformen un único concepto inventivo”*. Estos grupos inventivos carecen de las mismas o correspondientes características técnicas especiales para considerarse como un único concepto inventivo. Por tanto no se ajustan a lo que dicta la Norma en Colombia.

2.5. Ahora bien, con base en el artículo 14 de la Decisión 486 de la CAN consagra lo siguiente: *“Los países miembros otorgan patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel*

inventivo y sean susceptibles de aplicación industrial". Así las cosas, las invenciones objeto de patente deben ser nuevas y tener nivel inventivo. Bien, pues antes de la fecha de prioridad de esta solicitud se encuentran los siguientes documentos que afecta la patentabilidad de la presente solicitud:

- D1: US2010144617 publicada el 10 de octubre de 2010.
- D2: US20060171920 publicada el 3 de agosto de 2006.

2.6. Ahora bien, el documento D1, se refiere en el párrafo [0001] a derivados de oxintomodulina, su síntesis y su uso para el tratamiento de trastornos metabólicos como la diabetes y la obesidad. Particularmente, en el párrafo [0071] esta anterioridad D1 se refiere a derivados de OXM modificados. Según se informa, los derivados de OXM son desarrollados por PEGilación o conjugación a otros restos o proteínas portadoras para mejorar la estabilidad y la farmacocinética y/o mediante la incorporación de sustituciones de residuos de aminoácidos para hacer que los péptidos resistentes a la escisión DP-IV. Además, según D1, los derivados de OXM estabilizadas no exhiben actividad agonista del receptor de glucagón y de ese modo pueden ofrecer ciertas ventajas en el tratamiento de la hiperglucemia y la obesidad en los sujetos diabéticos o prediabéticos. En el párrafo [0127], D1 contempla el uso de derivados poliméricos multi-funcionales, como se ejemplifica por los derivados de PEG bifuncional N-maleimidilo y multiarm. Una amplia variedad de (PEG) especies de glicol polietileno puede ser utilizado según D1, para la PEGilación de los nuevos derivados de OXM. Igualmente se afirma que sustancialmente cualquier reactivo de PEG adecuado puede ser utilizado pero no se limitan a las que estén disponibles para la venta. D1 también define que los reactivos de PEG se prefieren a menudo en diversas realizaciones: multi-Arm PEG, mPEG(MAL)2, mPEG2(MAL), cualquiera de los SUNBRIGHT PEG activado (incluyendo pero no limitado a carboxilo-PEG, p-NP-PEG, Tresyl-PEG, PEG aldehído, acetal-PEG, amino-PEG, PEG-tiol, maleimido-PEG, hidroxilo-PEG-amina, amino-PEG-COOH, hidroxilo-PEG-aldehído, anhídrido carboxílico de tipo PEG, PEG funcionalizado (fosfolípido) y otros PEG reactivos similares y/o adecuados como seleccionados. Adicionalmente, en el párrafo [0128] D1 agrega que los péptidos derivados OXM también pueden contener dos restos de PEG que están unidos covalentemente a través de un carbamato o un enlace amida a un resto espaciador, en el que el resto

espaciador está unido covalentemente al enlazador de amida terciaria del péptido. Cada uno de los dos restos de PEG usados en tales formas de realización de D1 puede ser lineal y puede ser unidas entre sí en un solo punto de unión. D1 define cada resto de PEG que tiene un peso molecular de aproximadamente 10 kilodaltons (10K) a aproximadamente 60K. Cada uno de los dos restos de PEG puede tener un peso molecular de aproximadamente 20K a aproximadamente 40K. Un experto en la técnica será capaz de seleccionar el tamaño del polímero deseado basándose en consideraciones tales como la dosificación deseada; tiempo de circulación; resistencia a la proteólisis; efectos, si los hay, sobre la actividad biológica; facilidad de manejo; grado o falta de antigenicidad y otros efectos de la PEG conocida en un péptido terapéutico.

- 2.7. El documento D2, define una composición que consiste en una proteína, un polímero de polietilenglicol (PEG polímero) y 9 fluorenilmetoxicarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxicarbonilo (FMS), en el que dicha composición se logra mediante la conjugación de un polímero de polietilenglicol (polímero de PEG) al extremo amino terminal de la proteína a través de un enlazador flexible, tal como 9-fluorenilmetoxicarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxicarbonilo (FMS). En fármacos pegilados, el resto de PEG y el residuo de la droga no son vinculadas directamente entre sí, sino más bien ambos residuos están vinculados a diferentes posiciones de la estructura Fmoc o FMS que es altamente sensible a bases y es extraíble en condiciones fisiológicas. Igualmente se define en D2, que los fármacos son preferiblemente fármacos que contienen un grupo amino, lo más preferiblemente péptidos y proteínas de bajo o medio peso en el párrafo [0066] por lo que el resto de PEG y el residuo de drogas no están ligados directamente entre sí, como en los procedimientos de pegilación estándar, sino que ambos residuos están vinculados a diferentes posiciones de una estructura que son muy sensibles a bases y es extraíble en condiciones fisiológicas según los párrafos [0359] a [0360]. Así, basándose en los rendimientos de secuenciación, el grupo PEG.sub.40-FMS está asociado principalmente a la alfa N-terminal alpha-amino grupo de PYY.sub.3-36.
- 2.8. Así las cosas, un experto en la técnica encontraría que es obvio el uso de dicho enlazador flexible FMS o Fmoc para modificar el conjugado de PEG oxintomodulina divulgado en D1 de modo que las principales deficiencias de la tecnología de proteínas de pegilación, principalmente la pérdida de potencias biológicas y farmacológicas en los conjugados de PEG in vivo, pueden ser

superados, como se describe por D2 (párrafo [0015]) y llegar así de manera obvia al objeto de la presente solicitud. Por lo tanto, este documento objeto de oposición a la luz del documento D1 carece evidentemente de ALTURA INVENTIVA.

2.9. En consecuencia y de acuerdo a lo descrito anteriormente, se deriva que la solicitud colombiana 15-301152 no puede pretender un monopolio por patente toda vez que lo que pretende no cumple con dos de los tres requisitos necesarios para su protección.

2.10. Que por consiguiente, respecto a la solicitud pretendida se tiene que:

2.8.1 CARENCIA DE ALTURA INVENTIVA. Según la Decisión 486 de la Comunidad Andina de Naciones, Artículo 18: *"Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica."* A la fecha de prioridad, 2 de junio de 2011, a partir del documento D1, un experto en la materia puede llegar de manera evidente al uso de dicho enlazador flexible FMS o Fmoc para modificar el conjugado de PEG oxintomodulina de modo que las principales deficiencias de la tecnología de proteínas de pegilación, principalmente la pérdida de potencias biológicas y farmacológicas en los conjugados de PEG in vivo, como se describe por D2 (párrafo [0015]) y llegar a una composición que consiste en oxintomodulina, un polímero de polietilenglicol (PEG polímero) y 9-fluorenilmetoxycarbonilo (Fmoc) o 2-sulfo-9-fluorenilmetoxycarbonilo (FMS), sobre lo cual busca protección en el capítulo reivindicatorio.

2.8.2 CARENCIA DE APLICACIÓN INDUSTRIAL. La Decisión 486 de la CAN consagra en su artículo 14 lo siguiente: *"Los países miembros otorgarán patentes para las invenciones, sean de producto o procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial"*. La presente solicitud carece de aplicación Industrial ya que como se demostró anteriormente, algunas cláusulas reclaman un método de tratamiento y estos no son aplicables industrialmente porque primero no se obtiene un producto o proceso; y segundo no es

reproducible o repetible a escala industrial. Por lo tanto, el objeto de la presente invención referido a métodos de tratamiento, no cumple con el requisito de la aplicación industrial.

2.9 Dado lo anterior, es posible deducir que la solicitud y lo reivindicado no es objeto de patentabilidad pues es contrario a lo que dicta la Decisión 486 de la Comunidad Andina de Naciones, Artículo 14: *"Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial."* La solicitud pretendida no cumple con dos de los tres requisitos de patentabilidad.

2.10 Por todo lo anterior, atentamente solicito a usted que se sirva declarar fundada *"AGONISTAS DEL RECEPTOR DE GLUCAGÓN/GLP-1 DE ACCIÓN PROLONGADA"*, cuyo titular OPKO BIOLOGICS LTD., solicitud Colombiana que apareció publicada en la Gaceta de la Propiedad Industrial N° 698 del 27 de Junio de 2014.

3. PRUEBAS

De conformidad con lo dispuesto por las normas vigentes, solicito a usted tener como prueba en este asunto los siguientes:

- D1: US2010144617 publicada el 10 de octubre de 2010.
- D2: US20060171920 publicada el 3 de agosto de 2006.

4. FUNDAMENTO DE DERECHO

Fundamento esta observación en las normas legales siguientes:

- 4.1. Artículo 45 de la Decisión 486 de la Comisión del Acuerdo de Cartagena.
- 4.2. En las demás normas concordantes.

5. ANEXOS

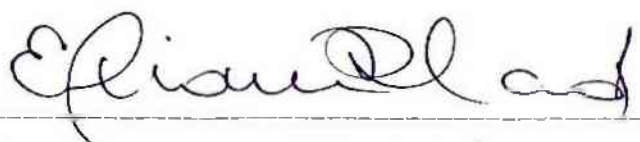
Para los efectos correspondientes anexo al presente escrito los siguientes documentos:

1. Poder para actuar en el presente.
2. Documento que acredita al representante legal de PROCAIS S.A.
3. Recibo de caja del respectivo pago de tasa.
4. Copias de los documentos:
 - D1: US2010144617 publicada el 10 de octubre de 2010.
 - D2: US20060171920 publicada el 3 de agosto de 2006.

6. NOTIFICACIONES

Para efectos de notificaciones que debe hacer su Despacho, informo que mi representada y la suscrita recibiremos notificaciones en la Secretaría de su Despacho o en mis oficinas situadas en la Carrera 13 N° 119-95, Of. 104 de esta ciudad de Bogotá.

Señor director,



ELIANA ISAURA MORENO BOHÓRQUEZ

C.C. 51'975.664 de Bogotá

T.P.A. 83823 del C.S. de la J.