

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

2020
Bogotá, D.C.,

Abogado
EMILIO FERRERO

ASUNTO	Radicación	07 078 139
	Trámite	011
	Evento	378
	Actuación	519
	Oficio	53
	Folios	53

1 06 6 4

Patente de Invención



Patente de Modelo de Utilidad

Vía Nacional

Vía PCT (Fase Nacional)



Capítulo I



Capítulo II

No. Solicitud Internacional

PCT/EP2006/050551

Fecha de Presentación Internacional

31/Enero/2006

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

1. Documentos estudiados

Descripción:	Folios 58 a 139	31/Julio/2007
Reivindicaciones:	Folios 140 a 148	31/Julio/2007
Dibujos:	Folios 150 a 173	31/Julio/2007
Prioridad:	NL 05100688.0	01/Febrero/2005

2. Análisis de la prioridad

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

3. Objeto de la invención

Título de la solicitud: "Conjugados de un polipéptido y un oligosacárido"

El problema planteado en esta solicitud consiste en la necesidad de proporcionar conjugados de proteína que tengan propiedades farmacocinéticas mejoradas.

Para resolver este problema técnico la solicitud en estudio proporciona conjugados de proteína con pentasacáridos sulfatados que mejoren las propiedades farmacocinéticas con respecto al polipéptido sin conjugar.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 47/48

4. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 D 486)

El uso de las reivindicaciones 25 y 26 no es patentable, de acuerdo con el Art 14.

5. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Expresiones como: "al menos" (Reivindicaciones 1, 19, 27), y "residuo ligado flexible inactivo farmacológicamente esencial" (Reivindicaciones 1, 6, 7, 17, 27, y 30) le restan claridad y concisión al capítulo reivindicatorio por cuanto son términos ambiguos y no permiten definir la invención.

La reivindicación 1 no es clara porque no define con exactitud el péptido, el espaciador y el oligosacárido que hacen parte del conjugado en mención. Se solicita definir dichos componentes específicamente de manera que dicho conjugado se pueda diferenciar del estado del arte, esto, teniendo en cuenta el soporte presentado dentro del capítulo descriptivo.

En las reivindicaciones 2, 3, 28, 29, 31 y 32 es necesario definir a que monosacárido de 4-6 unidades y a que pentasacárido está haciendo referencia respectivamente.

En las reivindicaciones 4 y 5 se está definiendo el conjugado mediante el resultado a alcanzar, se sugiere definirlo mediante sus características técnicas.

En las reivindicaciones 6- 8 se define el residuo espaciador-oligosacárido, pero las definiciones que se dan para R, Ra y Rb no permiten diferenciar la invención del estado del arte, ya que para cada uno se presentan definiciones poco específicas como alcoxi, monosacárido, oligosacárido y otras ambiguas como: "un residuo ligado flexible inactivo farmacológicamente esencial", se solicita definir exactamente y de manera clara la composición de dicho residuo espaciador oligosacárido y además definir los contraiones positivos a los que está haciendo referencia, teniendo en cuenta las modalidades realizadas en los ejemplos, en donde X= contraión = Na+. Así mismo, las reivindicaciones 16-19 muestran que el polipéptido esta monosustituido por un residuo espaciador-pentasacárido pero no lo definen claramente, el mismo argumento anterior se aplica también a esta reivindicaciones.

Las reivindicaciones 11-13 definen el polipéptido del conjugado de la reivindicación 1 mediante un inespecífico rango de peso molecular y por ende cualquier polipéptido podría cumplir el requisito para estar dentro de esta definición, por lo tanto se requiere que el polipéptido quede claramente definido mediante sus características técnicas, de manera que no haya lugar a confusión.

La reivindicación 24 presenta una composición farmacéutica que comprende el conjugado de la reivindicación 1, es necesario que dicha composición sea caracterizada a partir de los elementos que la componen y las proporciones de los mismos, de acuerdo con la información sustentada en la descripción.

Es necesario que los procesos de las reivindicaciones 27 y 30 se presenten como una serie consecutiva de pasos, especificando condiciones de reacción: solventes, temperaturas, tiempos de reacción, así como los reactivos de partida, cantidades, etc. aspectos que hacen que el proceso quede definido mediante sus características técnicas.

6. Unidad de invención (Art 25 D 486)

Mediante la presente solicitud, se plantea una solución al problema técnico planteado y consiste en la conjugación de un péptido con un oligosacárido, sin embargo ya es conocido en el estado de la técnica que la conjugación de diferentes moléculas con oligosacáridos sulfatados incrementa su permanencia en el organismo debido a la afinidad de estos últimos con AT-III, (documentos D1, y D2) y que la conjugación con pentasacáridos incrementa el tiempo de vida media de algunos polipéptidos como por ejemplo la insulina (documentos D3, D4 y D5). Por lo tanto, esta característica técnica no se puede usar como un único concepto inventivo común a todos los grupos, y es por esta razón que cada uno de los grupos mencionados a continuación se consideran como invenciones separadas.

Los Grupos Inventivos están definidos por las siguientes características esenciales:

- 1° Un conjugado que comprende un oligosacárido sulfatado y un polipéptido: insulina, y el proceso para su preparación.
- 2° Un conjugado que comprende un oligosacárido sulfatado y un polipéptido: ganirelix, y el proceso para su preparación.
- 3° Un conjugado que comprende un oligosacárido sulfatado y un polipéptido: [D-Ala⁸]-GLP-1(7-36), y el proceso para su preparación.
- 4° Un conjugado que comprende un oligosacárido sulfatado y un polipéptido: ADM(27-52), y el proceso para su preparación.
- 5° Un conjugado que comprende un oligosacárido sulfatado y un polipéptido: octreotida, y el proceso para su preparación.
- 6° Un conjugado que comprende un oligosacárido sulfatado y un polipéptido: Interleucina 2, y el proceso para su preparación.

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 6 grupos inventivos.

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

7. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: Febrero 01 de 2005 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	FEBS LETTERS, 1989, vol. 248, N° 1-2, páginas 111-114.	O- mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in <i>Saccharomyces cerevisiae</i>	Mayo/1989
D2	CARBOHYDRATE RESEARCH, 1995, vol. 272, N° 1, páginas 9-16.	Synthesis of the saccharide moiety of galactosylgloboside (SSEA-3) and its conjugation to bovine serum albumin and sepharose.	1995
D3	ADVANCED DRUG DELIVERY REVIEWS, 1999 NETHERLANDS, vol 35, N° 2-3, páginas 289-306.	Site-specific insulin conjugates with enhanced stability and extended action profile.	1999
D4	JOURNAL OF THROMBOSIS AND HAEMOSTASIS, BLACKWELL PUBLISHING OXFORD, 2003, vol 1, N° 9, páginas 1945-1954.	Antithrombotic properties of a direct thrombin inhibitor with a prolonged half-life and AT-mediated factor XA inhibitory activity.	Septiembre/2003
D5	BIOCONJUGATE CHEMISTRY, 1998, vol. 9, N° 2, páginas 176-183.	Extending insulin action in vivo by conjugation to carboxymethyl dextran.	Marzo 1998
D6	BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD GB, 1999, vol. 9 N° 14, páginas 2013-2018.	Design and synthesis of a novel synthetic NAPAP-penta-saccharide conjugate displaying a dual antithrombotic action.	19/Julio/1999
D7	ADVANCED DRUG DELIVERY REVIEWS, 2002, vol. 54, páginas 505-530	Effects of PEG conjugation on insulin properties.	2002
D8	US6486129B1	Antithrombotic compounds	26/Noviembre/2002

D1
Divulga (Pág. 111, 2da columna) un conjugado de IGF-I con un oligosacárido constituido principalmente por manosa.

D2
Divulga un conjugado de un pentasacárido: galactosilglobósido (SSEA-3) (página 11, compuesto 10) con albúmina sérica bovina (BSA) (Pág. 15, párrafo 2)

D3
Divulga un conjugado de Insulina monoglicosilada (Pág. 293, fig. 1).

D4
Divulga un incremento del tiempo de vida media de una molécula de un inhibidor de trombina al conjugarla con el pentasacárido usado en la presente solicitud (Pág. 1945, abstract, figuras 1-3, tablas 1-5).

D5
Divulga un conjugado de Insulina con carboximetil dextrano (CMD) el cual incrementa el tiempo de vida media comparado con la insulina sin conjuguar (Pág. 176, abstract).

D6
Divulga un conjugado de NAPAD con un pentasacárido, el cual tiene un tiempo de vida media incrementado con respecto a NAPAD solo. (Pág. 2013, abstract, Pág. 2014, fig. 1).

D7
Divulga un conjugado de Insulina con polietilenglicol (PEG) el cual incrementa el tiempo de vida media, y aumenta la resistencia al ataque proteolítico comparado con la insulina sin conjuguar (Pág. 506, 2da columna, Pág. 525, 2da columna).

D8
http://worldwide.espacenet.com/publicationDetails/biblio?DB=EPODOC&II=0&ND=3&adjacent=true&locale=en_EP&FT=D&date=20021126&CC=US&NR=6486129B1&KC=B1
Divulga un compuesto de fórmula 1, el cual es utilizado como agente antitrombótico (abstract y Compuestos IV, V y VI).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en un conjugado que comprende las siguientes características técnicas:

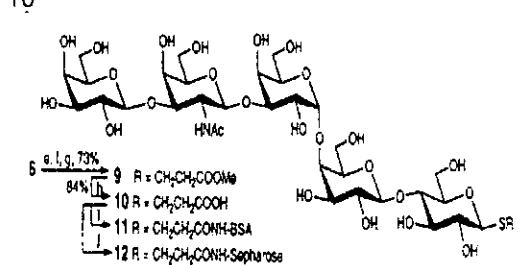
Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (D1):

Características esenciales	D1
Conjugado que comprende:	Conjugado que comprende:
Un polipéptido	Un polipéptido: Factor de crecimiento insulínico tipo I.
Un espaciador que puede ser un enlace o un residuo farmacológicamente inactivo	Un enlace
Un oligosacárido de 4-18 unidades de monosacárido y que tiene afinidad con Antitrombina III	tetrasacárido Manα1-3Manα1-2Manα1-2 Man.

Las características **esenciales** del conjugado de la reivindicación 1 habían sido divulgadas previamente en D1, el cual revela un conjugado de factor de crecimiento insulínico tipo I (Pág. 111, 2da columna) con un tetrasacárido Man α 1-3Man α 1-2Man α 1-2 Man (Pág. 114, 1ra columna) cuyas características técnicas se muestran en el cuadro anterior, las cuales se traslapan con las divulgadas en la solicitud en estudio.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica (D2):

Características esenciales	D2
Conjugado que comprende:	Conjugado que comprende (página 11, compuesto 11):
Un polipéptido	Una proteína: albúmina sérica bovina (BSA)
Un espaciador que puede ser un enlace o un residuo farmacológicamente inactivo	Un enlace
Un oligosacárido de 4-18 unidades de monosacárido y que tiene afinidad con Antitrombina III	pentasacárido galactosilglobósido (SSEA-3) compuesto 10 

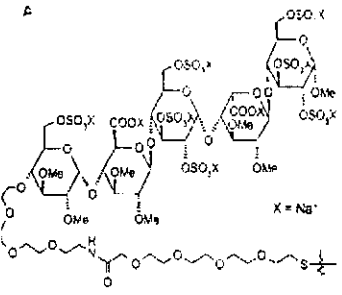
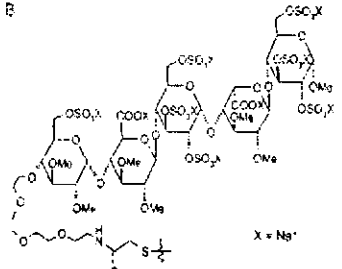
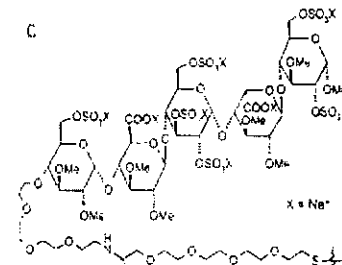
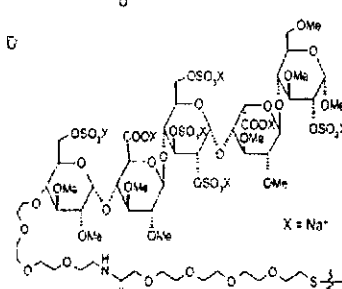
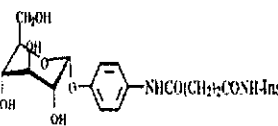
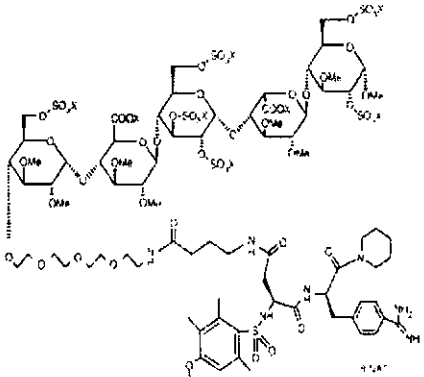
Las características **esenciales** del conjugado de la reivindicación 1 habían sido divulgadas previamente en D2, el cual revela un conjugado de un pentasacárido: galactosilglobósido (SSEA-3) (página 11, compuesto 10) con albúmina sérica bovina (BSA) (Pág. 15, párrafo 2) cuyas características técnicas se muestran en el cuadro anterior, las cuales se traslapan con las divulgadas en la solicitud en estudio.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D2.

Nivel Inventivo (Art18 D486)

Las reivindicaciones 2-24 consisten en un conjugado que comprende las siguientes características técnicas:

Comparación de las características técnicas esenciales, con las del Estado de la Técnica (D3 y D4)

Características esenciales	D3	D4
Conjugado que comprende:	Conjugado que comprende:	Conjugado que comprende:
Un polipéptido: Como la insulina	Insulina	Compuesto inhibidor de trombina.
Un espaciador que puede ser un enlace o un residuo farmacológicamente inactivo		Pentasacárido y espaciador.
Un oligosacárido de 4-18 unidades de monosacárido y que tiene afinidad con Antitrombina III		
A  X = Na⁺ B  X = Na⁺ C  X = Na⁺ D  X = Na⁺	p-succinamidofenil-glucopiranososa (SAPG), p-succinamidofenil-galactopiranososa (SAPGa), p-succinamidofenil-fucopiranososa (SAPF), o p-succinamidofenil-manopiranososa (SAPM). 	

El documento D3 se considera el estado de la técnica cercano a la invención definida en las reivindicaciones 2-21 y 24 porque divulga un conjugado de Insulina monoglicosilada (Pág. 293, fig. 1).

La diferencia entre la invención, definida en la reivindicación 1 y el conjugado D3
Expediente 07 078 139 1er Art 45

consiste en que el oligosacárido utilizado es un pentasacárido y no un monosacárido como se muestra en la tabla anterior.

El efecto que logra el incluir esta característica es que se incrementa el tiempo de vida media de la insulina en plasma, conservando su actividad farmacológica.

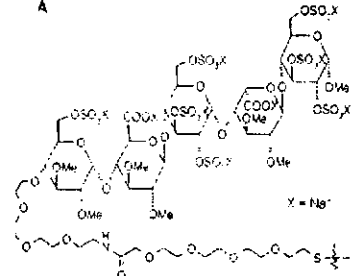
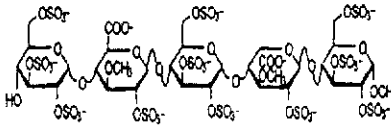
Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar el conjugado conocido en D3 para incrementar el tiempo de vida media de la insulina en plasma, conservando su actividad farmacológica.”

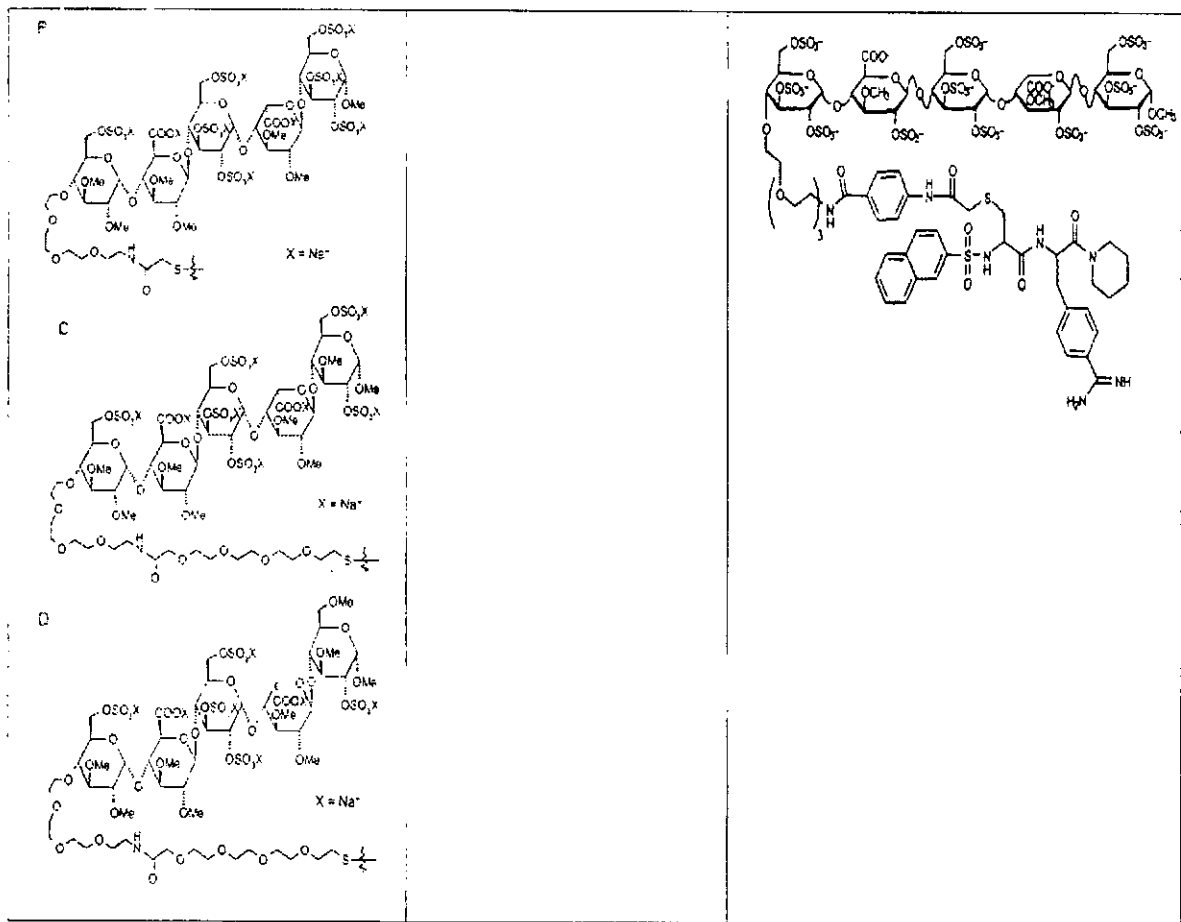
Sin embargo, la utilización de pentasacáridos para incrementar el tiempo de vida media ya está divulgada en el documento D4 el cual se refiere al incremento del tiempo de vida media de una molécula de un inhibidor de trombina al conjugarla con el pentasacárido usado en la presente solicitud (Pág. 1945, abstract, figuras 1-3, tablas 1-5).

En consecuencia, la persona del oficio normalmente versada en la materia, estaría motivada a incluir esta característica de D4 en la invención de D3 es decir, conjugar la molécula de insulina ya no con un monosacárido sino con un pentasacárido esperando conseguir un incremento del tiempo de vida media y además conservando su actividad farmacológica, por lo cual se considera obvio.

Conclusión:
Las reivindicaciones 2-24 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

Comparación de las características técnicas esenciales, con las del Estado de la Técnica (D5 y D6)

Características esenciales	D5	D6
Conjugado que comprende:	Conjugado que comprende:	Conjugado que comprende:
Un polipéptido: Como la insulina	Insulina	N-(2-naftalenesulfonil)-glicil)-D-4-aminofenil-alanil-piperidina (NAPAD)
Un espaciador que puede ser un enlace o un residuo farmacológicamente inactivo		Pentasacárido y espaciador.
Un oligosacárido de 4-18 unidades de monosacárido y que tiene afinidad con Antitrombina III		
A 	Carboximetil dextrano (CMD)	



El documento D5 se considera el estado de la técnica cercano a la invención definida en las reivindicaciones 2-21 y 24 porque divulga un conjugado de Insulina con carboximetil dextrano (CMD) el cual incrementa el tiempo de vida media comparado con la insulina sin conjugar (Pág. 176, abstract, figuras 2-6).

La diferencia entre la invención, definida en la reivindicación 1 y el conjugado de D5 consiste en que se realiza la conjugación con un pentasacárido y no con un dextrano.

El efecto que logra el incluir esta característica es que se incrementa el tiempo de vida media de la insulina en plasma, conservando su actividad farmacológica.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el conjugado conocido en D5 para incrementar el tiempo de vida media de la insulina en plasma, conservando su actividad farmacológica."

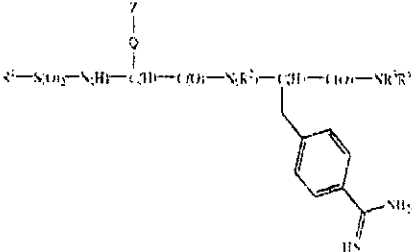
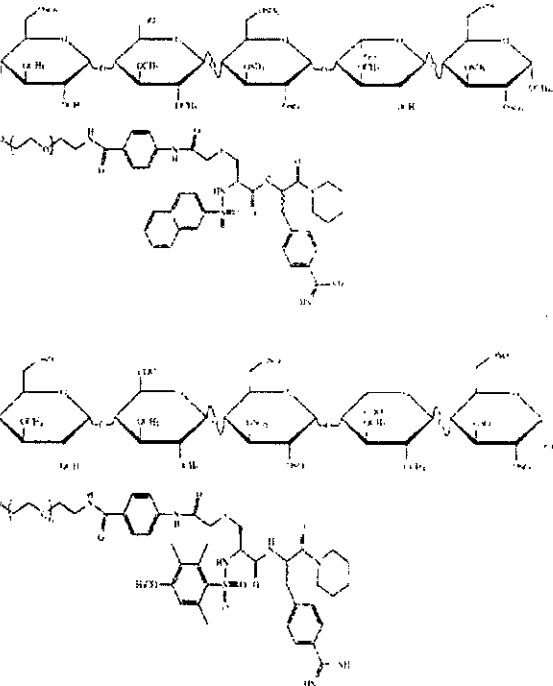
Sin embargo, la utilización de un pentasacárido para incrementar el tiempo de vida media ya está divulgada en el documento D6 el cual se refiere al incremento del tiempo de vida media de una molécula de N-(2-naftalenesulfonil)-glicil)-D-4-aminofenil-alanil-piperidina (NAPAD) al conjugarla con el pentasacárido usado en la presente solicitud (Pág. 2013, abstract, Pág. 2014, fig. 1).

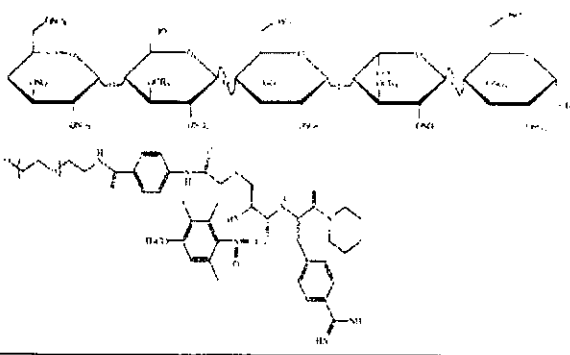
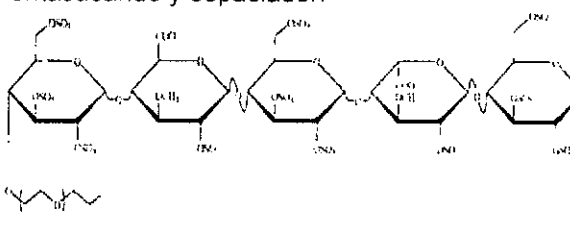
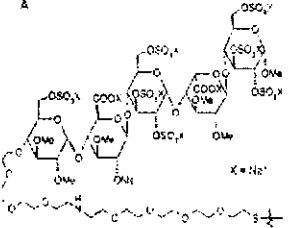
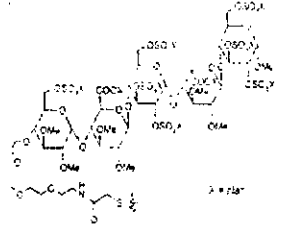
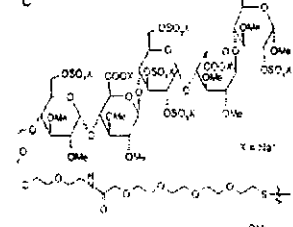
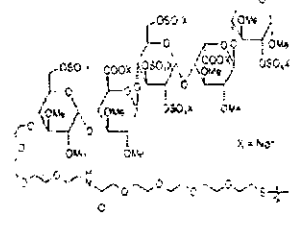
En consecuencia, la persona del oficio normalmente versada en la materia, estaría motivada a incluir esta característica de D6 en la invención de D5 es decir, conjugar la molécula de insulina ya no con un dextrano sino con el pentasacárido de D6

esperando conseguir un incremento del tiempo de vida media y además conservando su actividad farmacológica, por lo cual se considera obvio.

Conclusión:
Las reivindicaciones 2-24 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

Comparación de las características técnicas esenciales, con las del Estado de la Técnica (D7 y D8)

Características esenciales	D7	D8
Conjugado que comprende:	Conjugado que comprende:	Conjugado que comprende:
Un polipéptido: Como la insulina	Insulina	Una molécula de un agente antitrombotico:  Por ejemplo: compuesto IV, V y VI 

		
Un espaciador que puede ser un enlace o un residuo farmacológicamente inactivo Un oligosacárido de 4-18 unidades de monosacárido y que tiene afinidad con Antitrombina III		Pentasacárido y espaciador. 
A  X = Na⁺ B  X = Na⁺ C  X = Na⁺ D  X = Na⁺	Poli(etilenglicol) (PEG)	

El documento D7 se considera el estado de la técnica cercano a la invención definida en las reivindicaciones 2-21 y 24 porque divulga un conjugado de Insulina con polietilenglicol (PEG) el cual incrementa el tiempo de vida media, y aumenta la resistencia al ataque proteolítico comparado con la insulina sin conjugar (Pág. 506, 2da columna, Pág. 525, 2da columna).

La diferencia entre la invención, definida en la reivindicación 1 y el conjugado de D7 consiste en que se realiza la conjugación con una molécula de pentasacárido y no con una PEG.

El efecto que logra el incluir esta característica es que se incrementa el tiempo de vida media de la insulina en plasma, conservando su actividad farmacológica.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: "cómo modificar el conjugado conocido en D7 para incrementar el tiempo de vida media de la insulina en plasma, conservando su actividad farmacológica."

Sin embargo, la utilización de un pentasacárido para incrementar el tiempo de vida media ya está divulgada en el documento D8 el cual se refiere al incremento del tiempo de vida media de una molécula de NAPAD al conjugarla con el pentasacárido usado en la presente solicitud (compuestos IV, V y VI).

En consecuencia, la persona del oficio normalmente versada en la materia, estaría motivada a incluir esta característica de D8 en la invención de D7 es decir, conjugar la molécula de insulina ya no con PEG sino con el pentasacárido de D8 esperando conseguir un incremento del tiempo de vida media y además conservando su actividad farmacológica, por lo cual se considera obvio.

Conclusión:
Las reivindicaciones 2-24 27 y 30 cumplen el requisito de novedad (Art. 16), pero no cumplen con el requisito de nivel inventivo (Art. 18).

9. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	FEBS LETTERS, 1989, vol. 248, N° 1-2, páginas 111-114.	1	Novedad
D2	CARBOHYDRATE RESEARCH, 1995, vol. 272, N° 1, páginas 9-16.	1	Novedad
D3	ADVANCED DRUG DELIVERY REVIEWS, 1999 NETHERLANDS, vol 35, N° 2-3, páginas 289-306.	2-24	Nivel Inventivo
D4	JOURNAL OF THROMBOSIS AND HAEMOSTASIS, BLACKWELL PUBLISHING OXFORD, 2003, vol 1, N° 9, páginas 1945-1954.	2-24	Nivel Inventivo

D5	BIOCONJUGATE CHEMISTRY, 1998, vol. 9, N° 2, páginas 176-183.	2-24	Nivel Inventivo
D6	BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, OXFORD GB, 1999, vol. 9 N° 14, páginas 2013-2018.	2-24	Nivel Inventivo
D7	ADVANCED DRUG DELIVERY REVIEWS, 2002, vol. 54, páginas 505-530	2-24 27 y 30	Nivel Inventivo
D8	US6486129B1	2-24 27 y 30	Nivel Inventivo

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

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

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



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha 20 JUN. 2012

Realizado por: Ximena Andrade. *XIA*
Revisado por: Consuelo Leguizamón. *CL*

O-Mannosylation of recombinant human insulin-like growth factor I (IGF-I) produced in *Saccharomyces cerevisiae*

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A glycosylated form of recombinant human insulin-like growth factor I (IGF-I) expressed in *Saccharomyces cerevisiae* was shown to contain mannose as the only carbohydrate constituent. All oligosaccharide chains of the glycoprotein could be released by mild alkaline treatment, and separated from the protein by gel permeation chromatography on Bio-Gel P-2. The structures of these O-linked carbohydrate chains were determined by 500-MHz ¹H-NMR spectroscopy, affording the disaccharide Manα1-2Man as the major component and the trisaccharide Manα1-3Manα1-2Man as a minor component. Reference oligosaccharides were prepared from mannoproteins released from the cell wall of *S. cerevisiae* X2180 (α-wild type). In addition to previously reported structures, ranging from mannose to hexasaccharide, the pentasaccharide Manα1-3Manα1-2Manα1-2Man was identified in the cell wall mannoprotein.

Insulin-like growth factor I; Recombinant glycoprotein; Carbohydrate; O-linked; (Human, *Saccharomyces cerevisiae*)

1. INTRODUCTION

There is great interest in the yeast *Saccharomyces cerevisiae* as a host for the biotechnological production of recombinant human (glyco)proteins, since it is relatively easy for one to manipulate this eukaryotic organism which can be cultured in large amounts [1-3]. Especially for recombinant glycoproteins with a pharmacological application, knowledge of the exact structure of the carbohydrate chains is required. Glycosylation may influence the secretion efficiency [4], biological activity [4,5] and stability of the protein [6]. Furthermore, yeast-specific glycans on a human protein may introduce new immunological determinants. The glycosylation machinery in yeast can produce both N- and O-linked glycans [2]. N-linked glycans from yeast possess the same core structure as is found in human glycoproteins, but yeast N-glycans often contain highly branched

mannans. In contrast to most other eukaryotic organisms the O-glycosidic carbohydrate chains in yeast are linked via mannose to serine or threonine [7]. However, despite the rapid development in yeast gene-cloning research, there are no reports about the detailed structure of the glycans present on human proteins expressed in yeast [1].

Here, we report on the carbohydrate structures present in a glycosylated form of recombinant human insulin-like growth factor I (IGF-I or somatomedin C) expressed in *S. cerevisiae*. Human serum IGF-I is not glycosylated, but the recombinant human IGF-I from yeast can be separated into both an unglycosylated and a glycosylated form (Gellerfors, P., personal communication).

2. MATERIALS AND METHODS

2.1. Insulin-like growth factor I

The recombinant glycosylated human IGF-I preparation used here was provided by Dr. P. Gellerfors (Kabi, Stockholm, Sweden). The concentration of IGF-I was determined by radioimmunoassay using placental membrane, the specific activity being 15000 U/mg protein.

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2.2. Isolation of yeast cell wall mannoprotein

Reference oligosaccharides were prepared from yeast cell wall mannoprotein, solubilized from the cell wall of *S. cerevisiae* X2180 (α-wild type) by boiling in the presence of 2% (w/w) SDS, according to [8].

The O-linked carbohydrate chains were released by alkaline treatment as described [7]. Recombinant IGF-I (16 mg) was dissolved in 16 ml of 0.1 M NaOH and incubated for 18 h at 21°C. After neutralization with 2 M acetic acid and lyophilization, the released oligosaccharides were separated from the protein by gel filtration on a column (1.2 x 30 cm) of Bio-Gel P-2 (200 ÅQ mesh, Bio-Rad) using water as eluent at a flow rate of 6 ml/h. The carbohydrate-positive fractions (orcinol/sulfuric acid spot test) were pooled and desalted on a column (1.2 x 8 cm) containing equal layers of AG1 X-3 acetate form, 200-400 mesh, Bio-Rad) and AG 50W (H⁺ form) with water as eluent.

The reference saccharides were released from 405 mg yeast cell wall mannoprotein in 80 ml of 0.1 M NaOH. In addition to

the previously reported structures Man (M1), Manα1-2Man (M2), Manα1-2Manα1-2Man (M3) and Manα1-3Manα1-2Man (M4) [7], a carbohydrate-positive fraction was obtained with a larger retention volume on HPLC (Lichrosorb-NH) than M4. The anomeric region of the 500-MHz ¹H-NMR spectrum of M5 showed five signals of similar intensity. By comparing the chemical shift values of the anomeric protons with those from reference compounds [9], the oligosaccharide was identified as the pentasaccharide Manα1-3Manα1-3Manα1-2Manα1-2Man (M5).

2.4. HPLC

The oligosaccharide mixture obtained from *S. cerevisiae* cell wall mannoprotein was fractionated on a Kratos Spectroflow 400 HPLC system (Kratos Analytical) using a Lichrosorb-NH₂ 10 µm column (0.46 x 25 cm, Chrompack). Prior to injection, samples were dissolved in water (Milli-Q quality). Carbohydrates were eluted isocratically with acetonitrile/water (80:20, v/v) at a flow rate of 2 ml/min and monitored at 195 nm with a Spectroflow 783 absorbance detector (Kratos Analytical).

Manα1-2Man 1
2
Manα1-3Manα1-2Man 1
2
3
4

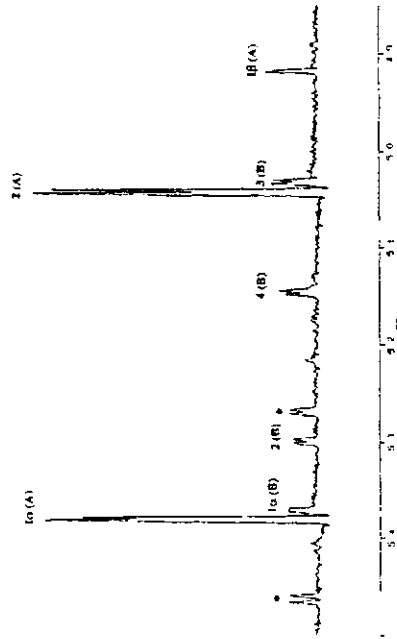


Fig. 1. Anomeric region of the resolution-enhanced 500-MHz ¹H-NMR spectrum of the pool of oligosaccharides obtained from recombinant human IGF-I. The low-intensity signals designated by the asterisks at δ 5.462 ppm (J_{1,2} = 3.7 Hz) and δ 5.369 ppm (J_{1,2} = 1.5 Hz) probably belong to as yet unidentified carbohydrate. However, the available amount of IGF-I was too low to permit further analysis of the minor compounds.

2.5. Monosaccharide analysis

Samples were subjected to the methanolysis procedure and analysed by GLC on CP-Sil 5, as described [10].

2.6. Reduction of oligosaccharides

Part of the oligosaccharide mixture obtained from IGF-1 was reduced with NaBH₄ in water (1 ml) for 14 h at room temperature. The reaction was terminated by the addition of AG-50W (H⁺-form) and the cation-exchange resin was removed by filtration. Acetic acid was removed by co-evaporation with 1% (v/v) acetic acid in methanol.

2.7. 500-MHz ¹H-NMR spectroscopy

Carbohydrate samples were repeatedly exchanged in ²H₂O (finally using 99.96 atom% ²H₂O, Aldrich) with intermediate lyophilization. Resolution-enhanced 500-MHz ¹H-NMR spectra were recorded in ²H₂O at 27°C on a Bruker AM-500 spectrometer (Department of Chemistry, Utrecht University). Chemical shifts (δ) are given relative to internal acetone (δ = 2.225 ppm) [11]. The two-dimensional homonuclear Hartmann-Hahn (HOHAHA) spectrum was obtained using a 100 ms MLEV-17 mixing sequence [12], recording 512 measurements of 2K data points with 16 scans per *t*₁ value. For the two-dimensional double-quantum filtered ¹H-¹H correlation spectrum (DQF-COSY) [13] 350 measurements of 2K data points were performed. The 90° ¹H pulse width was 32.6 μs and the residual HOHAHA signal was suppressed by preirradiation for 1.0 s.

3. RESULTS

Sugar analysis of glycosylated recombinant human IGF-1 revealed only mannose, and the total carbohydrate content was about 8% (w/w). After alkaline treatment of IGF-1, the pool of released oligosaccharides was isolated by gel-permeation chromatography on Bio-Gel P-2 (not shown), desalted, and analyzed by 500-MHz ¹H-NMR spectroscopy. As has been shown before, the chemical shifts of the H-1 and H-2 atoms of monosaccharides are characteristic of both the type of linkage and the sequence of the constituent monosaccharides [9,14]. The anomeric region of the ¹H-NMR spectrum of the mixture of IGF-1 oligosaccharides is depicted in fig. 1. The major component turned out to be Man^α1-2Man (compound A), reflected by two about equally intense H-1 signals at δ 5.380 ppm (*J*_{1,2} = 1.5 Hz) and δ 5.042 ppm (*J*_{1,2} = 1.5 Hz), and having corresponding H-2 signals at δ 3.96 and 4.073 ppm, stemming from the reducing and nonreducing Man residues, respectively. The structural reporter groups match completely those obtained for the reference M2, isolated from the cell wall mannoprotein of *S. cerevisiae* (see table 1), but are different from those

Table 1

¹H chemical shifts of structural reporter group protons of the constituent monosaccharides for the oligosaccharides A and B derived from recombinant human IGF-1 together with those for reference compounds M2, M5, prepared from yeast cell wall mannoprotein

Reporter Residue ^a	A	B	M2	M3	M4	M5
H-1						
1α	5.381	5.371	5.380	5.370	5.371	5.371
1β	4.918	n.d.	4.918	n.d.	n.d.	n.d.
2	5.042	5.297	5.042	5.299	5.297	5.296
3	-	5.032	-	5.064	5.032	5.016
4	-	5.147	-	-	5.142	5.121
5	-	-	-	-	-	5.137
H-2						
1α	3.96	3.96	n.d.	3.960	n.d.	n.d.
1β	4.073	n.d.	n.d.	n.d.	n.d.	n.d.
2	4.073	4.11	4.073	4.109	4.11	n.d.
3	-	4.23	-	4.065	4.221	n.d.
4	-	4.07	-	-	4.068	n.d.
H-3						
1	3.69	n.d.	n.d.	n.d.	n.d.	n.d.
2	3.86	n.d.	n.d.	n.d.	n.d.	n.d.

^a For numbering of monosaccharide residues, see fig. 1. n.d., not determined. Chemical shifts are given in ²H₂O at 300 K relative to internal acetone (δ 2.225 ppm).

reported for the α1-3, α1-4, and α1-6-linked mannosyl-mannoses [9]. The assignments of additional protons for the IGF-1 disaccharide, as given in table 1, were made using COSY and HOHAHA experiments. The disaccharide accounts for about 80 mol% of the oligosaccharides present in IGF-1, estimated by integrating the anomeric region of the ¹H-NMR spectrum of the mixture of oligosaccharides.

In addition to the disaccharide, small amounts of Man^α1-3Man^α1-2Man^α1-2Man (compound B) could be identified. The most indicative structural reporter group signals for this tetrasaccharide are the H-1 signals at δ 5.147 and 5.032 ppm, being characteristic for the terminal α1-3-linked Man-4 and the subterminal 3-substituted α1-2-linked Man-3, respectively. From the HOHAHA experiment the corresponding H-2 signals were assigned to δ 4.07 ppm (Man-4) and δ 4.23 ppm (Man-3). The signal at δ 5.371 ppm is assigned to H-1 of the α-anomer of Man-1, with the H-2 signal at δ 3.79 ppm, whereas the H-1 of Man-2 resonates at δ 5.297 ppm, with the H-2 signal at δ 4.11 ppm (cf. M4, table 1). The relatively high intensity of the signal at δ 5.147 ppm, as compared to the signal at

δ 5.297 ppm, is due to overlap with another, as yet unassigned signal, which was detected by a second cross-peak in the HOHAHA experiment. In the tetrasaccharide M3 and the tetrasaccharide M4 the chemical shift values of the H-1 atoms of Man-1 and Man-2 are the same. However, based on the intensities of the signals it can be concluded that only trace amounts, if any, of the trisaccharide Man^α1-2Man^α1-2Man can occur in IGF-1.

4. DISCUSSION

Here it is shown that Man^α1-2Man is the predominant oligosaccharide in a glycosylated form of human recombinant IGF-1 produced in *S. cerevisiae*. As a minor constituent Man^α1-3Man^α1-2Man^α1-2Man could be identified. Both oligosaccharides are typical yeast *O*-glycans, but it should be noted that the same carbohydrate epitope as in the disaccharide occurs as a terminal sequence in several human *N*-glycoproteins containing oligomannose-type carbohydrate chains [15]. On the other hand, the tetrasaccharide does not normally occur in human glycoproteins and strongly reacting antibodies against this yeast oligosaccharide have been raised in rabbit [16]. The *O*-linked pentasaccharide Man^α1-3Man^α1-3Man^α1-2Man^α1-2Man, which was found in yeast cell wall mannoprotein, was not found in the recombinant protein, but has previously been isolated from *S. italicus* [17].

In IGF-1 there are eight serine or threonine residues [18], being potential *O*-glycosylation sites. It has been shown that a proline residue in the vicinity of a serine or threonine residue markedly enhances the *O*-glycosylation efficiency [19,20], therefore the most likely glycosylation sites in IGF-1 are the threonines at positions 4, 29 and 41. The lack of *N*-linked saccharides in IGF-1 is explained by the absence of an Asn-X-Ser/Thr sequence, required for *N*-glycosylation [21].

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Synthesis of the saccharide moiety of galactosylgloboside (SSEA-3) and its conjugation to bovine serum albumin and Sepharose

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Abstract

The pentasaccharide glycoside corresponding to galactosylgloboside (SSEA-3), β -D-Gal p-(1 $\rightarrow$ 3)- α -D-Gal pNAc-(1 $\rightarrow$ 4)- β -D-Glc p-(1 $\rightarrow$ 4)- β -D-Glc p-1-OCH₂CH₂Si(CH₃)₃ (4), was synthesized via glycosylation (87%) of 2-(trimethylsilyl)ethyl 2,3,6-tri-O-benzyl-4-O-[2,3,6-tri-O-benzyl-4-O-(2,4,6-tri-O-benzyl- α -D-galactopyranosyl)- β -D-glucopyranoside (2) with the glycosyl donor methyl 4,6-di-O-acetyl-2-deoxy-2-phthalimido-3-O-(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl)-1-thio- β -D-galactopyranoside (1), followed by removal of protecting groups. Compound 4 was transformed into the spacer glycoside β -D-Gal p-(1 $\rightarrow$ 3)- α -D-Gal pNAc-(1 $\rightarrow$ 3)- α -D-Gal p-(1 $\rightarrow$ 4)- β -D-Glc p-(1 $\rightarrow$ 4)- β -D-Glc p-1-SCH₂CH₂COOH (10), which was coupled to bovine serum albumin (BSA), and Sepharose beads, to give the corresponding neoglycoprotein (11, 6 mol of saccharide/mol of BSA) and glycosylated Sepharose (12, 2.7 μ mol of saccharide/mL of sedimented beads), respectively. An improved synthesis of a protected globotetraoside β -D-Gal pNAc-(1 $\rightarrow$ 3)- α -D-Gal p-(1 $\rightarrow$ 4)- β -D-Glc p-(1 $\rightarrow$ 4)- β -D-Glc p-1-OCH₂CH₂SiMe₃ is also reported.

Keywords: Galactosylgloboside; Thiolglycoside donor; 2-(Trimethylsilyl)ethyl glycoside; BSA-conjugate; Sepharose-conjugate

1. Introduction

Galactosylgloboside [also called stage-specific embryonic antigen-3 (SSEA-3), globopentaosyl ceramide, and IV³GalGb₄Cer] is present in human [1] and green monkey [2] kidney tissue as well as in human teratocarcinoma cells [3]. The amount of

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galactosylgloboside on cell surfaces varies during embryogenesis [4], hence the acronym SSEA-3. Monoclonal antibodies raised against murine morula stage embryos [3] or an octasaccharidic glycosyl ceramide containing the galactosylgloboside structure [5] recognize galactosylgloboside. The heavy- and light-chain sequences of the latter antibodies have been determined [6].

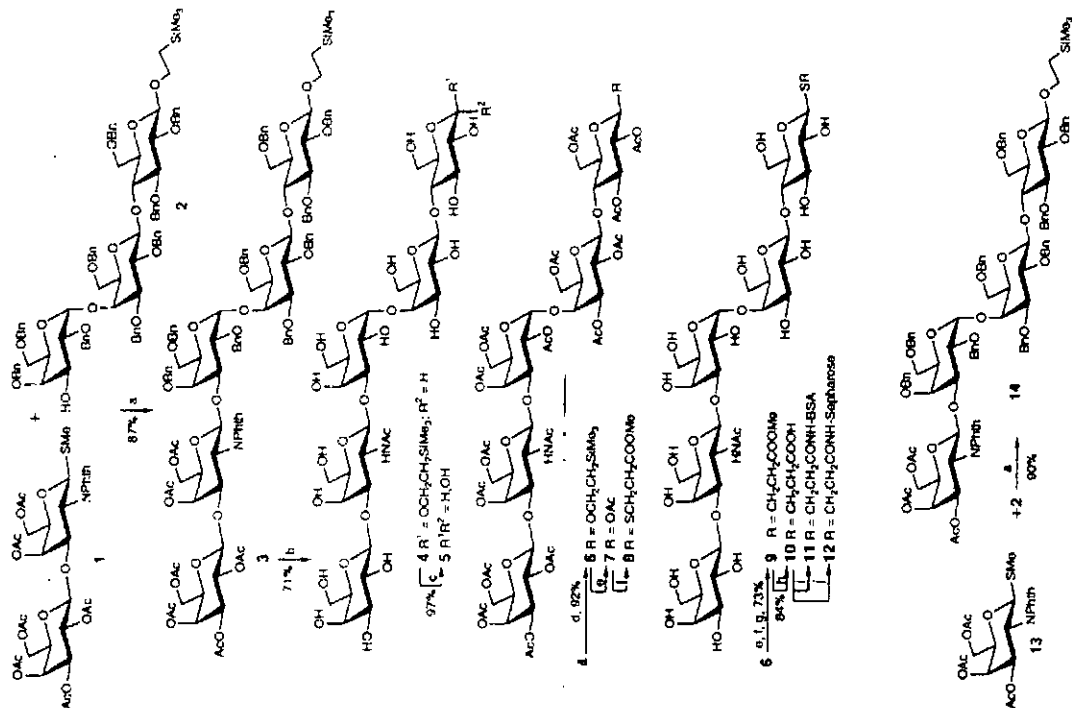
A synthesis of galactosylgloboside has been reported [7], as well as the corresponding silylated and sulfated galactosylglobosides [8]. We now report an improved synthetic route to the galactosylglobotetraose saccharide (GalGb₄), as well as the preparation of the corresponding neoglycoprotein (GalGb₄-BSA) and Sepharose conjugate (GalGb₄-Sepharose).

The glycosyl donor 1 (obtained in > 60% yield from the monosaccharide building blocks [9]) was used in the glycosylation of the known [10] acceptor 2. Thus, a mixture of 1 and 2 was treated in acetonitrile with *N*-iodosuccinimide and trifluoromethanesulfonic acid (NIS/TfOH) [11], which gave the desired pentasaccharide 3 in 87% yield after chromatographic purification. When triethylsilyl trifluoromethanesulfonate (TESOTf) was used instead of TfOH, the reaction was rather unpredictable and the yield varied. Thus, in a typical experiment, compound 3 was obtained in 49% yield together with 50% of silylated 2. Similar problems were encountered by Ogawa et al. [7], who used the trichloroacetimidate corresponding to 1 and obtained the desired pentasaccharide in only 22% yield, together with 44% of a trichloroacetamide (rearranged trichloroacetimidate). Another example of a less satisfactory glycosylation of 2 is found in our preparation [10] of the globotetraoside 14, using the β -glycosyl chloride corresponding to 13 in dichloromethane, which gave only a 35% yield (α/β 15:85) and a substantial amount of the phthalimidogalactal. In contrast, treatment of 2 and 13 [12] with NIS/TfOH in acetonitrile, as above, gave 14 in 90% yield as described in the Experimental section. The improved yield of the glycosylation may be due to the use of acetonitrile as solvent (nitrile effect) [13] (Scheme 1).

The pentasaccharide 3 was transformed into the glycoside 4 in one pot by sequential treatment with hydrogen/Pd-on-carbon, hydrazine hydrate, acetic anhydride/pyridine, and *O*-deacetylation in methanolic sodium methoxide. Compound 4 was obtained in 71% overall yield after chromatography. Treatment of 4 with trifluoroacetic acid/dichloromethane [14] gave the reducing pentasaccharide 5 in 97% yield after purification by gel filtration. Acetylation of 4 with acetic anhydride/pyridine gave the fully acetylated glycoside 6 in 92% yield after chromatography. Treatment of 6 with boron trifluoride etherate/acetic anhydride [14] gave a quantitative yield of the anomeric acetate 7 (β/α 96:4), which was used without further purification in the following reactions.

The choice of mercaptopropionic acid as spacer aglycon deserves some comment. A chemically pure oligosaccharide obtained by organic synthesis represents a substantial investment. Therefore, it is important that ensuing chemical reactions, leading to functional derivatives (such as various neoglycoconjugates [15]), proceed in high yield and stereoselectivity. Glycosides carrying a mercaptopropionic acid aglycon [16] (or other mercaptoalkanoic acids) are easily prepared and thioglycosides are normally more stable in the aqueous environment employed with most applications of glycoconjugates.

The crude acetate 7 (containing 4% of the corresponding α -acetate) was treated with



Scheme 1. (a) NIS, TIOH, MeCN, CH₂Cl₂, -50°C; (b) H₂, Pd/C, AcOH; (c) Ac₂O, pyridine; (d) Ac₂O, Et₃O, Ac₂O, CH₂Cl₂; (e) HSCl, CH₂COOMe, BF₃·Et₂O, CH₂Cl₂; (f) MeONa, MeOH; (g) NaOH, H₂O; (h) BSA, TSTU, Et₃N; (i) EAH-Sepharose, N-hydroxysuccinimide, EtN-C-N(CH₂)₄NMe₂.

methyl mercaptopropionate and boron trifluoride etherate to give **8** (88%, contaminated by 3% of the unreactive **7a**). Compound **8** was deacetylated to give **9** (73% overall yield from **6**) after removal of **5** by chromatography on silica gel. Hydrolysis of the methyl ester group in **9** gave the spacer glycoside **10** in quantitative yield. Attempted glycosylation of mercaptopropionic acid itself gave the acetylated derivative of **10** in only ~15% yield, in contrast to successful glycosylations with lower saccharides [16,17].

Coupling of compound **10** to bovine serum albumin (BSA) was performed using *N,N,N',N'*-tetramethylsuccinimiduronium tetrafluoroborate [18] as the promoter, to give the corresponding conjugate **11**. Gel electrophoresis of **11** showed a major band corresponding to a molecular mass of 73–75 kDa, as compared to the band for unconjugated BSA (67 kDa). In a similar coupling reaction, aminohexyl-Sepharose was conjugated with **10**, using *N*-hydroxysuccinimide/1-(3-dimethylaminopropyl)-3-ethylcarbodiimide as promoter, to give the Sepharose conjugate **12**. Sulfur combustion analysis of **11** and **12** showed the saccharide contents to be 6 mol/mol of BSA and 2.7 μmol/ml of sedimented Sepharose beads, respectively. The calculated molecular mass of a BSA conjugate carrying six saccharide residues is ~73 kDa, which is in good agreement with the gel electrophoresis data.

2. Experimental

General methods.—Melting points are uncorrected. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. The ¹H- and ¹³C-NMR spectra were recorded with a Varian XL-300 or a Bruker ARX 500 spectrometer. 1,4-Dioxane was used as internal reference (67.4 ppm) in ¹³C-NMR experiments in D₂O. Concentrations were made using rotary evaporation with bath temperature at or below 40°C. Anhydrous Na₂SO₄ was used as drying agent for the organic extracts in the work-up procedures. TLC was performed on Kieselgel 60 F₂₅₄ plates (Merck). Column chromatography was performed using SiO₂ (Marex LC-gel; 60 Å, 35–70 MY, Grace). BSA and EAH-Sepharose were obtained from Sigma and Pharmacia, respectively.

2-(Trimethylsilyl)ethyl 2,3,6-tri-O-benzyl-4-O-(2,3,6-tri-O-benzyl-4-O-(2,4,6-tri-O-benzyl-3-O-(4,6-di-O-acetyl-2-deoxy-2-phthalimido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl)-β-D-galactopyranosyl)-β-D-glucopyranoside (3).—Methyl 4,6-di-O-acetyl-2-deoxy-2-phthalimido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-1-thio-β-D-galactopyranoside [9] (**1**; 210.3 mg, 0.28 mmol), 2-(trimethylsilyl)ethyl 2,3,6-tri-O-benzyl-4-O-(2,3,6-tri-O-benzyl-4-O-(2,4,6-tri-O-benzyl-α-D-galactopyranosyl)-β-D-galactopyranosyl)-β-D-glucopyranoside [10] (**2**; 282.1 mg, 0.20 mmol), and acid-washed molecular sieves AW-300 (0.2 g) were stirred in dry CH₂Cl₂ (1.4 mL) and dry MeCN (4.1 mL) under Ar for 75 min. The solution was cooled to -50°C and a solution of *N*-iodosuccinimide (90 mg, 0.40 mmol) and trifluoromethanesulfonic acid (13.6 μL, 0.06 mmol) in dry MeCN (0.6 mL) was added. After 1 h, *N,N*-diisopropylethylamine (1 mL) was added and the mixture was filtered and concentrated. Flash chromatography of the residue (2:1 heptane-EtOAc) gave **3** and concentrated. Flash chromatography of the residue (500 MHz, CDCl₃): δ (367.7 mg, 87%); [α]_D²⁵ +10.3° (c 1, CHCl₃). ¹H-NMR data (500 MHz, CDCl₃): δ 5.31 (br d, 1 H, J 2.9 Hz, H-4^W), 5.27 (dd, 1 H, J 1.1, 3.4 Hz, H-4^W), 5.23 (d, 1 H, J 8.2 Hz, H-1^W), 5.03 (d, 1 H, J 10.9 Hz, PhCH₂), 4.98 (d, 1 H, J 11.4 Hz, PhCH₂).

α -D-galactopyranosyl-2,3,6-tri-O-acetyl- β -D-galactopyranosyl-2,3,6-tri-O-acetyl- β -D-glucopyranoside (6).—Compound 4 (40.8 mg, 0.0434 mmol) was stirred in Ac₂O (1.5 mL) and pyridine (1.5 mL) for 23 h, then concentrated. Flash chromatography (10:1 toluene–EtOH) of the residue gave 6 (61.5 mg, 92%), [α]_D²⁵ +48° (c 1, CHCl₃). ¹H-NMR data (500 MHz, CDCl₃): δ 5.65 (c, 1 H, J 7.1 Hz, NH), 5.59 (dd, 1 H, J 0.8, 3.3 Hz, H-4''), 5.42 (br d, 1 H, J 3.3 Hz, H-4''), 5.34 (dd, 1 H, J 0.9, 3.4 Hz, H-4''), 5.19 (dd, 1 H, J 3.6, 10.5 Hz, H-2''), 5.18 (d, 1 H, J 8.0 Hz, H-1''), 5.18 (t, 1 H, J 9.4 Hz, H-3), 5.13 (dd, 1 H, J 7.8, 10.4 Hz, H-2''), 5.10 (dd, 1 H, J 7.7, 10.9 Hz, H-2'), 4.96 (dd, 1 H, J 3.4, 10.4 Hz, H-3''), 4.92 (d, 1 H, J 3.6 Hz, H-1''), 4.87 (dd, 1 H, J 8.0, 9.4 Hz, H-2), 4.75 (dd, 1 H, J 2.6, 10.9 Hz, H-3'), 4.68 (dd, 1 H, J 3.3, 10.8 Hz, H-3''), 4.61 (d, 1 H, J 7.8 Hz, H-1''), 4.52 (d, 1 H, J 7.7 Hz, H-1'), 4.49 (d, 1 H, J 7.9 Hz, H-1), 4.00 (br d, 1 H, J 2.6 Hz, H-4'), 3.78 (t, 1 H, J 9.3 Hz, H-4), 3.22 (dt, 1 H, J 8.0, 10.8 Hz, H-2''), 2.13, 2.11, 2.10, 2.09, 2.08, 2.07, 2.062, 2.056, 2.05, 2.04, 2.03, 1.96, 1.93 (13 s, 48 H, Ac), 0.89 (m, 2 H, CH₂Si), 0.00 (s, 9 H, SiMe₃). Anal. Calcd for C₆₇H₉₇NO₂₄Si: C, 50.3; H, 6.1; N, 0.9. Found: C, 50.6; H, 6.1; N, 0.7.

2-(Methoxycarbonyl)ethyl 4-O-(4-O-{3-O-(2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl)- β -D-galactopyranosyl}-2,4,6-tri-O-acetyl- α -D-galactopyranosyl)-2,3,6-tri-O-acetyl- β -D-glucopyranosyl)-2,3,6-tri-O-acetyl- β -D-glucopyranoside (8).—Compound 6 (58.4 mg, 0.0365 mmol) was treated with BF₃·Et₂O (47 μ L, 0.37 mmol) and Ac₂O (152 μ L, 1.66 mmol) in dry CH₂Cl₂ (1.9 mL) for 45 min under Ar, then diluted with CH₂Cl₂, washed with saturated aqueous NaHCO₃, dried (Na₂SO₄), and concentrated to give a quantitative yield of crude 7 (α / β 4:96), which was used without further purification in the synthesis of 8. ¹H-NMR data (300 MHz, CDCl₃): δ 6.22 (d, 1 H, J 3.6 Hz, H-1 α), 5.67 (d, 1 H, J 7.8 Hz, H-1 β). Crude 7 was treated with methyl 3-mercaptopropionate (15.8 μ L, 0.0146 mmol) and BF₃·Et₂O [19] (17.5 μ L, 0.22 mmol) in dry CH₂Cl₂ (0.3 mL) for 90 min, diluted with CH₂Cl₂, washed with saturated aqueous NaHCO₃, dried (Na₂SO₄), and concentrated. Flash chromatography (9:1 toluene–EtOH) gave a mixture of 8 and unreacted α anomer of the acetate 7 (52.3 mg; 8/7 97:3). ¹H-NMR data (300 MHz, CDCl₃): δ 5.64 (d, 1 H, J 7.1 Hz, NH), 5.59 (dd, 1 H, J 0.6, 3.4 Hz, H-4''), 5.42 (d, 1 H, J 3.4 Hz, H-4''), 5.34 (dd, 1 H, J 3.4 Hz, H-4''), 4.75 (dd, 1 H, J 2.4, 10.9 Hz, H-3'), 4.67 (dd, 1 H, J 3.4, 11.1 Hz, H-3''), 4.61 (d, 1 H, J 7.8 Hz, H-1''), 4.52 (d, 1 H, J 10.3 Hz, H-1), 4.51 (d, 1 H, J 7.4 Hz, H-1'), 3.69 (s, 3 H, OMe), 3.22 (dt, 1 H, J 7.6, 10.9 Hz, H-2''), 2.89 (m, 2 H, SCH₂), 2.67 (br t, 2 H, J 7.9 Hz, CH₂CO), 2.14 2.11, 2.10, 2.08, 2.07, 2.063, 2.056, 2.05, 2.04, 1.96, 1.93 (11 s, 48 H, Ac).

2-(Methoxycarbonyl)ethyl 4-O-(4-O-{3-O-(2-acetamido-2-deoxy-3-O- β -D-galactopyranosyl)- β -D-galactopyranosyl)- α -D-galactopyranosyl)- β -D-galactopyranosyl-1-thio- β -D-glucopyranoside (9).—Compound 8, containing ca. 3% of 7 α (52.3 mg) was treated with methanolic NaOMe (1.2 mL, 13 mM) for 85 min, and the mixture was neutralized with Duolite C26 (H⁺) resin, filtered, and concentrated. Flash chromatography (5:5:1 CH₂Cl₂–MeOH–H₂O) of the residue gave 9 (25.9 mg, 73% overall yield from 6), [α]_D²⁵ +72° (c 1.1, H₂O). ¹H-NMR data (300 MHz, D₂O): δ 4.88 (d, 1 H, J 3.7 Hz, H-1'), 4.66 (d, 1 H, J 8.3 Hz, H-1''), 4.55 (d, 1 H, J 10.0 Hz, H-1), 4.48 (d, 1 H, J 7.6 Hz, H-1'), 4.42 (d, 1 H, J 7.6 Hz, H-1''), 4.35 (br t, 1 H, J 6.4 Hz, H-5'), 4.22 (br s, 1 H, H-4'), 4.15 (br d, 1 H, J 2.7 Hz, H-4''), 3.69 (s, 3 H, OMe), 3.32 (br t,

4.98 (dd, 1 H, J 7.8, 10.3 Hz, H-2''), 4.91 (d, 1 H, J 11.1 Hz, PhCH₂), 4.80 (d, 1 H, J 3.6 Hz, H-1''), 4.79 (dd, 1 H, J 3.5, 10.3 Hz, H-3''), 4.69 (d, 1 H, J 11.1 Hz, PhCH₂), 4.67 (dd, 1 H, J 3.2, 11.2 Hz, H-3''), 4.59 (dd, 1 H, J 8.2, 11.2 Hz, H-2''), 4.46 (d, 1 H, J 7.6 Hz, H-1'), 4.42 (d, 1 H, J 7.8 Hz, H-1''), 4.37 (d, 1 H, J 7.7 Hz, H-1), 4.16 (dd, 1 H, J 2.9, 10.3 Hz, H-3'), 3.88 (t, 1 H, J 9.2 Hz, H-3), 3.78 (dd, 1 H, J 3.6, 10.3 Hz, H-2'), 2.12, 2.08, 2.06, 1.93, 1.88, 1.47 (6 s, 3 H each, Ac), 1.03 (m, 2 H, CH₂Si), 0.03 (s, 9 H, SiMe₃). Anal. Calcd for C₁₁₈H₁₃₃NO₃₁Si: C, 66.8; H, 6.3; N, 0.7. Found: C, 66.6; H, 6.5; N, 0.6.

2-(Trimethylsilyl)ethyl 4-O-(4-O-{3-O-(2-acetamido-2-deoxy-3-O- β -D-galactopyranosyl)- β -D-galactopyranosyl)- α -D-galactopyranosyl)- β -D-galactopyranosyl- β -D-glucopyranoside (4).—Compound 3 (500.4 mg, 0.235 mmol) was hydrogenated over Pd/C (10%, 375 mg) in AcOH (25 mL) for 7 h, then filtered through Celite and concentrated. The residue was treated with H₂NNH₂·H₂O (150 μ L) in EtOH (11 mL) at 80°C for 2.5 h, concentrated and co-concentrated with three portions of EtOH and one portion of toluene to remove excess of H₂NNH₂·H₂O. Acetylation with Ac₂O (7.5 mL) and pyridine (7.5 mL) overnight followed by O-deacetylation (0.08 M MeONa in MeOH, 20 mL) for 2 h gave crude 4. Flash chromatography (65:35:5 CH₂Cl₂–MeOH–H₂O) furnished pure 4 (162.2 mg, 71%), according to TLC analysis and ¹H- and ¹³C-NMR; [α]_D²⁵ +52° (c 1, H₂O). ¹H-NMR data (500 MHz, D₂O): δ 4.85 (d, 1 H, J 3.9 Hz, H-1'), 4.62 (d, 1 H, J 8.5 Hz, H-1''), 4.45 (d, 1 H, J 7.8 Hz, H-1'), 4.43 (d, 1 H, J 8.1 Hz, H-1), 4.38 (d, 1 H, J 7.7 Hz, H-1''), 4.31 (br t, 1 H, J 6.5 Hz, H-5'), 4.19 (br d, 1 H, J 2.9 Hz, H-4'), 4.11 (d, 1 H, J 3.1 Hz, H-4''), 3.51 (dd, 1 H, J 7.8, 10.2 Hz, H-2), 3.46 (dd, 1 H, J 7.7, 9.9 Hz, H-2''), 3.22 (br t, 1 H, J 8.4 Hz, H-2), 1.96 (s, 3 H, Ac), 1.01 (dd, 1 H, J 5.5, 12.9 Hz, CH₂Si), 0.91 (dt, 1 H, J 5.3, 12.9 Hz, CH₂Si), –0.04 (s, 9 H, SiMe₃). ¹³C-NMR data (500 MHz, D₂O): δ 176.2, 105.9, 104.4, 104.1, 102.5, 101.6, 80.7, 79.9, 79.8, 78.4, 76.6, 76.1, 75.9, 75.8, 75.7, 74.1, 73.6, 73.3, 72.1, 71.8, 71.4, 70.1, 69.7, 69.5, 69.1, 68.8, 62.14, 62.09, 61.5, 61.4, 61.2, 52.6, 23.4, 18.7, –1.4. Mass spectrum: Calcd for C₃₇H₄₈NO₁₆Si (M + 1): m/z 970.3799. Found: m/z 970.3785.

4-O-(4-O-{3-O-(2-acetamido-2-deoxy-3-O- β -D-galactopyranosyl)- β -D-galactopyranosyl)- α -D-galactopyranosyl)- β -D-glucopyranosyl)- β -D-glucopyranose (5).—Compound 4 (10.2 mg, 10.5 μ mol) was dissolved in CH₂Cl₂ (70 μ L) and CF₃COOH (70 μ L) under N₂. After 22 min, n-propyl acetate (0.95 mL) and toluene (2 mL) were added and the mixture was concentrated. Gel filtration (Sephadex G25) gave pure 5 (8.9 mg, 97%), according to TLC analysis and ¹H- and ¹³C-NMR; [α]_D²⁵ +67° (c 0.3, H₂O). ¹H-NMR data (300 MHz, D₂O): δ 5.20 (d, 1 H, J 3.7 Hz, H-1 α), 4.89 (d, 1 H, J 3.9 Hz, H-1'), 4.66 (d, 1 H, J 8.1 Hz, H-1''), 4.63 (d, 1 H, J 7.8 Hz, H-1 β), 4.49 (d, 1 H, J 7.7 Hz, H-1'), 4.42 (d, 1 H, J 7.6 Hz, H-1''), 4.36 (br t, 1 H, J 6.4 Hz, H-5'), 2.00 (s, 3 H, Ac). ¹³C-NMR data (300 MHz, D₂O): δ 177.9, 107.5, 106.0, 105.7, 103.1, 98.5, 94.6, 82.3, 81.6, 81.5, 79.99, 79.95, 78.2, 77.7, 77.6, 77.4, 77.3, 77.2, 76.7, 75.3, 75.2, 74.9, 74.2, 74.0, 73.6, 73.3, 73.0, 72.9, 71.7, 71.3, 70.7, 70.4, 68.8, 68.3, 65.2, 63.7, 63.3, 63.13, 63.05, 62.8, 62.7, 54.2, 25.0. Mass spectrum: Calcd for C₃₇H₄₈NO₁₆Si (M + Na): m/z 892.2910. Found: m/z 892.2885.

2-(Trimethylsilyl)ethyl 4-O-(4-O-{3-O-(2-acetamido-4,6-di-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl)- β -D-galactopyranosyl)-2,4,6-tri-O-acetyl-

1 H, *J* 9.0 Hz, H-2), 2.96 (m, 2 H, SCH₂), 2.76 (t, 2 H, *J* 6.9 Hz, CH₂CO), 2.00 (s, 3 H, Ac).

2-Carboxyethyl 4-O-(4-O-(3-O-(2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl)-β-D-galactopyranosyl)-α-D-galactopyranosyl)-β-D-galactopyranosyl-1-thio-β-D-glucopyranoside (10).—Compound 9 (16.7 mg, 0.0172 mmol) was treated with aqueous NaOH (1 mL, 20 mM) for 5 h 40 min and the mixture was neutralized with Duolite C26 (H⁺) resin, filtered, and concentrated to give 10 (13.9 mg, 84%), [α]_D²⁰ −50° (c 0.7, H₂O). ¹H-NMR data (300 MHz, D₂O): δ 4.89 (d, 1 H, *J* 3.6 Hz, H-1''), 4.66 (d, 1 H, *J* 8.1 Hz, H-1''), 4.56 (d, 1 H, *J* 9.9 Hz, H-1), 4.49 (d, 1 H, *J* 7.7 Hz, H-1'), 4.44 (d, 1 H, *J* 7.5 Hz, H-1''), 4.37 (br t, 1 H, *J* 6.4 Hz, H-5''), 4.23 (br d, 1 H, *J* 2.2 Hz, H-4''), 4.16 (br d, 1 H, *J* 2.5 Hz, H-4''), 2.93 (m, 2 H, SCH₂), 2.58 (t, 2 H, *J* 6.9 Hz, CH₂CO), 2.01 (s, 3 H, Ac).

Galactosylglucoside-BSA conjugate (11).—Compound 10 (7.7 mg, 0.008 mmol) and *N,N,N',N'*-tetramethylsuccinimiduronium tetrafluoroborate [18] (TSTU; 3.76 mg, 0.0126 mmol) were dissolved in 1,4-dioxane/water (0.22 mL, 4:1) and triethylamine (1.1 μ L, 0.008 mmol) was added. After 1 h the solution was added to BSA (14.9 mg, 0.23 μ mol) in sodium borate buffer (6 mL, 0.1 M, pH 8.5) and the mixture was left for 2.5 h at room temperature and then dialysed for 72 h against distilled water (4 $\times$ 1 L). Freeze-drying gave 11 (15.4 mg). Differential combustion analysis of BSA and 11 showed the sulfur content due to the saccharide unit to be 0.1%. This amount corresponds to a degree of binding (number of pentasaccharide molecules per BSA) of 6. SDS-PAGE showed a major band with a molecular mass of 73–75 kDa (BSA showed a different mobility and had a molecular mass of ~67 kDa).

Galactosylglucoside-Sepharose affinity beads (12).—To a solution of compound 10 (13.9 mg, 0.008 mmol), *N*-hydroxysuccinimide (12 mg, 0.1 mmol), and EAH-Sepharose (15 mL sedimented volume; washed with 0.3 L distilled water on a glass filter funnel) in distilled water (10 mL) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (20 mg, 0.1 mmol). After 17.5 h the Sepharose beads were washed on a glass filter funnel with distilled water (0.3 L divided in small portions) to give 12. Combustion analysis of EAH-Sepharose and 12 showed the sulfur content to be 0.067 and 0.093%, respectively. The difference corresponds to 2.7 μ mol of pentasaccharide molecules per mL of sedimented beads.

2-(Trimethylsilyl)ethyl 2,3,6-tri-O-benzyl-4-O-(2,3,6-tri-O-benzyl-4-O-(2,4,6-tri-O-benzyl-3-O-(4,6-di-O-acetyl-2-deoxy-2-phthalimido-β-D-galactopyranosyl)-α-D-galactopyranosyl)-β-D-glucopyranoside (14).—A mixture of methyl 3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-1-thio-β-D-galactopyranoside [12] (13; 53.3 mg, 0.11 mmol), 2-(trimethylsilyl)ethyl 2,3,6-tri-O-benzyl-4-O-(2,3,6-tri-O-benzyl-4-O-(2,4,6-tri-O-benzyl-α-D-galactopyranosyl)-β-D-galactopyranosyl)-β-D-glucopyranoside [10] (2; 115.8 mg, 0.082 mmol), and molecular sieves AW-300 (0.1 g) was stirred in dry CH₂Cl₂ (0.6 mL) and dry MeCN (1.7 mL) under Ar for 75 min. The solution was cooled to −50°C and a solution of *N*-iodosuccinimide (37 mg, 0.16 mmol) and trifluoromethanesulfonic acid (5.6 μ L, 0.025 mmol) in dry MeCN (0.25 mL) was added. After 1 h *N,N*-diisopropylethylamine (0.5 mL) was added and the mixture was filtered and concentrated. Flash chromatography of the residue (3:1 heptane–EtOAc) gave 14 (135.6 mg, 90%). Analytical data were in agreement with those previously published [10].

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Two different hydrophobic moieties, carboxyl derivatives of monosaccharide (Cite, Gal, Man, Fuc) glycosides and methoxyethyl low glycosyls of varying MW, were covalently attached to the insulin GhAI. PheI and/or LysB-29 amino groups (seven possible derivatives), and resulting insulin conjugates purified to homogeneity. *In vivo* bioactivity in rats of most derivatives was preserved while disubstituted PEG₁₀ insulins showed decreased potency. Only site-specific modification of PheI amino group with either moiety resulted in pronouncedly increased resistance of insulin to fibrillation, indicating that the B-chain N-terminus of the insulin molecule is mechanistically involved in the fibrillation process. Immunogenicity *in vivo* and *in vitro* of monoglycosylated insulins was comparable to that of insulin, diglycosylated insulins showed immunogenicity enhancement. Immunogenicity of PEG₁₀ insulins was significantly suppressed. PheI-glycosylated insulins administered subcutaneously in dogs displayed extended action profiles, the most effective being PheI1-galactosylated insulin, resembling the pharmacodynamic response of intermediate-acting insulin preparations. The pharmacokinetic parameters of these insulin derivatives were not significantly different from that of insulin even though absorption and residence time and clearance were increased, providing some explanation for prolonged action profile. Leucin-specific binding as a retardation basis is not likely involved. In support of this, subcutaneously administered PheI1-PEtC(400) insulin showed an even more pronounced action profile, suggesting that the basis of retardation is physical and nonspecific. This implies that by increasing PEG chain MW, further delay/prolongation of action can be achieved to yield new soluble basal insulin substitutes with potential clinical applications. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Glycosylated insulin. Polyethylene glycol. Polyethylene glycol insulin conjugate, PEG insulin, Insulin stability, Insulin immunogenicity, Pharmacokinetics, Pharmacodynamics, Parenteral delivery

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13. References

1. Introduction

The therapeutic benefit of insulin for the treatment of diabetes has been recognized for more than 70 years [1]. Since then, insulin has been the only drug approved for the treatment of insulin-dependent diabetes mellitus (IDDM; Type 1 diabetes). Conventional insulin treatment is basically a substitution therapy, in which exogenous insulin is administered subcutaneously to mimic, as close as possible, insulin secretion of a healthy pancreas. However, conventional subcutaneous insulin therapy does not result in normal physiological daily concentrations of insulin in blood and target tissues [2,3]. As a result, metabolic regulation in diabetic patients is compromised, which is mainly reflected in frequent hypoglycaemic episodes during/after meals and hyperglycaemia between meals. This became even more apparent after the introduction of patient self-monitoring of blood glucose and laboratory measurement of glycosylated haemoglobin [4]. These findings led to the use of conventional intensive insulin therapy which provides a better glycaemic control in IDDM patients and slows down the progression of retinopathy, nephropathy and neuropathy as demonstrated by the results of Diabetes Control and Complications Trial [5]. It involves different regimens with multiple daily injections of insulin, usually before meals, to simulate physiological insulin secretion [6]. This is, however, difficult to achieve using short-acting neutral regular insulin preparations because the absorption from the sub-

cutaneous tissue is delayed [7]. This can lead to elevated plasma insulin levels in the post-absorptive periods and, consequently, the risk of hypoglycemia between meals and at night is enhanced [8]. To compensate for the inadequacies of conventional mealtime neutral insulin, the time of the injection should precede the meal by at least 30 min [9], but this requirement is commonly ignored. The lag of appearance of subcutaneously administered regular zinc insulin in the plasma results from the slow dissociation of insulin hexamer [10,11]. It is a very well documented and accepted fact that in neutral solution in the presence of Zn^{2+} at pharmacologically relevant insulin concentration (U-100, 0.6 mM), three insulin dimers assemble to form a hexamer of the insulin monomers [12]. Insulin hexamers are the predominant association species of zinc insulin, at least down to a concentration of 0.1 mM [13]. Thus, it was reasoned that preparing fast-acting monomeric insulin analogs which would have a rapid absorption and onset of action, as well as disappearance from subcutaneous injection site, would obviate the need for administration of soluble insulin 0.5–1 h before meal, as well as reducing postprandial hypoglycemia, and also minimizing risk of delayed hypoglycemia [14,15]. Recombinant DNA technology and protein engineering methods were successful in preparing human insulin analogs that displayed stable monomeric or dimeric forms by changing some key amino acid residues along the polypeptide chain involved either in monomer or dimer dimer interface [16,18].

is a sine qua non for fibrillation to occur. This seems to be quite an important issue for the stability of fast-acting monomeric insulin analogs even though such data are not explicitly available [16]. Indeed, very recently fast-acting insulin analog LysPro (Eli Lilly) is still formulated in the presence of Zn²⁺ and phenol, presumably increasing insulin stability, and thus is administered in hexameric form [15,18]. Fast absorption of the analog from the injection site has been explained by faster dissociation kinetics of the analogs as compared to a regular insulin hexamer [49]. The issue of chemical stability has not been thoroughly addressed, presumably because no major differences as compared to regular insulin can be expected or can be predicted depending on the primary structure changes introduced. Finally, the immunogenicity issue, both humoral and cellular, of insulin analogs and conjugates must not be neglected and should be investigated in appropriate animal model(s) before any clinical studies are initiated [16].

Even highly purified human insulin formulations are slightly immunogenic which can be explained by the way of antigen administration as well as chemical alteration of the molecule that develops during storage or after injection [50,51]. Naturally, introduction of chemically foreign groups or mutation of insulin sequence can have a substantial impact on immunogenicity of a given insulin analog/conjugate.

Our group has been working for a long time on a unique insulin delivery system based on competitive binding, namely the displacement of glycosylated insulin by glucose from concanavalin A enclosed in a polymeric membrane device that is permeable to glucose and glycosylated insulin and non permeable to concanavalin A [52,53], which can be implanted into the peritoneal cavity. As a natural part of the project, we synthesized, purified and characterized seven glycosylated insulin derivatives by the site-specific attachment of *p*-succinamidophenyl glucopyranoside to the three available insulin amino groups GlyA1, PheB1 and/or LysB29 [54,55]. Physico-chemical stability in solution, immunological properties and pharmacokinetic/pharmacodynamic characteristics [55, 57] were also investigated. Although selected glycosylated insulins proved beneficial for some properties, especially stability and action profile (see below), further improvements on the therapeutic and stability properties of the protein, such as decreased

protection of insulin action for potential use in basal insulin delivery utilizing Co²⁺ insulin has recently been reported [52,53]. Insulin hexamers containing Co²⁺ are extraordinarily stable and practically unchanged in terms of regard to other transition cations [51,54] and thus, have a very reduced tendency to subunit dissociation. Only after *in vivo* reduction of Co²⁺ to Co⁺ in the bloodstream is the active insulin monomer released from Co²⁺ hexamer formed. The degree of protection of Co²⁺ insulin effect is similar to that of NPH insulin when evaluated in a euglycemic glucose clamp in pigs [54].

Pharmacokinetic and pharmacodynamic factors are not the only parameters to be considered with regards to insulin drug performance and safety. Other important parameters include physical and chemical stability, and immunogenicity [22]. The issue of physical stability of aqueous insulin formulations became of primary importance with the introduction of early continuous subcutaneous insulin infusion devices (insulin pumps) which frequently malfunctioned because of formation of insoluble insulin precipitate [36]. The main component of the precipitate is fibrous insulin, as was shown previously by electron microscopic studies [37]. Insulin, like numerous other proteins [38], can undergo a not well understood conformational change which results in successive, linear macroscopic aggregation and formation of a viscous gel or insoluble precipitates [39]. Basic building blocks of this phase are insulin fibrils or their twisted bundles with diameter ranging from 3 to 15 nm and length up to several microns [37,40]. The structural arrangement of insulin molecules in fibrils is still a matter of controversy even though, very recently, a structural model has been published suggesting that only limited structural changes of the insulin molecule enables the fibrillation process to proceed [41]. This model, however, contradicts a generally accepted view, and there is considerable evidence that amyloid fibrils from different sources and unrelated proteins, including insulin, share a common ultrastructure, the so called cross- β structure [42,43]. On the other hand, there is strong evidence that formation of insulin fibrils requires the presence of insulin monomer [44,45]. Hydrophobic surfaces like air-water interface as well as increased temperature promote aggregation [46, 48], indicating that a conformational change of the insulin molecule

Another part of a post-absorptive hypoglycemic risk comes from the non-physiological pattern of insulinemia and substantial absorption variability of currently available basal insulins [19, 21], which are always administered as suspensions [22]. The disadvantages of administration of intermediate and long-acting insulin as a suspension of microcrystals are several. First, at least a portion of variability appears to be due improper mixing of the vial or pen prior to withdrawal into the syringe [23]. Second, local variations in the subcutaneous tissue may influence the dissolution process, further increasing variability. Third, nonspecific defense mechanisms like the attraction of macrophages after injection of crystalline insulin suspension add on the variability of endocytosis of insulin microcrystals occurs [23]. Finally, because the rate of dissolution depends on the local blood flow in the capillaries at the injection site, the rate of dissolution is influenced by exercise [24] and local temperature [25]. Evidently, the variability of absorption based on the above factors could be decreased with soluble basal analogs that exhibit a protracted action profile. First attempts to overcome this concentrated on the first two drawbacks. Insulin analogs were designed to be soluble at low pH during storage but precipitate at neutral pH after injection. This was achieved by manipulation of the insulin isoelectric point from 5.4 to 7.3 by adding two positive charges [26]. Nevertheless, this derivative proved unsatisfactory in the clinical setting [27]. Similar experience resulted from using human proinsulin as a basal insulin substitute in clinical trials [21]. Very recently, soluble, fatty acid-acylated insulins with protracted action in pigs and diabetic dogs were introduced [23,28-31]. These analogs are stored soluble at neutral pH and stay soluble after injection. The retardation principle is based on specific binding of a fatty acid-insulin conjugate to serum albumin present in the interstitial fluid, which increases insulin derivative absorption time [23], as well as in the bloodstream, which substantially decreases its clearance rate [31]. As a result, subcutaneously administered fatty acid-acylated insulins show blood glucose lowering time profile more extended than those of intermediate-acting conventional insulin formulations NPH [23] or Humulin I [31], while inter-subject variability is very significantly decreased. Another novel principle to achieve

immunogenicity and increased elimination half-life and so, consequently decreased clearance and protracted action profile, could be possible by site-specific coupling of inert poly(ethylene glycol) (PEG) chains to insulin molecule. PEGs and monomethyl-PEG (mPEG) are non-toxic and have been approved by the US FDA for internal use [58]. Conjugation of proteins with PEGs has been shown to increase circulation half-life [59] and reduce immunogenicity of parent immunogen [59,60]. Thus, we have recently initiated studies involving PEG-insulin conjugates which are also discussed in detail further.

2. Synthesis and purification of insulin conjugates

2.1. Synthesis and purification of glycosylated insulin derivatives

Originally, we hypothesized that glycosylation of insulin molecule at PheB1 and/or LysB29 amino groups should lead to insulin derivatives with a suppressed self-association tendency and consequently faster subcutaneous absorption [14,16,18]. This is due to the fact that LysB29 is located on the edge of the dimerization surface of the monomer and PheB1 residue is involved in the dimer-dimer interactions of the hexamer [12]. Moreover, by incorporating the hydrophilic carbohydrate moiety onto the insulin molecule, the nonspecific aggregation process driven by hydrophobic forces could be suppressed, while maintaining the bioactivity of the insulin derivative.

First, the free carboxyl group of *p*-succinamidophenyl glucopyranoside (SAPG) was activated by the mixed anhydride method and the activated intermediate reacted with insulin at a 3:1 molar ratio in 50% DMF, pH 8.7 (Fig. 1). Under these conditions, the relative reactivity of insulin amino groups decreased in the order GlyA1 > LysB29 > PheB1. However, at pH 9.5 and higher, the reactivity of LysB29 becomes dominant [51,55]. The low reactivity of PheB1 amino group, which pK_a is <7.0 [55], was overcome by the reversible protection of GlyA1 and/or LysB29 amino groups by *t*-butoxycarbonyl groups (tBoc), with the subsequent coupling of Boc-insulin intermediates in

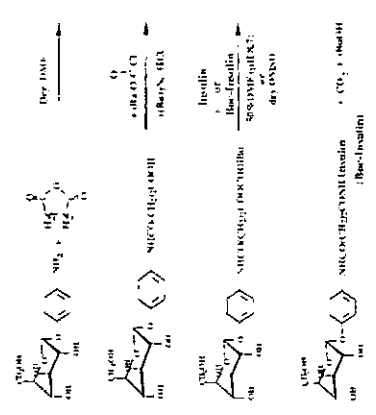


Fig. 1. Synthetic scheme of SAPG insulin derivatives.

DMSO with a 10 molar excess of activated SAPG and, finally, removal of Boc groups) with trifluoroacetic acid [55,61]. All described synthetic schemes yielded mixtures of SAPG insulin derivatives. Individual SAPG insulins were purified by two consecutive ion-exchange chromatography steps, first on a Mono Q column equilibrated at pH 7.9 and second on a Mono S column equilibrated at pH 3.0. Both purification steps required the presence of 7 M urea to achieve efficient separation [55]. SAPG insulins prepared through Boc chemistry (all PheB1-SAPG substituted insulins) required only one purification step on a Mono S column, since Boc insulin derivatives had already been purified on a Mono Q column. In parallel, glycosylated (SAPG), fucosylated (SAPF) and mannosylated (SAPM) insulin derivatives were synthesized and purified to be used in pharmacokinetic and pharmacodynamic studies.

2.2. Synthesis and purification of pegylated (PEG) insulin derivatives

Another interesting chemical moiety recently introduced for protein conjugation/modification is monomethylene glycol (mPEG) [62], and its monovalent derivative monomethoxyethyl(ethylene glycol) (mPEG). The attachment of (PEG chains) to a

protein molecule has been shown to alter hydrophobicity of the molecule through the increased molecular motion and dynamics of the PEG moiety [63], which substantially increases protein solubility and stability. It also has other beneficial effects on protein drugs in general, most importantly it leads to a very significant increase of elimination half-life and immunogenicity suppression [64-66]. Thus, a number of the drug properties can be improved by insulin pegylation.

First, we prepared monomethoxycarbonyl derivatives of mPEG (MW 600, 2000) by the reaction of its free OH group with succinic anhydride [67]. The free carboxyl group introduced at the one end of the succinylated mPEG molecule was activated by mixed anhydride method and reacted with amino groups of insulin as described above for glycosylated insulins. To prepare PheB1-substituted pegylated insulins, Boc-protected insulin intermediates were used in the same manner as for SAPG insulins [55,61]. The glycosylated insulins purification scheme as detailed above was applied to prepare pure PEG insulin conjugates. Later, we found that the presence of ester bond in the succinyl mPEG moiety renders these PEG insulin conjugates chemically unstable under the conditions required for long-term storage of aqueous insulin formulations [68]. We therefore synthesized carboxyl derivatives of mPEG (MW 600, 800, 2000, 5000) by alkylation of mPEG molecule with ethyl bromoacetate [68,69] (Fig. 2). Unreacted mPEG and colored byproducts were extracted from an alkaline water phase into an organic phase composed of chloroform and toluene mixture (9/1 by volume). Alkaline phase was acidified and mPEG carboxylic acid product (mPEG-COOH) was extracted into chloroform. The carboxylic group presence was confirmed by FTIR and its content quantified by titration (always >98%). For the later PEG insulin conjugates, we did not study the effect of pegylation of GlyA1 and/or LysB29 amino groups due to prior findings that attachment of hydrophobic groups at these sites did not improve insulin stability substantially [55,70]. Only PheB1 substituted mPEG-carboxylic acid insulin conjugates were synthesized starting from (Boc)-GlyA1 LysB29 insulin and purified to homogeneity on a Mono S column equilibrated at pH 3.0.

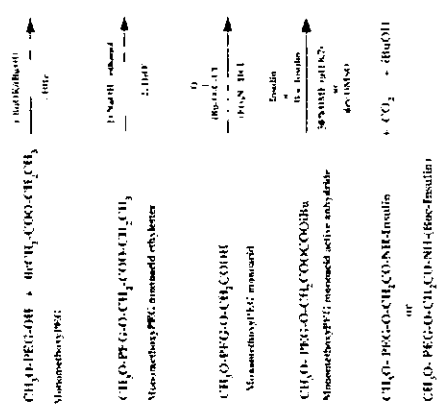


Fig. 2. Synthetic scheme of mPEG-monoacid and PEG-insulin conjugates.

3. Physicochemical properties, biological activity, and physical stability

3.1. Glycosylated insulins

The site of attachment of SAPG moieties (in individual SAPG insulins) was determined by N-terminal group analysis [55]. The number of coupled SAPG groups was independently determined by saccharide content and UV-spectrophotometric analysis since *p*-amidophenyl group of glycosyl moiety has an absorption maximum at 244 nm [53]. No significant changes in secondary/tertiary structure of SAPG-insulins occurred because of modification. This is demonstrated in Fig. 3 where far-UV CD spectrum of PheB1-SAPG-insulin is compared to that of Zn-free insulin. Similar results were obtained for other SAPG insulins. Biological activity of SAPG-insulins was tested *in vivo* by blood glucose depression test in rats (Table 1) after an intravenous dose of 3.3 μmol/mg/kg of a specific derivative [55]. Clearly, the bioactivities of most of the derivatives are close to that of native insulin, with the exception

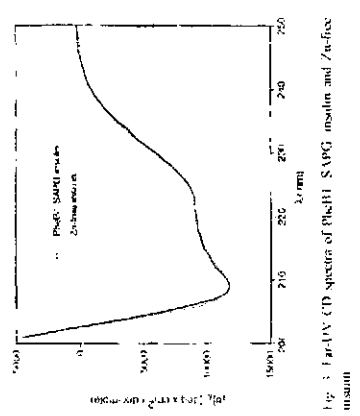


Fig. 3. Far-UV CD spectra of PheB1-SAPG-insulin and Zn-free insulin.

of GlyA1-LysB29 (SAPG)₂ insulin which showed significantly decreased bioactivity in rats.

Specific association of the monomers of glycosylated insulin derivatives into dimers and hexamers was studied by size-exclusion chromatography (SEC) and dynamic light scattering (DLS). SEC was used to evaluate association properties under non-equilibrium dynamic conditions (dissociation kinetics). Each derivative was injected at several different concentrations and constant volume and association state (number), and its concentration dependency was determined [55]. We concluded that GlyA1-PheB1-LysB29 (SAPG)₂-insulin is the most monomeric derivative because it showed the least concentration dependency of its association state. More importantly, the results clearly demonstrated that glycosylation of PheB1 is crucial to suppressing the association of dimers into tetramers/hexamers. The DLS method, which measures the average hydrodynamic radius of macromolecules, was used to corroborate these results under equilibrium conditions. Several concentrations were examined and the results obtained supported the conclusion drawn from SEC data that glycosylation of PheB1 is most influential in suppressing the hexamerization of synthesized insulin derivatives. These results correlate with X-ray structure of insulin hexamer in the *T*₀ conformational state, where PheB1 amino group was found to be a part of dimer-dimer interface [71] forming a salt

Table 1
Biological activity of SAPG insulin derivatives

SAPG insulin derivative	Biological activity (% SAPG)
Insulin	100 ± 1.7
GlyA1 SAPG insulin	97 ± 1.8
LysB29 SAPG insulin	94 ± 1.6
PheB1 SAPG insulin	93 ± 1.4
GlyA1-PheB1 (SAPG) ₂ insulin	92 ± 1.3
GlyA1-LysB29 (SAPG) ₂ insulin	91 ± 1.2
PheB1-LysB29 (SAPG) ₂ insulin	89 ± 1.1
GlyA1-PheB1-LysB29 (SAPG) ₃ insulin	87 ± 1.0

Determined with at least six animals

bridge with GluA17 of the neighbouring dimer: in the presence of Ni^{2+} cations, such as Zn^{2+} or Co^{2+} , and phenolic excipients, the hexamer undergoes a conformational change yielding the more stable R_2 hexamer, where this salt bridge is abolished [72]. Thus, modification of the PheB1 amino group should help stabilize the R_2 form of the hexamer when Ni^{2+} and phenolic compounds are added. The concentration of Co^{2+} R_2 hexamer can be directly measured by visible absorption spectroscopy [73]. Indeed, under these conditions PheB1 SAPG insulin covalently produced a more pronounced visible absorption spectrum than insulin derivatives glycosylated at one or two of the other amino groups, and the spectrum was comparable to that of insulin [55]. Consequently, pharmaceutical formulation of PheB1 SAPG insulin which will require the presence of Zn^{2+} and phenol should be as stable as ordinary soluble neutral Zn insulin formulation.

Table 2
Concentration dependency of physical stability of SAPG insulins

SAPG insulin derivative ^a	0.5 mg/ml	1.0 mg/ml	2.0 mg/ml	4.0 mg/ml
Zn insulin	6.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)
Zn-free insulin	0.6 (0.2)	1.0 (0.5)	1.0 (0.5)	1.2 (0.3)
GlyA1 SAPG insulin	3.4 (2.3)	2.2 (0.3)	1.7 (0.4)	1.2 (0.7)
PheB1 SAPG insulin	12.7 (2.9)	11.0 (4.7)	9.8 (3.0)	9.6 (3.5)
GlyA1-LysB29 (SAPG) ₂ insulin	4.8 (3.5)	3.5 (1.3)	ND ^b	ND ^b
PheB1-LysB29 (SAPG) ₂ insulin	15.0 (1.2)	8.2 (2.0)	6.6 (1.6)	6.6 (1.7)
GlyA1-PheB1-LysB29 (SAPG) ₃ insulin	12.5 (7.1)	10.1 (5.0)	3.4 (1.8)	2.3 (1.2)

^aZn, Zn²⁺, no phenol, only 0.01 M PHS, pH 7.1

^bValue is ± 1.2 , insulin/SAPG insulins in 0.1 M PHS, pH 7.1 were shaken at 100 strokes/min and 37°C

^cNot determined

PheB1-LysB29 (SAPG)₂ insulin as described above. As analytical concentration of these derivatives increases, so does the concentration of corresponding insulin derivative monomer required for fibrillation to take place and, thus, the stabilization period decreases. This does not hold true for PheB1 SAPG insulin which is mostly dimeric in the concentration range investigated, and so stabilization effect is preserved. Generally and regardless of concentration, these data indicate that N-terminus of B-chain is directly mechanistically involved in fibrillation mechanism, perhaps to hydrophobic interface binding. The enhancement of physical stability through the stabilization of R-state-like conformation as previously suggested [55] can be excluded since recent structural studies have shown that insulin monomer in solution takes up a T-state-like structure [74].

3.2 PheB1 insulin conjugates

As in the case of SAPG insulins, the site of attachment of PEG moieties in individual purified PEG insulins was determined by N-terminal group analysis using dansyl chloride [55]. The number of coupled PEG chains was independently confirmed by cation-exchange chromatography on a Mono S column in 1 M acetic acid and 7 M urea, which provides an excellent group separation for insulin

and its amidated mono-, di- and trisubstituted derivatives [53]. To find out if changes in secondary tertiary of the insulin molecule occurred because of PEG chain(s) coupling, far-UV CD of mono- and disubstituted PEG insulins (PEG MW 600, 2000) were compared to that of Zn-free insulin. Similarly to SAPG insulins, the spectra practically overlapped regardless of PEG MW, indicating that no significant spatial structural changes occurred. A preliminary note on PheB1-PEG 5500 insulin [69] reported attenuation of near-UV CD signal which can be very likely explained by the perturbation of a monomer-dimer hexamer equilibrium in the monomer/dimer direction [75] (see further).

Biological activity of PEG insulins was tested in vivo by blood glucose depression test in rats (Table 3) after an intravenous dose of 3.4 mmol/ml/kg of a specific derivative. Mono-substituted PEG(600) insulins retained almost 100% of the biological activity as compared to native insulin regardless of the substitution site. For a longer PEG(2000) chain, only the bioactivity of GlyA1-PEG(2000) insulin was significantly decreased ($P < 0.02$, Student's *t*-test). This reflects a generally accepted fact that modifications at the GlyA1 amino group yield insulin derivatives with reduced affinity for insulin receptor [16,76]. Indeed, recent binding studies of GlyA1-SAPG insulin and R1N5S1 rat insulinoma cell line showed that the insulin receptor binding constant is 3

Table 3
Biological activity and physical stability of PEG insulins

PEG insulin derivative	Biological activity, $\pm$ SD (IU/mg)	Days to fibrillation, $\pm$ SD
Zn-insulin	25.2 $\pm$ 4.7	0.5 $\pm$ 0.2
GlyA1-PEG(600) insulin ^a	22.2 $\pm$ 4.0	4.7 $\pm$ 1.4
PheB1-PEG(600) insulin ^a	23.8 $\pm$ 4.2	22.0 $\pm$ 4.6
LysB29-PEG(600) insulin ^a	25.0 $\pm$ 5.2	ND ^b
GlyA1-PheB1-PEG(400)-insulin ^a	19.2 $\pm$ 3.2	36.1 $\pm$ 5.6
GlyA1-LysB29-PEG(600)-insulin ^a	16.1 $\pm$ 3.6	7.4 $\pm$ 1.8
GlyA1-PheB1-LysB29-PEG(600)-insulin ^a	ND ^c	29.9 $\pm$ 5.3
GlyA1-PEG(2000) insulin ^a	15.7 $\pm$ 3.1	4.8 $\pm$ 1.6
GlyA1-LysB29-PEG(2000)-insulin ^a	13.4 $\pm$ 2.6	6.6 $\pm$ 1.5
PheB1-PEG(600) insulin ^a	24.8 $\pm$ 3.8	21.1 $\pm$ 5.5
PheB1-PEG(2000) insulin ^a	20.8 $\pm$ 3.5	26.9 $\pm$ 4.9
PheB1-PEG(5000) insulin ^a	17.8 $\pm$ 2.2	ND ^b

^aIn PHS, succinylated insulin conjugates

^bIn PHS, O², CO² insulin conjugates

^cDetermined with at least six animals

^dValue is ± 1.2 , protein concentration 87 μ M in 0.1 M PHS, pH 7.1 were shaken at 100 strokes/min and 37°C

^eNot determined

times lower compared to native insulin [61]. On the other hand, the bioactivities of PheB1-PEG(2000) insulin and PheB1-PEG(5000) insulin are also decreased, even though the decrease is not significant. Since PheB1 and its vicinity are not involved in the receptor binding [16,76], the negative impact on bioactivity can be most likely explained by non-specific steric hindrance to the receptor binding by the PEG moiety caused by its large hydrodynamic volume [58]. This effect should also contribute to decreased bioactivity of GlyA1-PEG(2000) insulin discussed above, and becomes even more important for disubstituted PEG insulin derivatives (Table 3). Nevertheless, to make these assumptions more strong, the receptor binding constants for PEG insulin derivatives remain to be determined. Others have also shown that blood glucose lowering activity *in vivo* of PheB1-PEG insulins is more or less preserved as compared to insulin [28,77]. These authors moreover, found that these insulin derivatives cause lower utilization of glucose by fat cells *in vitro* as well as by adipose tissues *in vivo*, than does the native insulin, and suggested that, by using certain modified insulins, it may be possible to manipulate the extent of glucose metabolism by lipid tissues in diabetic patients [77].

Specific association of the monomers of PEG insulin conjugates into dimers and hexamers was studied by SEC method only. We arrived at conclusions very similar to those for SAPG insulins [70], that pegylation of PheB1, regardless of the length of PEG chain, is crucial to suppressing the association of dimers into tetramers/hexamers. Thus, PheB1-PEG insulins can be considered dimeric inside the range of pharmaceutically relevant concentrations (U-40 and U-100). The long-term solution physical stability of PEG-insulins was studied in an accelerated shaking test [16] as with SAPG insulin conjugates, but only at one selected concentration of 0.5 mg/ml (Table 3) [68,70]. Again, PEG moiety to PheB1 had much far significant impact on insulin stability increase that coupling of PEG chains to the two other insulin amino groups (GlyA1/LysB29). The length and number of PEG chains are only of secondary importance with regards to the stabilization effect. In general, when taking into account SAPG insulins stability data, the site-specific attachment of a hydrophilic moiety to PheB1 amino group

of insulin substantially increases physical stability of insulin in solution. This conclusion is supported by the stability data of PheB1-substituted insulin, which showed a very similar stability increase [78].

4. Immunogenicity

4.1 Studies *in vivo*

A number of unwanted side effects in insulin therapy including allergic reactions and prothrombotic action of injected insulin can be attributed to the production of insulin-specific antibodies. Insulin antibodies circulating within the vascular compartment bind insulin absorbed from subcutaneous injection site and formed complexes act as an undrained insulin reservoir, delaying insulin action, thus increasing risk of postprandial hyperglycemia [79] and delayed hypoglycemia [80,81]. Since even pure homologous human insulin chronic administration gives rise to production of insulin specific antibodies [82], it is necessary to investigate immunogenicity of insulin conjugates where the attachment of a foreign chemical moiety creates new potential immunogenic epitopes.

The ability of glycosylated insulins to induce *in vivo* antibody response was evaluated with two genetically different inbred strains of mice: A/J (haplotype H-2^k), which was shown to be low anti-insulin responder, and C57BL/10ScSn (haplotype H-2^d), which is high anti-insulin responder [56,61]. Glycosylated insulin derivatives (GlyA1 and/or LysB29 substituted) were administered 3 times (0, 3, 6 weeks) at two different doses of 5 and 50 µg per immunization intraperitoneally as alum precipitate or subcutaneously with complete Freund's adjuvant. Specific IgG antibodies were measured by ELISA indirect double-layer method with antigen adsorbed microplates [56]. Both immunization methods revealed the same trend while, generally, a lower antibody response was induced by the intraperitoneal administration route. The immunogenicity of monoglycosylated derivatives was found comparable to the native insulin irrespective of the sugar moiety attached (Glc, Man, Gal, Fuc), while GlyA1-LysB29-disubstituted insulins induced a higher response. Moreover, site-specific glycosylation (GlyA1

and/or LysB29) did not change the high and low insulin responsiveness pattern of the two inbred mice strains, suggesting that the immunodominant epitopes were not affected by glycosylation.

The *in vivo* immune response to PheB1-substituted glycosylated insulins was investigated using the same experimental protocol described above. Only lower antigen doses and nonparenteral administration route were examined (Table 4). While PheB1-SAPG insulin immunogenicity surprisingly decreased substantially as compared to native insulin, disubstituted PheB1-LysB29-SAPG insulin showed comparable immunogenicity and the immunogenicity of tri-substituted glycosylated insulin was higher than that of the native insulin. In the case of PheB1-glycosylated insulins, the nature of sugar substituent influenced immune response. Thus, glucosyl moiety was the least immunogenic, followed by mannosyl and galactosyl derivatives, and finally by PheB1-thiosyl insulin, the immunogenicity of which was higher than that of insulin. Interestingly, A/J mice gave consistently higher antibody titer than C57BL/10ScSn mice for all PheB1-glycosylated insulins tested. This indicates that original immunodominant epitope of insulin has been changed by the site-specific glycosylation of PheB1 amino group.

Finally, we studied immunogenicity of PEG insulin conjugates (Table 4). The effect of PEG chain

length was evaluated for PheB1-pegylated insulins only, while the effect of the number of PEG chains attached was estimated using trisubstituted (PEG(400))₃ insulin. The effect of pegylation of GlyA1 and/or LysB29 on the immunogenic potential was not separately studied because of stability issues (see Section 3.2). In general, pegylation suppressed immunogenicity of the insulin molecule very substantially. As the length and/or number of PEG chains increased, immunogenicity diminished. These results are within the lines of a generally accepted fact that pegylation of protein immunoconjugates leads to significant reduction of their immunogenicity [64]. In accord with the fact that PEG itself is not immunogenic, the immune response pattern of still immunogenic PheB1-PEG(400) insulin was the same as for insulin and GlyA1-LysB29-glycosylated insulins (see above). Evidently, no new immunogenic determinant is formed by pegylation but, rather, the immunogenic effect of existing determinants is suppressed.

4.2 Studies *in vitro*

We studied the effect of GlyA1- and/or LysB29-substituted glycosylated insulins (2, 4, 20 and 40 µg/ml) on *in vitro* spontaneous, Con A-induced T-cell and SpA-induced B-cell proliferative responses of mouse splenocytes and human peripheral

Table 4
Antibody titers induced in inbred mice by glycosyl insulins and PEG insulins applied intraperitoneally as alum precipitate

Insulin derivative	Mice strain	
	A/J	C57BL/10ScSn
Native insulin	10.2 ^a ± 1.8	11.1 ± 1.6
PheB1-SAPG insulin	2.9 ± 0.9	4.2 ± 0.6
PheB1-LysB29-SAPG ₁ insulin	11.8 ± 2.2	10.1 ± 2.0
GlyA1-PheB1-LysB29-SAPG ₁ insulin	12.1 ± 2.1	11.2 ± 1.0
PheB1-SAPG ₂ insulin ^b	11.9 ± 2.3	11.7 ± 2.1
PheB1-SAPG ₃ insulin ^b	14.5 ± 3.0	13.8 ± 2.5
PheB1-SAPM insulin ^c	13.7 ± 2.7	6.9 ± 1.5
PheB1-PEG(400) insulin ^d	6.6 ± 1.9	8.8 ± 1.8
PheB1-PEG(2000) insulin ^d	1.2 ± 0.8	1.6 ± 1.0
GlyA1-PheB1-LysB29-PEG(400) ₃ insulin	0	0

^aExpressed as log₁₀ of serum dilution (S/D) and determined with at least 10 animals

^bSAPG₁, succinamido-phenyl glycosylcarbamate

^cSAPG₂, succinamido-phenyl glucopyranoside

^dSAPM, succinamido-phenyl mannopyranoside

^ePEG, O-CH₂-CH₂ insulin conjugates

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5. Pharmacokinetic and pharmacodynamic studies

Pharmacokinetic and pharmacodynamic properties of glycosylated insulin derivatives after subcutaneous administration were studied in healthy mongrel dogs weighing 25-30 kg. First, selected SAPG insulins were investigated. Soluble neutral Zn insulin was used as a general control. GlyAl SAPG insulin was included as a "glycosylated insulin control" since it was not expected to produce glucose response profile different from that of insulin because of its association properties. PheB1 SAPG insulin and (SAPG)₁ insulin were selected because of their "dimeric" and "monomeric" character, respectively, and were expected after subcutaneous administration to produce a faster hypoglycemic response curve similar to other monomeric insulins [11,18]. Unexpectedly, PheB1 SAPG insulin showed extended time action profile (Fig. 4). Very similar protracted action profiles were found for (SAPG)₁ insulin and PheB1 LysB29 (SAPG)₁ insulin. These two last derivatives were, however, not investigated further simply because no available RIA insulin kit gave a binding curve that would enable us to reliably measure their pharmacokinetics. GlyAl SAPG insulin gave, as expected, a pharmacodynamic profile (blood glucose depression curve) very similar to that of Zn insulin [57]. Having realized the potential

of blood lymphocytes in primary cell culture by measuring ³H-TdR incorporation, the effect of monosubstituted derivatives at low doses (2 and 4 µg/ml) on proliferation was comparable to that of native insulin while disubstituted derivatives usually triggered increased antigen-specific proliferation in accordance with *in vivo* data. Evidently, glycosylated insulins substituted at two amino groups are stronger immunogens than those substituted on one amino group only. Interestingly, high concentration (40 and 200 µg/ml; monoglycyl, diglycyl and diflucosyl derivatives) had a negative effect on spontaneous proliferation of peripheral human lymphocytes but not mouse splenocytes [56]. Con A-induced T-cell proliferation was enhanced in cultures of mouse splenocytes only, predominantly by disubstituted insulins again in accord with *in vivo* data. SpA-induced B-cell proliferation for both human lymphocytes and mouse splenocytes was reduced at high concentrations of a number of mono- and diglycosylated insulin derivatives, similarly to the findings for spontaneous proliferation. Low concentrations (2 and 4 µg/ml) of diglycosylated insulins, on the other hand, increased B-cell proliferation triggered by SpA. Immunogenicity studies *in vitro* with PheB1-substituted glycosylated insulins are in progress and, thus, no comparisons can be made so far.

Finally, we have just completed preliminary *in vitro* immunogenicity experiments with PEG insulin conjugates using A/J mouse splenocytes. Only PEG insulins shown in Table 4 were studied. PEG insulin derivatives concentration was varied from 0.01 to 10 µg/ml. PEG insulins, as well as native insulin, had no effect on spontaneous proliferation of splenocytes. PheB1 PEG insulins reduced Con A-induced T-cell proliferation to a similar extent as insulin. Only irsubstituted PEG insulin showed no effect, suggesting that insulin receptor-mediated effects may be involved. On the other hand, PEG-insulins did not have a significant effect on SpA-induced B-cell proliferation as compared to control regardless of PEG chain length or the number of PEG chains attached. Generally, no stimulation of immunocompetent cell proliferation by PEG insulin derivatives was detected further supporting findings *in vivo*, that PEG insulins are less immunogenic than parent insulin.

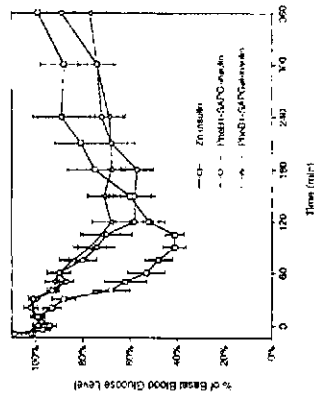


Fig. 4 Blood glucose level changes after subcutaneous administration of 0.7 mmol/kg of Zn insulin, PheB1 SAPG insulin or PheB1 SAPG insulin in six dogs (mean $\pm$ SEM).

benefits of PheB1 SAPG insulin as a prolonged soluble insulin derivative, we further explored this possibility with other PheB1-glycosylated insulin derivatives such as PheB1-galactosylated (SAPG)₁, fucosylated (SAPF) and mannosylated (SAMP) insulins. We hoped that by specific binding of these insulin derivatives to lectin-type proteins [83,84] with specificity for these sugar moieties (does not apply for glycosylated insulin derivatives) we could further extend the pharmacodynamic profile of these derivatives as was recently achieved with fatty acid insulin conjugates which specifically bind to albumin [30-32]. Especially, PheB1 SAPG insulin showed further prolonged and more shallow blood glucose depression curve (Fig. 1). PheB1 SAPG insulin action profile was comparable to that of PheB1 SAPG insulin, even though slightly more extended. PheB1 SAPM insulin, on the other hand, did not show such an extended action profile. Quantitative pharmacodynamic parameters for glycosylated insulins are shown in Table 5. Thus, PheB1

SAPG insulin demonstrated about 2 times extended action profile as compared to Zn insulin. PheB1 SAPM insulin appeared to be the only glycosylated insulin derivative less potent than subcutaneously administered human insulin, based on the area under the blood glucose concentration versus time curve (depressed AUC).

To get a deeper insight into the mechanism, the pharmacokinetic curves after subcutaneous administration were studied and pharmacokinetic parameters determined (Table 6) using statistical instant-derived noncompartmental analysis. While PheB1-glycosylated insulin plasma concentration changes exhibited profiles not very different from Zn insulin (Fig. 5, shown for PheB1 SAPG insulin only), the GlyAl SAPG insulin plasma profile after subcutaneous administration was markedly higher than the control and the corresponding AUC was significantly greater than that for insulin (Fig. 5, Table 6). We made similar observation during phase I-type clinical trial with six non-insulin-dependent diabetes mellitus

Table 5 Pharmacodynamic parameters for glycosylated insulins after subcutaneous administration (0.7 mmol/kg) in dogs

Insulin derivative	Depressed AUC (1 $\pm$ S.E.M. mg $\cdot$ dl ⁻¹ $\cdot$ min)	t_{max} (min)	S.E.M. (min)
Zn-insulin	8330 $\pm$ 1357	100 $\pm$ 19	
GlyAl SAPG insulin	8991 $\pm$ 4483	150 $\pm$ 16	
PheB1 SAPG insulin	6611 $\pm$ 1222	166 $\pm$ 16	
PheB1 SAPG-al-insulin	6621 $\pm$ 1576	200 $\pm$ 31	
PheB1 SAPF insulin	5312 $\pm$ 873	165 $\pm$ 22	
PheB1 SAMP-insulin	3488 $\pm$ 1640	125 $\pm$ 16	

*Area under the blood glucose concentration versus time curve

†Time to reach minimum blood glucose concentration

‡Statistically different from Zn insulin at $P < 0.05$

Table 6 Pharmacokinetic parameters for glycosylated insulins after subcutaneous administration (0.7 mmol/kg) in dogs

Insulin derivative	AUC ⁰⁻³ (1 $\pm$ S.E.M. in M $\cdot$ min)	MAIT (1 $\pm$ S.E.M. min)	MRT (1 $\pm$ S.E.M. min)	CL (1 $\pm$ S.E.M. ml/min/kg)
Zn-insulin	18 $\pm$ 3	106 $\pm$ 8	115 $\pm$ 8	13.1 $\pm$ 1.3
GlyAl SAPG insulin	113 $\pm$ 16	79 $\pm$ 12	100 $\pm$ 11	6.5 $\pm$ 1.1
PheB1 SAPG insulin	62 $\pm$ 9	127 $\pm$ 7	136 $\pm$ 7	12.2 $\pm$ 1.5
PheB1 SAPG-al-insulin	62 $\pm$ 11	153 $\pm$ 26	165 $\pm$ 10	11.7 $\pm$ 1.9
PheB1 SAPF insulin	59 $\pm$ 12	126 $\pm$ 3	134 $\pm$ 7	13.5 $\pm$ 2.2
PheB1 SAMP-insulin	78 $\pm$ 15	111 $\pm$ 26	126 $\pm$ 26	9.3 $\pm$ 1.5

*Area under the insulin derivative plasma concentration versus time curve

†Mean residence time

‡Total clearance

§Statistically different from Zn insulin at $P < 0.01$

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Table 7

Pharmacokinetic parameters for glycosylated insulins after bolus intravenous administration to 7 normal dogs in 3 days

Insulin derivative	AUC _{0-∞} × 10 ³ M ⁻¹ min ⁻¹	MRT ^a × 10 ³ min	CL × 10 ³ L min ⁻¹ kg ⁻¹
Zn insulin	101 ± 23	8.7 ± 0.9	8.8 ± 0.9
GlyAl SAPG insulin	177 ± 11	10.8 ± 1.1	6.0 ± 0.1
PheBI SAPG insulin	100 ± 6	9.5 ± 0.6	7.1 ± 0.5
PheBI SAPG insulin	176 ± 13	12.8 ± 0.6	5.8 ± 0.6
PheBI SAPG insulin	131 ± 11	15.0 ± 2.7	5.1 ± 0.5
PheBI SAPM insulin	166 ± 17	11.6 ± 1.1	7.6 ± 1.4

^aArea under the insulin derivative plasma concentration versus time curve

Mean residence time

Local clearance

^bStatistically different from Zn insulin at *P* < 0.01

^cStatistically different from Zn insulin at *P* < 0.05

as in the case of PheBI PEG insulins. Similar plasma levels and profiles after subcutaneous administration of PheBI-glycosylated insulins, as compared to Zn insulin, support this assumption. Nevertheless, noncompartmental pharmacokinetic analysis revealed some differences, even though not significant (*P* > 0.05) (Table 6). PheBI-glycosylated insulins generally exhibited increased mean absorption time (MAT), mean residence time (MRT), and lowered clearance (CL) in accord with retarded action profile. Among these, PheBI SAPG insulin revealed the slowest absorption rate and medium-delayed clearance from the vascular compartment, and also the most delayed pharmacodynamic response, indicating that lectin-specific binding could be involved. This is, however, not supported by PheBI SAPG insulin pharmacokinetic and pharmacodynamic data that were very close to those of PheBI SAPG insulin, the derivative of which cannot be expected to have a lectin-binding capability [83,84]. PheBI SAPM insulin data do not support lectin-binding hypothesis either and so the issue remains controversial.

Blood glucose pharmacodynamic profiles after intravenous administration in dogs for glycosylated insulins studied were not significantly different from that of insulin control. Pharmacokinetic analysis revealed the same trend as for subcutaneous administration. Thus, only GlyAl-SAPG insulin showed significantly increased pharmacokinetic parameters AUC^c, clearance and mean residence time (Table 7). However, contrary to the subcutaneous administration route, MRT values for PheBI SAPG insulin, PheBI SAPM insulin and PheBI SAPM insulin were significantly elevated.

A number of observations indicate a delay in insulin action at the cellular level. In other words, the effect course of insulin is more prolonged than the insulin course [7,85]. A possibility exists that the receptor mediated transcytosis over the endothelial cells significantly delays insulin action [86]. This will also have, of course, a significant impact on the action profile of insulin analogs and derivatives, such as glycosylated insulins. An alternative explanation for delayed insulin response assumes that a substantial part of administered insulin is degraded in the liver and kidneys [87], whereas only a smaller part is taken up by muscles where most of the glucose

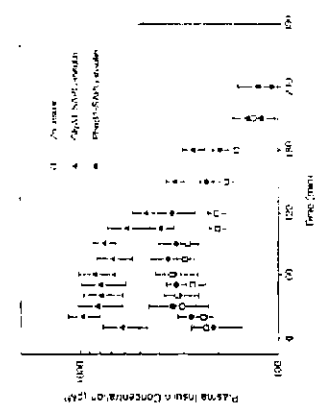


Fig. 5. Plasma immunoreactive insulin concentration (pM) after subcutaneous administration of 0.7 nmol/kg of Zn insulin, GlyAl SAPG insulin or PheBI SAPG insulin in six dogs (mean ± SEM).

patients without complications [61]. After subcutaneous administration of equimolar doses of either GlyAl SAPG insulin or Zn insulin randomly (1-week resting period between the two injections), we observed significantly elevated GlyAl SAPG insulin plasma levels, while the potency of the derivative was preserved and action profile was not changed. Previous results have shown that protein engineered insulin analogs, having affinities of 20-300%, are equipotent on a molar basis *in vivo* [7]. The analogs with lower insulin receptor binding constant concomitantly display higher plasma levels and consequently lower clearance than does native insulin. Higher blood levels likely compensate for lower receptor affinity, preserving overall bioactivity of such insulin derivatives [85]. GlyAl SAPG insulin falls into this category since its insulin receptor binding constant, $2.46 \times 10^6 \text{ M}^{-1}$, is more than 3 times lower than that of insulin ($7.64 \times 10^6 \text{ M}^{-1}$) [61]. Similar phase I-type clinical trial with GlyAl SAPG insulin gave the same results (unpublished), indicating that the sugar moiety in GlyAl-substituted insulins is not important with regards to pharmacodynamics of these derivatives.

It is reasonable to assume that PheBI-glycosylated insulins' affinity for insulin receptor is not significantly changed for the reasons explained above [16,76], but this remains to be experimentally proven

which should also have more or less preserved bioactivity [16,76] and can serve as an alternative to PheBI PEG insulins with regard to the protraction of insulin action. Obviously, these specific PEG insulin derivatives have potential clinical application as new soluble substitutes for insoluble basal insulin formulations, similar to fatty acid insulin conjugates [23,31] but without the requirement for specific binding to serum albumin.

6. Conclusion

Two different types of insulin conjugates are the subject of this review: glycosylated insulins and PEG insulin conjugates. Specific synthetic routes were designed to prepare seven glycosylated insulin derivatives by site-specific attachment of SAPG moieties to insulin amino groups and the derivatives purified by two ion-exchange chromatography steps. Galactosylated, mannosylated and fucosylated insulins were synthesized and purified analogously. PEG insulin conjugates were prepared in the same manner using carbonyl derivatives of methoxy-polyethylene glycols with different MW. Bioactivity of monosubstituted derivatives in rats was mostly preserved while disubstituted PEG insulin conjugates showed significantly decreased *in vivo* potency. Only site-specific covalent attachment of hydrophilic glycoside or PEG moieties to PheBI amino group of insulin substantially increased insulin resistance to fibrillation. The effect of modification at GlyAl or LysB29 amino groups was much less pronounced. This suggests that N-terminus of B-

ORIGINAL ARTICLE

Antithrombotic properties of a direct thrombin inhibitor with a prolonged half-life and AT-mediated factor Xa inhibitory activity

C. M. T. VOGEL, G. MEULMAN, F. G. VAN DEN HOF, R. BUIJSMAN, A. W. M. PRINCING
and M. J. SMIT

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Summary Richard: Infection generation after successful oncometabolism could be related to intracellular term autocatalytic therapy, and to the inability of hepatic derivatives to inhibit cell-bound chemotherapy. To meet these short-circuiting, a common mechanism is to use a pentachloride compound, which is used to affect infection inhibitor. This compound (C₁₂H₁₀) has a 10 times larger half-life compared with the original half-life of the infection inhibitor, while the infection inhibitor activity is maintained. An extra advantage of this product is the inhibitory activity on infection generation via anti-infection (HAI)-mediated factor (HAI) inhibitors. C₁₂H₁₀ antibodies (HAI) *in vitro* could bound infection with similar activity to the direct infection inhibitory agent. In experimental model, in vivo, C₁₂H₁₀ showed an in vitro low similar antitumor activity to

PXA inhibition of arterial thrombolysis. Finally, Dp-72675 was less than 10% as active as tPA in the inhibition of arterial thrombolysis and was more active than fondaparinux sodium/AT in regard to the ability to offset tPA-induced fibrinolysis, was more active than streptokinase in the ability to offset tPA-induced fibrinolysis and was more active than alteplase in the ability to offset tPA-induced fibrinolysis. In addition, Dp-72675 was more active than the three reference compounds in an experimental thrombolysis model in rabbits. These properties of Dp-72675, with its PXa and cathepsin B/thrombin inhibitory activity in combination with its long half-life, make this compound a potential drug that is likely to be effective in the prevention of re-occlusion after successful thrombolysis in man.

Keywords: direct thrombin and XI-mediated Factor Xa inhibition, thrombolysis.

introduction

In the (TTM4)-9B, GUSTO-2B and OASIS-2 trials, the direct thrombin inhibitor bivalirudin was compared with heparin as adjuncts to thrombolytic therapy in patients with myocardial

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infection and unstable angina [17]. The results suggest that

thrombin is at least as effective as heparin. It was expected that thrombin would be more active, because of its ability to inactivate bound thrombin, which is exposed after thrombolysis [5, 7]. According to Weber and Richter [8] better results were obtained if the antithrombotic treatment is started soon, combined with the thrombolytic therapy and the treatment lasts at least 6 months. This was achieved because more thrombin is expressed during the thrombolytic process [9,10] and because of indirect evidence that exposed activation occurs after short-term treatment with heparin or thrombin once therapy is stopped [11]. In agreement with thrombin generation assays (or at least in 6 months after onset of acute coronary syndrome) [3]. However, in long-term therapy, the half-lives of the current direct thrombin inhibitors on the market are too short.

Legrand and low molecular weight heparins, such as LMW heparin, inhibit thrombin activity by accelerating its inactivation by α_1 -antitrypsin and α_2 -macroglobulin [13,14]. However, these AT -mediated thrombin inhibitors are only able to inhibit free thrombin, but are not able to sequester thrombin bound to a cell [15,16]. On the other hand, direct thrombin inhibitors, such as hirudin and apixiban, are able to neutralize thrombin bound to a cell or fibrin [7]. An important limitation of direct thrombin inhibitors is their inability to prevent new generation of thrombin [7]. This study summarizes the antithrombotic and antitrombotic properties of Org-42675, a synthetic compound existing of a pentasaccharide with AT -mediated F_1X_2 -dependent inhibitory activity which was conjugated to a Malgate steroidal direct thrombin inhibitor in a test space. The synthesis of the new compound of this series was described by Baigún *et al.* [15]. It was hypothesized that this compound (i) could inhibit thrombin generation simultaneously with the inhibition of thrombin thrombin, and (ii) would show a prolonged elimination half-life. Binding of the compound to AT (the process of thrombin inhibition) was studied by fluorescence resonance energy transfer experiments. Sodium shows an elimination half-life of 13 h [15]. Org-42675 was tested in experimental thrombosis in man [16]. Org-42675 was tested in experimental thrombosis models with thrombin developing under various flow conditions in vivo, representing different forms of venous and arterial thrombosis. The inhibitory activities of Org-42675 in these models were compared with those obtained with heparinase, a natural anticoagulant.

of the 40 subjects were compared with a standard population of 40 subjects in an examination of each item, irrespective of which the predominant state induced by them. In 1946, B. A. [redacted] et al.

Materials and methods

Asymptotic oscillations[illegible]

Female Beagle dogs (body weight 6–8 kg) and male New Zealand rabbits were used. Rabbits were anesthetized by a mixture of 35 mg kg⁻¹ Ketamine and 5 mg kg⁻¹ Sedamipron (a mixture of 35 mg kg⁻¹ Ketamine and 2 mg kg⁻¹ *h*⁻¹ followed by a mixture of 10 and 2 mg kg⁻¹ *h*⁻¹, respectively). The Ethics Committee of Animal Welfare in accordance with Dutch guidelines approved all procedures.

Veronix Therapeutics Inc. 178

Thrombus formation in rats was induced by i.v. tissue thromboplastin followed by stasis of the vena cava for 10 min. Thrombus was removed and weighed. Test compound or placebo was administered intravenously 5 min prior to the induction of thrombosis.

Venous thrombosis induced with partial stasis (19)

Venous thrombosis in rats was induced by insertion of a silk thread (size 4/0) into the vena cava of anaesthetized rats using a guide cannula (see Fig. 1a) (Kawachi *et al.*, 1992). The cannula was inserted caudally (easier). Applying a light force beneath the right renal vein exposed the vena cava. The cannula was inserted into the vena cava with a 10–20 mm cannula induced partial thrombosis after withdrawal of the cannula. Test compound or placebo was administered intravenously 2 min before. Fifteen minutes later the silk thread with thrombus was exactly removed and weighed.

Arterial thrombosis in an intact vessel [20]

Thrombus formation was induced on a silk thread (size 4/0) positioned in the rat aortic artery via the common carotid artery by the use of a guideline catheter. Test compound or placebo was injected intravenously 2 min prior to thrombus induction. After 60 min of blood circulation, the silk thread covered with thrombus was carefully removed and the weight was determined.

Arterial thrombosis after vessel wall damage (dose-response experiment) [19]

A silicone cuff was placed around the carotid artery to anesthetized rats. blood flow (Doppler) was recorded proximally. Test compounds were administered with a loading infusion.

suggesting no 'priming' effect of Mg^{2+} concentration on tyrosine phosphorylation of the Ca^{2+} channel. In the Ca^{2+} threshold experiment, the main index of the type of action potential (AP) that the Ca^{2+} channel was subjected to was the number of APs. The action potential waveform was applied on the Ca^{2+} current, presented in the cell, to induce internal damage. The Ca^{2+} current was recorded within 2 min to a complete level of steady-state activation and within 10 min in view of exclusion of external Ca^{2+} and within 1 h after the Ca^{2+} channel was closed. The Ca^{2+} current was recorded to change in these time-lapsed flow was monitored according to the Ca^{2+} threshold reduction.

Incumbency model in rational

[illegible]

Anticoagulant activity

Plasma c-fos activity was measured immunocytochemically using the chromogenic substrate 5-2222 (21). Several dilutions of Triton X-100 buffer, 1H123 plasma samples, thus ensuring that with equally prediluted pooled rat plasma, this staining was absent. At concentrations and to reaction time concentrations within the range of 0.006–0.2 and 1–30 s, the X-to-Y distance was stable, 50(11.5)1 ml, 1% bovine factor (Xa) was added to sample, 50(11.5)1 ml, 1% bovine factor (Xa) was added to sample, 2 min later followed by 100(10.5)22, 5.2222 (0.75)2 ml, and c-fos activities (nmol, 1) were read from a calibration curve of test compound in spiked plasma by measuring the difference in optical density after 2 and 22 min at 405 nm. c-fos activities were used as standard.

NdA1 isolates of phages samples 3, 6, 7, 10, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 511, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 524, 525, 526, 527, 528, 529, 530, 531, 532, 533, 534, 535, 536, 537, 538, 539, 540, 541, 542, 543, 544, 545, 546, 547, 548, 549, 550, 551, 552, 553, 554, 555, 556, 557, 558, 559, 560, 561, 562, 563, 564, 565, 566, 567, 568, 569, 570, 571, 572, 573, 574, 575, 576, 577, 578, 579, 580, 581, 582, 583, 584, 585, 586, 587, 588, 589, 590, 591, 592, 593, 594, 595, 596, 597, 598, 599, 600, 601, 602, 603, 604, 605, 606, 607, 608, 609, 610, 611, 612, 613, 614, 615, 616, 617, 618, 619, 620, 621, 622, 623, 624, 625, 626, 627, 628, 629, 630, 631, 632, 633, 634, 635, 636, 637, 638, 639, 640, 641, 642, 643, 644, 645, 646, 647, 648, 649, 650, 651, 652, 653, 654, 655, 656, 657, 658, 659, 660, 661, 662, 663, 664, 665, 666, 667, 668, 669, 670, 671, 672, 673, 674, 675, 676, 677, 678, 679, 680, 681, 682, 683, 684, 685, 686, 687, 688, 689, 690, 691, 692, 693, 694, 695, 696, 697, 698, 699, 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716, 717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 827, 828, 829, 830, 831, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 84

[illegible]

The activated partial thromboplastin clotting time (APTT) was measured using Biotek[®] Acum[®] 1[®] FS, the prothrombin time (PT) using Stomplasia[®] Ecl and the thrombin clotting time (TCT) using 2.5 (Cui),¹ human thrombin in Barrow plasma at a KC10 coagulometer.

Neutrality studies

Compounds were dissolved in TBE buffer at 1.0 concentration (mmol/L). Thus, 50 mM NaCl, 174 mM Na₂BTA, 75 mM and 0.7% polyethylene glycol 6000 to a final concentration of 250 mM and incubated with equal volumes of different human recombinant P4a concentrations (1.5, 3333, 6666, 13333, and 26666) during 30 min at room temperature. Then these mixtures were diluted with 1 volume of TBE buffer containing 2.5 mM EDTA and 1 volume of the anti-P4a and anti P4a antibodies. The results were determined as described for the determinations, in relation and compared with the results obtained in a control incubation mixture without P4a.

Cross-reactivity with serum from hepatitis induced

Thrombocytopenic (TTP) patients

The cytotoxicity of compounds with HT antibodies was assessed by the release of ^{51}Cr -erythrocytes from pretreated human platelets [23]. HT serum was different patients (donation of Dr A. Genschel from Bonn, Germany; University, Kiel) and was used. Test compound (5 μL), concentrations of 0.01, 0.1, 1 and 10 $\mu\text{g mL}^{-1}$ and H_2O (2 μL) was added to 20 μL HT serum or Watson buffer pH 7.4 in a microtitre plate; then 15 μL washed human platelets from healthy volunteers and prelabelled with ^{51}Cr -erythrocytes were added. After 60 min of incubation at room temperature under continuous shaking, at 3700 rpm, the reaction was blocked with EDTA and the radioactivity was counted.

Pharmacokinetics

The jugular vein of anesthetized rats was cannulated (17,50) and exteriorized in the neck. After a recovery period of at least 16 h, test compound was administered via the cannula. Blood was collected at several time points from the cannula in 0.1 volume, 3.8% (w/v) sodium citrate and centrifuged (Thermo Biosciences).

The tested compound was administered into the saphenous vein to dogs, after which blood was collected from the jugular vein to 1.5 mg/ml. ¹ KCl/17A and centrifuged

The extra volume of ribbles was calculated for blood sampling. The volume of the experimental Test compound was injected into the intramuscular vein, after which blood was sampled for the calculation of caffeine. A 8% sodium citrate acid centrifuged. A 6.4 positive results were stored at -20°C until use.

According to the author [159]

A part of blood serum was injected (10 µl) into each well in the first row of sensitized rats. Four days after the administration of the test compound. The sera were agglutinated with anti-bovine serum albumin (BSA) and the agglutination was observed with the naked eye. The serum was put in the ongoing and removed immediately. Afterward, blood was extracted in water, after which the hemoglobin content was estimated spectrophotometrically.

1. *Onipourins*

Leipheim (M_w 15,000) was obtained from Dusseldorf AG (GAS, 42% nitrogen). For molecular structure studies, oligomers (M_w 2,000–10,000) were synthesized within the collaboration of NV Organon (GAS, the Netherlands, and Santon Research Inc. (Trompsburg, Virginia, Argonnean, Missouri¹), was purchased from Mitsubishi Kasei Chemical Industries Ltd (Yokohama, Japan).

Index evaluation

Thrombotic weights were expressed in mg or mg kg^{-1} , means $\pm$ SEM. ED_{50} values were calculated from the linear part of the curves and the curves were plotted by linear regression analysis according to Brown and Randol [234]. The ED_{50} with 95% confidence limits in the bivariate model was calculated with the Spearman-Kärner method [235]. Also using the hemoglobin content of the extracted blood was assessed bleeding. Means $\pm$ SEM were calculated for the affected group and expressed as percentage of control. The dose which caused bleeding to 50% of the control value (ED_{50}) was calculated by linear regression analysis of log-dose-response [234]. The pharmacokinetic parameters were calculated using the Mon Compartmental Analysis (MC A) of WinNonlin (Pharsight Corp, Alameda View, CA, USA).

Results

In vitro activities

The anti-IL-6 activity of Crg-126/75 in bovine PBMCs was 1.1 ± 0.11 IU/ml⁻¹, which was similar to that of pentoxifylline (Table 2), but lower than that of heparin. The anti-IL-6 activity of Crg-126/75 was 17.0 nmol L⁻¹, which was similar to that of Crg-126/75 derivative itself and approximately 10 times lower than that of agmatine (Table 2). Compared with heparin, the anti-IL-6 activity in human and rat plasma was 3.5 and 0.9 times less, respectively. Heparin Crg-126/75 and agmatine had similar effects on the release of IL-6 from PBMCs stimulated with LPS. The release of IL-6 was inhibited dose dependently by heparin (IC₅₀ 0.81–0.2 and 0.61–0.3 µg mL⁻¹ in human and rat PBMCs, respectively) and Crg-126/75 (IC₅₀ 0.001–0.0001 and 0.0001–0.00001 µg mL⁻¹ in human and rat PBMCs, respectively).

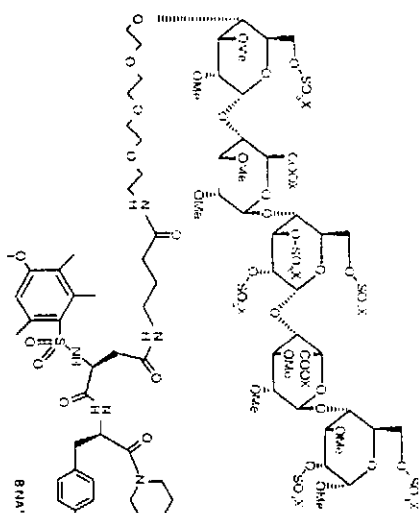


Fig. 1. The general structure of the 1,4-bis-*N*-methyl-2-pyrrolidone-2,5-bis(2,6-dimethyl-4-oxo-1,3,5-triazine-3-yl)-1,3,5-triazine-2,4,6-trisulfonate (1) (1,4-bis-*N*-MP-2,5-bis(2,6-DMT-3-*S*)-1,3,5-T-2,4,6-TS) monomer, a typical structural unit in the polymers. The structure of the monomer is shown in the inset.

Table 1. Acid-catalyzed and ^{125}I - and $\text{min-}^{125}\text{I}$ -labeled reactions involving N^{15} of the tested compounds in buffer at pH 7.4

Iron compound	Nucleolar weight	Area $\times 10^3$ (femol)	Area $\times 10^3$ (femol μ)	Area $\times 10^3$ (femol μ)	Area $\times 10^3$ (femol μ)
Depleted	1520*	2.85 ± 0.12	172 ± 3	1.71 ± 0.04	0.4
Replenished	1727	1.27^b	700 ^c		
Depleted + 0.5% ^{59}Fe	2007	1.01 ± 0.04	210 ± 4		

*Activity set to 700 dpm Na²²Cl mg⁻¹. And Na²² activities were determined from a calibration curve of an internal standard of trisubstituted acetone. The amino-alkaloids were determined from a calibration curve of the internal standard of Nephron IV. Error in activity is the error of at least two experiments ($n = 3$ per experiment).

Pharmacokinetics and pharmacodynamics

The pharmacokinetics of 500 nmol/kg ¹ Oxy 42675 was determined in rats using functional assays (Fig. 3) after i.v. or s.c.

Org 12675, like teslaparin, did not cross-react with H17 antibodies, in contrast to heparin. Heparin showed the typical bell-shaped dose-response for template-mediated release; complete release at concentrations of 0.005 and 0.05 $\mu\text{mol L}^{-1}$ and no release at 5 $\mu\text{mol L}^{-1}$ (data not shown).

Anti-thrombotic activities in rat experimental thrombosis models

Title activity (log_{10} -ED₅₀) had a mean inhibition half life of 10.1 (0.1) h, and after oral administration a bioavailability of 13.7% and 86% for the anti- $\beta\text{-TxA}_2$ and anti-thrombin activities, respectively (Table 5). In rodents, rats and dogs also similar AECs were obtained for the two activities (Table 5). The elimination half-lives obtained of the four tested reference compounds were 0.29, 4.00, 3.91 and 1.00 h for the non-complicated thrombin inhibitor of Org. 32675, 11013 + 00102, the angiotensin II antagonist of Org. 32675, 11013 + 00102 and the angiotensin II receptor antagonist of Org. 32675, 11013 + 00102, respectively. These results indicate that the anti-thrombotic effect of the studied compound was completely mediated by its specific inhibition of thrombin.

Anti-mediated inhibitors only heparin did not show complete biochemical inhibition of thrombin activity after oral administration.

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Үлэсүрэнзүх а тавь үхүүтэй нь.

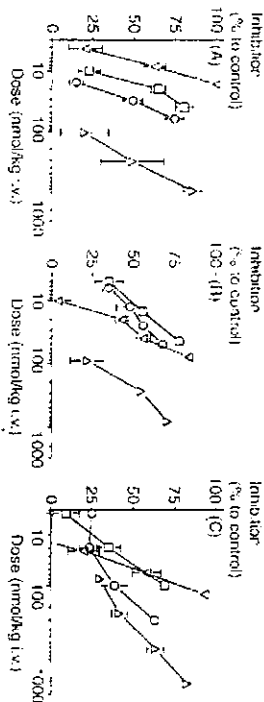


Fig. 4. Dose response to inhibition of fibrinogen synthesis by heparin (A), heparin sulfate (B), and argatroban (C) in the rat. Data are expressed as percentage inhibition (mean $\pm$ SEM) of the control value. The values obtained under control conditions are represented by the 100% value.

Table 6. Inhibition of fibrinogen synthesis by heparin, heparin sulfate, and argatroban in the rat. Data are expressed as percentage inhibition (mean $\pm$ SEM) of the control value after dosing with heparin.

Inhibitor	Starting dose (nmol/kg i.v.)	Maintenance dose (nmol/kg i.v.)	Time response (min)	% Inhibition
Heparin	100	100	10	100
Heparin sulfate	100	100	10	100
Argatroban	100	100	10	100
Heparin	100	100	10	100
Heparin sulfate	100	100	10	100
Argatroban	100	100	10	100

The anti-FXa as well as the anti-FIIa activity of Oxy-42675 were partially (40%) neutralized by high concentrations of PEG. This neutralization seems not specific, in contrast to the concentration-dependent neutralization of heparin. There is no explanation for this partial neutralization because no neutralization was observed after testing the pentasaccharide in Oxy-42675 itself (data not shown). Oxy-42675 does not activate platelets in the presence of HIT serum, whereas heparin shows the typical bell-shaped dose response of platelet activation, indicating its template activation pattern [23].

In rats, Oxy-42675 showed an elimination half-life of 3.0 h and an AUC of 16.0 nmol h⁻¹ l⁻¹ for both anti-FXa and anti-FIIa activities, which indicates that the compound is chemically stable in vivo. This half-life was 10 fold longer than that of the anti-coagulated thrombin inhibitor part of Oxy-42675 (80.29 h) and also longer compared with fondaparinux, argatroban and heparin. Also in rabbits and dogs the pharmacokinetics of anti-FXa as well as anti-FIIa activity were the same in each species, again confirming the stability of Oxy-42675 in these animals. Based on the elimination half-lives of Oxy-42675 in these species and on the elimination half-life of fondaparinux sodium in man of 13–15 h [10], the half-life in man is predicted to be

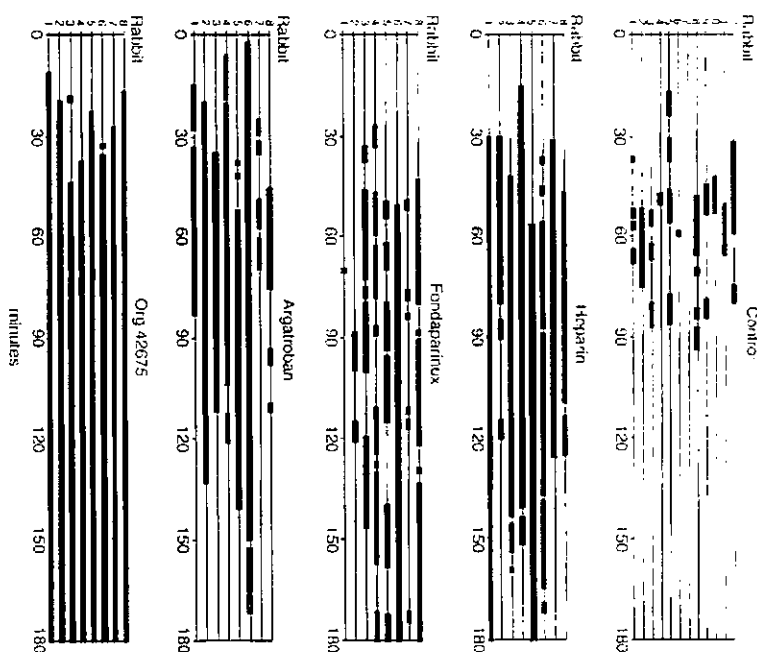


Fig. 6. Bar graphs showing the presence of blood flow after thrombolytic treatment in the rat. The values are expressed as percentage of blood flow remaining after thrombolysis. The values are expressed as percentage of blood flow remaining after thrombolysis.

30–60 h, based on allometric scaling, which would allow a once a day treatment. Oxy-42675 exerts potent, dose dependent inhibitory activity in different rat models of venous and arterial thrombosis. The contribution of platelet plug formation differs in the different models, in experimental models of venous thrombosis the participation of platelets is low, whereas in models of arterial thrombosis at high shear rates the involvement of platelets is high. Heparin displays relatively less antithrombotic activity in arterial type of thrombosis, presumably due to the higher sensitivity to neutralization of heparin in thrombin formation, in which platelets are involved. Compared with fondaparinux, Oxy-42675 predominantly gains its potency in the arterial models. Especially in the venous thrombotic model, in which aggressive vessel wall injury resulted in 100% occlusion within

30 min in the control rats, fondaparinux showed no dose dependent response and was not able to prevent occlusion for more than 50% of the tested animals. Compared with heparin, Oxy-42675 was somewhat less active in the venous stasis model but was similarly active in arterial thrombosis. Argatroban showed a similar activity in the three models with different flow, independently of whether thrombus formation was under venous or arterial conditions. This indicates that similar thrombin levels are generated in these models and that platelet involvement and differences in FXa generation cause the difference in effects in the presence of Oxy-42675, heparin and fondaparinux sodium. We examined whether Oxy-42675 also showed activity when the thrombotic process was ongoing. The tested compounds were administered at 5 min after the carotid artery was damaged

Table 7 Pre- and post-thrombotic inhibition with pre- and post-thrombotic

Time	Pre-thrombotic	Post-thrombotic	Pre-thrombotic
Time	Pre-thrombotic	Post-thrombotic	Pre-thrombotic
1	1.0	1.0	1.0
2	1.0	1.0	1.0
3	1.0	1.0	1.0
4	1.0	1.0	1.0
5	1.0	1.0	1.0
6	1.0	1.0	1.0
7	1.0	1.0	1.0
8	1.0	1.0	1.0
9	1.0	1.0	1.0
10	1.0	1.0	1.0
11	1.0	1.0	1.0
12	1.0	1.0	1.0
13	1.0	1.0	1.0
14	1.0	1.0	1.0
15	1.0	1.0	1.0
16	1.0	1.0	1.0
17	1.0	1.0	1.0
18	1.0	1.0	1.0
19	1.0	1.0	1.0
20	1.0	1.0	1.0
21	1.0	1.0	1.0
22	1.0	1.0	1.0
23	1.0	1.0	1.0
24	1.0	1.0	1.0
25	1.0	1.0	1.0
26	1.0	1.0	1.0
27	1.0	1.0	1.0
28	1.0	1.0	1.0
29	1.0	1.0	1.0
30	1.0	1.0	1.0
31	1.0	1.0	1.0
32	1.0	1.0	1.0
33	1.0	1.0	1.0
34	1.0	1.0	1.0
35	1.0	1.0	1.0
36	1.0	1.0	1.0
37	1.0	1.0	1.0
38	1.0	1.0	1.0
39	1.0	1.0	1.0
40	1.0	1.0	1.0
41	1.0	1.0	1.0
42	1.0	1.0	1.0
43	1.0	1.0	1.0
44	1.0	1.0	1.0
45	1.0	1.0	1.0
46	1.0	1.0	1.0
47	1.0	1.0	1.0
48	1.0	1.0	1.0
49	1.0	1.0	1.0
50	1.0	1.0	1.0
51	1.0	1.0	1.0
52	1.0	1.0	1.0
53	1.0	1.0	1.0
54	1.0	1.0	1.0
55	1.0	1.0	1.0
56	1.0	1.0	1.0
57	1.0	1.0	1.0
58	1.0	1.0	1.0
59	1.0	1.0	1.0
60	1.0	1.0	1.0
61	1.0	1.0	1.0
62	1.0	1.0	1.0
63	1.0	1.0	1.0
64	1.0	1.0	1.0
65	1.0	1.0	1.0
66	1.0	1.0	1.0
67	1.0	1.0	1.0
68	1.0	1.0	1.0
69	1.0	1.0	1.0
70	1.0	1.0	1.0
71	1.0	1.0	1.0
72	1.0	1.0	1.0
73	1.0	1.0	1.0
74	1.0	1.0	1.0
75	1.0	1.0	1.0
76	1.0	1.0	1.0
77	1.0	1.0	1.0
78	1.0	1.0	1.0
79	1.0	1.0	1.0
80	1.0	1.0	1.0
81	1.0	1.0	1.0
82	1.0	1.0	1.0
83	1.0	1.0	1.0
84	1.0	1.0	1.0
85	1.0	1.0	1.0
86	1.0	1.0	1.0
87	1.0	1.0	1.0
88	1.0	1.0	1.0
89	1.0	1.0	1.0
90	1.0	1.0	1.0
91	1.0	1.0	1.0
92	1.0	1.0	1.0
93	1.0	1.0	1.0
94	1.0	1.0	1.0
95	1.0	1.0	1.0
96	1.0	1.0	1.0
97	1.0	1.0	1.0
98	1.0	1.0	1.0
99	1.0	1.0	1.0
100	1.0	1.0	1.0

by active chloride. The vessel diameter was the 100% diameter in the dose response experiment (Table 1) resulted in occlusion within 10 min in the rats treated with arginine and in the rats treated with the combination of Arg-42675/phenylephrine and a Krapap derivative both simultaneously administered. The arteries of rats that were treated with heparin occluded within 10 min whereas no occlusion was observed in the rats treated with Arg-42675 alone (more than 100 min). The large difference in effect obtained with Arg-42675 versus other configurations of the two compounds to one compound renders a highly potent drug. Differences in kinetics among the tested compounds are the main explanation for the different effects, but also the addition of FXa generation inhibitory activity to a direct inhibitor would contribute to the beneficial effect of Arg-42675. Retained thrombin generation was shown after cessation of heparin therapy in unstable angina and in patients with coronary syndromes [26-28].

The effect on thrombotic re-occlusion following thrombolysis was investigated in a rabbit model. Ligation of the artery and application of t-PA resulted within 30 min in damage to the endothelial layer and mural thrombus formation and in shrinkage of the vessel wall within the next 5 min (unpublished observations). Applying saline of the artery during a period of 30 min formed occlusive thrombus. Thrombolysis was subsequently induced by a streptokinase infusion. It was not aim to reach similar plasma levels for the tested compounds during the infusion period. Plasma levels varied from 1.5 µmol L⁻¹ for Arg-42675 to 2.7 µmol L⁻¹ for heparin. The arterial thrombi in all 12 control animals lysed within 10 min but reocclusion occurred within 30 min. Treatment with Arg-42675 or argatanon induced a rapid opening of the occluded vessels compared with control, fondaparinux and in a lesser extent compared with heparin. Argatanon is known to enhance endogenous fibrinolysis [29], but it may also be that plasminogen activator activity is more important than FXa inhibition after the start of fibrinolysis. All arteries of the rabbits treated with argatanon occluded within the next 20 min after arrest of the infusion, probably because of the rapid reduction of anti-FIIa activity. In contrast, all arteries of the rabbits treated with Arg-42675 remained open during the experiment. Fondaparinux showed an irregular pattern of flow but at the end of the experiment 50% of the rabbits showed reflow. This could mean that in the second half of the

course re-occlusion inhibition of FXa was mainly important. Because of the lack of direct fibrinolytic activity of phenylephrine, heparin prevented more prolonged reocclusion than heparin particles that at the end of the experiment reocclusion occurred in 50% of the rabbits despite higher plasma levels. It could well be that FIIa release in the environment of the thrombus causes re-occlusion and possibly rebound thrombogenesis occurs [26, 27].

Re-occlusion through the occlusions frequently occur following successful thrombotic therapy [7, 17]. This thrombotic re-occlusion is probably induced by the thrombotic therapy itself. Plasmin mediated mechanisms may induce continuing systemic thrombin activation [30, 32], while clot lysis itself produces local thrombin formation. Current antithrombotic therapy displays limited efficacy; clot bound thrombin is relatively resistant to heparin [5, 6]. Direct thrombin inhibitors are able to inhibit this bound thrombin, but cannot prevent new thrombin generation [7]. Moreover, the renewed thrombin induction encourages for a considerable time which requires already anticoagulant plasma levels for a prolonged time, thus putting emphasis on anticoagulants displaying a long half-life.

This study demonstrates that Arg-42675, a dual-inhibitor analogue of an AT-inhibited FXa inhibitor to a direct thrombin inhibitor via an ester spacer, has potent anticoagulant and anti-thrombotic properties in experimental thrombosis. It also inhibits the activity of clot bound thrombin. Arg-42675 is functionally stable in vivo and displays half-life prolonged half-life in comparison with its parent, non-conjugated thrombin inhibitor. Arg-42675 is highly efficacious in the prevention of thrombotic reocclusion following thrombolysis of occlusive arterial thrombi in an experimental model. This improved efficacy in comparison with the conjugate drugs heparin, argatanon and fondaparinux is probably caused by its prolonged efficacy in inhibiting the bound thrombin and thrombin generation.

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Extending Insulin Action in Vivo by Conjugation to Carboxymethyl Dextran

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INTRODUCTION

Since 1980s, many patents with great therapeutic potential have become available due to enormous progress in recombinant DNA techniques. Their clinical efficacy, however, is often limited (somewhat due to a short half-life *in vivo*, instability, or immunogenicity). One general approach for overcoming these shortcomings is conjugation of a protein drug to a stable polymer, carrying out such as poly(vinyl alcohol) (P), poly(ethylene glycol) (PEG), or dextran (3), which usually results in a significantly longer molecular half-life, increased stability, and attenuated immunogenicity.

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⁵1H NMR (370 K, CDCl₃): δ 7.15 (m, 1H), 6.92 (m, 1H), 6.82 (m, 1H), 6.72 (m, 1H), 6.62 (m, 1H), 6.52 (m, 1H), 6.42 (m, 1H), 6.32 (m, 1H), 6.22 (m, 1H), 6.12 (m, 1H), 6.02 (m, 1H), 5.92 (m, 1H), 5.82 (m, 1H), 5.72 (m, 1H), 5.62 (m, 1H), 5.52 (m, 1H), 5.42 (m, 1H), 5.32 (m, 1H), 5.22 (m, 1H), 5.12 (m, 1H), 5.02 (m, 1H), 4.92 (m, 1H), 4.82 (m, 1H), 4.72 (m, 1H), 4.62 (m, 1H), 4.52 (m, 1H), 4.42 (m, 1H), 4.32 (m, 1H), 4.22 (m, 1H), 4.12 (m, 1H), 4.02 (m, 1H), 3.92 (m, 1H), 3.82 (m, 1H), 3.72 (m, 1H), 3.62 (m, 1H), 3.52 (m, 1H), 3.42 (m, 1H), 3.32 (m, 1H), 3.22 (m, 1H), 3.12 (m, 1H), 3.02 (m, 1H), 2.92 (m, 1H), 2.82 (m, 1H), 2.72 (m, 1H), 2.62 (m, 1H), 2.52 (m, 1H), 2.42 (m, 1H), 2.32 (m, 1H), 2.22 (m, 1H), 2.12 (m, 1H), 2.02 (m, 1H), 1.92 (m, 1H), 1.82 (m, 1H), 1.72 (m, 1H), 1.62 (m, 1H), 1.52 (m, 1H), 1.42 (m, 1H), 1.32 (m, 1H), 1.22 (m, 1H), 1.12 (m, 1H), 1.02 (m, 1H), 0.92 (m, 1H), 0.82 (m, 1H), 0.72 (m, 1H), 0.62 (m, 1H), 0.52 (m, 1H), 0.42 (m, 1H), 0.32 (m, 1H), 0.22 (m, 1H), 0.12 (m, 1H), 0.02 (m, 1H).

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interacted (6). In this case, the enzyme was coupled by peroxidase oxidation. Others took advantage of functional carboxyl groups on CMB to prepare stable, enzyme conjugates by using water soluble crosslinkers as the crosslinking agent (7–9). The use of carbodiimide activation is, however, problematic because of the well recognized toxicity of the activated intermediate to undergo an O- to N-acyl shift to form more stable, nonreactive N-acyl derivatives (10).

flonidil, a polypeptide hormone discovered more than 70 years ago [1], is the only drug used to control type 1 diabetes. As for any polypeptide hormone in general, its half-life in circulation is short, ranging from about 10 min to about 43 min for humans [12, 13]. As a consequence, soluble insulin action after exogenous (synthetic or subcutaneous but) administration is relatively short-

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By using well-established Ca^{2+} and insulin signalling pathways, we have developed a robust assay to measure insulin-like effects in the absence of endogenous insulin. In these assays, insulin-like effects are coupled to substrates that have been shown to be involved in the insulin signalling pathway. For example, insulin stimulation of the long-term effect of glucose Ca^{2+} release is mediated by a mixed anhydride method to selectively activate Ca^{2+} release from a mixed anhydride defined conditions so that we could control the site of activation of the Ca^{2+} release. We have characterized the Ca^{2+} release pathway in the insulin-stimulated state of the insulin-stimulated state, and studied its insulin response, binding, and its effect on *in vitro* smooth muscle features as well as its effect on *in vivo* smooth muscle features and the hyperglycemic effect in type 2 diabetes mellitus. Finally, we evaluated the pharmacokinetic properties of the Ca^{2+} insulin conjugate in rats and found that its elimination rate is significantly slower than that of insulin alone, consequently, the Ca^{2+} insulin conjugate profile shows a delayed, prolonged response, resembling the intermediate-acting insulin preparations (14).

EXPERIMENTAL, CLINICAL, RISK

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Synthesis and Purification of the CMI Insulin Conjugate. One hundred milligrams of freeze-dried CMI (degree of substitution, 0.6) and of carboxymethyl groupment of glucose (electrum unit) (CMI) formal, subsequently dried under high vacuum over P_2O_5 , was

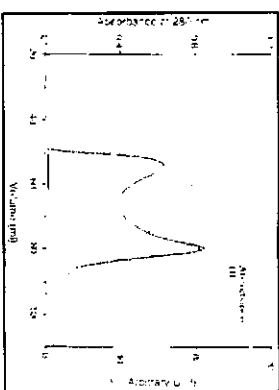


Figure 1. Purification of the CMO fraction containing the putative factor sequence 75 column (pH 5.5) in ARI. The product fraction containing the conjugate is purified by a two-step column. See Experimental Procedures for details.

mineral, 50, or 75 mmol of TEA and 25, 50, or 75 mmol of HEP, were added to the reaction mixture, and the mixture was stirred for 15 min under N₂. Similarly, monomers, three fixed amounts of linibin (115, 225, or 440 mmol) were added to the reaction mixture, and the mixture was stirred for 15 min under N₂. The monomers were dissolved in 3 mL of dry DMSO, and mixed well with the activated CMB solutions, irrespective of the HEP or TEA amount used for activation. Additional TEA (25, 50, or 75 mmol) was added, and the resulting solutions were stirred overnight at room temperature under N₂. The stirred mixtures were then diluted with 3 volumes of the solvent, dialyzed exhaustively against 0.02M NH₄HCO₃ water, dialyzed exhaustively against 0.02M NH₄HCO₃ and then freeze-dried. Separation of the conjugate and unreacted linibin was achieved using FPLC on a HiTrap Superdex S75 column (Pharmacia AB) at a flow rate of 1 mL/min. The column was equilibrated with 0.02M Tris (pH 7.4) buffer with 8.0% containing 0.5 M NaCl at a flow rate of 1 mL/min. Fifty milligrams of the mixture in 2 mL of water was injected, and the refractive index (RI) detector and flow of the eluent were continuously monitored. The first major peak eluted playing both 45 and 60 min (Figure 1) was pooled, dialyzed against 0.02M NH₄HCO₃ and lyophilized. To remove traces of physiological buffer, ion-exchange chromatography under identical conditions was necessary.

CMD has been conjugated to Chemical Characterization. The relative distribution of baselin coincident attached to polydispersed CMD in the purified conjugation fraction was examined by size exclusion chromatography on an analytical Supragel 12 column (10 mm × 30 cm) using Phenanthrene AIB using FPLC. The column was eluted with 0.05 M Tris-HCl buffer (pH 8.0) at a flow rate of 1 mL/min at 25°C. A 0.2 and sample (~1 mg of CMD-bound) were injected, and the RI and Abs of the eluent were continuously monitored.

UV absorption spectra were obtained on a Pye Unicam UV-6, Lambda 19 spectrophotometer. (1) Protein absorption spectra for CMD baselin conjugates were measured in phosphate buffered saline (PBS) containing 0.1% Triton X-100 at pH 7.4 (containing 0.01% azide, and the efficacy of the solution was measured in a typical quadruplicate) 1 cm optical path length).

For N-terminal amino acids in insulin conjugates, CMT were determined by the N-terminal dansylation technique. Released dansyl amino acids were identified after acid hydrolysis (6 N HCl, 105 °C, 8 h) by two-dimensional thin layer chromatography (23).

The insulin content in the conjugates was determined by two independent methods. First, 1 μ l of each CMI insulin conjugate of known concentration was measured and the insulin content calculated assuming that 1 μ l of the 0.1% insulin solution is 100 U/20. Alternatively, the insulin amount attached to CMI-2 was determined by amino acid analysis.

In Vitro Cell Growth Studies. Thoracic aorta smooth muscle cells (SACs) from Sprague-Dawley rats were isolated as previously described [22]. SACs grown from aorta explants were cultured in MEM supplemented with 10% FCS and 2 mM glutamine at 37 $^{\circ}$ C in a humidified atmosphere containing 5% CO₂. Cultures of third and fourth passage were used throughout the study. The nature of SACs was confirmed by the aspect of confluent cultures (fully and easily potted) and by positive immunofluorescence for α -smooth muscle actin [22].

SACs (10⁴) were plated into 16 mm multiwell plates in MEM containing 10% FCS. After 48 h, cell growth was assessed (cell by plating them in medium with 0.1% FCS for 72 h). Cell growth was initiated by changing the medium to MEM with 2% FCS and adding insulin (CMI), or the CMI-insulin conjugate (C2). The control cells were exposed to medium with 2% FCS and positive control cells to 10% FCS. After 3 days, cells in quadruplicate wells were harvested after dislodging them with 0.05% trypsin/3 mM EDTA treatment and then counted using a Counter Counter JM (Coultronics, Inc., USA). The net growth of SACs in culture was obtained by subtracting the cell number at the end of the experiment from the starting cell number (at the time the cells were released from CMI) and results were expressed as the net growth above the control.

The effect on cell proliferation was also assessed with the tritiated thymidine uptake method as previously described [26]. Confluent SACs were placed in MEM and 0.4% FCS for 48 h. Then, the cells were incubated with or without (control) the tested compounds for 24 or 48 h. Cells were pulsed with 1 μ Ci per well of [³H] thymidine for the last 3 h. At the end of the assay, the plates were washed, DNA was precipitated with 10% trichloroacetic acid and solubilized and radioactivity was determined by β -liquid scintillation counting (4000 Pharmacia, Uppsala, Sweden).

Insulin Receptor Binding Studies. A rat insulin human cell line (RCH-1) was used for CMI-insulin conjugate insulin receptor (IR) binding studies. The cells were grown in 75 ml flasks in MEM supplemented with 10% fetal inactivated FCS, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin. The cells were harvested at the exponential growth phase, 4 days after seeding, using a calcium- and magnesium free medium at 4 $^{\circ}$ C. The harvested cells were washed and resuspended in the binding buffer (15 mM Tris, 120 mM NaCl, 12 mM MgSO₄, 5 mM KCl, 10 mM glucose, 15 mM sodium acetate, 1% bovine serum albumin, 20 μ g/ml bovine insulin, and 50 μ g/ml ovalbumin) at a final concentration of 1.5×10^6 cells/ml. The cell suspension (200 μ l) was mixed with 200 μ l of insulin solution (insulin concentration varying from 10 nM to 1×10^{-10} M). The insulin solution consisted either of pure [¹²⁵I]insulin having an activity of ~3000 cpm/10³ Biotinylated insulin. Free or a mixture of [¹²⁵I]insulin and a particular insulin derivative, specifically unlabeled insulin (CMI), glucose insulin (G2) or the CMI-insulin conjugate. The insulin ratio of 2:1, 1:1, and 1:2 between unlabeled insulin or the insulin conjugate and [¹²⁵I] insulin were usually used. The incubation was per-

formed at 4 $^{\circ}$ C for 1 h. (In the formation of bound and free insulin were separated by centrifugation. The cell pellet was rinsed, washed in 400 μ l of distilled water and the whole procedure repeated five times to remove insulin nonspecifically adsorbed. The bound radioactivity was measured in a γ counter (4000 Beckman). First, the autophosphorimetry for pure [¹²⁵I]insulin was measured and the results were replicated according to Seabolt [28] to determine its affinity constant for IR. In the next step, the binding (insulin for a mixture of [¹²⁵I]insulin and the insulin derivative with their molar ratio fixed at 2:1, 1:1, or 1:2 was acquired, and the affinity constant for a particular insulin derivative was then calculated.

Insulin Glucose Depression Assay. The biological activity of CMI-insulin conjugates was tested in a rat model (male Sprague-Dawley rats, 200 $\pm$ 25 g). In measuring blood glucose depression levels. All procedures in handling animals adhered to the *Principles of Laboratory Animal Care* (NIH Publication 85-23, revised 1986). The animals fasted overnight (16 h) prior to the experiment. Water was placed in *ad libitum*. In the morning, the rats (9–10 seven animals) with sodium pentobarbital anaesthesia (120 mg/kg) and mid-reversed an anaesthesia (by injecting tail vein) of insulin (3 U/ml) or the CMI-insulin conjugate (3 U/ml) (NIDDK or the CMI) insulin conjugate (equivalent amount attached insulin derivative NS-36). Blood samples were taken through a catheter introduced into the jugular vein 15 and 5 min before injection and 15, 30, 60, 90, 120, 180, and 240 min after injection. Blood glucose levels (BGLs) were measured with an Accutrack III blood glucose monitor (Beckman Instruments, Indianapolis, IN). Baseline of CMI-insulin conjugates was calculated from AUC (area under the curve) values and the AUC value for the same dose of unmodified insulin.

Pharmacokinetic and Pharmacodynamic Studies. Male Sprague-Dawley rats weighting 250–350 g were used throughout the studies. At least six animals were used in individual experiments. The basic animal protocol was the same as that described above for the bioactivity determination.

For the in vivo administration and pharmacokinetic (PK) and pharmacodynamic (PD) analysis of the CMI-insulin conjugate, blood samples were taken at 0, 3, 7, 15, 30, 60, 90, 120, 180, 240, and 300 min. EDTA-plasma samples were prepared and stored at 20 $^{\circ}$ C until analysis. Blood sample volumes were replaced with the same volume of saline (37 $^{\circ}$ C). Three different CMI-insulin doses of 0.125, 0.25, and 0.5 mg/kg dissolved in 0.1 M PBS (pH 7.4) were administered through a tail vein of rats that fasted overnight. Insulin reference for PK analysis (0.5 U/ml) was injected at 0, 1, 3, 5, 7, 10, 15, 20, 30, and 45 min. For PD analysis, blood samples were taken at 0, 15, 30, 60, 90, 120, 180, 240, and 300 min.

For the subcutaneous (sc) route of administration and PK analysis, blood samples were taken at 0, 10, 20, 30, 45, 60, 90, 120, 180, and 240 min, while for PD analysis, blood samples were obtained at 0, 30, 60, 90, 120, 180, 240, 300, and 360 min. Three different CMI-insulin doses of 0.125, 0.25, and 0.5 mg/kg 4% in PBS were administered sc into the cage of the rat. Insulin reference was injected at three 0.5 and 10 U/kg/kg. Locally, both BGLs and plasma insulin concentrations (IPIS) were determined. BGLs were measured with a glucometer (Chemocheck HC, glucometer strips with an Accutrack III blood glucose monitor, Chemocheck and CMI-insulin concentrations were determined with

a commercial insulin colorimetric immunoassay kit (Eitest) (Eitest, Norway). The CMI-insulin standard curve was used for calibrating the process of plasma CMI-insulin. Statistical Analysis. Data of administered insulin and CMI results appear to follow a two-compartment model. Thus, the data were fitted as a nonlinear least squares fit using the compartmental pharmacokinetic and experimental curves have the units substituted, and run χ^2 respect value.

$$I = I_0 + I_1 e^{-k_1 t} + I_2 e^{-k_2 t} \quad (1)$$

Data were analyzed using the PKModel software package (MicroMath, Salt Lake City, UT) that provided basic PK parameters, including biological plasma half-life and area under the curve (AUC). Based insulin levels were considered to be 20 μ mol/L, and the area below this threshold was subtracted from AUC.

In previous PK data of the sc as well as iv administered CMI-insulin conjugate and reference insulin, statistical moment derived from compartmental analysis was applied [29]. The AUCs for insulin and CMI-insulin plasma concentration-time curves above controls were calculated for the linear trapezoidal method. If the insulin levels were still higher than those of control experiments (20 μ mol/L), the last two points were extrapolated to a time point where the plasma insulin concentration resolved (20 μ mol/L) and this region was also included. The extrapolation for the concentration curve (AUC) were also calculated and used to calculate the mean residence time (MRT) of CMI-insulin and insulin after iv as well as sc administration. Finally, mean absorption times (MAT) for sc administered CMI-insulin and insulin were calculated, using eq 2.

$$MAT = MRT_{sc} - MRT_{iv} \quad (2)$$

The time needed to reach the maximum plasma insulin and CMI-insulin concentration (t_{max}) after sc administration was also used as an independent PK parameter. For PD analysis, two parameters reflecting the time dependency of BGL, after CMI-insulin or insulin administration and prolongation of the blood glucose lowering effect were selected. The most important PD parameter measured was the time to reach minimum BGL (t_{min}). The duration over which BGL remained below 70% of the initial value (t_{low}) was taken as the second PD parameter.

For the assessment of the statistical significance of the difference, the Student's *t* test, with one- and two-way analysis of variance, was used. Results were termed significant when $P < 0.05$. To pool data for different insulin or CMI-insulin conjugate doses (see Results and Discussion), t had to be >0.2 .

RESULTS AND DISCUSSION

CMI-insulin Conjugate Synthesis, Purification, and Characterization. Previously, we have shown that the CMI-insulin amino group is a preferential site of reaction and attachment of any structural moiety containing a carboxylic group activated by a mixed anhydride method [22]. We decided to use this activation method after having established that the free and (CMI) form of CMI is easily soluble in dry DMSO. Exclusion of water enabled us to activate only a small number of reaction of CMI carboxylic groups. In the first experiment, only 8 and % of available carboxylic groups in the polymer, which corresponded to about 12 groups per CMI molecule, was activated with BOC and reacted

with an equimolar insulin amount to achieve a cross linkage. No residue attached was found, indicating no cross linking occurred. Separation of the conjugate from unreacted insulin was accomplished by size exclusion chromatography on a Superose 25 column (Figure 1). The elution order (elution) of CMI and 2% insulin of the eluent were monitored. The first peak eluted containing the free insulin, and the second peak eluted containing the conjugate was eluted and dialyzed, freeze dried, and lyophilized (table 1). The insulin amount in the conjugate was determined by amino acid analysis as well known for the tendency to be in physical mixtures and monomers [24]. The conjugate emerged from the Superose 25 column however at the void volume peak, so there was a possibility that a smaller high molecular weight insulin cross linked material was present in the same fraction (see below). Using analytical Superose 6 and 12 columns that have high molecular weight exclusion limits for both proteins and dextrans, we established that purified conjugate is chromatographically homogeneous (single peak) and that the 60 to 450 signal ratio was practically constant, which indicates that the insulin moiety was evenly distributed (uniformly) with regard to the polydispersity of CMI. This means that a constant insulin amount was attached per arbitrary unit length of linear CMI chain. We excluded the possibility that the insulin moiety behaved like a cross-linking agent bridging two or more CMI molecules, because no significant difference between the retention volume of free CMI and the CMI-insulin conjugate was found. In large dilute feed that no physically bound insulin was present in the conjugate feed, the conjugate was again examined on Superose 6 and 12 columns with the column buffer containing a 50 mM Tris buffer with conditions, the insulin molecule moieties can no longer form dimers and/or hexamers. No peak corresponding to the unlinked insulin monomer was detected. Thus, the structure of the conjugate corresponds to the linear CMI chain with grafted insulin moieties. For future preparative production, repetitive size exclusion chromatography is not an ideal method for the conjugate purification because of its limited capacity. An obvious simple and efficient alternative method is pH dependent isoelectric precipitation of free insulin (pH range 5.0–6.0), because the solubility of the CMI-insulin conjugate is not significantly affected (as compared to the solubility at pH 8.0), by using this procedure, at least one intermediate chromatographic step can be avoided.

The UV spectrum of the CMI-insulin conjugate was identical to the UV spectrum of insulin, so no chemical modification of aromatic amino acids occurred. The far UV CD spectrum was indistinguishable from that of free insulin or CMI (Figure 12), indicating that the attached insulin molecule is properly folded (data not shown). We have also found the presence of the insulin molecule attachment to CMI (cysteine) groups with the N-terminal amino acid technique. Insulin is a two chain molecule with two N-terminal amino acids, Glycyl and Prolyl. The third insulin amino group comes from Lys22 [20]. Since with BHS free and BHS + Lys were detected by two-dimensional HPLC [23], we concluded that insulin moieties were attached to the CMI chain by an amide bond through the CMI amino group. Insulin molar content in the CMI-insulin conjugate was determined by UV absorption spectrometry and amino acid analysis. As an average, 3.2% mol of insulin was bound per mole of CMI. Thus, only 2% of activated CMI groups reacted with insulin, even though equimolar amounts of insulin and activated CMI groups were used. To shed more light on the limiting step, we



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LETTERS

DESIGN AND SYNTHESIS OF A NOVEL SYNTHETIC NAPAP-PENTA-SACCHARIDE CONJUGATE DISPLAYING A DUAL ANTITHROMBOTIC ACTION

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Abstract: The synthesis of a novel antithrombotic consisting of a heparin pentasaccharide conjugated to the active site inhibitor *N*-(2-naphthalenesulfonyl)-glycyl-(*O*)-4-aminophenyl-alanyl-piperidine (NAPAP) (i.e. compound 1) is reported. This conjugate shows a unique pharmacological profile both in vitro and in vivo having direct anti-thrombin and A1111-mediated anti-Xa activity. Furthermore, conjugate 1 has a prolonged in vivo half-life compared to NAPAP (1.5 h vs 9 min). © 1999 Elsevier Science Ltd. All rights reserved.

Introduction

The serine proteases thrombin and factor Xa play a pivotal role in thrombosis and haemostasis. Factor Xa catalyzes the conversion of prothrombin into thrombin, whereas thrombin is involved in activation of blood platelets and the cleavage of fibrinogen into clottable fibrin. Therefore factor Xa and thrombin have become highly interesting pharmaceutical targets for the treatment of thrombotic disorders.

The sulfated glycosaminoglycan heparin has been a subject of research for many years.¹ Heparin enhances the inhibitory potency of antithrombin III (A1111) towards both thrombin and factor Xa. A unique and well defined pentasaccharide (PS) domain² in heparin is responsible for the activation of A1111. This PS domain is sufficient to promote the inhibition of factor Xa, while the activity of thrombin remains unaffected. For effective thrombin inhibition³ heparin sequences are required which contain in addition to the PS domain ten to twelve consecutive saccharide units in order to form a ternary complex together with A1111 and thrombin. Based on this mechanism of ternary complex formation a variety of heparin mimics⁴ were designed comprised of an *O*-sulfated/*O*-methylated PS-analog⁵ (see Figure 1) tethered via a polychylene glycol spacer to a negatively charged thrombin binding domain. These heparin mimics proved to be effective A1111-mediated inhibitors of factor Xa and thrombin in vitro.

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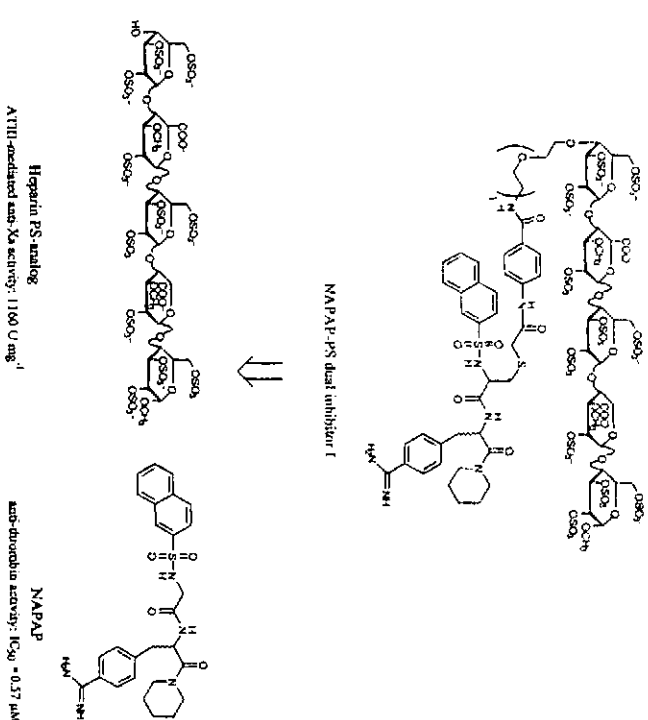


Figure 1 Structures of NAPAP, PS, and NAPAP-PS conjugate.

In this paper we now present a novel antithrombotic, which consists of a PS-analog conjugated to a derivative of *N*-(2-naphthalenesulfonyl)-glycyl-(*O*)-4-aminophenyl-alanyl-piperidine⁶ (NAPAP) (i.e. compound 1, see Figure 1). NAPAP is a potent representative (EC₅₀ = 0.75 μM) of the low molecular weight thrombin inhibitors acting directly on thrombin's active site. The NAPAP-PS conjugate 1 is expected to elicit new pharmacological properties as it may stimulate the A1111-mediated anti-Xa activity on the one hand (see Figure 2, A), while on the other hand it may inhibit thrombin directly (see Figure 2, B). Since earlier pharmacokinetic studies⁷ clearly demonstrated that the half-life of a heparin pentasaccharide is governed by its affinity for A1111, this novel dual inhibitor should possess a prolonged antithrombin activity compared to NAPAP. Moreover, this approach opens the way to the preparation of NAPAP-PS conjugates having tailor-made half-lives.⁸ Furthermore, this conjugate has a higher solubility in aqueous media than NAPAP making the former more suitable for parenteral administration.⁹

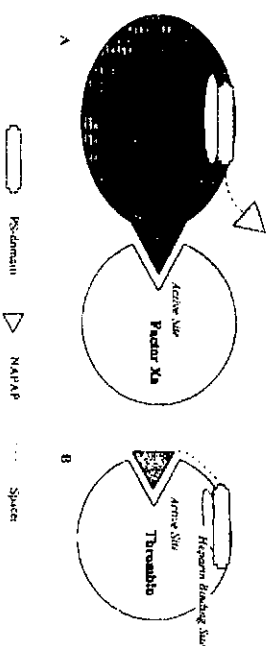
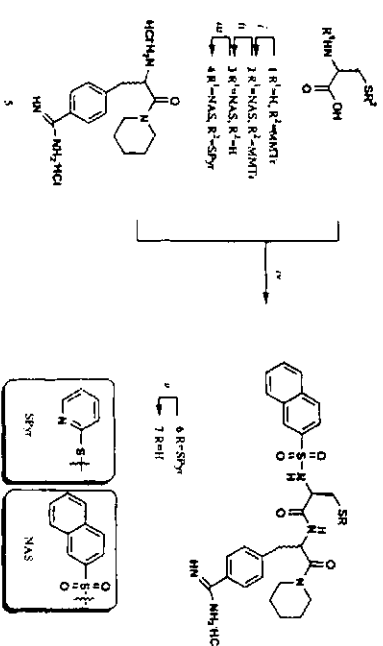


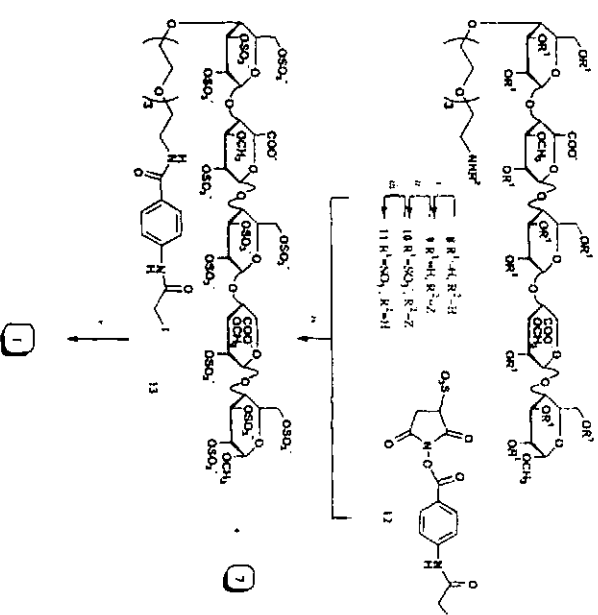
Figure 2. Schematic representation showing the action of a NAPAP-PS dual inhibitor. The PS-domain stimulates (A) the inhibitory potency of a T111 towards factor Xa by binding to AT11, whereas the NAPAP part inhibits thrombin directly (B) by interaction with the active site.

Results and Discussion

The construction of conjugate **1** required suitably derivatized NAPAP and PS building blocks. Earlier investigations³ indicated that derivatization at the α -carbon of the glycine moiety of NAPAP did not influence its inhibitory potency against thrombin. Therefore, we synthesized NAPAP derivative **7** (see Scheme 1), the thiol-function of which allowed conjugation with thiophilic groups like maleimides and bromo- or iodoacrylamides.¹⁹ Synthesis of compound **7** commences with sulfonylation of *N*-(4-monomethoxybenzyl)-L-cysteine¹¹ (**1**) with 2-naphthalenesulfonyl chloride (NAS-Cl) under Schotten-Baumann conditions.



Scheme 1. (i) NAS-Cl, dioxane/0% Na₂CO₃ (1/1), v/v, 1 h, 76%; (ii) TEA/n-methoxybenzylamine/CH₂Cl₂ (1/1/1), v/v/v, 20 min; (iii) AldricholTM, isopropanol/2 N AcOH (1/4), v/v, 1 h, 55% (2 steps); (iv) EDCI, HOBT, *N*-ethylmorpholine, DMF, 16 h, 70%; (v) Bu₄P⁺, MeOH, 1 h.



Scheme 2. (i) Z-OSu, DMF/H₂O (1/4), *N*-ethylmorpholine, 15 min, 91%; (ii) a. (C₆H₅)₃N⁺SO₃⁻, DMF, 55 °C, 16 h, 0.2 M HCl, 4 °C, 16 h; (iii) H₂, Pd/C, *tert*-BuOH, H₂O, 3 h, 65% (3 steps); (iv) 0.1 M Na₂HPO₄ (pH 7.5), 3 h; (v) DAP, MeOH/0.1 M Na₂HPO₄ (pH 7.0) (1/4/5, v/v/v), 3 h, 52% (2 steps).

Derivatization¹¹ of the resulting sulfonamide **2** gave the free thiol **3**, which was subsequently treated with an excess of dihydroisophthalic (AldricholTM) to yield thiopyridyl protected **4**. Condensation of **4** with the known¹⁰ (μL)-4-antidimethylamino-1-pyridine dihydrochloride (5, H-(μL)-AdP-PI₂) using the well established peptide coupling agent 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) in the presence of 1-hydroxybenzotriazole (HOBt) and *N*-ethylmorpholine as a base afforded compound **6** as a mixture of diastereoisomers in an overall yield of 29% (from **1**). Compound **6** was reduced using a slight excess (1.1 equiv) of tri-*n*-butylphosphine in methanol to give the free thiol **7**. In order to gain access to the NAPAP-PS conjugate **1** (iodoacetyl) containing PS **13** had to be synthesized, which could be obtained from known¹⁴ **8** in five consecutive steps (see Scheme 2). Protection of the amine in **8** with *N*-(benzyloxycarbonyloxy)-succinimide (Z-OSu) yielded **9**. Sulfation of the free hydroxyls in **9** with triethylamine/sulfur trioxide complex and subsequent treatment with 0.2 N HCl (to remove the *N*-SO₃⁻ group formed during sulfation) gave **10**. Reductive cleavage of

the 7-group in 10 and condensation of the corresponding amine 11 with the thiophilic *sulfo*-SIAB¹⁴ linker 12 gave the required PS 13. Compound 13 was reacted with a two-fold excess of an equimolar mixture of NAPAP derivative 7, which afforded the NAPAP-PS conjugate 1 as a mixture of diastereoisomers in an overall yield of 23% (based on 8). The homogeneity and the identity of compound 1 was firmly established by mass spectrometry and NMR-spectroscopy.¹⁵ Conjugate 1 indeed displayed a unique antithrombotic profile (in vitro) in that it shows both high anti-thrombin (IC₅₀ = 0.35 μ M) and ATIII-mediated anti-Xa activity (885 U mg⁻¹),¹⁶ we were anxious to find the pharmacological implications of this unprecedented profile in an *in vivo* model. Measurement of the antithrombotic activity in an aorta-flow model¹⁴ revealed that conjugate 1 is a stronger inhibitor than a combination of the free pentasaccharide and (3)-NAPAP. In addition, preliminary pharmacokinetic studies indicated that the elimination half-life of conjugate 1 is prolonged significantly compared to NAPAP (~1.5 h vs 9 min in rat)⁸, thus confirming that the half-life of this novel class of antithrombotics is determined by the interaction of the PS with ATIII.

Acknowledgement: We wish to thank Gerard Vogel for measuring the antithrombotic activity and the pharmacokinetic behavior of compound 1.

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12. ¹H NMR (D₂O, 600 MHz, 300 K, HH-COSY): δ (HOD = 4.76) 3.60, 3.53, 3.43 (3 x s, 9H, CH₃O₂); *ring D*: 5.53 (m, 1H, H1), 4.15 (m, 1H, H2), 4.58 (m, 1H, H3), 3.56 (m, 1H, H4), 3.92 (m, 1H, H5), 4.26, 4.13 (2 x m, 2H, H6, H6'); *ring E*: 4.70 (d, 1H, H1, *J*_{1,2} = 8.1 Hz), 4.21 (m, 1H, H2), 3.62 (m, 1H, H3), 3.92 (m, 1H, H4), 3.74 (m, 1H, H5), *ring F*: 5.39 (d, 1H, H1, *J*_{1,2} = 3.8 Hz), 4.22 (m, 1H, H2), 4.56 (m, 1H, H3), 3.83 (t, 1H, H4, *J*_{4,5} = 9.8 Hz), 4.12 (m, 1H, H5); *ring G*: 5.15 (m, 1H, H1), 4.35 (m, 1H, H2), 3.76 (m, 1H, H3), 4.21 (m, 1H, H4), 4.80 (m, 1H, H5); *ring H*: 5.10 (d, 1H, H1, *J*_{1,2} = 3.6 Hz), 4.31 (m, 1H, H2), 4.54 (m, 1H, H3), 4.21 (m, 1H, H4); *spacer*: 7.51, 7.53, 7.13, 7.12 (4 x d, 4H, H_{gem} SIAB), 3.73 (m, 2H, CH₂CH₂NH), 3.66 (m, 12H, OCH₂ TEG), 3.31 (m, 2H, CH₂NH); *peptide*: 8.27, 8.22 (2 x s, 1H, H_{gem} NAS), 7.98–7.60 (m, 6H, H_{gem} NAS), 7.71, 7.64, 7.46, 7.44 (4 x d, 4H, H_{gem} Ad), 4.60, 4.45 (2 x t, 1H, α CH Ad), *Leu*: 6.6 (H α), 4.00, 3.97 (2 x m, 1H, α CH Cys), 3.10–2.85 (m, 4H, CH₂N pipidine), 2.82–2.70 (m, 3H, BCH₂ Cys), BCH Ad), 2.61 (m, 1H, BCH Ad), 1.55–1.15 (m, 6H, CH₃ pipidine); ES-MS: [*M*+H]⁺ 2680.6.
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Effects of PEG conjugation on insulin properties

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Abstract

The goal of this research was to determine whether the site-specific attachment of poly(ethylene glycol) to insulin could enhance the physical and pharmacological properties of insulin without negatively affecting its biological activity or immunological properties. Electrostatically activated derivatives of low-molecular-weight monomethoxypoly(ethylene glycol) (mPEG) were chemically coupled to insulin via its amino groups at positions phenylalanine-B1 or tyrosine-B29, with an amide bond being formed between the polymer and protein. The site-specific attachment of mPEG to insulin did not substantially alter insulin's secondary/tertiary structure, self-association behavior, or potency *in vivo*. However, mPEG attachment did significantly enhance insulin's resistance to aggregation. In addition, the conjugation of insulin almost completely eliminates the resultant conjugate's immunogenicity, allergenicity, and antigenicity. Finally, the conjugates were observed to remain in the systemic circulation for longer periods of time than unmodified insulin after subcutaneous administration. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Diabetes; Poly(ethylene glycol); PEG; protein conjugates; Protein delivery; Pharmacokinetics; Immunogenicity; Protein application; Pharmacokinetics

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1. Introduction

Parenteral administration of insulin formulations remains the primary therapy utilized for the treatment of insulin-dependent diabetes mellitus (IDDM) since insulin's discovery over 75 years ago [1]. Many of the factors that make the current therapies inadequate are inherent shortcomings intrinsic to the insulin molecule. Contemporary researchers are applying genetic and protein engineering techniques to further probe the structure-function relationships of insulin in the continuing quest to produce pharmaceutical insulin preparations that closely mimic the natural secretion of insulin and, therefore, maximize the likelihood of achieving a euglycemic state in those afflicted with diabetes. Specifically, insulin faces many problems typical to protein pharmaceuticals, including poor physical and chemical stability, increased susceptibility to proteolysis, increased immunogenicity, and antigenicity, and a relatively short plasma half-life [2].

Poly(ethylene glycol) (PEG) is a linear or branched neutral polyether with the chemical formula $\text{HO}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{H}$. PEG is highly soluble in water and many organic solvents (e.g., methylene chloride, ethanol, toluene, acetone, and chloroform), and is readily available in various sizes (molecular weights) and functionalized architectures (e.g., amino-, carboxyl-, and sulhydryl-terminated). Low-molecular-weight (< 1000 Da) PEGs are viscous and colorless liquids, whereas higher-molecular-weight PEGs are waxy solids whose melting points are proportional to their molecular weights with an upper limit near 67°C [3,4]. PEG has been found to be nontoxic and is approved by the FDA for use in drugs (parenterals, topicals, suppositories, nasal sprays), foods, and cosmetics [5]. Molecular weights of PEGs used in biomedical applications usually range between a few hundred to 20,000 Da [6]. The molecular weight distribution (polydispersity) of these PEG polymers is generally low, but the commonly used monomethyl ethers of PEG exhibit broader molecular weight distributions [7]. In solution PEG is also a

highly hydrated polymer, where each monomethyl ether unit can bind three molecules of water [3].

Linn and Herson executed molecular simulations of the behavior of single surface-bound PEG (45, 160 Da chains in water [8]. Their results show the polymer exhibiting a large degree of segmental flexibility in aqueous solution. In addition, it is thought that PEG has the ability to influence the structure of several molecular layers of more loosely associated hydrating water molecules [9]. These findings taken together might explain why PEG is remarkably effective in excluding other polymers (natural and synthetic) from its presence in aqueous environments. The exclusion of other polymers is the primary driving force behind PEG's ability to reject proteins from two-phase systems with other synthetic polymers, and makes this polymer both nonimmunogenic and nonantigenic [6]. When PEG is covalently attached to a protein, it typically transfers many of the polymer's favorable characteristics to the resultant conjugate. Because of the many beneficial properties mentioned above, PEG is ideally suited for protein modification. A non-exhaustive list of examples of PEG protein conjugates described in the literature includes the conjugates of proteins such as adenosine deaminase [10,11], bovine liver catalase [12], urease [13], collagen [14], human growth hormone [15], nerve growth factor [16], lysinase [17], alkaline phosphatase [18], asparaginase [19], superoxide dismutase [20], hemoglobin [21], interleukin-2 [22], granulocyte colony-stimulating factor [23], ovalbumin [24], ribonuclease A [25], and insulin [26,28].

This conjugation technology has been used to improve the therapeutic efficacy of recombinant human proteins because it alleviates many problems associated with therapeutic protein formulations. First, most parenterally administered proteins are rapidly cleared from the body by the reticuloendothelial system (RES), kidney, spleen, or liver [29]. Moreover, clearance depends on molecular size, charge, and the presence of specific cellular receptors for the proteins of interest. The attachment of PEG to

group that is covalently bound to PEG. An N-terminal amino acid molar ratio of [Ile]Ala: [PheB1] = 1 is indicative of conjugation to residue LysB29 (or none at all) and [GlyAla: [PheB1] = 1 is indicative of conjugation to residue PheB1. We found that the mPEG-PheB1-insulin conjugates had a [Ile]Ala: [PheB1] molar ratio at least three orders of magnitude larger than either Zn²⁺-insulin or the mPEG-LysB29-insulin conjugates. Thus, the combined MALDI-TOF mass spectrometry and Edman degradation results provide conclusive evidence that only one mPEG chain was attached to each insulin molecule and the appropriate site-of-substitution was achieved with a high degree of purity (>95%) by RP-HPLC.

Circular dichroism studies were utilized to gain a semi-quantitative understanding of how the pegylation of insulin at two different sites affects its three-

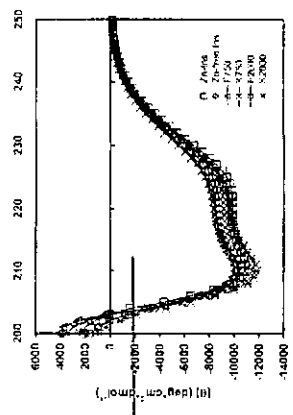


Fig. 2. Far UV CD spectra of insulin and its pegylated derivatives at 1.0 x 10⁻⁴ M in PBS.

Table 1
Secondary structure elements for various insulins calculated from far-ultraviolet CD spectra*

	Zn-ins	Zn-free	F750	F2000	K750	K2000
% Helix	48.1	17.1	16.6	45.3	15.6	48
% Antiparallel	6.1	5.9	7.3	7.1	7.2	5.1
% Parallel	5.5	5.3	5.8	5.4	5.7	5.2
% Beta-sheet	13.2	14.3	13.0	14.9	13.6	14.7
% Random coil	24.8	25.2	25.4	26.6	25.7	25.0
Total	98.1	98.2	98.1	97.3	97.8	98.3

*Expressed as the percent of total secondary structure elements. Average error of the neural network was 6.4%.

used for the fatty acid acetylation of insulin [55]. Briefly, Zn²⁺-insulin was dissolved in 60% dimethylformamide (DMF) (pH_{app} 9.5) at room temperature. At this pH the LysB29 amino group should be most able to react with the electrophilically activated PEG. An excess of mPEG SPA (750 or 2000 Da) was added by immediate injection to the Zn²⁺-insulin solution, keeping the apparent pH_{app} of the reaction solution constant by NaOH addition. The same methods were used to isolate both types (PheB1 and LysB29) of the mPEG-insulin and derivatives. The conjugates will be referred to in all subsequent studies as follows: F750 for mPEG(750)-PheB1-insulin, F2000 for mPEG(2000)-PheB1-insulin, K750 for mPEG(750)-LysB29-insulin, and K2000 for mPEG(2000)-LysB29-insulin.

2.3. Physicochemical characterization

Each conjugate's purity was assessed using a reversed-phase high-performance liquid chromatography (RP-HPLC) system lined with an analytical C₁₈ column. The presence of a single chromatographic peak confirmed the conjugates' purity was >95% in all cases. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis was used to determine the number of mPEG chains attached and the conjugate's molecular weight. Monomeric insulin has a calculated molecular weight of 5807.2 Da, and the number-average molecular weights of mPEG(750) SPA and mPEG(2000) SPA used in the conjugation reactions were 827 and 2096 Da, respectively. The mass spectrum of both mPEG(750) insulin (average molecular weight 6600 Da) and both mPEG(2000) insulin (average molecular weight 7790 Da) conjugates clearly demonstrated that only one mPEG chain was attached to insulin. In addition, the individual ion peaks consistently differed by 44 Da (the molecular weight of an ethylene oxide monomer unit). These results confirm that only one mPEG chain was attached to insulin in all of the conjugates prepared and that their polydispersity is solely due to the polydispersity intrinsic to PEG.

N-Terminal protein sequence analysis was utilized to indirectly determine the site of PEG conjugation, with the knowledge that the Edman degradation reaction will not proceed at any N-terminal amino

group (ethanedithiolate) (mPEG) to the PheB1 (A₁) or LysB29 (A₁) amino groups of insulin to negatively impact its bioactivity since it is known that neither of these residues are essential to its bioactivity [50-52]. Conjugates substituted at positions B1 (mPEG-B1-insulin) and B29 (mPEG-B29-insulin) were prepared and characterized according to methods described in a previous report from our group [53]. For the sake of completeness, we have provided a brief overview of these methods followed to prepare and characterize the conjugates used in subsequent studies intended to determine the effect of pegylation on insulin's properties.

The preparation of N¹⁰⁰-methoxyacetylmethylglycyl-insulins (mPEG-PheB1-insulins) began by synthesizing and purifying a di-N¹⁰⁰-methoxyacetyl-insulin (diboc-insulin) intermediate according to procedures described earlier [54]. Briefly, *tert*-boc-insulin was reacted with insulin in a dimethyl sulfoxide-methylamine (DMSO:MeA) mixture. The crude reaction mixture containing mono-, di-, and tri-substituted boc-insulins was extensively dialyzed and lyophilized before isolation of diboc-insulin. A fast protein liquid chromatography (FPLC) system fitted with a preparative cation-exchange column was equilibrated with a 7 M urea 1 M acetic acid mobile phase and a 0.1 to 0.3 M NaCl gradient was used to elute the various boc-insulin species. The purity of diboc-insulin was assessed using a Mono S analytical cation-exchange column under the same conditions. Once the diboc-insulin was determined to be highly pure (single chromatographic peak), it was ready for pegylation.

The mPEG(750)-PheB1-insulin (F750) and mPEG(2000)-PheB1-insulin (F2000) conjugates were prepared by reacting 750 or 2000 Da mPEG-SPA (Shearwater, AL, USA) with diboc-insulin in DMSO:MeA. The mPEG(750 or 2000)-PheB1-diboc-insulin solutions were then extensively dialyzed and lyophilized. Quantitative deprotection of the lyophilized product was achieved by reaction with trifluoroacetic acid (TFA) containing 10% anisole at 0 °C. Finally, the conjugates were purified to homogeneity using the same chromatographic procedure described above for diboc-insulin.

Both N¹⁰⁰-methoxyacetylmethylglycyl-insulin (mPEG-LysB29-insulin) conjugates were synthesized according to a modified version of a method

dimensional structure. A large number of studies have used circular dichroism spectroscopy measured in the far-ultraviolet range to examine the conformation of insulin [56-62]. Fig. 2 shows the far-ultraviolet CD spectra of the various conjugates and two control species. In general, the features of the spectra for insulin and its pegylated derivatives are similar in some cases there are slight differences in the dichroic intensity of the spectra. The deconvolution program CDNN (version 3.1) was used to further analyze the data [63]. This program compared each samples' far-ultraviolet dichroic spectra to a set of 33 basis spectra representing the major secondary structure elements (e.g., α -helix, β -sheets, β -turn, random coil) commonly seen in peptides and proteins. Furthermore, the neural network chosen to analyze the data accounted for both aromatic and disulfide contributions to a sample's dichroic intensity in the far-ultraviolet region. The results given in Table 1 confirm that the secondary structure of native insulin is largely conserved after the covalent attachment of low-molecular-weight mPEG (750 or 2000). Due to either site of substitution. All of the samples were composed of roughly the same proportions of the elements: 46% α -helix, 11% β -sheets, 14% β -turn, and 25% random coil. Any differences between the conjugates and insulin were within the error limits of the software ($\pm 6.45\%$). The values we calculated were very close to the values of Melberg and Johnson who determined that Zn-free-insulin was composed of 57% α -helix, little or no β -sheets, 18% β -turn, and 24% random coil elements [58]. In fact our results more closely match values estimated from the crystal structure of insulin (53% α -helix, 8% β -sheets, 18% β -turn, and 21% random coil) [64]. The spectral characteristics of the conjugates provide

the absence of zinc that insulin exists as a dimer. However, in the presence of zinc, insulin exists as a hexamer at the concentrations used in these experiments. Lyspro[®] (Lilly, USA) is a mutant form of insulin where two amino acid residues (ProB28 and LysB29) are transposed, which results in an increased rate of subcutaneous absorption because the Lyspro[®] hexamers more rapidly dissociate into monomers that are easily absorbed into systemic circulation. As expected, Lyspro[®] was monomeric in solution. This is consistent with many reports indicating Lyspro[®] is predominantly monomeric at high concentrations without the addition of stabilizers like phenol, Zn²⁺, and glycerol. Finally, all of the mPEG-insulin derivatives appear to be dimeric in solution, regardless of their molecular weight or site-of-substitution.

The residuals from the curve fits were observed to be small and randomly distributed around zero, indicating the model chosen was able to accurately describe the data. The fits and residuals from three different concentration distributions were used in the final calculation of the values in Table 2. The presence of the mPEG made it difficult to assign an accurate *r* value to the pegylated insulin derivatives. Because the molecular weights obtained for the control samples were comparable to previously published reports and the weights of the conjugates were reasonable, the use of native insulin's *r* value seems justified in the realm of calculating an average association-state. A likely explanation for this is that mPEG behaves as a random-coil attachment on the protein and does not change *r* significantly.

An accelerated shake test was used to assess the physical stability of the four mPEG insulin conjugates in comparison to both Zn²⁺- and Zn-free-insulin. This test is commonly described in the literature as providing an accurate measure of an insulin preparation's physical stability in an accelerated manner. The fibrillation kinetics of the four insulin conjugates, Zn²⁺-insulin and Zn-free insulin (control samples) were studied by subjecting the solutions to horizontal shaking (100 strokes/min) at 37 °C. At prescribed time points, individual samples were withdrawn filtered (to remove the insoluble aggregates), and then analyzed for residual insulin (temperature) concentration using RP-HPLC. The time elapsed until 50% of the initial protein concentration remained in solution (*t*_{50%}) was taken as a relative measure of the samples' physical stability.

The results are summarized in Table 3 and corroborate previous results from our group for both glycosyl- and glycoyl-PEG insulin conjugates [10,51]. The PheB1-insulin conjugates possess substantially higher (36–40%) physical stabilities and the LysB29-insulins were somewhat more stable (4–8%) than control samples. We propose that the increased fibrillation-resistance of LysB29 and LysB29 is due to two complementary effects. One is the specific steric blocking of the N-terminus in the B-chain by mPEG conjugation resulting in the prevention of this surface from participating in the hydrophobic interactions that drive insulin fibril growth [75,76]. The second contributing effect is PheB1-insulin's increased physical stability is nonspecific and steric in nature and increases with an increase in mPEG molecular weight. Both K750 and K2000 exhibited increased physical stability, showing a heightened resistance to fibrillation, but not to the same extent as the conjugates substituted at PheB1. This can be explained by the lack of LysB29's participation in fibrillation reactions, with any stabilizing effects caused by nonspecific steric hindrance of the intermolecular interactions that are involved in fibrillation.

Table 3
Long-term physical stability of various insulins evaluated by shake testing

Sample ^a	<i>t</i> _{50%} (days)
Zn ²⁺ -insulin	0.5 ± 0.3
Zn-free insulin	6.4 ± 0.2
P750	18.4 ± 2.8
P2000	20.7 ± 4.1
K750	13 ± 1.1
K2000	8.6 ± 1.7

^a Accelerated shake-test done at 100 strokes/min and 37 °C.
^b Protein solutions prepared in PBS (30 mM, pH 7.4) containing 0.1% Na-azide.
^c Elapsed time to 50% of the initial protein concentration remaining (mean ± standard deviation).

one or more sets of ultracentrifuge data according to the following equation:

$$\frac{1}{M} = \frac{2RT}{\omega^2 r^2} \ln \frac{dr}{r^2}$$

where $\frac{dr}{r^2}$ is the solute concentration as a function of radial position within the centrifuge cell, ω is the angular velocity, r is the solute's partial specific volume (ml/g), V is the volume of water displaced by 1 g of solute, ρ is the density of the solution, R is the gas constant, T is the thermodynamic temperature, Δf is the solute's molecular weight, and r is the radial position of the measurement within the centrifuge cell. In each case, the final fit resulted from the simultaneous fitting of three to six different concentration distributions. The partial specific volume (V) was fixed at 0.72 ml/g representing the measured value for insulin [57].

Table 2 gives the apparent molecular weights and average association-states for the unmodified insulin species and pegylated insulin derivatives. In every case a single-species (monomer, dimer, or hexamer) model best described the data, where the final fit comprised three separate data files with varying speeds and concentrations. In the presence of zinc, insulin appears to be primarily hexameric in solution. The data from Zn-free insulin indicate that its association-state is dimeric. It is well known that in

Table 2
Molecular weights of various insulins determined from sedimentation equilibrium experiments

Sample ^a	<i>M</i> _{app} × 10 ³ (kDa)	<i>M</i> _{app} × 10 ³ (kDa)
Zn ²⁺ -insulin	30.0 ± 0.0	5.17 ± 1.55
Zn-free insulin	10.4 ± 2.0	1.79 ± 0.14
Lyspro [®]	5.26 ± 0.60	0.98 ± 0.10
P750-insulin	13.3 ± 3.0	2.0 ± 0.45
P2000-insulin	13.33 ± 3.0	1.71 ± 0.38
K750-insulin	14.41 ± 2.0	2.17 ± 0.50
K2000-insulin	15.98 ± 3.0	2.05 ± 0.38

^a All samples prepared in Tris-EDTA buffer (90 mM Tris, 5 mM EDTA, pH 7.4) except Zn²⁺-insulin, which was prepared in Tris buffer only.
^b *M*_{app} is the calculated molecular weight of the derivatives' monomeric species.
^c Nonheparin Lyspro[®] was prepared after extensive dialysis against distilled water to remove any stabilizing substances (phenol, glycerol, and Zn²⁺) from the commercial formulation.

evidence that attachment of mPEG to insulin does not materially alter its secondary/tertiary structure.

It is commonly known that the average association-state of an insulin preparation substantially affects its pharmacological disposition [2,65–69]. Aqueous insulin solutions contain mixtures of monomeric, dimeric, and hexameric association-states that coexist under a dynamic equilibrium. The average association-state in insulin preparations depend on protein concentration, temperature, ionic strength, and the presence of stabilizing molecules such as Zn²⁺, phenol, chloride, or *m*-cresol [2,30,68,70,74]. Insulin monomers have two nonpolar surfaces that are directly involved in the reversible self-association process and dimerization proceeds through inter-monomer interactions involving residues ValB12, TyrB16, PheB24, TyrB26, and ProB28 [30]. The ProB28 result in an antiparallel β -sheet conformation between the monomers. Insulin hexamers are formed through both hydrogen bonding and hydrophobic interactions between surfaces formed by residues LeuA13, TyrA14, PheB1, ValB2, LeuB6, AlaB14, LeuB17, and ValB18. Addition of Zn²⁺-ions further stabilizes the insulin hexamer through complexation of a single Zn²⁺-ion with an imidazole group from each of three dimers that constitute the insulin hexamer [30,56]. It is important to note that the hexameric form of insulin is not bioactive and is only partly absorbed across the capillary endothelium into the systemic circulation [68]. In addition, the dissociation of insulin hexamers into dimers and monomers is seen as the rate-limiting barrier to subcutaneous absorption that directly affects the preparation's pharmacological response [66,72].

Investigations into the effects of insulin pegylation on its self-association were carried out using analytical ultracentrifugation. Sedimentation equilibrium experiments were used to gain insight into the association states of various insulin preparations including the four mPEG-insulin derivatives. Data analysis describing the concentration distribution was achieved by fitting the final absorbance versus radius data to various models of insulin self-association using nonlinear least-squares techniques and the analysis program NONLIN [73,74]. This software performs simultaneous nonlinear least-squares fits to

2.4. *In vivo* bioactivity studies

The *in vivo* biological activity of the conjugates was measured using a blood glucose depression assay in a rat model (Male Sprague-Dawley (SD) rats were kept under specific pathogen-free conditions in the animal facility Humulink® (11b)) or the conjugate preparations were injected at a dose of 0.3 (1 kg) into the jugular vein. Blood samples were obtained at prescribed time points and assayed for glucose concentration using a commercially available fluorometer. The blood glucose level (BGL) versus time area-under-curve (AUC₀₋₁₂₀), nadir BGL (t_{min}), % of basal value, and time to t_{min} (t_{min}) were determined from averaged experimental data. For assessment of the statistical significance of the differences, the Student *t*-test was employed and *P*-values for significance were set to 0.05.

Table 4 compares the bioactivities (AUC₀₋₁₂₀) of each of the four mPEG insulin conjugates to that of Humulink® (11b) regular insulin formulation) control samples. We found that F750 and K750 conjugates had equivalent bioactivities to Humulink® after intravenous (i.v.) administration. However, the F2000 and K2000 conjugates possessed bioactivities that were slightly lower than Humulink®. The attenuated glucose depression of the F2000 and K2000 conjugates is most likely due to increased steric hindrance of the larger polymer (relative to the smaller one) on the insulin receptor interactions. As expected, no differences in bioactivity were observed between conjugates with different sites-of-substitution because neither PheB1 nor LysB29 are directly involved in receptor binding [51].

3. Immunological properties of PEG-insulin conjugates

Insulin is typically administered to diabetics via subcutaneous injection, and once the insulin has absorbed from the injection site into the systemic circulation it is cleared from target tissues primarily through receptor-mediated events. Clinical experience has shown that many insulin-dependent diabetics patients produce anti-insulin antibodies, mostly of the IgG and IgE subclasses [34]. High levels of anti-insulin IgG and IgE antibodies are commonly found in diabetic patients with insulin allergy [32,36] and insulin resistance syndrome [37,38], respectively. Consequently, the therapeutic effectiveness of pharmaceutical insulin preparations may be counteracted by their rapid elimination from the patient's circulation in the form of immune complexes and/or anaphylactic symptoms.

We studied the immunological properties of mPEG insulin conjugates in two types of mice: A/J (low insulin responders) and C57BL/6J (high insulin responders). The humoral immunogenicity, cellular immunogenicity, antigenicity, and allergenicity of conjugates with different sites of substitution and molecular weights of mPEG attached were compared to those of unmodified recombinant human insulin. To avoid any confusion, in the context of these studies, immunogenicity is defined as the *in vivo* capacity of an antigen or allergen to induce the corresponding immune response. Similarly, in the context of this paper, antigenicity is defined as the ability of an antigen to bind to circulating antibodies. Finally, allergenicity is defined as the

capacity of an allergen to combine *in vivo* with homologous or foreign antibodies, thus triggering systemic anaphylaxis or local-skin reactions whether in direct skin tests or in passive cutaneous anaphylaxis (PCA) assays [77].

Here we compare the immunological properties of conjugates with distinct sites of substitution (N-terminus of the A-chain, N-terminus of the B-chain, and amino group of lysine in the C-terminal region of the B-chain) and varying sizes of low-molecular-weight mPEG (600, 750, and 2000 Da) to those of recombinant human insulin. Only derivatives with one mPEG chain/insulin monomer were used in this study because conjugates containing two or three mPEG chains/insulin monomer were previously found to have significantly reduced biological activities and, therefore, were not considered to be clinically relevant candidates for further study [78]. Likewise, only low molecular weight mPEG insulin conjugates were used in these studies because the attachment of higher-molecular-weight mPEG (5000 Da) was found to considerably depress the conjugate's bioactivity [78].

Four experimental end-points were examined to determine the subjects' immunological response to insulin-mPEG-insulin immunization. First, the effect of site-specific pegylation on the humoral immunogenicity of insulin was ascertained by measuring the amount of antigen-specific antibodies (IgG and IgE) present in the sera of immunized mice. Second, the effect of insulin pegylation on insulin's ability to interact with anti-insulin IgG antibodies (antigenicity) was assessed by enzyme-linked immunosorbent assay (ELISA) measurements using insulin or its pegylated derivatives as the detecting antigens. Third, the effect of insulin pegylation on delayed-type hypersensitivity reactions (cellular immunogenicity) was investigated by measuring hind footpad swelling in antigen-primed animals. Finally, a passive cutaneous anaphylaxis test was used to determine the effect of pegylation on immediate-type hypersensitivity reactions that include local allergy and systemic anaphylaxis.

Experiments described in this section were performed under the supervision of Professor Blanka Rihova in her laboratories at the Institute of Microbiology of the Czech Academy of Sciences in Prague. The authors wish to explicitly acknowledge

her contribution to these studies. Table 4 shows the insulin species used in these experiments. All of these conjugates were prepared as described in the previous section except F600 (mPEG A1-insulin), which was prepared as previously described by Uchida et al. [78].

3.1. Humoral immunogenicity and antigenicity

The experimental groups of mice of inbred strain A/J and C57BL/6J were selected (10 mice per one experimental group per cage) and 100 µl of blood was taken as a control before immunization. The preimmunization level of antibodies (so-called initial antibodies) was taken as a baseline and subtracted from all sera subsequently tested. The first immunization in a dose of 5 µg of sample/mouse/immunization was given intraperitoneally as an alum preparation to increase the animals' response to immunization. A second dose was given after 3 weeks and 12 days later the mice were exsanguinated. Serum levels of insulin-specific IgG and IgE antibodies were measured by ELISA indirect double-layer method with antigen-adsorbed microplates, as previously reported [79,80].

It should be noted that in all of the mice immunized with any one of the conjugates, both unmodified insulin (Zn-ins) and the respective mPEG-insulin conjugates (C600, F750, K750, F2000) were used as detecting antigens in the ELISA tests. However, in all of the cases (except when the mice were twice immunized with Zn-ins) where the conjugates were used as the detecting antigens, no measurable levels of antibodies were measured. Therefore, all of the data presented in this section (with the exception of the antigenicity data) refer to levels of insulin-specific IgG and IgE antibodies detected using unmodified insulin as the antigen.

In both types of mice we tested 12 days after the last immunization, the highest levels of insulin-specific IgG and IgE antibodies were found in the mice immunized solely with unmodified human insulin. Significantly, no insulin-specific IgG or IgE antibody responses were observed in mice immunized at least once F2000.

In C57 mice (high insulin responders), the experimental group immunized with only unmodified insulin produced from 1.4 to 3.15 times more insulin-

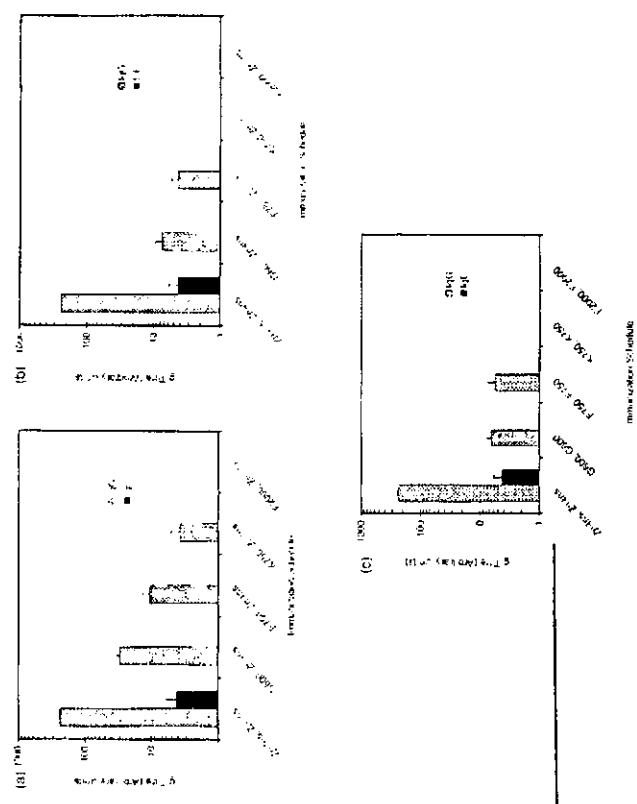


Fig. 1. (a) Total levels of insulin-specific antibodies in A/J mice following immunization at 0 and 3 weeks.

insulin-specific IgG antibodies. These findings are in full agreement with those of other groups who assessed the immunogenicity of pegylated proteins like 11-2 [22], bovine liver catalase [12], bovine serum albumin [81], ovalbumin [77], and hen egg lysozyme [82]. Also, pegylation of recombinant human insulin resulted in the elimination of any detectable IgE antibody response in the test sera measured. Both of these conclusions endorse the concept that pegylation of human insulin results in a nonspecific reduction in the conjugate's immunogenicity. These conclusions are supported by the findings of other groups who have studied the effects of pegylation on the immunogenicity of protein antigens and found that conjugates containing increased amounts (size or number of mPEG chains)

exhibited lower immunogenicities than their less-modified or unmodified counterparts [10,12,22,77,81,83,85].

To assess the antigenicity of the PEG-insulin conjugates relative to unmodified insulin, all of the conjugates (G600, F750, K750, F2000) and Zn-insulin were used as the detecting antigens in ELISA tests used to quantify the amounts of anti-insulin IgG antibodies in those mice who were immunized solely with unmodified insulin. The results of these experiments are given in Table 5 and clearly demonstrate that the covalent attachment of PEG to insulin substantially (by two to three orders of magnitude) diminishes the conjugates' ability to bind to anti-insulin IgG antibodies.

B cell epitopes, or antigenic determinants, are the

permeant group immunized with only unmodified insulin produced from 8 to 61% more insulin-specific IgG antibodies than any other group studied (Fig. 3a-c). In A/J mice immunized with insulin followed by a conjugate always produced higher titers than mice immunized in the opposite order, although the differences were not as distinct as those found in C57 mice. Differences did exist between the low molecular weight mPEG-insulin conjugates with different sites of PEG-substitution, although these differences were not significant.

In general, we found that pegylation of human insulin, irrespective of the site of substitution or molecular weight of mPEG attached, resulted in a 10-1000 times reduction in the amount of circulating

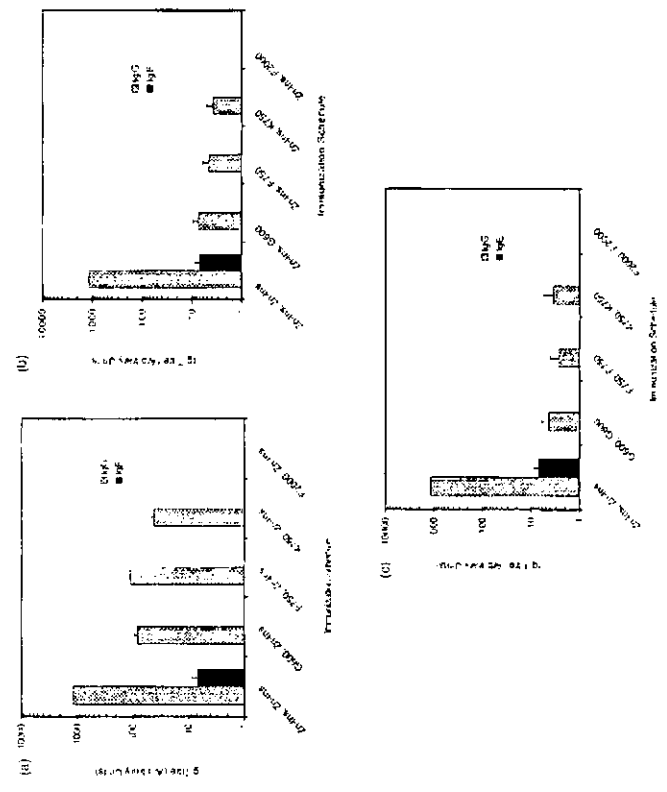


Fig. 3. (a) Total levels of insulin-specific antibodies in C57BL/10 mice following immunization at 0 and 3 weeks.

specific IgG antibodies than any other group studied (Fig. 3a-c). In C57 mice there were at least 10% more insulin-specific IgG antibodies detected in groups immunized with insulin followed by a conjugate (G600, F750, K750, F2000) than in groups immunized in the opposite order. In addition, there were no significant differences in the immunogenicities of low-molecular-weight (600 or 750 Da mPEG) conjugates possessing totally distinct sites of substitution (N-terminus of the A-chain, N-terminus of the B-chain, and C-terminus of the B-chain). The only measurable levels of circulating insulin-specific IgE antibodies found in C57 mice were from those immunized exclusively with unmodified insulin.

In A/J mice (low insulin responders) the ex-

Table 1. Antigenic and insulin (PIG) insulin at A/J and C57BL/6J mice.

Antigen	lgG titer	Relative antigenicity (%)
Zn-ins	11.0 ± 1.5	100
G600	5.0 ± 1.0	45
F750	2.1 ± 1.1	19
K750	1.0 ± 1.5	9
F2000	0	0

Relative antigenicity of the conjugates determined by comparing IgG titers detected using these antigens relative to those detected using Zn-ins as the antigen.

regions on an antigen molecule to which antibodies bind and are usually found on the surface of protein antigens. B cell epitopes on protein antigens are of variable size comprising up to about 20 amino acids. Antibodies bind in a more or less exact three-dimensional fit with an epitope. Thus, the three-dimensional structure of a protein antigen may be essential for antibody binding. Because the production of specific antibodies against protein antigens is mediated through the recognition of conformationally-distinct regions of insulin by membrane-bound antigen receptors (IgM and IgG) on the B cell surface, it can be deduced that the decreased number of circulating antibodies in mice immunized with the PEG insulin conjugates might be due to a nonspecific 'masking' of one or more of insulin's B cell-recognition epitopes by the polymer. Thus, an increase in the molecular weight or number of PEG chains attached to insulin would be expected to result in a more pronounced steric 'masking' effect, as was seen in this study.

A qualitative ranking of the conjugates' humoral immunogenicity, from highest to lowest, is as follows: G600 > F750 > K750 > F2000. From our results there seems to be a direct correlation between a conjugate's molecular weight of attached mPEG and reduction in its humoral immunogenicity. When the amount of mPEG attached approaches 2000 Da (F2000), the immunogenicity of the conjugate is

virtually non-existent (Table 1, underexposed). Taken together with the fact that a threshold amount of conjugate with 600 Da mPEG attached to positions A1, B1, and B29 [mP-(A1B1B29)-insulin] did not elicit any measurable immune response in A/J and C57BL/6 mice [78] we assert that a threshold amount of mPEG attached insulin monomer of roughly 2000 Da is necessary for a conjugate to neither elicit immune responses or be recognized by anti-insulin antibodies in the animal models tested here. This assertion is supported by the work of other analyzing the immunological properties of PEG protein conjugates [13,86,87]. These groups also found that a minimum amount of PEG needs to be coupled to a protein for the conjugate to be rendered non-immunogenic and nonantigenic. Conversely, we did not observe any relationship between a conjugate's site of substitution and its immunogenicity, antigenicity

Cellular immunogenicity

Cellular immunogenicity of insulin and its pegylated derivatives was examined by measuring hind footpad swelling in mice caused by delayed-type hypersensitivity (DTH) reactions [88]. The term delayed-type hypersensitivity includes the delayed skin reactions associated with type IV hypersensitivity and is defined as an immunologically specific inflammatory reaction involving the infiltration of mononuclear cells that is maximal at 24-48 h after sensitization [89]. On day 14 after the last immunization with insulin or its derivatives, the DTH response was assessed by a hind footpad-swelling test. This assay is widely used for detecting DTH in rodents and measures the gross manifestation of DTH reactions [88,89].

Table 6 gives a summary of the results of the DTH experiment. As seen in the antigen-specific IgG and IgE experiments, there was no DTH reaction in any of the mice immunized with F2000. The most significant DTH response was measured in animals immunized solely with Zn-ins, but low responses were also observed in animals immunized with unmodified insulin prior to immunization with one of the mPEG insulin derivatives (G600, F750, K750, F2000). When mice were immunized first with one of the conjugates no measurable DTH reactions occurred, regardless of the type of antigen used in

Table 6. DTH and PCA responses in mice A/J and C57BL/6J mice immunized with insulin or one of its pegylated derivatives.

Immunization schedule	Footpad swelling (mm)	PCA (%)
Zn-ins	8.1 ± 1.3	89 ± 9
G600	9.0 ± 1.1	91 ± 11
F750	3.1 ± 1.1	21 ± 16
K750	4.1 ± 1.1	0
F2000	0	0

DTH responses were tested on day 14 after the last immunization. Concomitant with the DTH test, 0.75 µl NaCl only. The data are presented as the mean ± SD (non-differences in thickness between antigen and saline injected footpad) ± SD of four mice per group.

The antigen, here was used as the recipient of the further dilution of the antigen, at least 5 mm in diameter.

subsequent immunizations. Thus unmodified recombinant human insulin is the only antigen, when intradermally injected into previously sensitized individuals, capable of eliciting strong delayed-type hypersensitivity reactions in the mouse models used in this study.

Because DTH is a T cell-mediated event [88-90], the results of these experiments suggest that there must be differences in the way unmodified insulin and its pegylated derivatives are either recognized, processed intracellularly, or presented to antigen-specific T cells by antigen presenting cells (APCs). Although the mechanism by which the mPEG insulin conjugates do not evoke DTH responses was not characterized here, we contend that pegylation might nonspecifically 'mask' one or more of insulin's T cell epitopes and thus prevents it from being recognized normally by the cellular immune system. We believe the effects are nonspecific because there were no observable differences in the DTH responses between mice immunized with

conjugates having distinct sites of substitution and sizes of mPEG attached. We do acknowledge that the footpad test, although being widely used for detecting DTH reactions in rodents, can be justifiably criticized for its lack of sensitivity and objectivity.

3.3. Allergenicity

PCA is a skin test that involves the *in vivo* passive transfer of heterocytotropic antibodies that mediate type I immediate hypersensitivity (e.g., IgE in man) from a sensitized animal to a previously non-sensitized individual by injecting the antibodies intradermally [80]. After injection, these antibodies become anchored to mast cells through their Fc receptors. This is followed by intravenous injection of antigen mixed with a dye such as Evan's Blue. Crosslinking of the cell-fixed (e.g., IgE) antibody receptors by the injected antigen induces a type I hypersensitivity reaction in which histamine and other pharmacological mediators of immediate hypersensitivity are released. Vascular permeability factors act on the vessels to permit plasma and dye to leak into the extravascular space, forming a blue area that can be measured with calipers.

In order to confirm the presence of insulin or mPEG-modified insulin specific reaginic (IgE) antibodies, representative samples were also subjected to a PCA test, the results of which are depicted in Table 6. Sera that were positive in the insulin-specific IgE ELISA, e.g., sera from mice immunized with unmodified insulin only, also showed slightly positive PCA results. All other sera that had negative or very low positive responses in the IgE ELISA tests were also negative in the PCA test.

It has been demonstrated that IgE plays an important role in mediating immediate hypersensitivity reactions in response to antigen in humans [91,92] as well as in animal models [93,94]. Allergic reactions of the immediate type are triggered by multivalent allergens on account of their ability to cross-link IgE antibodies fixed to mast cells and basophils and the resultant release of vasoactive constituents from these cells [77]. For pegylated allergens (like insulin) to effectively be used in a clinical setting, it would be desirable that they not only suppress an ongoing IgE response but also be devoid of the capacity to

cross-link IgG antibodies and elicit unwanted allergic reactions.

The elimination of IgG responses to insulin was achieved in all of the cases evaluated here. The only mice, from both models investigated, that demonstrated PCA reactions were those animals that received sera from donor mice exclusively immunized with unmodified insulin (20-Ins). Thus it appears that the pegylation of recombinant human insulin, at any of the attachment sites studied, precludes conjugates that are non-allergenic and do not elicit immediate-type hypersensitivity reactions. These findings are very much in line with those from another group who examined the allergenicity of pegylated ovalbumin and found that although the native protein stimulated strong PCA reactions, the PEG-modified albumin failed to elicit any significant PCA reaction [27, 95, 96]. The results of the present study are encouraging because they suggest that mPEG conjugates could be administered to patients who have insulin allergies or high levels of circulating IgG antibodies in large doses without the risk of undesirable systemic anaphylactic reactions.

The present study also attempted to measure the immunogenicity (humoral and cellular), antigenicity, and allergenicity of human insulin covalently modified with mPEG. The initial hypothesis was that distinct differences would be observed between the immunological properties of conjugates with different sites of substitution (N-terminus of A-chain, B-terminus of B-chain, C-terminus of B-chain) and/or size of polymer attached (600, 750, or 2000 Da). However, the results of these studies indicate that the effects of mPEG-conjugation on insulin's immunological properties are nonspecific in nature. This conclusion was found to hold true in all of the experimental end-points we examined. In the experiments done to characterize the humoral (circulating IgG and IgE levels) and cellular (DTH reaction) immunogenicity of mPEG insulin derivatives, no significant differences were observed between the conjugates. In addition, no significant differences were observed between the conjugates in their abilities to interact with anti-insulin IgG antibodies. Likewise, when the effects of mPEG-modification on insulin's allergenicity (PCA) were examined, identical results were obtained for all of the conjugates. Thus, our original hypothesis that the site-specific

pegylation of human insulin will result in conjugates with distinct immunological properties proved to be inaccurate.

Because protein antigens generally possess both B cell and T cell epitopes, it appears from the results of these studies that the attachment of low-molecular-weight mPEG (600–2000 Da) to insulin may serve to nonspecifically 'mask' both types of epitopes. Additionally, there did appear to be some molecular weight-dependence observed in the studies evaluating the humoral (B cell-mediated) immunogenicity and antigenicity of the conjugates, but no such dependence was observed in the experiments characterizing either the cellular (T cell mediated) immunogenicity or allergenicity of the conjugates. This suggests that insulin could contain two or more B cell epitopes that are spatially separated and the efficient masking of these epitopes by mPEG requires the polymer to be approximately 2000 Da in size.

Two clinical manifestations of circulating anti-insulin antibodies are postprandial hyperglycemia and extended periods of hypoglycemia, both of which are extremely undesirable. In addition, it is clear that diminishing the immunogenicity and antigenicity of an insulin preparation is an important factor in increasing the therapeutic efficacy of that preparation. These results, although not mechanistically characterized, imply that the pegylation of recombinant human insulin effectively suppresses the immunological properties of this important therapeutic protein in a way that is safe, nontoxic, and previously US Food and Drug Administration (FDA) approved (in other PEG protein conjugates). Finally, the PEG insulin conjugates studied here could be safely used in diabetic patients that have pre-existing anti-insulin antibodies due to their extremely low antigenicities.

4. Pharmacological properties of PEG-insulin conjugates

Two PEG insulin conjugates (F750 and F2000) were injected via both intravenous and subcutaneous routes into male beagle dogs and their biological activities, and pharmacokinetic parameters were compared to Lilly's regular human insulin formula-

tion HumulinR[®] (Hum). Model-independent non-compartmental methods are commonly used to calculate the pharmacokinetic parameters of insulin preparations [97], and in this study we have also utilized this approach to compare the pharmacological properties of insulin and two of its pegylated derivatives. A chronic dog model was used to evaluate the changes in some pharmacokinetic (PK) and pharmacodynamic (PD) parameters due to modification of insulin with 750 or 2000 Da mPEG at residue PhcB1. Healthy beagle dogs (17–18 kg) were maintained at the Animal Resource Center and given at least 3 weeks between treatments.

Prior to insulin (conjugate) injection, samples of blood were taken to establish the basal levels of insulin (conjugate), canine C-peptide, and glucose. Insulin (F750 and F2000) preparations (0.3 U/kg) were administered via i.v. injection into the jugular vein in order to assess total bioavailability of the insulin preparations. The three insulin preparations (0.3 U/kg) were also injected in the nape of the neck of dogs to examine their absorption characteristics from a subcutaneous depot. Serum samples were analyzed for insulin, glucose, and C-peptide content according to the methods described below.

4.1. Biological activity and pharmacodynamic parameters

The serum samples obtained from treated dogs were assayed prior to freezing for glucose concentration. The blood glucose depression abilities of the insulin derivatives after intravenous administration are depicted in Fig. 5. This plot shows that there are some minor differences between the glucose-lowering abilities of native insulin and its pegylated derivatives after intravenous injection into dogs. The onset of action (lowering of blood glucose) and duration of action for HumulinR[®] (Hum), F750, and F2000 are similar which suggests that the pegylation of human insulin does not result in any unfavorable effects on its biological activity *in vivo* if directly injected into systemic circulation. After intravenous administration of these insulin derivatives the dogs' glucose levels rebounded to their initial levels within about 3 h.

The blood glucose lowering effects of Hum, F750, and F2000 after subcutaneous injection of 0.3 U/kg

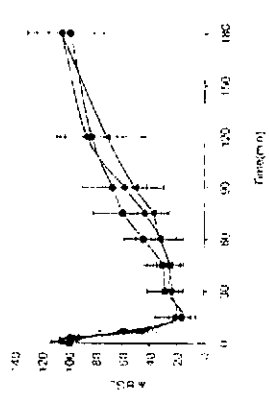


Fig. 5 Blood glucose levels in male beagle dogs following intravenous administration (0.3 U/kg) of HumulinR[®] (○), or conjugates F750 (▲), or F2000 (●). Data are means ± S.D. n = 6.

are depicted in Fig. 6. The main conclusions that may be drawn from these data are the pegylation of insulin at residue B1 results in a slower onset of glucose depression in the animal model studied. A relationship between time to onset of activity and molecular weight was observed between the two conjugates and insulin. The higher-molecular-weight conjugate (F2000) required the longest amount of time to decrease blood glucose levels, while the lower-molecular-weight conjugate (F750) exhibited an intermediate (between Hum and F2000) amount of time to decrease blood glucose levels. After the initial lag time to full action, the duration of biological activity (ability to lower blood glucose) is comparable between the three insulin derivatives.

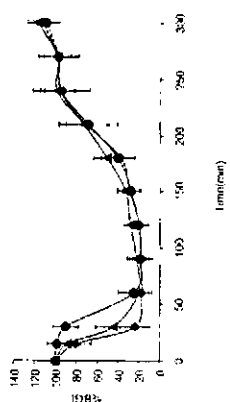


Fig. 6 Blood glucose levels in male beagle dogs following subcutaneous administration (0.3 U/kg) of HumulinR[®] (○), or conjugates F750 (▲), or F2000 (●). Data are means ± S.D. n = 6.

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Thus, the conjugates and unmodified insulin behave similarly once they reach systemic circulation.

Values of the IC_{50} versus time AUC_{0-12} were calculated by the trapezoidal method. The IC_{50} ($IC_{50} = 10^{-5}$ of basal value) and the time to IC_{50} ($T_{IC_{50}}$) were selected to be the pharmacodynamic parameters compared between preparations, and these values were determined from the experimental data. For assessment of the statistical significance, a two-tailed Student's *t*-test (paired two samples mean) was used, and mean values were considered to be statistically different when $P < 0.05$.

Table 7 summarizes the pharmacodynamic parameters calculated for the different insulin treatments after intravenous and subcutaneous administration. When the area under the blood glucose depression curves (AUC_{0-12}) were calculated for the three derivatives after intravenous or subcutaneous administration, we found that F750, F2000 and Hum were the same ($\sim 10^6$) biological effect. Notably, the differences in the biological activities of Hum, F750, and F2000 are statistically indistinguishable from each other; therefore, all intravenous and subcutaneous treatments are considered to be biologically equivalent within the errors of measurement. PEGylation of human insulin did not affect the time to onset of biological action (glucose depression) after subcutaneous administration with an increase in the time to onset with increasing molecular weight of mPEG attached.

The equivalent biological activities found between both of the mPEG-insulin conjugates and unmodified human insulin, as measured their ability to lower blood glucose concentrations, are consistent with the findings of another group who examined

similar PEG-insulin conjugates in male Wistar rats and domesticated male rabbits [48]. These authors also found that conjugates substituted at position PheB1 with PEG (M_n 1500 Da) were equally capable of lowering the animals' blood glucose levels after intravenous administration. The intentioned authors also determined that their PEG(1500) PheB1-insulin conjugate was fully bioactive after administration to both male Wistar rats and male domesticated rabbits via the subcutaneous route [48]. The fact that two independent research groups came up with the same results in very different animal models suggests that the conjugation of low-molecular-weight PEG ($M_n < 2000$ Da) to insulin at residue PheB1 does not adversely affect its biological activity *in vivo*.

4.2 Pharmacokinetic parameters

Conventionally available human insulin and canine C-peptide radioimmunoassay (RIA) kits were used to measure serum concentrations of insulin (mPEG-insulin conjugates) and endogenous insulin secretion, respectively. Each of the insulin kits used in these studies was carefully validated for the analysis of unmodified human insulin, F750 and F2000 using appropriate reference solutions prepared in insulin-free matrix. Both types of assays were considered valid for the individual treatments if the calculated values of two levels of control samples were found to fall within 2 standard deviations of their known value.

Serum insulin (mPEG-insulin) concentrations were adjusted for endogenous insulin using canine C-peptide concentrations as an indicator of endogenous

insulin secretion. This is necessary because human and canine insulin have identical primary sequences while the primary sequences of their connecting-peptides are distinct. Representative samples of serum from animals injected with unmodified insulin or its pegylated derivatives were assayed for their concentrations of canine C-peptide. Because there were no distinguishable differences in the levels of serum C-peptide observed between animals injected via *iv* or *sc* routes and no differences between animals treated with insulin or its pegylated derivatives were observed, average values of serum C-peptide concentrations were used to account for endogenous insulin secretion. Consequently, serum insulin levels depicted in the figures are truly considered to be the levels of exogenously administered insulin (Hum) or its pegylated derivatives (F750 and F2000).

Serum concentrations of insulin (mPEG-insulin conjugates) after correction for endogenous insulin secretion were used to calculate several pharmacokinetic parameters, including apparent volume of distribution (V_d/F), apparent total systemic clearance (CL/F), terminal rate constant (k_{el}), and half-life ($t_{1/2}$). In addition, the area under the serum insulin (conjugate)-versus-time curves (AUC_{0-12}) and MRTs for insulin and its pegylated derivatives were determined. Calculation of these values was performed using the noncompartmental analysis features of WinNonlin™ (Pharsight, USA). For assessment of statistical significance, a two-tailed Student's *t*-test (paired two samples mean) was used and mean values were considered to be statistically different when $P < 0.05$.

Fig. 7 shows the serum insulin (PEG-insulin) levels following intravenous administration. Circulating levels of both conjugates (F750 and F2000) were slightly higher than those of Hum at almost every time point after 15-min postinjection, which indicates that the conjugates may be cleared more slowly than Hum. However, the differences in the terminal portions of these pharmacokinetic curves did not generally result in statistically relevant differences in the calculated pharmacokinetic parameters given in Table 8, except in two cases: (1) F750 had a significantly longer elimination half-life than Hum and (2) the mean residence times (provide measure of the average length of time a molecule resides

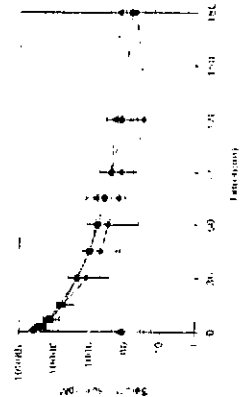


Fig. 7 Serum insulin (mPEG) levels (ng/ml) versus time (min) following intravenous administration of 0.3 U/kg of Hum (○), F750 (▲), or F2000 (●). Data are means $\pm$ S.D. $n = 4$.

within the body) of both conjugates were significantly longer than Hum after intravenous injection. The increased MRTs of the conjugates relative to Hum might be explained by PEG's ability to interact with cell membranes [6,29]. Caliceti et al. demonstrated that PEG-SOD was able to bind to red blood cells via interaction of the PEG-chains and cell membranes [38]. By interacting with the animals' red blood cells, the conjugates possibly will remain in the circulation longer than unmodified insulin, as seen in our results.

The serum insulin (PEG-insulin derivative) levels following subcutaneous administration of 0.3 U/kg are shown in Fig. 8. This plot provides clear evidence that there are substantial differences in the circulating levels of native insulin (in the form of Hum) and its pegylated derivatives (F750 and F2000) after subcutaneous injection into dogs. Both of the conjugates exhibited increased serum concentrations at most time points tested. As can be seen in Table 9, the conjugates rose to significantly higher (C_{max} levels (~ 1400 pM) than Hum (~ 1000 pM), and it took longer for the conjugates to achieve their maximum concentrations (T_{max}) than Hum. However, the differences in calculated T_{max} values were not statistically significant. Analysis of the AUC_{0-12} values calculated for F750 and F2000 suggest that both conjugates were absorbed to a significantly ($P < 0.02$) greater extent than the Hum insulin hexamers following subcutaneous injection. A clear trend was also observed between the relative availability, defined as $(AUC_{0-12})_{conjugate} / (AUC_{0-12})_{Hum}$, of Hum

Table 7 Summary of dog model *in vivo* biological activity data for various insulins*

Parameter	Treatment	Hum	F750	F2000
Intravenous AUC_{0-12} (mg $\cdot$ min/dl)		959 $\pm$ 1661 (10 ⁶)	929 $\pm$ 1521 (10 ⁶)	10320 $\pm$ 1612 (10 ⁶)
Subcutaneous AUC_{0-12} (mg $\cdot$ min/dl)		8083 $\pm$ 809 (10 ⁶)	9998 $\pm$ 591 (10 ⁶)	10653 $\pm$ 1244 (10 ⁶)

* Area under glucose depression curves (AUC_{0-12}) calculated by trapezoidal method.

* Doses given were 0.3 U/kg. Data are means $\pm$ S.D. $n = 6$. Values in parentheses are percent bioactivity relative to Hum.

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ments suffered from a high degree of inter- and intra-subject variability, so more definitive differences in the pharmacokinetic parameters were not observed. Notably, the bioequivalence found between the conjugates and insulin is supported by the work of another group who studied similar conjugates in male Wistar rats and male domesticated rabbits [48].

Many unresolved issues remain critical for the development of a clinically feasible insulin substitute in the form of a pegylated derivative. Several issues have not been adequately resolved by studies reported to date, and need to be resolved before these conjugates could be considered for Phase I clinical trials. Some of these issues include: (1) preparation of conjugates with branched PEG species, (2) evaluation of optimized formulation conditions, (3) assessment of chemical stability, and (4) a more comprehensive pharmacological characterization of the optimal conjugate formulations.

Although some beneficial properties were imparted to insulin through covalent attachment of low-molecular-weight PEG species, other conjugates should be prepared using commercially available branched (e.g., PEG₂-Lys) PEG derivatives. These different PEG species, which are specifically attached to insulin, could conceivably give rise to conjugates with improved physical, immunological, and pharmacological properties compared to the conjugates studied here.

Circulating insulin exists as a complex mixture of monomers, dimers, and hexamers under the influence of a dynamic equilibrium. Furthermore, many variables such as protein concentration, pH, temperature, ionic strength, and the exact composition of the insulin formulation itself influence this dynamic equilibrium. Depending on what properties are desired (prolonged or rapid action, enhanced physical and chemical stability, etc.) in the final product, formulation conditions and compositions will need to be adjusted accordingly.

Another very important issue that must specifically be addressed before any candidate PEG insulin conjugate could be considered for clinical development is chemical stability. Insulin is known to undergo many nonenzymatic reactions including deamidation, β -isomerization, oxidation, covalent dimerization and polymerization, and disulfide ex-

change. It is also known that formulation conditions affect which of these reactions takes place and to what extent. Therefore, careful choosing of optimal formulation conditions will be largely dependent on the findings of real-time chemical stability studies. Results of this research to date suggest that the site-specific pegylation of recombinant human insulin produces conjugates that are physically more stable, non-immunogenic and nonantigenic, fully bioactive, and cleared from the circulation more slowly following subcutaneous administration. Thus, many of the beneficial properties reported for proteins by the attachment of the water-soluble, nontoxic, and FDA-approved polymer poly(ethylene glycol) were also common to PEG-insulin conjugates. This evidence provides a 'proof-of-concept' for the continued development of these conjugates with the hopes that one day their full clinical benefits might be realized.

A recent article by Kozlowski and Harris [101] provides strong testimony that the clinical relevance of pegylated proteins or peptides is finally being realized. Currently two FDA-approved pegylated forms of proteins developed by Enzon (NJ, USA) that are currently on the market: (1) PEG-albumin deaminase (Adagen[®]) used for the treatment of severe combined immunodeficiency syndrome (SCIDS), and (2) PEG-asparaginase (Oncaspar[®]) marketed for treatment of acute lymphoblastic leukemia [101]. In addition, several pegylated proteins are currently in advanced-stage clinical trials and it is expected that some of these conjugates will receive approval within the next year. Examples of these conjugates include soluble PEG-TNF-receptor from Amgen [102], a pegylated antibody fragment that binds TNF- α from Celtech [103] for the treatment of rheumatoid arthritis, PEG-G-CSF from Amgen to be used as an adjuvant for chemotherapy of breast cancer, PEG- α -IFN from Shering-Plough [104,105] and Hoffmann-La Roche [106,107] for the treatment of hepatitis C, and PEG-RGIF receptor agonist from Pharmacia-Sensis [108] for the treatment of acromegaly. In light of the recent progress being made in the area of protein pegylation, this technique seems to be gaining acceptance from both the pharmaceutical industry and federal regulatory agencies as an advantageous method of increasing the therapeutic efficacy of protein pharmaceuticals.

of passive cutaneous anaphylaxis tests. We propose that the attachment of PEG to human insulin prevented these conjugates from interacting with anti-insulin antibodies in any substantial manner, as assessed by an ELISA method. These results are very promising in terms of finding a safe method for immunizing the immunogenicity, antigenicity, and allergenicity of recombinant human insulin. Circulating anti-insulin IgG and IgE antibodies can cause unwanted and dangerous problems in diabetes patients and can result in severe systemic anaphylactic reactions. On a more general note, the presence of anti-insulin antibodies negatively affects the clinical efficacy of insulin treatment regimens by causing postprandial hyperglycemia and prolonged hypoglycemia, both of which can lead to more serious complications if left unchecked.

Finally, we investigated the effects of pegylation on insulin's biological activity and pharmacokinetics after intravenous and subcutaneous administration in a dog model. The blood glucose lowering abilities of the conjugates relative to soluble regular human insulin were used to determine the conjugates' bioequivalences. In addition, noncompartmental methods were utilized to calculate several pharmacokinetic parameters, and statistical comparisons were made between the values of the parameters calculated for each treatment group. We found that the blood glucose lowering abilities of F750 and F2000 were statistically indistinguishable from insulin after intravenous or subcutaneous administration. Likewise, the terminal clearance rates of F750 and F2000 were also statistically indistinguishable from insulin after intravenous administration, but the pegylated insulin derivatives had significantly slower clearance rates following subcutaneous administration. The elimination half-life of F750 was statistically significantly longer than Humulin after both intravenous and subcutaneous administration, whereas the half-life of F2000 was significantly longer only in the case of subcutaneous administration. The MRT of F2000 was statistically significantly longer than Humulin after injection via both routes, whereas the MRT of F750 was significantly longer only after intravenous injection. These studies demonstrate there are measurable increases in the circulation half-lives of the PEG insulin conjugates relative to insulin. However, the data collected in these experi-

imental success. In addition, it seems an almost insurmountable challenge to obtain efficacious insulin preparations administrable by a nonparenteral route. Many of the problems that insulin therapy encounters are due to the instability intrinsic to insulin. Insulin, along with most other therapeutic peptides and proteins, possesses many unfavorable attributes that make the development of effective therapeutic preparations particularly challenging. These unfavorable attributes include chemical and physical instability, inherent immunogenicity and antigenicity, and rapid clearance from the systemic circulation. The fundamental hypothesis underlying this work was that the site-specific covalent attachment of PEG to human insulin would impart beneficial physical, immunological, and pharmacological properties to this important therapeutic protein. To this end, conjugates with different sites of substitution and molecular weights of PEG attached were prepared. Following purification to homogeneity, a broad-spectrum approach was used to assess the physicochemical, physical, biological, immunological, and pharmacological properties of the conjugates.

Site-specific attachment of low-molecular-weight PEG to insulin via its N-terminal (PheB1) amino group and its penultimate C-terminal ϵ -amino group (LysB29) modified the physical, biochemical, and biological properties of this important therapeutic protein to varying degrees. Specifically, the effects of PEG conjugation on native insulin's conformation, average association-state, and *in vivo* biological activity were minimal. However, when insulin was pegylated at residue PheB1 the resultant conjugates were two orders of magnitude more physically stable.

The conjugates' humoral immunogenicity, cellular immunogenicity, antigenicity, and allergenicity were then examined in two mouse models (A/J and C57BL/10). The attachment of low-molecular-weight PEG to any of the residues resulted in a significant decrease in the conjugates' humoral and cellular immunogenicities, as characterized by circulating IgG and IgE antibody levels and delayed-type hypersensitivity tests, respectively. The PEG insulin conjugates were unable to elicit any measurable allergic reactions in immunized mice, as determined from circulating IgE antibody levels and the results

