

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente N° 12-020.409

Solicitud de Patente de Invención titulada: "L-ASPATAGINASA TRATADA CON PEG"

Solicitante: ALIZE PHARMA II.

Nuestra Ref.: P2012/000012

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

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RECURSO DE REPOSICIÓN

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, D.C., identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante, y dentro del término legal previsto por el artículo 51 del Código Contencioso Administrativo, me permito interponer RECURSO DE REPOSICIÓN en contra de la Resolución No. 8157, notificada por edicto desfijado el 03 de abril de 2015.

1. ANTECEDENTES

1.1 La solicitud de patente ventilada en este expediente fue presentada el 06 de febrero de 2012, con un Capítulo Reivindicatorio de 39 reivindicaciones.

1.2 En respuesta al examen de forma, Oficio No. 4036, notificado por la SIC en lista No. 121 de 13 de marzo de 2012, se presentó un Capítulo Reivindicatorio Modificado con 22 reivindicaciones (radicado en 09 de mayo de 2012).

1.3 En respuesta al primer examen de fondo, Oficio No. 23171, notificado por la SIC en la lista No. 51 del 24 de diciembre de 2013, se presentó un Capítulo Reivindicatorio Modificado con 14 reivindicaciones (radicado el 23 de abril de 2014), con el fin de superar las objeciones de claridad, novedad y nivel inventivo elevadas por la Examinadora.

1.4 En respuesta al segundo examen de fondo, Oficio No. 9664, notificado por la SIC en la lista No. 032 de 12 de agosto de 2014, se presentó un Capítulo Reivindicatorio Modificado con 7 reivindicaciones (radicado el 18 de diciembre de 2014), con el fin de superar las objeciones de claridad y nivel inventivo elevadas por la Examinadora.

1.5 Según la Resolución No. 8157, el Despacho niega las Reivindicaciones 1 a 7 por supuesta falta de nivel inventivo.

2. OBJETO DEL RECURSO

Que se revoque la resolución recurrida, y en su lugar se conceda el privilegio de patente de invención para las reivindicaciones contenidas en el **Capítulo Reivindicatorio Modificado adjunto**, o aquellas que el Despacho considere cumplen con los requisitos de patentabilidad.

3. FUNDAMENTO DEL RECURSO

3.1 a- En primer lugar, con el ánimo de brindar mayor claridad a la presente solicitud, me permito adjuntar un **Capítulo Reivindicatorio Modificado**, que reemplaza en su totalidad al Capítulo Reivindicatorio Modificado presentado el 18 de diciembre de 2014 en respuesta al segundo examen de fondo, en donde la nueva Reivindicación 1 incluye la actividad L-asparaginasa *in vitro* del conjugado de la invención.

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial. Por el contrario, dichas modificaciones corresponden a una limitación de la materia reclamada.

3.2 Con referencia a los argumentos presentados por la SIC para desvirtuar el nivel inventivo de la presente solicitud en la resolución aquí recurrida, respetuosamente difiero del análisis expuesto, y procedo a refutar cada uno de dichos argumentos, exponiendo las motivaciones técnicas y legales que desvirtúan la posición de ese Despacho.

a- El Despacho insiste en que los documentos "Modification of Recombinant Asparaginase from *Erwinia carotovora* with Polyethylene Glycol 5000"¹ (D1) y US5310670 (D3) afectan el nivel inventivo de la presente invención.

La SIC argumenta que D1 es el documento más cercano a la presente invención, porque divulga un conjugado que comprende L-asparaginasa de *Erwinia carotovora* y polietilenglicol con peso molecular de 5000Da, cuya diferencia con la invención es que la L-asparaginasa del conjugado reclamado es de *Erwinia chrysanthemi* caracterizada por la SEQ ID NO: 1, mientras que en D1 la L-asparaginasa del conjugado es de *Erwinia carotova*. Sin embargo, dicha diferencia no confiere ningún efecto técnico, por lo que el problema técnico que pretende resolver la invención es cómo modificar el conjugado divulgado en D1 para obtener un conjugado alternativo para el tratamiento de leucemia linfoblástica aguda.

Así, el Despacho sostiene que D3, por su parte, divulga la L-asparaginasa de *Erwinia chrysanthemi* caracterizada por la SEQ ID NO: 1, donde la L-asparaginasa presenta un porcentaje de homología del 100% con respecto a la L-asparaginasa de la invención. Por lo tanto, la persona medianamente versada en la materia sin ningún esfuerzo modificaría el conjugado de D1, sustituyendo la L-asparaginasa de *Erwinia carotovora* de D1 por la L-asparaginasa de *Erwinia chrysanthemi* como lo sugiere D3, para obtener el conjugado de la invención.

b- Frente a mis argumentos presentados en respuesta al segundo examen de fondo, el Despacho señala que para la persona medianamente versada en la materia, la inclusión de PEG en una molécula no modifica la actividad de la misma, puesto que ésta es inherente a la molécula y su interacción con el objetivo farmacológico (farmacodinamia), sino que modifica la distribución y el metabolismo de la molécula (farmacocinética), por lo que no considera correcto señalar que la conjugación de una molécula con PEG modifica la actividad de la misma. Para la persona medianamente versada en la materia es totalmente conocido que las composiciones farmacéuticas que contienen moléculas pegiladas se diseñan para ser administradas vía intramuscular o subcutánea, con el propósito de que la liberación de la molécula pegilada al torrente sanguíneo sea retardada y sostenida, además de que la presencia del PEG en la molécula impide una pronta degradación de la molécula, retardando así el metabolismo de la misma, y logre ejercer la actividad terapéutica por más tiempo.

¹ A. V. Kuchumova et al., "Modification of Recombinant Asparaginase from *Erwinia Carotovora* with Polyethylene Glycol 5000," *Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry* 1, no. 3 (September 2007): 230–32, doi:10.1134/S1990750807030092.

Adicionalmente, la SIC señala que si bien es cierto que la L-asparaginasa de *Erwinia chrysanthemi* de la solicitud es diferente a la L-asparaginasa de *Erwinia carotovora* de D1 y teniendo en cuenta que D3 sugiere una L-asparaginasa de *Erwinia chrysanthemi* con la SEQ ID NO 1, la persona medianamente versada en la materia no tiene la opción de elegir un universo de L-asparaginasas, sino que tiene la posibilidad de emplear la L-asparaginasa *Erwinia chrysanthemi* de la SEQ ID NO 1 como la opción sugerida en el arte previo para desarrollar los conjugados de la invención.

Frente a las comparaciones de los perfiles farmacocinéticos, el Despacho señala que la L-asparaginasa de *E. coli* es diferente a la L-asparaginasa de *Erwinia* y que puede diferir en las propiedades farmacocinéticas e inmunogénicas (Pág. 8, párrafo siguiente a la Tabla 1), por lo que no se considera pertinente la comparación entre los conjugados que contienen L-asparaginasa de dos especies filogenéticamente diferentes. Además, el Despacho menciona que las ventajas del conjugado de L-asparaginasa de la solicitud con respecto al conjugado Oncaspar® no se pueden considerar del todo ciertas, porque si se compara el tiempo de vida media del conjugado pegilado PEG 5kDa con el tiempo de vida media de Oncaspar®, se observa que el intervalo de vida media obtenido para el Oncaspar® está contenido dentro del intervalo establecido para el tiempo de vida media del conjugado de la solicitud, por lo que hay coincidencia de valores y por lo tanto se determina que existen valores similares de tiempo de vida media para los dos conjugados. De igual manera, para el tiempo de agotamiento de la L-asparaginasa, ocurre el mismo fenómeno, donde los intervalos de tiempo coinciden, así que el tiempo de agotamiento de L-asparaginasa en los dos conjugados es similar y por lo tanto no se puede reconocer una ventaja técnica evidente al conjugado de la solicitud con respecto al conjugado de referencia, en este caso el Oncospar®.

Finalmente, la SIC señala que los conceptos de D1 y D3 se consideran suficientes para obtener el conjugado de la invención con las mismas características reclamadas. Agrega que, si bien es cierto que la diferencia establecida entre el conjugado de la invención con respecto al conjugado de D1 es que el conjugado de la solicitud contiene L-asparaginasa de *Erwinia chrysanthemi* caracterizada por la SEQ ID NO 1, mientras que el conjugado divulgado en D1 contiene L-asparaginasa de *Erwinia carotovora* sin una secuencia específica, para lo cual el documento divulga una L-asparaginasa de *Erwinia chrysanthemi* caracterizada por la SEQ ID NO 1, la persona medianamente versada en la materia tendría la motivación suficiente para incluir la L-asparaginasa sugerida en D3 al conjugado divulgado en el documento D1, para obtener de manera evidente el conjugado de la invención.

3.3. En respuesta a los argumentos presentados por el Despacho, me permito argumentar lo siguiente:

i) En primer lugar, me permito llamar la atención del Despacho sobre el Capítulo Reivindicatorio Modificado adjunto, en particular, la nueva Reivindicación 1, la cual, en aras de mejorar la claridad, reclama que el conjugado tiene al menos 70% en la actividad *in vitro* frente a L-asparaginasa de *Erwinia chrysanthemi* no conjugada.

ii) Por otro lado, me permito señalar que los argumentos planteados por el Despacho son erróneos. En primer lugar, la SIC desestimó la característica técnica relacionada con una actividad *in vivo* 50 veces más potente que la actividad de la L-asparaginasa de *Erwinia chrysanthemi* no conjugada, característica que incluso se encuentra en la Reivindicación 1 de la invención, que define que el conjugado L-asparaginasa tiene al menos cincuenta veces más actividad *in vivo* en comparación con la actividad L-asparaginasa de *Erwinia chrysanthemi* no conjugada. Este importante incremento se encuentra soportado en al menos la Figura 4 y el párrafo [074] de la página 41, en el Ejemplo 5 de la página 67, y en el Ejemplo 7 de la página 70 a 71 del Capítulo Descriptivo.

Sin embargo, la Tabla 2 se dirige a la actividad *in vitro* de modo que el Despacho considera erróneamente esta información y desestima su divulgación y la definición referida a un incremento de 50 veces en la actividad *in vivo* en la Reivindicación 1. Para distinguir mejor la invención de la divulgación de D1, se ha introducido una característica técnica adicional en la Reivindicación 1, que señala que el conjugado tiene al menos 70% de la actividad *in vitro* en comparación con L-asparaginasa de *Erwinia chrysanthemi* no conjugada. Como señalé anteriormente, D1 da a conocer una retención de a lo sumo 57% en la actividad *in vitro* para el conjugado pegilado en comparación con el conjugado L-asparaginasa no pegilado muy inferior en comparación con los valores de la invención reivindicada.

Por lo tanto, el conjugado de la presente invención es completamente distinguible de la divulgación de D1, como se refleja por las distintivas actividades *in vitro* e *in vivo*.

Además, el Despacho afirma que la vida media de Oncaspar® está dentro del rango de los conjugados reivindicados. Sin embargo, de acuerdo a la Tabla 5 del Capítulo Descriptivo, para una dosis particular, las vidas medias de los conjugados, ya sea con PEG de 2.000Da o PEG de 5000Da, **son más largas en comparación con la vida media de Oncaspar®**. Por lo tanto, el Despacho ha cometido otro error sustancial en el análisis que condujo al rechazo de la presente invención.

Al hacer su análisis el Despacho señaló que los efectos asociados con la conjugación de PEG son bien conocidos por la persona medianamente versada en la materia, como se describe en D1. Sin embargo, me permito argumentar que D1 no da a conocer los efectos técnicos de la pegilación,

específicamente el aumento de la media vida, la reducción de la inmunogenicidad y la actividad enzimática *in vivo*. Por el contrario, D1 se limita a mencionar la actividad *in vitro* de L-asparaginasa de *Erwinia catarova* pegilada, con resultados muy inferiores en comparación con la presente invención. De acuerdo con lo anterior, las conclusiones del Despacho fueron derivadas a partir de un análisis en retrospectiva, ya que no explica de forma técnica por qué la persona medianamente versada en la materia llegaría a partir de las enseñanzas de D1 al conjugado de la invención.

Por otra parte, el Despacho sostiene que D3 describe el uso de L-asparaginasa *Erwinia chrysanthemi*, por lo que la persona medianamente versada en la materia se vería motivada a pegilar esta molécula para mejorar sus propiedades, lo que a todas luces evidencia un análisis en retrospectiva. No obstante, D3 es una patente que fue publicada en 1994 y la tecnología de conjugación con PEG fue descubierta por lo menos hace más de treinta años (ver ANEXO 1: publicación de Ashihara). La presente invención, por otro lado, tiene una fecha de prioridad del 2009. Por lo tanto, hay al menos una brecha de quince años entre el uso de L-asparaginasa de *Erwinia chrysanthemi* y la conjugación de L-asparaginasa de *Erwinia chrysanthemi* con PEG para uso clínico. Tal significativo lapso de tiempo indica que no es obvio para la persona medianamente versada en la materia hacer una L-asparaginasa de *Erwinia chrysanthemi* pegilada.

De hecho, cuando la persona medianamente versada en la materia se enfrenta al problema de desarrollar una L-asparaginasa con mejores propiedades, debería considerar una serie de factores tales como la retención de la actividad *in vitro*, una alta actividad *in vivo*, una vida media más larga y una menor inmunogenicidad. Una solución a este problema requiere un delicado equilibrio de estos factores y los resultados no serían meros efectos adicionales.

En particular, como se señala en la publicación Fishburn, adjunta para referencia del Despacho (ANEXO 2), la pegilación a menudo conduce a una pérdida de potencia, y mientras que la mayoría de los enfoques para el desarrollo de nuevos fármacos intentan aumentar la actividad del fármaco directamente, la creación de fármacos pegilados busca balancear las propiedades farmacodinámicas (PD) y farmacocinéticas para producir nuevas terapias que reúnan ambos requerimientos relativos al aumento de la eficacia y a un mayor cumplimiento en el ámbito clínico (ver resumen). Tal balance de las propiedades farmacodinámica y farmacocinéticas, por lo tanto, requiere que la persona medianamente versada en la materia adopte un enfoque cauteloso en el desarrollo de una L-asparaginasa pegilada, lo que demuestra las cualidades superiores e inesperadas de la presente invención.

A partir de las enseñanzas de D1, la persona medianamente versada en la materia no hubiese tenido una expectativa razonable de que podrían ser alcanzados los resultados con relación a estas propiedades. Más aún, dicha persona tampoco hubiese sabido cómo lograr estos resultados. Como se ha indicado, D1 no hace referencia a las propiedades de conjugado L-asparaginasa de *Erwinia carotovora*, excepto para la actividad *in vitro*.

Por consiguiente, D1 y D3 no llevarían a la persona medianamente versada en la materia hacia la conjugación de asparaginasa de *Erwinia chrysanthemi* con PEG para producir un conjugado de L-asparaginasa que tiene al menos 70% de la actividad *in vitro* y al menos 50 veces más actividad *in vivo* en comparación con la L-asparaginasa de *Erwinia chrysanthemi* no conjugada.

Como ya lo indiqué durante el trámite de la presente invención, los resultados sorprendentes de L-asparaginasa de *Erwinia chrysanthemi* de la SEQ ID NO: 1 conjugada con PEG de 5000Da muestran un incremento de la actividad enzimática residual *in vivo* en comparación con L-asparaginasa de *Erwinia chrysanthemi* conjugada con PEG de 10.000 Da.

Adicionalmente, el Despacho debe tener en cuenta la divulgación en la Figura 4 y los párrafos [0116] a [0118], que demuestran que el aumento del tamaño de PEG a 10.000Da no aumentó aún más la actividad enzimática *in vivo* del conjugado sino más bien disminuyó la actividad en comparación con el conjugado de PEG 5000 Da, lo que corresponde a un efecto inesperado, ya que la persona medianamente versada en la materia hubiese esperado encontrar una actividad mejorada con el conjugado de mayor 10.000 Da en comparación con el conjugado más pequeño de 5000 Da.

Asimismo, ninguno de los documentos citados por el Despacho describen que el tamaño del polietilenglicol es crítico en la mejora de perfil farmacocinético, la potencia y duración de acción del conjugado, y la reducción de la inmunogenicidad.

De acuerdo con lo anterior, me permito señalar que el conjugado reivindicado posee propiedades superiores y sorprendentes, las cuales resumo a continuación:

- a) aumento de la vida media de la L-asparaginasa de *Erwinia chrysanthemi* pegilada a la luz de la vida media más corta cuando esta no se conjuga, (ver el párrafo [0011] y en la Tabla 5);
- b) alto nivel de retención para la actividad *in vitro* después de la pegilación, (ver la Tabla 2 y el Ejemplo 5);
- c) alta actividad residual del conjugado de PEG 5000 Da en comparación con el conjugado de PEG 10.000 Da, (ver las Tablas 3 y 4 y Ejemplo 9); y

d) la inesperada actividad mejorada *in vivo* de la L-asparaginasa de *Erwinia chrysanthemi* pegilada en comparación con la L-asparaginasa no conjugada, (ver Ejemplo 7).

iii) Adicionalmente a los argumentos presentados anteriormente, se adjunta una declaración experta del Dr. Thierry Abribat (ver ANEXO 3). La Declaración Abribat se ha presentado durante el trámite de la solicitud equivalente en la oficina de patentes de Los Estados Unidos (US 13/382/276) e incluye datos relacionados con los efectos inesperados y sorprendentes de la L-asparaginasa de *Erwinia chrysanthemi* conjugada.

A continuación me permito llamar la atención del Despacho sobre algunos apartados de la declaración del Dr. Abribat, donde se establece que:

“Los siguientes comentarios se presentan como evidencia que apoya los resultados inesperados y superiores para la farmacocinética y las propiedades inmunológicas de la L-asparaginasa de *Erwinia chrysanthemi* pegilada como se establece en las reivindicaciones de la presente la solicitud.

He estudiado los perfiles farmacocinéticos de L-asparaginasa de *E. coli* y *Erwinia chrysanthemi* pegiladas. El Ejemplo 9 de la presente solicitud demuestra la media vida sorprendentemente superior de la L-asparaginasa de *Erwinia chrysanthemi* pegilado. Las actividades enzimáticas de L-asparaginasa pegilada de *E. coli* y *Erwinia chrysanthemi* con PEG 5000 Da se determinaron con un ensayo cromogénico y las vidas medias de estos conjugados se calcularon como se establece en el la Tabla 5 del Capítulo Descriptivo.

Es bien sabido que la L-asparaginasa de *E. coli* no conjugada tiene una vida media más larga que la L-asparaginasa *Erwinia chrysanthemi* no conjugada. Por lo tanto, la expectativa original fue que la L-asparaginasa de *E. coli* pegilada tendría una vida media más larga en comparación con L-asparaginasa de *Erwinia chrysanthemi* que está pegilada de manera similar.

Los resultados experimentales, sin embargo, fueron distintos a las expectativas originales porque la L-asparaginasa de *Erwinia chrysanthemi* conjugada con PEG 5000Da tiene una vida media superior que la L-asparaginasa de *E. coli* pegilada con PEG 5000Da. Ver Tabla 5 del Capítulo Descriptivo.

Se estudió la retención de la actividad L-asparaginasa *in vitro* de la L-asparaginasa de *Erwinia chrysanthemi* después de la pegilación. En esencia, la actividad de L-asparaginasa *in vitro* de la L-asparaginasa de *Erwinia chrysanthemi* conjugada con PEG se midió con el ensayo de Nessler y se

comparó con la L-asparaginasa de *Erwinia chrysanthemi* no conjugada tal como se expone en el Ejemplo 5 del Capítulo Descriptivo.

Se encontró que el conjugado L-asparaginasa retiene al menos 78% de la actividad aminohidrolasa in vitro del conjugado L-asparaginasa de *Erwinia chrysanthemi*. Ver Tabla 2 del Capítulo Descriptivo. Este hallazgo es inesperado porque la pegilación de L-asparaginasa de otras especies resultó en una mucho más significativa pérdida de actividad enzimática.

También se realizaron experimentos para examinar los efectos del tamaño de PEG sobre la actividad L-asparaginasa. Los experimentos mostraron que la L-asparaginasa de *Erwinia chrysanthemi* conjugada con PEG 5000 Da tiene una actividad enzimática residual in vivo más prolongada en comparación con los conjugados con PEG 2000 Da y 10.000 Da. Ver párrafo [0017] y la Figura 5 del Capítulo Descriptivo.

Es sorprendente encontrar que los conjugados con PEG 10.000 Da fallan en aumentar la actividad enzimática residual de L-asparaginasa, dado que una persona experta en la materia hubiese esperado que un aumento del tamaño de PEG mejore la vida media mediante una reducción de la degradación. Este resultado es sorprendente debido a que el incremento del tamaño del conjugado de 2.000 Da a 5000 Da PEG resulta en una mayor actividad pero un conjugado PEG 10.000 Da no produce un incremento adicional similar.

Otros experimentos se realizaron en ratones para examinar la actividad enzimática en vivo de L-asparaginasa pegilada y no pegilada. La actividad de referencia de todos los elementos de prueba, incluyendo L-asparaginasa de *Erwinia chrysanthemi* pegilada y no pegilada, se determinó por un ensayo Nessler. Una (unidad) U del elemento de prueba oxida un micromol de L-asparagina por minuto a 37 ° C y un pH de 8,6. Los elementos de prueba se administraron a ratones con una única inyección intravenosa. Desde que L-asparaginasa cataliza la desamidación de L-asparagina a ácido L-aspartico y de L-glutamina a ácido L-glutámico, se midieron los niveles de L-asparagina y L-glutamina en el plasma de los ratones con un Kit de análisis de aminoácidos PICO-TAG como indicadores de la actividad de la L-asparaginasa *in vivo*.

Como se muestra en la Figura 1 en el presente documento adjunto, dosis inferiores a 125 U/ kg de peso corporal de asparaginasa de *Erwinia chrysanthemi* no pegilada (Erwinase®) no fueron capaces de reducir la L- asparagina plasmática a cero. Además, incluso a dosis más altas, tales como 125 U/kg y 250 U/kg, Erwinase® no pudo lograr que el nivel plasmático de L-asparagina permanezca en cero durante el periodo de prueba completa de 48 horas. Por otra parte, como se muestra en las Figuras 2 y 3, todas las L-asparaginasas pegiladas (conjugados de mPEG-r-crisantaspasa) indujeron

OLARTEMOURE

OLARTE MOURE & ASOCIADOS LTDA.

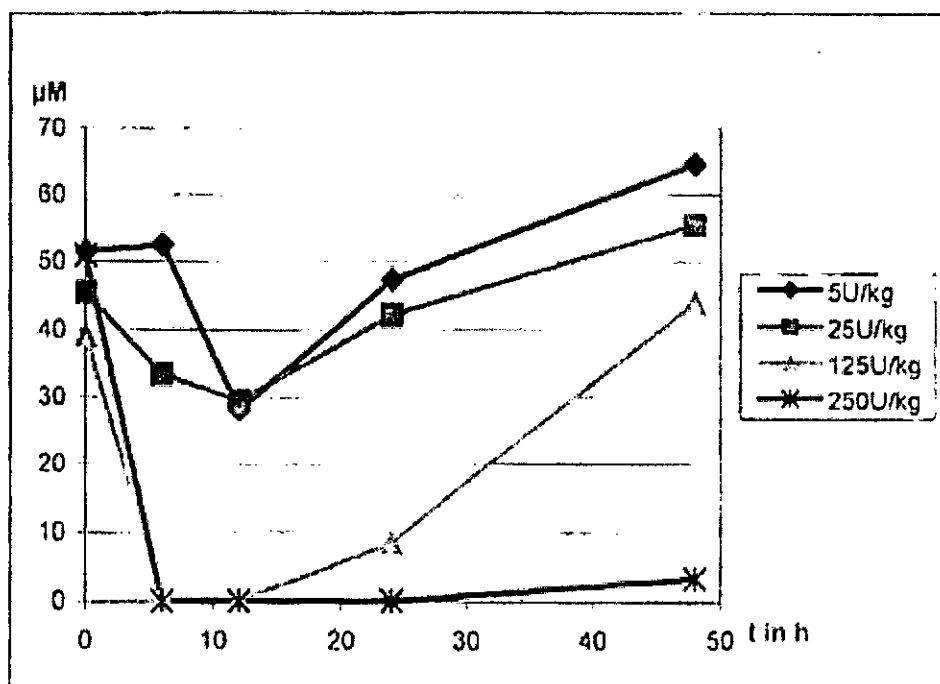
una disminución del nivel en plasma de L-asparagina a cero dentro de las 6 horas después del tratamiento en todas las dosis probadas.

Además, el nivel cero se mantuvo durante el período de prueba completo. Por lo tanto, la L-asparaginasa de *Erwinia chrysanthemi* conjugada con PEG 2.000 Da o 5000 Da probado a 5 U/kg fueron al menos tan potente como Erwinase® ensayado a 250 U/kg, lo que sugiere que la pegilación mejora la potencia en por lo menos 50 veces.

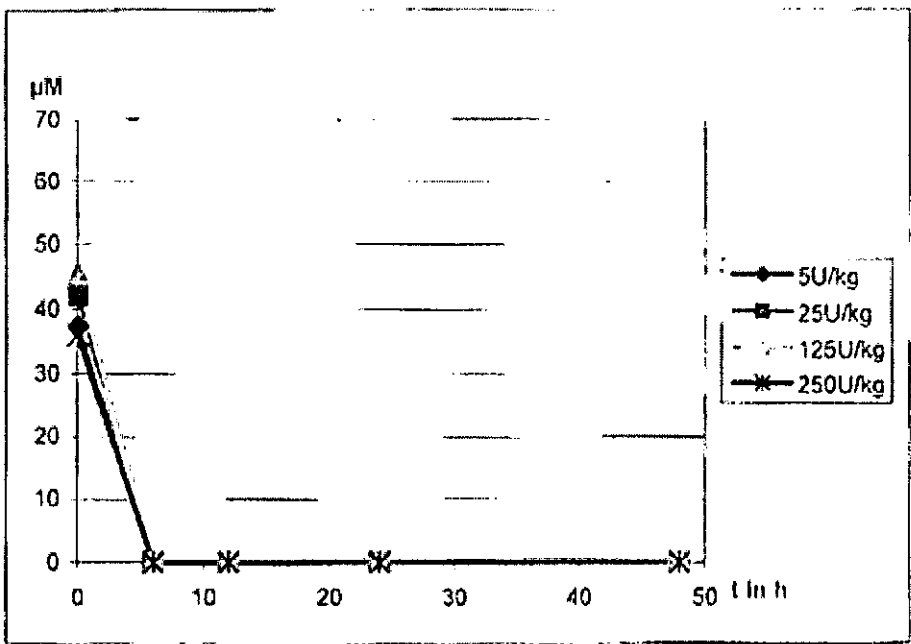
Como se muestra en las Figuras 4-6 en el presente documento adjunto, la pegilación de L-asparaginasa de *Erwinia chrysanthemi* resultó en una mejora similar en la actividad *in vivo* por la reducción de los niveles de glutamina. Mientras que Erwinase® falló esencialmente en afectar los niveles de L-glutamina en plasma, todas las L-asparaginasa pegiladas de *Erwinia chrysanthemi* redujeron significativamente los niveles en plasma de L-glutamina con todas las concentraciones ensayadas.

En particular, el tratamiento con 125 U/kg o 250 U/kg de L-asparaginasa de *Erwinia chrysanthemi* conjugada con 2.000 Da o PEG 5000 Da disminuyó a cero los niveles en plasma de L-glutamato después de 6 horas y los mantuvo cerca de cero durante los períodos de prueba.

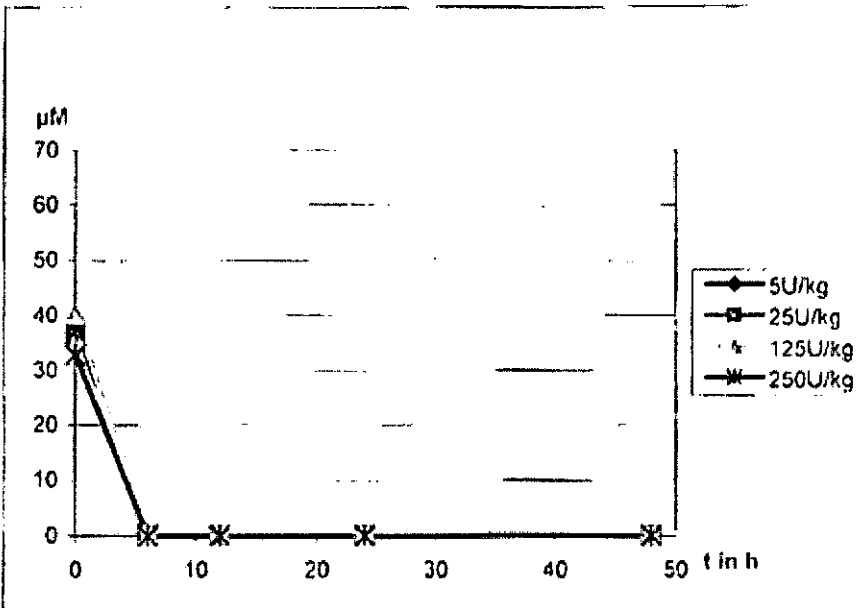
Estos resultados para la reducción de L-asparagina y L-glutamina son sorprendentes debido a la dramática mejora de la actividad enzimática *in vivo* después de la pegilación.



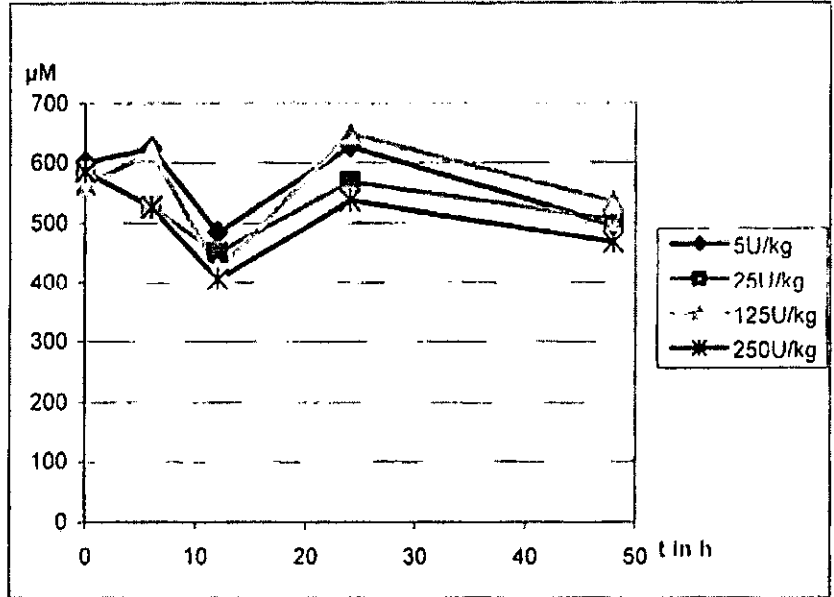
La figura 1 muestra los niveles en plasma de L-asparagina después de una dosis intravenosa única de asparaginasa de *Erwinia chrysanthemi* no pegilada (Erwinase®) sobre los niveles plasmáticos de L-asparagina en ratones. Los datos se expresan como la media (N = 2).



La Figura 2 muestra los niveles en plasma de L-asparagina después de una dosis intravenosa única del conjugado de L-asparaginasa de *Erwinia chrysanthemi* (r-crisantaspase) 2 kDa / 40% sobre los niveles plasmáticos de L-asparagina en ratones. Los datos se expresan como la media (N = 2).



La Figura 3 muestra los niveles en plasma de L-asparagina después de una dosis intravenosa única de un conjugado r-crisantaspase 5 kDa / 100% sobre los niveles plasmáticos de L-asparagina en ratones. Los datos se expresan como la media (N = 2).



La Figura 4 muestra los niveles de L-glutamina en plasma después de una dosis intravenosa única de Erwinase® sobre los niveles en plasma de L-glutamina en ratones. Los niveles plasmáticos de L-glutamina se mantuvieron esencialmente no afectado después del tratamiento con cualquiera de las dosis de Erwinase®. Los datos se expresan como la media (N = 2).

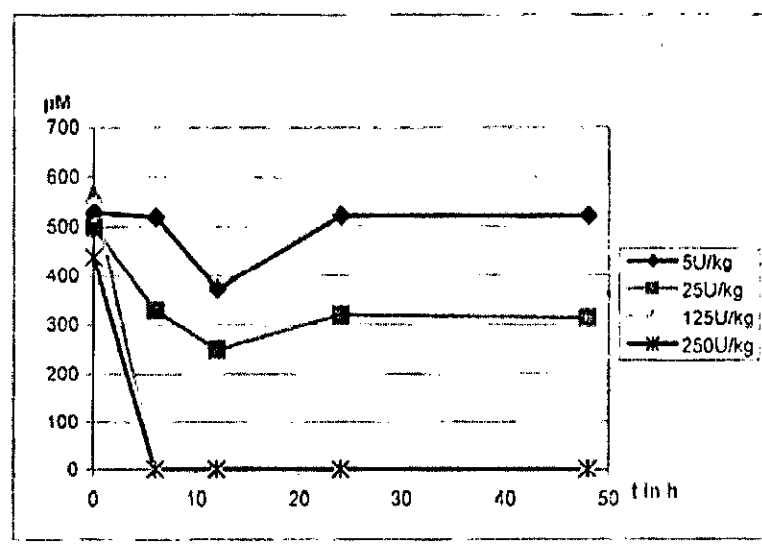


Figura 5 muestra los niveles en plasma de L-glutamina después de una dosis intravenosa única de un conjugado de r-crisantaspase 2 kDa / 40% sobre los niveles plasmáticos de L-glutamina en ratones. Después del tratamiento con 125 U/ kg y 250 kg U/Kg de peso corporal los niveles plasmáticos de glutamina se redujeron hasta el nivel cero dentro de las primeras 6 horas y se mantuvieron bajos durante el período de prueba. Los datos se expresan como la media (N = 2).

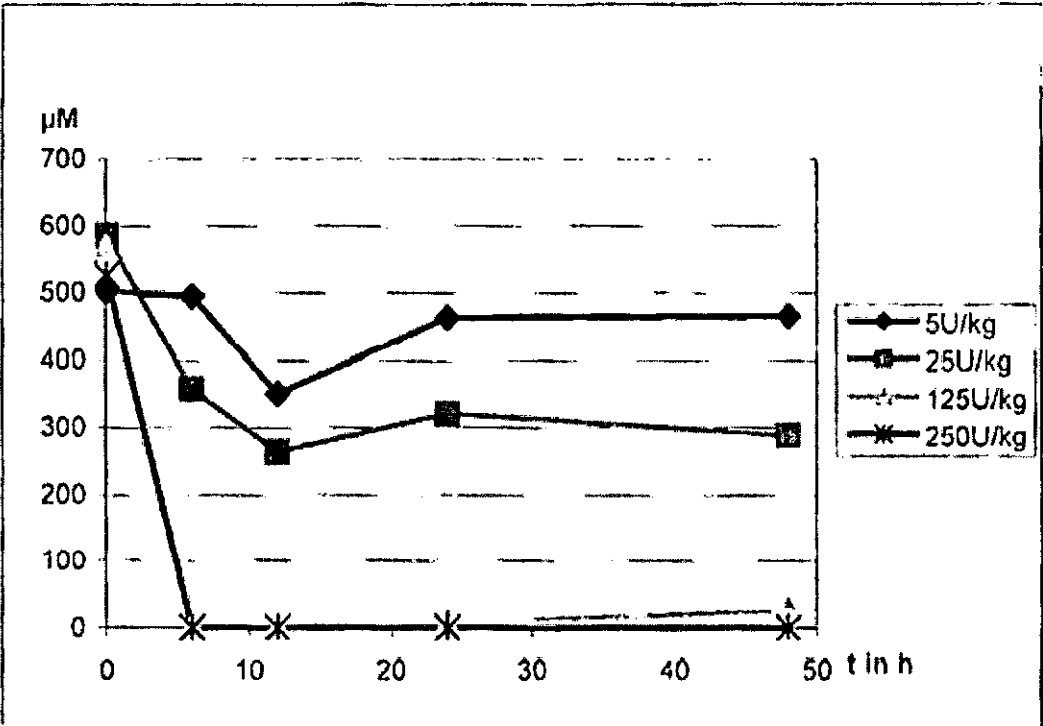


Figura 6 muestra los niveles en plasma de L-glutamina después de una dosis intravenosa única de un conjugado de r-crisantaspasa 5 kDa /100% sobre los niveles plasmáticos de L-glutamina en ratones. Los niveles de L-glutamina disminuyeron a cero en seis horas y permanecieron bajos después del tratamiento con 125 U/kg y 250 U/kg de peso corporal. Los datos son expresados como la media (N = 2)."

iv) En conclusión, de acuerdo con el análisis realizado en favor del nivel inventivo de la presente solicitud, éste se reconoce claramente, ya que cualquier persona medianamente versada en la materia reconocería que el conjugado aquí reclamado resulta ser no predecible del arte previo citado, lo cual, sumado al hecho que el arte previo en efecto no es suficiente para brindar indicaciones acerca de la posibilidad de desarrollar dicho conjugado de manera específica, sustenta la idea que la materia reclamada en la presente solicitud reviste nivel inventivo.

4. Para terminar, de conformidad con lo establecido en el artículo 80 del Código de Procedimiento Administrativo y de lo Contencioso Administrativo, solicito respetuosamente que todos y cada uno de los puntos planteados en el presente recurso sean resueltos de manera individual, completa y motivada, respecto de las razones de hecho, derecho y/o conveniencia que lleven a esta Superintendencia a adoptar la decisión que estime frente a la solicitud aquí formulada.

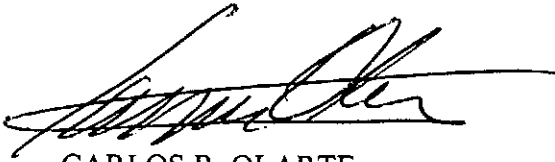
5. NOTIFICACIÓN

OLARTEMOURE

OLARTE MOURE & ASOCIADOS LTDA.

Recibiré notificaciones en mis oficinas ubicadas en la Carrera 5 No. 34-03, de Bogotá.

Atentamente,



CARLOS R. OLARTE

C.C. No. 79.782.747 de Bogotá

T.P. 74.295

Anexos:

- Capítulo Reivindicatorio Modificado
- Recibo de pago de tasa por modificaciones al Capítulo Reivindicatorio
- ANEXO 1: Declaración del Doctor Thierry Abribat.
- ANEXO 2: Modification of E. coli L-asparaginase with polyethylene glycol: Disappearance of binfing ability to anti-asparaginase serum.
- ANEXO 3: The pharmacology of PEGylation: Balancing PD with PK to generate novel therapeutics.

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CAPÍTULO REIVINDICATORIO MODIFICADO

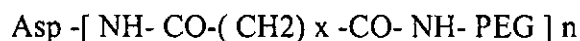
(Recurso de Reposición)

1. Un conjugado, CARACTERIZADO porque comprende una L - asparaginasa de *Erwinia chrysanthemi* que comprende la secuencia de aminoácidos SEQ ID NO: 1 y polietileno glicol (PEG), en donde el PEG tiene un peso molecular de 5.000 Da o de 2000 Da y en donde dicho PEG está unido a los grupos amino accesibles en los residuos de aminoácidos de lisina y/o en los aminoácidos N-terminales en la L-asparaginasa y donde dicho conjugado tiene una actividad L-asparaginasa *in vitro* de al menos 70% de la actividad de la L-asparaginasa de *Erwinia chrysanthemi* cuando no está conjugada con PEG y una actividad de agotamiento de L-asparagina *in vivo* que es al menos 50 veces más potente que la L-asparaginasa de *Erwinia chrysanthemi* cuando no está conjugada con PEG.

2. El conjugado de la Reivindicación 1, CARACTERIZADO porque dicha L-asparaginasa es idéntica a la SEC ID NO: 1 y dicho PEG tiene un peso molecular de 5000 Da.

3. El conjugado de la Reivindicación 1, CARACTERIZADO porque el PEG está unida a dichos grupos amino mediante un enlace amida.

4. El conjugado de una de las Reivindicaciones 1 a 3, CARACTERIZADO porque tiene la fórmula:



en la que Asp es L-asparaginasa, NH es uno o más de los grupos NH de los residuos de lisina y/o aminoácidos N-terminal en la Asp, PEG es un resto de polietilenglicol, n es un número que representa desde el 40 % al 100% de los grupos amino accesibles de la Asp, y x es un número entero que varía de 2 a 5.

5. El conjugado de cualquiera de las Reivindicaciones 1 a 4, CARACTERIZADO porque dicho PEG es monometoxi - polietileno.

6. El conjugado de cualquiera de las Reivindicaciones 1 a 5, CARACTERIZADO porque el PEG está vinculado al 40 % a 90 % de grupos amino accesibles.

7. El conjugado de cualquiera de las Reivindicaciones 1 a 6, CARACTERIZADO porque el PEG está unido a los grupos amino por enlaces amida.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800.176.089-2

-/-



RECIBO DE CAJA

No. 15 - 0035273

Bogotá D.C., Marzo 27 de 2015 - 15:20:29

RECIBIDO DE : OLARTE MOURE & ASOCIADOS

NI 830.122.870

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	601309004	27/03/2015	6.700.000.00

*** Conceptos Pagados ***

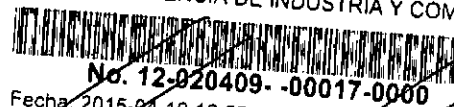
CANT.	RENTISTICO	CONCEPTO	Vr. UNDITARIO	Vr. CONCEPTO
1	50005-01-03 CAMBIO DE MODALIDAD Y MODIFICACIONES	EN N. CREACION CORRECCION A SOLICITUD EN TRAMITE / MODIFICA	132.000.00	132.000.00
				\$132.000.00

SON: **CIENTO TRENTA Y DOS MIL PESOS MONEDA CORRIENTE***

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

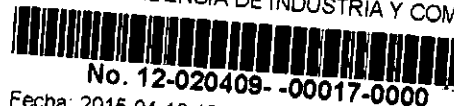
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No. 12-020409-00017-0000

Fecha: 2015-04-10 16:55:00 Dep. 2003 G. TRAB. VIA. GUI
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Act. 412 REPOSPRESRECU Folios: 50

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Act. 412 REPOSPRESRECU Folios: 50

-51

PATENT
Attorney Docket 061834-5062

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: **Thierry Abribat**

Application No. **13/382,276**

Examiner: **Hope A. Robinson**

Confirmation No. **1719**

Group Art Unit: **1652**

For: **Pegylated L-Asparaginase**

DECLARATION UNDER 37 C.F.R. 1.132

I, Thierry Abribat, declare that:

1. I obtained a Doctorate in Veterinary Medicine from the National Veterinary School of Toulouse and a PhD in Biological Sciences from the National Polytechnic Institute of Toulouse. From 2008 until the present, I have held the position of President of Alizé Pharma II SAS, assignee of the present application.
2. I have been involved with the discovery and development of PEGylated recombinant *Erwinia chrysanthemi*-derived L-asparaginase conjugate products and am the inventor on the above application.
3. I have reviewed the Office Action dated July 23, 2014 that issued in the above application. In particular, I have reviewed the Examiner's rejection of claims 1-2, 4, 31-33, 35, 39 and 95-135 under 35 U.S.C. § 103 as obvious in view of publications Keating, Minton and Miller as detailed on pages 8-10 of the Office Action. The following comments are being submitted as evidence supporting the unexpected and superior results for the pharmacokinetic and immunological properties of the pegylated L-asparaginase from *Erwinia chrysanthemi* as set forth in the claims of the present application.
4. I have studied the pharmacokinetic profiles of pegylated L-asparaginase from *E. coli* and *Erwinia*

chrysanthemi. Example 9 of the present application demonstrates the surprisingly superior half life of the pegylated L-asparaginase from *Erwinia chrysanthemi*. The enzymatic activities of *E. coli* and *Erwinia chrysanthemi* L-asparaginase pegylated with 5,000 Da PEG were determined with a chromogenic assay and the half-lives of these conjugates were calculated as set forth in the Specification of the present application on pages 31-36.

5. It is well known that unconjugated *E. coli* L-asparaginase has a longer half life than the unconjugated *Erwinia chrysanthemi* L-asparaginase. Therefore, the original expectation was that pegylated *E. coli* L-asparaginase would have a longer half life compared with *Erwinia chrysanthemi* L-asparaginase that is similarly pegylated. The experimental results, however, were distinct from the original expectation as *Erwinia chrysanthemi* L-asparaginase conjugated with 5,000 Da PEG has a superior half-life than the *E. coli* L-asparaginase pegylated with 5,000 Da PEG. See Table 5 of Specification.

6. The retention of *in vitro* L-asparaginase activity of L-asparaginase from *Erwinia chrysanthemi* after pegylation was studied. In essence, the *in vitro* L-asparaginase activity of *Erwinia chrysanthemi* L-asparaginase conjugated with PEG was measured with the Nessler assay and compared with the unconjugated *Erwinia chrysanthemi* L-asparaginase as set forth in Example 5 on page 30 of the Specification. We found that the conjugated L-asparaginase retained at least 78% of the *in vitro* aminohydrolase activity of the unconjugated L-asparaginase from *Erwinia chrysanthemi*. See Table 2 of the Specification. This finding is unexpected as pegylation of L-asparaginase from other species resulted in much more significant loss of enzymatic activity.

7. Experiments to examine the effects of PEG size on L-asparaginase activity were also conducted. The experiments showed that *Erwinia chrysanthemi* L-asparaginase conjugated with 5000 Da PEG has a longer residual enzymatic activity *in vivo* compared to 2000 Da and 10,000 Da PEG conjugates. See paragraph [0017] and Figure 5 of the Specification. It is surprising to find that the 10,000 Da PEG conjugate fails to increase the residual enzymatic activity of L-asparaginase given the increase in PEG size which would be expected by the skilled person to enhance half life by reducing degradation. This result is surprising because the increased size of the conjugate from 2000 Da to 5000 Da PEG results in enhanced activity but the 10,000 Da PEG does not produce a similar further increase.

8. Further experiments were conducted in mice to examine the *in vivo* enzymatic activity of

pegylated and unpegylated L-asparaginase. The baseline activity of all test items, including pegylated and unpegylated L-asparaginase from *Erwinia chrysanthemi*, was determined by Nessler assay. One U (unit) of test item oxidizes one micromole of L-asparagine per minute at 37°C and a pH of 8.6. The test items were administered to mice with a single intravenous injection. Since L-asparaginase catalyzes the deamidation of L-asparagine to L-aspartic acid and L-glutamine to L-glutamic acid, the L-asparagine and L-glutamine levels in the plasma of the mice were measured with PICO-TAG Amino Acid Analysis Kits as indicators of L-asparaginase *in vivo* activity.

9. As shown in Figure 1 herein attached, doses lower than 125 U/kg body weight of unpegylated *Erwinia chrysanthemi* asparaginase (Erwinase®) were not capable of reducing plasma L-asparagine to zero. In addition, even at higher doses such as 125 U/kg and 250 U/kg, Erwinase® could not keep plasma L-asparagine level to remain at zero for the full test period of 48 hours. On the other hand, as shown in Figures 2 and 3, all pegylated L-asparaginase (mPEG-r-crisantaspase conjugates) induced a decrease of plasma L-asparagine level to zero within 6 hours after treatment at all doses tested. In addition, zero level was maintained over the full test period. Thus, *Erwinia chrysanthemi* L-asparaginase conjugated with 2,000 Da or 5,000 Da PEG tested at 5 U/kg were at least as potent as Erwinase® tested at 250 U/kg, suggesting that Pegylation improved potency at least by 50 times.

10. As shown in Figures 4-6 herein attached, the pegylation of *Erwinia chrysanthemi* L-asparaginase resulted in similar *in vivo* activity improvement for the reduction of glutamine levels. While Erwinase® essentially failed to affect plasma L-glutamine levels, all pegylated *Erwinia chrysanthemi* L-asparaginase significantly reduced plasma L-glutamine with all concentrations tested. In particular, treatment with 125 U/kg or 250 U/kg *Erwinia chrysanthemi* L-asparaginase conjugated with 2,000 Da or 5,000 Da PEG decreased plasma L-glutamate levels to zero after 6 hours and maintained it at close to zero throughout the test periods.

11. These results for L-asparagine and L-glutamine reductions are surprising due to the dramatic improvement of *in vivo* enzymatic activities after pegylation.

12. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced

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application or any patent issuing thereon.

Signature:

Date:

Figure 1.

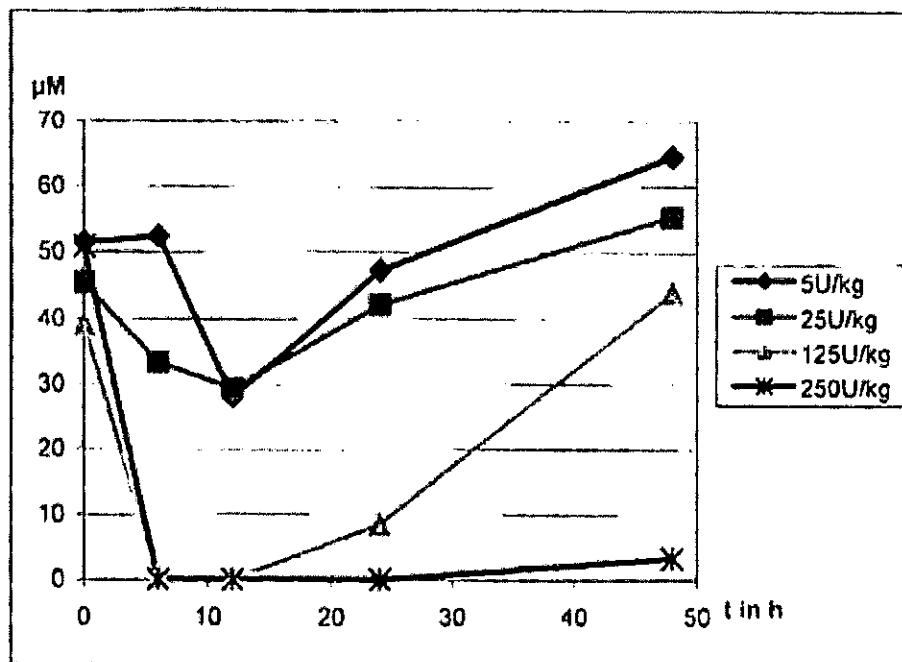


Figure 1 shows plasma L-asparagine levels following a single intravenous dose of unpegylated *Erwinia chrysanthemi* asparaginase (Erwinase®) on plasma L-asparagine levels in mice. Data are expressed as means (N=2).

Figure 2.

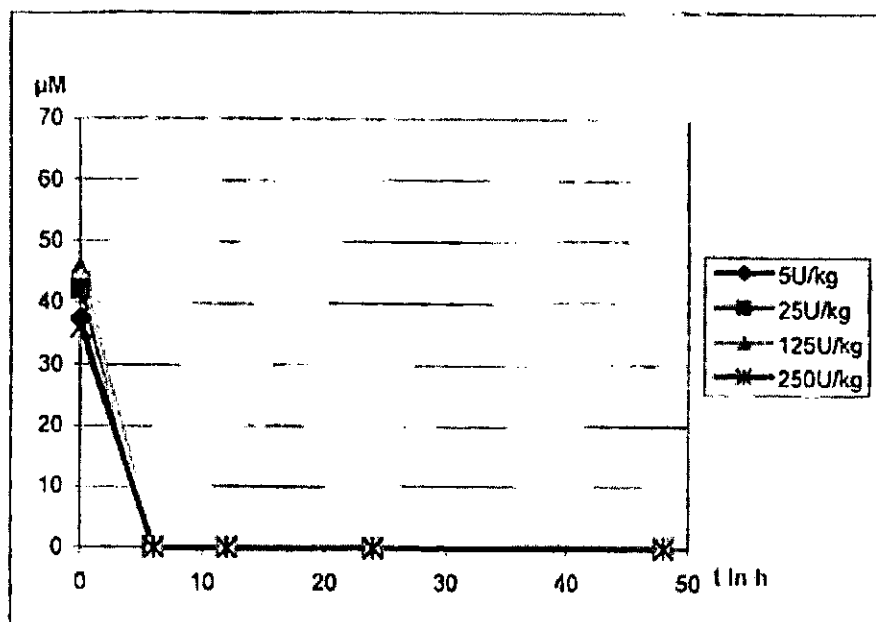


Figure 2 shows plasma L-asparagine levels following a single intravenous dose of the 2 kDa / 40% conjugate of *Erwinia chrysanthemi* L-asparaginase (r-crisantaspase) on plasma L-asparagine levels in mice. Data are expressed as means (N=2).

Figure 3

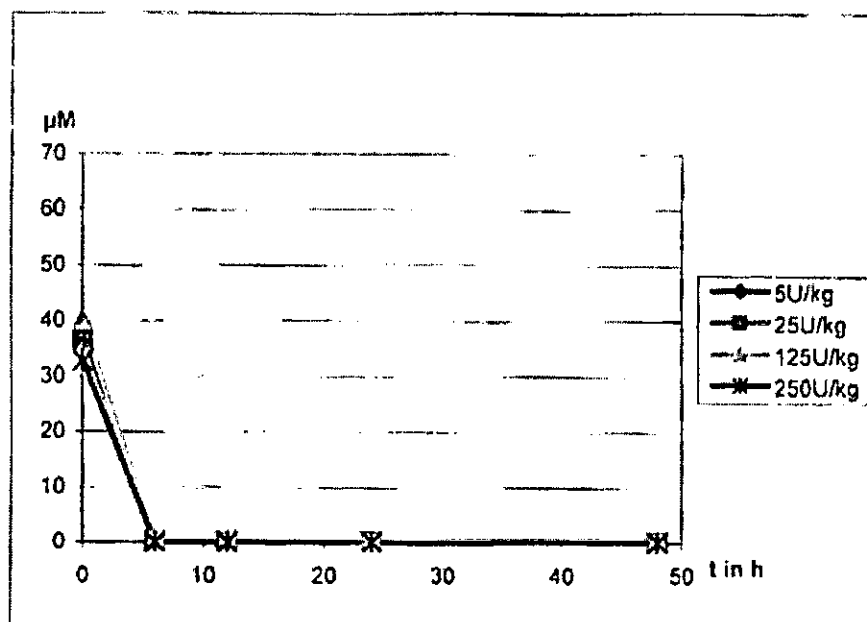


Figure 3 shows plasma L-asparagine levels following a single intravenous dose of 5 kDa / 100% conjugate of r-crisantaspase on plasma L-asparagine levels in mice. Data are expressed as means (N=2).

Figure 4

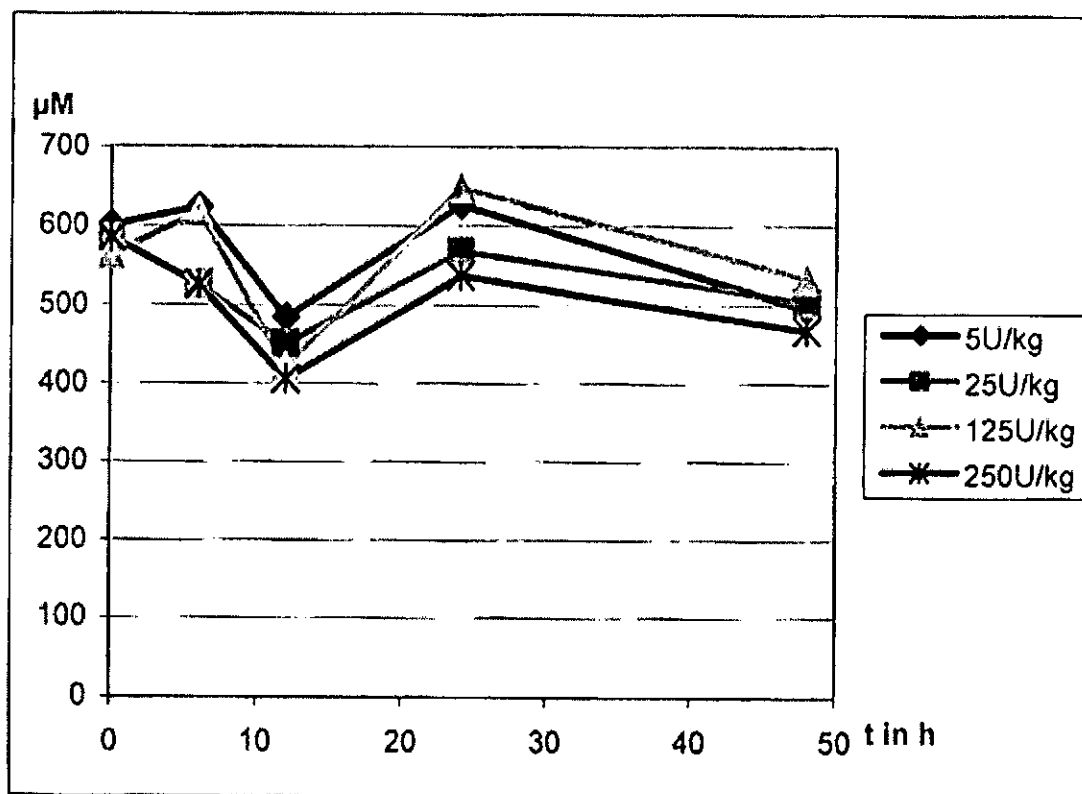


Figure 4 shows plasma L-glutamine levels a single intravenous dose of Erwinase® on plasma L-glutamine levels in mice. Plasma levels of L-glutamine remained essentially unaffected after treatment with any dose of Erwinase®. Data are expressed as means (N=2).

Figure 5

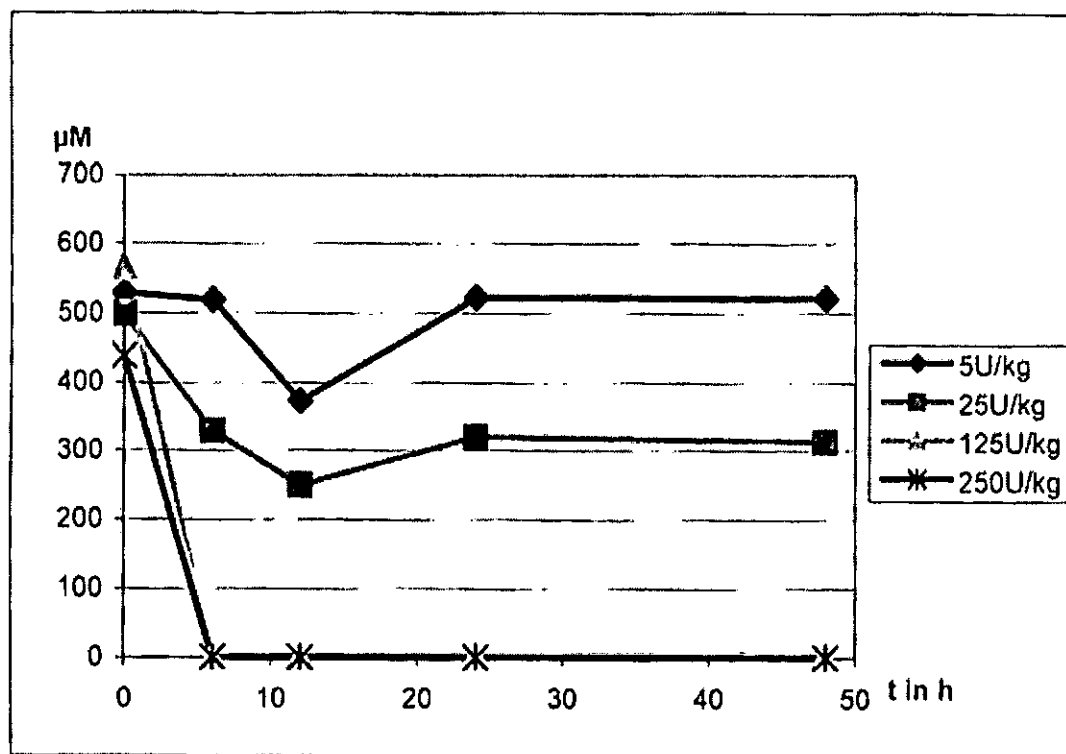


Figure 5 shows plasma L-glutamine levels following a single intravenous dose of the 2 kDa / 40% conjugate of r-crisantaspase on plasma L-glutamine levels in mice. After treatment with 125 U/kg and 250 U/kg bw plasma L-glutamine level decreased to zero level within the first 6 hours and remained low over the test period. Data are expressed as means (N=2).

Figure 6

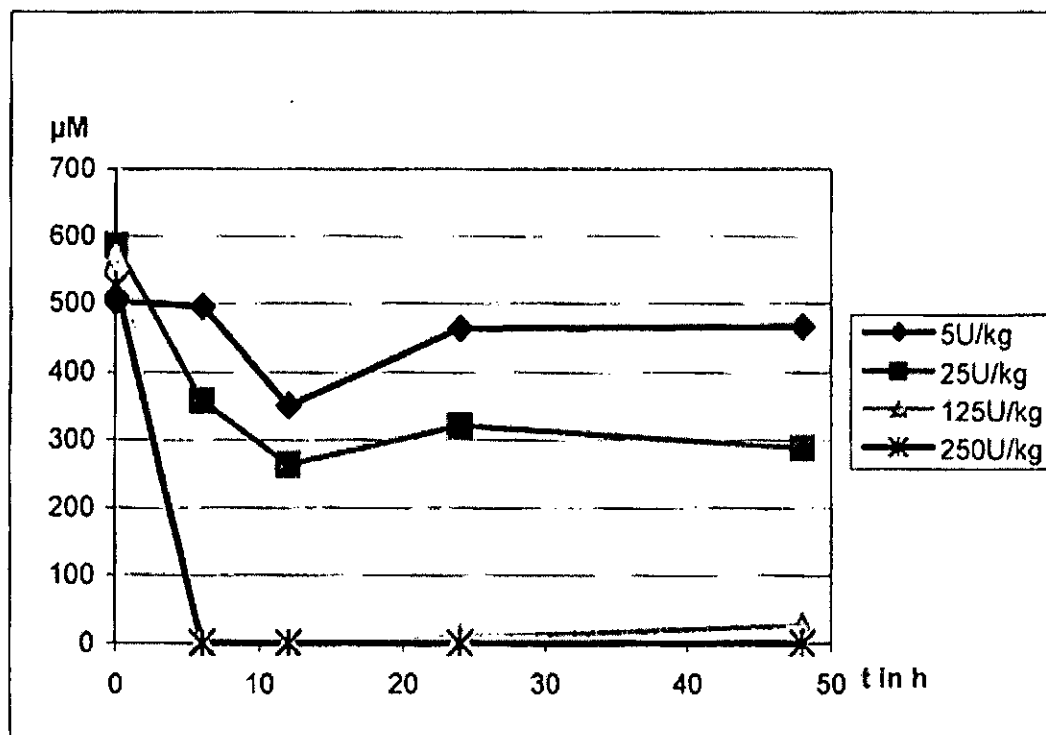


Figure 6 shows plasma L-glutamine levels following a single intravenous dose of the 5 kDa / 100% conjugate of r-crisantaspase on plasma L-glutamine levels in mice. The levels of L-glutamine decreased to zero within six hours and remained low after treatment with 125 U/kg and 250 U/kg bw. Data are expressed as means (N=2).

MODIFICATION OF E. COLI L-ASPARAGINASE WITH POLYETHYLENE GLYCOL:
DISAPPEARANCE OF BINDING ABILITY TO ANTI-ASPARAGINASE SERUM

Yoshihiro ASHIHARA, Takashi KONO, Shojiro YAMAZAKI and Yuji INADA

Laboratory of Biological Chemistry, Tokyo Institute of Technology,

Ookayama, Meguroku, Tokyo 152, Japan

Received May 16, 1978

SUMMARY

L-Asparaginase from *Escherichia coli* A-1-3 was modified with activated polyethylene glycols (2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine) with molecular weights of 750, 1900 and 5000. The modification of asparaginase to 73 amino groups out of the total 92 amino groups in the molecule with polyethylene glycol of 5000 daltons gave rise to a complete loss of the binding ability towards anti-asparaginase serum from rabbit. This modified asparaginase retained the enzymic activity (7%) and had a resistivity against trypsin. Asparaginases modified with polyethylene glycols of 750 and 1900 daltons did not show a substantial change of the immunogenic properties.

L-Asparaginase is effective for remission in patients with lymphoblastic leukemia, and its physicochemical properties and the clinical effects have been extensively studied by many investigators (1-3). The chemical modification of L-asparaginase has been studied in order to clarify the active site of the enzymic activity (4-7) and also to reduce the antigenic reactivity towards anti-asparaginase antibodies (8-11). Tamaura *et al.* (9) demonstrated that the photooxidation of asparaginase in the presence of methylene blue causes the reduction of the antigenicity (85%) and a complete loss of the enzymic activity, accompanied with photooxidation of histidine, methionine and tyrosine residues and with the reduction of α -helix content of asparaginase. Makino *et al.* (8) reported that acetylation of amino groups in asparaginase gives rise to the reduction of antigenicity (20%) without conformational change.

Recently, Abuchowski *et al.* (12,13) reported that covalent attachment of serum albumin or catalase to polyethylene glycol, nonimmunogenic polymer,

leads to a complete loss of its antigenicity with retaining its enzymic activity. Their reports led to the present study on the reduction of the antigenic reactivity of L-asparaginase towards anti-asparaginase serum, with a hope of a possible clinical use of the modified asparaginase.

MATERIALS AND METHODS

A crystalline L-asparaginase (EC 3.5.1.1) obtained from *E. coli* A-1-3 was kindly gifted from Kyowa Hakko Kogyo Co. and its specific activity was 200 IU/mg of protein. Monomethoxypolyethylene glycols of 750, 1900 and 5000 daltons were purchased from Polyscience Inc. and Union Carbide Co.

The modification of asparaginase was carried out by the modified method of Abuchowski *et al.* (12); monomethoxypolyethylene glycol (1.9 mmoles) was coupled with cyanuric chloride (5.7 mmoles) to form 2-O-methoxypolyethylene glycol-4,6-dichloro-s-triazine, activated polyethylene glycol. To an asparaginase solution (10 mg) in 0.1 M borate buffer (pH 9.2) was added activated polyethylene glycols with molecular weights of 750, 1900 and 5000. The mixture was incubated for 1 hr at 4°C and then filtrated with an ultrafiltration apparatus and an XM-50 membrane to remove free activated polyethylene glycol. The modified asparaginases were synthesized by changing the molar ratio of activated polyethylene glycol (PEG) to amino groups in the asparaginase molecule (PEG/-NH₂; 0.5, 1.0, 3.0, 5.0 and 10.0). The total amino groups in the asparaginase molecule is 92, including 88 ε-amino groups in lysine residues and 4 terminal amino groups (14). The degree of modification of amino groups in the molecule was determined by measuring the amount of free amino groups with trinitrobenzene sulfonate (15). Protein concentrations were determined by the biuret method (16).

The enzymic activity of asparaginase was determined spectrophotometrically by using a synthetic substrate, aspartic acid-β-p-nitroanilide, ANA (17), or by measuring the amount of aspartyl-β-hydroxamate formed from L-asparagine and hydroxylamine by the action of the enzyme (18). The quantitative precipitin reaction curve was obtained by the method of Kabat and Mayer (19). Anti-asparaginase serum was obtained from rabbit immunized three times by subcutaneous injection of asparaginase (3.3 mg x 3) and stored at -20°C (8). Modified and non-modified asparaginases were digested with trypsin (crystallized, Sigma) (weight ratio, 1:300) for a given time at pH 7.3 and 37°C. The digestion was stopped by the addition of trypsin inhibitor (Miles Lab.) and the enzymic activity of asparaginases was determined. Chromatography of modified asparaginase was carried out using Sephadex G-200 equilibrated with 15 mM phosphate buffer (pH 7.0) containing 150 mM sodium chloride.

RESULTS AND DISCUSSION

Native asparaginase was modified with polyethylene glycol of 5000 daltons in molar amounts equal to 0.5, 1.0, 3.0, 5.0 and 10.0 equivalents of the available amino groups in the asparaginase molecule. These modifications resulted in products with 18, 23, 38, 48 and 73 amino groups substituted by polyethylene glycol.

Native asparaginase and modified asparaginases prepared as above were

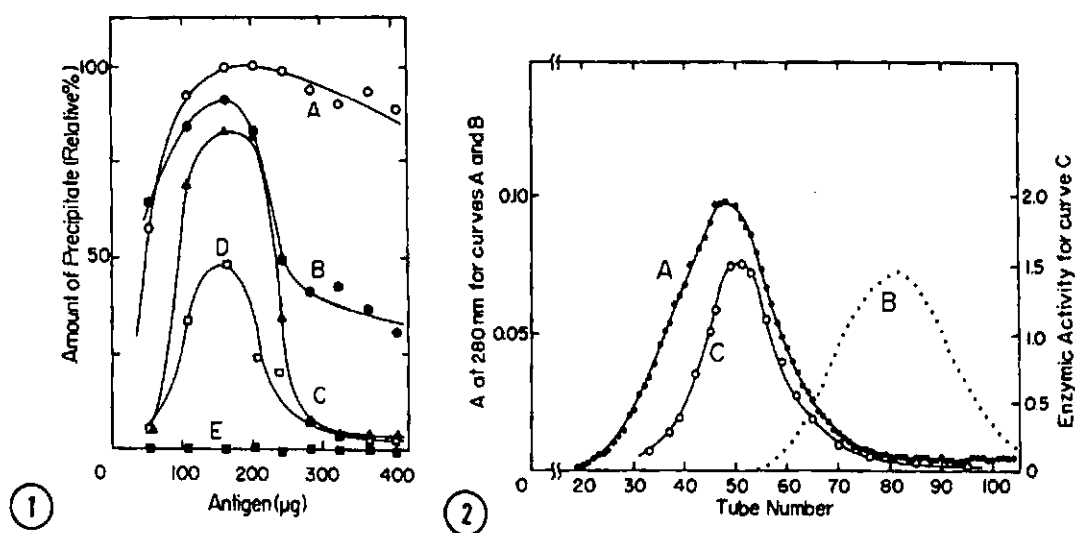


Fig. 1. Precipitin reaction of native asparaginase and the asparaginases modified with polyethylene glycol (mw; 5000) towards anti-asparaginase serum. Curve A; precipitin reaction curve of native asparaginase. Curves B, C, D and E; precipitin reaction curves of the modified asparaginases in which 18, 38, 48 and 73 amino groups out of the total 92 amino groups in the asparaginase molecule have been substituted by polyethylene glycol, respectively.

Fig. 2. Chromatogram of the modified asparaginase, in which 73 amino groups have been substituted by polyethylene glycol, using Sephadex G-200 column (2 x 40 cm). Curve A; elution pattern of the modified asparaginase (1.6 mg). Curve B; elution pattern of native asparaginase (1.5 mg). Curve C; enzymic activity measured for each protein fraction of the modified asparaginase eluted from Sephadex G-200 column. Elution was carried out with 15 mM phosphate buffer (pH 7.0) containing 150 mM NaCl.

subjected to studies on the binding ability of them with anti-asparaginase serum. The result is shown in Fig. 1. Curve A shows the precipitin reaction curve obtained for native asparaginase towards anti-asparaginase serum. The amount of precipitate, antigen-antibody complex, was increased markedly with raising asparaginase concentration and then gradually decreased. The maximum amount of precipitate was taken as 100%. The precipitin reaction curves obtained for modified asparaginases, in which 18, 38, 48 and 73 amino groups in the molecule have been substituted by polyethylene glycol of 5000 daltons, are shown by curves B, C, D and E, respectively. Increasing the degree of substitution of amino groups by polyethylene glycol resulted in the reduction of the binding ability of asparaginase against its antibodies.

Table 1. Immunogenic properties(binding ability towards anti-asparaginase serum), enzymic activities and degrees of substitution of amino groups by polyethylene glycol(PEG)(mw; 5000) of modified asparaginases

Molar Ratio PEG/-NH ₂	n ^{a]}	Enzymic Activity(%)		Binding Ability ^{d]} (%)
		Asn ^{b]}	ANAC ^{c]}	
0	0	100	100	100
0.5	18	40	50	90
1.0	23	25	37	94
3.0	38	6	22	83
5.0	48	7	17	39
10.0	73	7	15	0

a] Number of amino groups substituted by polyethylene glycol in the asparaginase molecule. The total number of amino groups in the molecule is 92.

b] Determined by measuring the formation of asparatyl hydroxamate.

c] Determined by using aspartic acid β -p-nitroanilide as a synthetic substrate.

d] Relative value of the amount of the maximum precipitate on each precipitin reaction curve in Fig. 1.

No binding ability was observed for one of the modified asparaginases, in which 73 amino groups had been substituted(curve E).

The enzymic activity of the modified asparaginase was measured by two separate methods. Although the enzymic activity was decreased by increasing the degree of modification, the modified asparaginase in which 73 amino groups had been substituted retained the enzymic activity above 7% of non-modified asparaginase. These results are summarized in Table 1.

The effects of the molecular weight of polyethylene glycol on the binding ability of modified asparaginase towards anti-asparaginase serum was tested. The result is shown in Table 2. Polyethylene glycol with the molecular weight of 750 or 1900 was not effective for preparing the modified asparaginase with a complete loss of the binding ability, although the degree of modification of amino groups was almost the same as that obtained for polyethylene glycol of 5000 daltons.

In order to check a homogeneity of the asparaginase modified with poly-

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7/

Table 2. Effect of molecular weight of polyethylene glycol(PEG) on the binding ability and the enzymic activity of the modified asparaginases.

PEG(mw)	Molar Ratio PEG/-NH ₂	n ^{a]}	Enzymic Activity(%)		Binding d] Ability(%)
			Asn ^{b]}	ANA ^{c]}	
750	10	77	12	20	83
1900	10	70	14	25	40
5000	10	73	7	15	0

a], b], c] and d]; See Table 1.

ethylene glycol of 5000 daltons, in which 73 amino groups had been substituted, the modified asparaginase was subjected to chromatography with Sephadex G-200 column. Its elution pattern is shown by curve A in Fig. 2, in which a single sharp band appeared with a peak at the tube number of 50. Curve B shows the elution pattern of native asparaginase obtained under the same experimental condition as above and its peak position was quite different from that obtained for the modified asparaginase. From the position of the elution pattern, the molecular weight of the modified asparaginase was assumed to be approximately 500,000, which is far greater than that of native asparaginase, 136,000. The enzymic activities measured for each protein fraction of the modified asparaginase eluted from Sephadex G-200 column are shown by curve C. Its profile was in good agreement with the elution pattern obtained for the modified asparaginase(curve A). The above result indicates that the modified asparaginase, in which 73 amino groups have been substituted, is a homogeneous preparation with the enzymic activity and does not contain native asparaginase as a contaminant.

Modification of amino groups in the asparaginase molecule with polyethylene glycol give rise to the high resistivity against trypsin. The result is demonstrated in Fig. 3. Native asparaginase and modified asparaginase in which 73 amino groups were substituted by polyethylene glycol of 5000 daltons were digested with trypsin. The enzymic activity of native asparaginase

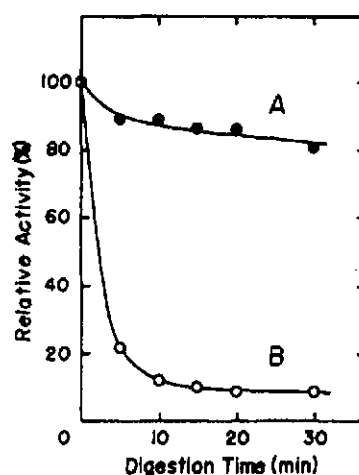


Fig. 3. Resistivity of the modified asparaginase in which 73 amino groups have been substituted by polyethylene glycol(mw; 5000) against trypsin. Curve A; the modified asparaginase. Curve B; non-modified asparaginase. Digestion with trypsin was carried out at pH 7.3 and 37°C.

was rapidly reduced with time (curve B), while that of the modified asparaginase was retained more than 80% of the original activity (curve A).

With the purpose of clinical use of the modified asparaginase without antigenicity and with enzymic activity, various modified asparaginases have been prepared in our laboratory for past five years, applying chemical modification reagents such as acetylimidazole, acetic anhydride, glyoxal, hydrogen peroxide, methyl-p-nitrobenzene sulfonate and 2-hydroxy-5-nitrobenzylbromide(8-11).

A partial reduction of the antigenicity was observed for each modified asparaginase, accompanied with a complete loss of the enzymic activity. The preparation of a modified asparaginase, which has no antigenicity, retains the enzymic activity and has resistivity against trypsin, may be useful in patients with lymphoblastic leukemia and the modified asparaginase obtained in the present study may be currently available in a medical field.

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The Pharmacology of PEGylation: Balancing PD with PK to Generate Novel Therapeutics

Anexo 3 35
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ABSTRACT: Conjugation of macromolecules to polyethylene glycol (PEG) has emerged recently as an effective strategy to alter the pharmacokinetic (PK) profiles of a variety of drugs, and thereby to improve their therapeutic potential. PEG conjugation increases retention of drugs in the circulation by protecting against enzymatic digestion, slowing filtration by the kidneys and reducing the generation of neutralizing antibodies. Often, PEGylation leads to a loss in binding affinity due to steric interference with the drug–target binding interaction. This loss in potency is offset by the longer circulating half-life of the drugs, and the resulting change in PK–PD profile has led in some cases to enabling of drugs that otherwise could not be developed, and in others to improvements in existing drugs. Thus, whereas most approaches to drug development seek to increase the activity of drugs directly, the creation of PEGylated drugs seeks to balance the pharmacodynamic (PD) and pharmacokinetic properties to produce novel therapies that will meet with both increased efficacy and greater compliance in the clinical setting. This review examines some of the PEGylated drugs developed in recent years, and highlights some of the different strategies taken to employ PEG to maximize the overall PK–PD profiles of these compounds. © 2008 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 97:4167–4183, 2008

Keywords: biotechnology; conjugation; drug delivery; macromolecular drug delivery; PEGylation; pharmacokinetics/pharmacodynamics; renal clearance

INTRODUCTION

Polyethylene glycol (PEG)-conjugated drugs first appeared on the pharmaceutical scene in 1990 with the FDA approval of Adagen® (pegademase: PEGylated adenosine deaminase), as enzyme replacement therapy for patients with severe combined immunodeficiency disease (SCID), an inherited disorder in which deficiency of adenosine deaminase causes accumulation of metabolites and prevents lymphocyte maturation. The

approval of Adagen followed more than a decade of research, precipitated by the first description of protein PEGylation by Abuchowski et al.¹ which documented the ability of PEG to prolong the half-life and reduce the immunogenicity of a conjugated protein. When applied to adenosine deaminase to produce pegademase, the technology yielded a drug that enabled twice-weekly intramuscular injections to replace multiple blood transfusions, and, by avoiding the transfusion-associated risks of viral infection and iron overload, provided both a better pharmacological profile and a considerable improvement in convenience for patients.

The following years saw the approval of additional PEGylated therapeutics for a number of prevalent conditions including Hepatitis C,

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chemotherapy-associated neutropenia and leukemia (Tab. 1). Several more PEG-conjugated compounds are currently in clinical and pre-clinical development, reflecting the emergence of this platform as a dominant strategy for enabling or improving macromolecule drugs.

The currently approved PEGylated products are all macromolecules that, between them, cross a number of therapeutic classes, including oncology, metabolic diseases and infectious diseases. The first five approved products were proteins or peptides; the most recently approved, Macugen® (pegaptanib), is an RNA aptamer, while studies on PEG-conjugates of small molecules such as the $\alpha 4\beta 1$ integrin inhibitor demonstrate that the technology can extend beyond biologics and macromolecules.² Furthermore, clinical and pre-clinical studies have been reported which use PEG to make prodrugs for small molecules such as irinotecan,^{3,4} doxorubicin⁵⁻⁷ and camptothecin.^{8,9}

Pharmacodynamic (PD) properties of a drug can be measured at the molecular level by parameters such as receptor binding affinity or enzyme activity. While PEG characteristically prolongs the plasma circulating time of a drug, a seminal PK parameter, this often comes at the expense of reduced binding affinity for the target receptor or enzyme. Thus PEG operates to alter the balance between pharmacodynamic and pharmacokinetic properties, compensating for reduction in binding affinity by extension of systemic exposure (Fig. 1). The earlier PEGylated conjugates aimed simply to use PEG to increase systemic exposure of the drug or reduce adverse reactions, without optimizing the effect on potency. More recent approaches,

however, integrate the pharmacological properties of the drug and of PEG to minimize the loss of potency while maximizing exposure.

This review will: (i) describe the pharmacological properties of PEGylated drugs, (ii) highlight two case-studies, Somavert® (pegvisomant) and PEGASYS® (peginterferon- $\alpha 2a$), and (iii) analyze some of the different classes of drug that can benefit from PEGylation, to demonstrate how incorporating PEG conjugation into the design of a drug is emerging as an effective strategy for improving or enabling novel therapeutics.

PHARMACOLOGICAL PROPERTIES OF PEGYLATED DRUGS

PEG polymers are composed of repeating units of ethylene glycol, which can be produced as linear or branched chains, with functional groups at one or more termini to enable a variety of conjugation possibilities (Fig. 2). Chemical strategies for conjugating PEG to macromolecules are beyond the scope of this review and have been described in detail recently.^{8,10} Further diversity for PEG conjugation arises from the use of either stable or hydrolyzable linkages, the latter resulting in the generation of pro-drugs. In both cases, the conjugated molecule benefits from the pharmaceutical properties of PEG, which include increased solubility, stability over a wide range of temperature and pH, and high mobility in solution.¹¹ However, whereas for prodrugs the activity lies in the released parent molecule, stable conjugates constitute a new active species. This new molecule possesses different pharma-

Table 1. FDA Approved PEGylated Drugs

Commercial Name	Drug Name	Parent Drug	PEG Size (Da)	Indication	Year of Approval
Adagen®	Pegadamasase	Adenosine deaminase	5000	SCID ^d	1990
Oncaspar®	Pegaspargase	Asparaginase	5000	Leukaemia (ALL ^e , CML ^f)	1994
PEG-INTRON®	Peginterferon- $\alpha 2b$	IFN- $\alpha 2B$	12000	Hepatitis C	2000
PEGASYS®	Peginterferon- $\alpha 2a$	IFN- $\alpha 2A$	40000	Hepatitis C	2001
Neulasta®	Pegfilgrastim	GCSF ^a	20000	Neutropenia	2002
Somavert®	Pegvisomant	GH ^b antagonist	4–5 × 5000	Acromegaly	2003
Macugen®	Pegaptanib	Anti-VEGF ^c aptamer	40000	Age-related macular degeneration	2004

^aGCSF, granulocyte-colony stimulating factor.

^bGH, growth hormone.

^cVEGF, vascular endothelial growth factor.

^dSCID, severe combined immunodeficiency disease.

^eALL, acute lymphoblastic leukemia, acute lymphocytic leukemia.

^fCML, chronic myeloid leukemia.18pt

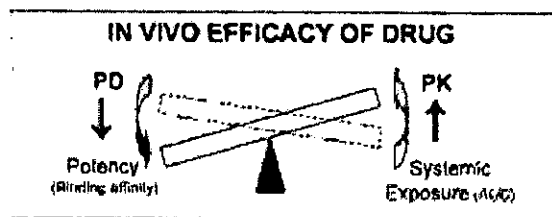


Figure 1. PEG alters the *in vivo* efficacy of drugs by altering the balance between their pharmacodynamic (PD) and pharmacokinetic (PK) properties. A decrease in potency caused by reduced binding affinity is compensated for by an increase in the overall systemic exposure caused by the prolonged plasma circulating time. The resulting change in the PK–PD profile provides an overall improved therapeutic efficacy that enables the generation of new drugs.

cokinetic and pharmacodynamic properties from the parent drug although it may act at the same target receptor or enzyme. Steric hindrance created by the large PEG polymer often underlies the reduced binding affinity that results from PEG conjugation. Compensation for this is achieved through the prolonged circulation time, which, together with the reduced immunogenicity often afforded by PEG, creates an overall improved pharmacological profile that can translate not only to improved efficacy but also to reduced dosing frequency and increased patient compliance. Table 2 outlines comparative PK and PD parameters for PEGylated molecules and their unmodified parent drugs for a number of compounds. These data demonstrate how the combination of decreased activity at the target receptor or enzyme with increased plasma half-life can translate to increase efficacy in an *in vivo* animal model.

In addition, it is worth noting that PEG conjugation, by limiting diffusion across membranes, often retains drugs in the plasma com-

partment and results in a reduced volume of distribution.¹¹ For PEGylated prodrugs, therefore, controlled-release from the plasma compartment can be achieved by using the appropriate PEG linkers, as discussed in recent reviews.^{8,10}

Prolonged Circulation Time

The characteristic prolonged circulation time that PEG endows on proteins and peptides arises through two principal effects; a decrease in the rate of kidney clearance and an increase in protection from proteolytic degradation, both of which decrease the overall clearance of the drug. Since PEG polymers are highly hydrated, with two water molecules per ethylene glycol unit, their hydrodynamic radii are approximately 5- to 10-fold greater than would be predicted by their nominal molecular weight,¹¹ underlying a dramatic increase in the effective molecular size of the PEG conjugate. At lower molecular weights of PEG, clearance occurs primarily by the kidneys; above a molecular weight of approximately 20 kDa renal filtration decreases in favor of excretion by the bile, and above approximately 50 kDa, hepatobiliary clearance dominates.^{12,13} It is worth noting that PEGylation of proteins that are normally cleared by receptor-mediated endocytosis does not generally alter the route of elimination, although some slowing of this process has been observed.^{14,15}

For many proteins and peptides, rapid proteolytic degradation by circulating enzymes represents one of the principal challenges in producing viable therapeutics. PEG provides protection from proteases and peptidases by impairing access for proteolytic enzymes. Despite this, the continual mobility of the PEG domain provides sufficient flexibility to enable high affinity interactions between the target receptor and the drug moiety and thus yield a biological effect. Consequently, the PEGylated drug retains efficacy while acquiring greater stability in plasma.

The most likely model to explain the protection from proteolysis involves a dynamic process in which the highly hydrated but mobile PEG moiety creates steric obstruction over the domain of the protein that serves as an enzyme substrate, thus reducing the frequency of favorable collisions. Figure 3 shows two scenarios, one involving the action of a proteolytic enzyme (top panel), and the other the action of a high affinity binding molecule (bottom panel). The two are distinguished

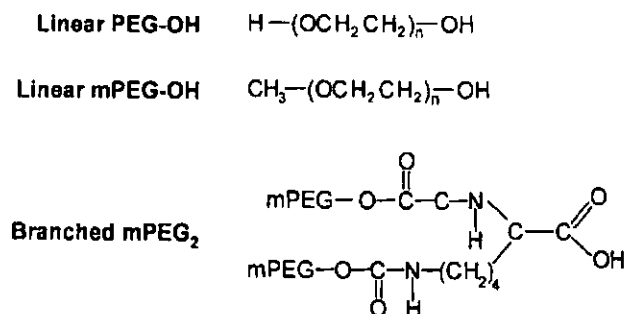


Figure 2. Structural formulae of PEG molecules. mPEG, monomethoxy PEG.

Table 2. Comparative Pharmacokinetic and Pharmacodynamic Parameters for PEGylated Molecules and Their Parent Unmodified Drugs

Measured Parameter	PK		PD		In Vivo Efficacy		
	Half-Life ($t_{1/2}$)		In Vitro Activity		In Vivo Activity		
	Parent PK Species ^a	Drug $t_{1/2}$ (h)	PEG-Drug $t_{1/2}$ (h)	Fold Difference	% Activity Retained ^b	PEG Drug Compared with Parent Drug in Animal Models	References
Enzymes							
PEG-arginine deiminase	m	2.8	50	$\uparrow \times 18$	48	Sustained reduction of plasma arginine to undetectable levels in mice	63
PEG-catalase	m	1	10	$\uparrow \times 10$	95	10^3 – 10^4 -fold reduction in immunogenic response in mice	1,64
PEG-methioninase	m	2	38	$\uparrow \times 19$	70	12-fold longer depletion of plasma methionine levels; 10^4 -fold decrease in IgG titer in mice	65,66
PEG-superoxide dismutase	r	0.01	38	$\uparrow \times 380$	51	Protection from oxygen toxicity in rats and 10^3 – 10^4 -fold reduction in immunogenic response in mice	20,64,67
PEG uricase	m	3	72	$\uparrow \times 24$	100	Reduction in urate levels and protection of renal function in uricase-deficient mice. Reduced immunogenic response	44,68
Cytokines							
PEG-GCSF ^c	r	1.8	7.0	$\uparrow \times 3.9$	41	14-fold longer effect in sustaining neutrophil count in rats	69
PEG-IFN- α 2a	m	0.7	51	$\uparrow \times 73$	7	Increased anti-tumor activity, elimination of neutralizing antibody response in mice	34
PEG-IFN- β 1a	r	0.98	13	$\uparrow \times 13$	50	2-fold increase in anti-angiogenic properties in murine tumor model	70
PEG-interleukin-6	m	0.05	48	$\uparrow \times 960$	51	10-fold increase in thrombopoietic	71
PEG-TNF α^d	m	0.07	0.7	$\uparrow \times 10$	80	Increased tumor regression using lower doses of drug in mice	72
Polypeptide hormones							
PEG-calcitonin	r	3.31	15.4	$\uparrow \times 4.6$	50	3–6-fold increase in hypocalcaemic efficacy in rats	51,73
PEG-GLP-1 ^e	r	0.04	0.56	$\uparrow \times 14$	83	Stabilization of glycemia in diabetic mouse model and prolonged glucose-lowering effect in mice	50,52
PEG-hGH ^f	r	0.34	10	$\uparrow \times 29$	24	2–3-fold prolongation of growth promoting activity in hypophysectomized rats	49
Antibodies							
Fab' fragments	r	0.33	9.05	$\uparrow \times 27$	100	Reduced tissue distribution and greater concentration in plasma in mice. Reduced immunogenic response	58,59
Nucleic acids							
Anti-VEGF RNA aptamer	mo	24 ^g	94	$\uparrow \times 3.91$	25	3-fold increased inhibition of VEGF-induced vascular permeability in guinea pigs	62,74

^am, mouse; r, rat; mo, monkey.^bPercent parent drug activity retained in *in vitro* assay (enzyme–substrate, receptor-binding, etc.).^cGCSF, granulocyte colony-stimulating factor.^dTNF, tumor necrosis factor.^eGLP-1, glucagon-like peptide-1.^fhGH, human growth hormone.^gHalf-life values for parent and PEG refer to clearance from vitreous humor in the eye in monkeys.

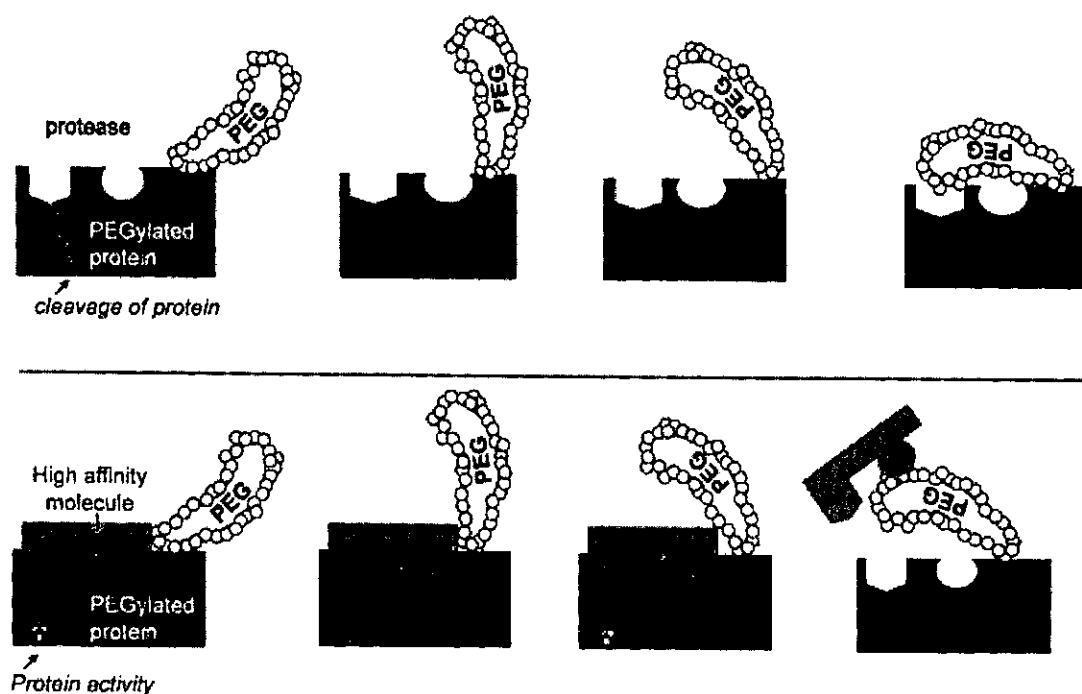
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Figure 3. Model for mechanism by which PEG provides proteolytic protection. Top panel: Binding of a plasma protease (light blue) to a PEGylated protein (dark blue) is impaired by the presence of the highly hydrated PEG domain (yellow, with white circles). The mobile PEG domain generates different configurations which reduce the probability of a favorable collision leading to enzyme-substrate interaction and protein cleavage. Lower panel: For the intended target binding molecule (pink) the higher affinity of the interaction drives the equilibrium to increase the probability of a productive interaction, and thus more configurations are permissive for yielding biological efficacy.

by the affinity of the interaction. Thus for the lower affinity protease, accessing the necessary configuration for binding and cleavage is made considerably harder by the presence of the PEG domain. For the target binding molecule (which could be a receptor, enzyme substrate or other macromolecule), the higher affinity of the interaction increases the probability of a productive interaction, and thus biological efficacy is still achieved. Note that the presence of PEG does create steric hindrance to some degree also for this interaction, and this is reflected in the lower intrinsic activity of PEGylated molecules compared with their parent native molecule seen in Table 2.

Reduced Immunogenicity and Toxicity

The same steric effect of the hydrated PEG chain that impairs access of proteolytic enzymes also underlies the reduced immunogenicity of PEGylated proteins. The PEG moiety minimizes the exposure of antigenic determinants, thereby reducing or preventing the generation of neutra-

lizing antibodies. The outcome is thus not only increased circulating half-life but also reduced toxicity of the conjugated drug. PEG itself has been approved by the FDA for use in food and cosmetics, and itself is considered essentially nontoxic.^{16,17} Uptake of PEGylated drugs into Kupffer cells in the liver has also been reported,¹² and while some reports indicate that such intracellular uptake of PEGylated molecules can lead to vacuolization, no toxic consequences of this phenomenon have been observed.¹⁸⁻²⁰ Researchers have attempted to capitalize on this postulated cellular uptake property of PEG by using it to enhance uptake of enzymes such as catalase and superoxide dismutase into cells and deliver to their site of action.²¹ To date, however, this has not yet proved sufficiently effective to transition to full drug development.

TWO CASE STUDIES

The traditional paradigm for creating drugs involves synthesis of new molecular entities in

the Discovery phase which are optimized according to their activity *in vitro* and in animal models *in vivo*. Formulation approaches are commonly included later in Preclinical Development, and are employed to obtain the most favorable biopharmaceutical properties and PK profile. PEGylation provides an alternative strategy in which both the efficacy and desired PK profile are built into the design of the molecule during the Discovery phase. The first of the two case-studies describes the creation of the drug pegvisomant (Somavert®) in which PEGylation was incorporated into the molecular design of the drug at the outset, and in which PEG enabled a drug whose rapid elimination would otherwise have made it not viable as a therapeutic. The second case-study describes the development of peginterferon- α 2a (PEGASYS®), in which PEG conjugation was utilized on an existing drug not merely to provide a more convenient dosing regimen, but to alter the overall pharmacological efficacy of the drug as a result of its altered PK profile. Both cases highlight the potential for using PEG to alter the balance of PD and PK in a coordinated manner to provide valuable and effective new drug entities.

SOMAVERT®: Pegvisomant—A Drug “Enabled” by PEGylation

Pegvisomant was developed as a growth hormone (GH) antagonist to block the actions of hypersecreted GH in acromegaly, and represents a first-in-class therapy in the treatment of this disorder. Acromegaly is a growth disorder characterized by abnormal enlargement of the hands, feet, skull and jaw, and is caused by excessive secretion of GH, in most cases arising from a pituitary adenoma.²² The hypersecreted GH activates signaling pathways that lead to overproduction of the insulin-like growth factor IGF-1 and related growth factors, whose mitogenic properties underlie the growth and metabolic clinical manifestations of acromegaly.

First-line therapy for acromegaly involves surgical removal of the adenoma, but this is successful in less than 60% of cases.²³ Alternative treatments, including radiation therapy and drug treatment with dopaminergic or somatostatin agonists show limited success and cause significant side-effects.^{22,24} The development of pegvisomant as a GH antagonist therefore represented a breakthrough therapy and provided physicians

with a potent tool for treating this endocrine disorder.

Growth Hormone is a 22 kDa protein, secreted by somatotrophs of the anterior pituitary gland, that promotes growth and affects metabolism.²⁵ The hormone binds to a preformed dimer of the GH receptor, leading to activation of the JAK/STAT-MAP kinase signaling pathway (Fig. 4). GH contains two distinct binding sites for the receptor, termed BS1 and BS2, which display high and low affinity binding respectively. *In vitro* binding studies suggest that high affinity binding to a single receptor via the BS1 site enables interaction of the lower affinity BS2 site with a second receptor in the preformed dimer, leading to activation of the intracellular signaling cascade.²⁶

Site-directed mutagenesis experiments demonstrated that a single point glycine to lysine mutation in the BS2 site (G120K) generates an antagonist able to bind the GH receptor with high affinity but unable to activate signal transduction.²⁴ This G120K-GH antagonist displayed a very short plasma half-life of approximately 30 min, similar to the 15 min half-life reported for the parent GH.²⁴ To prolong the plasma circulating time, 5 kDa PEG was conjugated to the antagonist using a random conjugation approach that attached PEG at free amine groups on lysine residues. This yielded a protein with a dramatically longer half-life at more than 100 h, but which lost 186-fold potency in receptor binding studies compared with GH (Tab. 3). To restore some binding potency, eight additional mutations were introduced into BS1 to increase its affinity further and compensate for the loss in affinity at BS2. Two of these, K168R and K172R, mutated lysine residues to arginine to remove PEGylation sites and thus reduce the steric hindrance generated by PEG around the binding site. The other six mutations optimized binding based on structure-function analyses of the ligand-receptor interaction.²⁵ The resulting mutated form of GH, termed B2036, behaved as a GH antagonist with similar binding affinity to that of the parent GH. PEGylation of B2036 yielded a markedly improved profile over that of the previously PEGylated antagonist. The same prolonged plasma half-life of ~100 h was produced, but the binding affinity was reduced by only 28-fold, suggesting greater retention of activity. Interestingly, binding studies using the soluble extracellular portion of the receptor demonstrated only a 4.5-fold lower affinity for PEG-B2036 than the non-PEGylated antagonist, and comparable

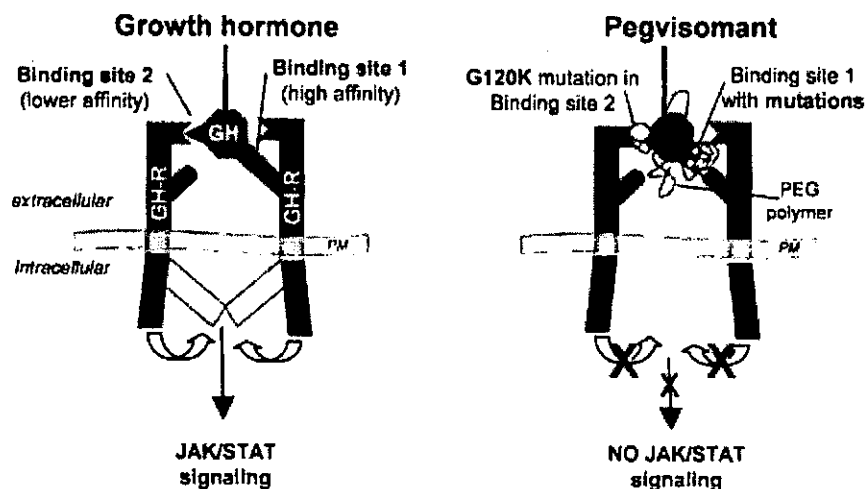


Figure 4. Model for binding of growth hormone receptor to GH (left) and pegvisomant (right). Binding of high affinity and low affinity sites on the hormone (blue shape) to sites on the extracellular domain of the GH-R in its dimer configuration causes a conformational change that activates signaling through the JAK/STAT pathway. Pegvisomant, by contrast, contains the G120K mutation (large yellow circle) and additional mutations (small yellow circles) which enable high affinity binding but cause no conformational change and hence no signal transduction, thus acting as an antagonist. GH, growth hormone; GH-R, growth hormone receptor; JAK, janus kinase; PM, plasma membrane; STAT, signal transducers and activators of transcription.

binding affinity to the parent GH,²⁵ strongly supporting the notion that the majority of the effect of PEG on binding occurs due to steric interference with the membrane-bound configuration of the receptor.

Clinical studies on pegvisomant demonstrated prolonged efficacy for up to 12 weeks,²⁷ evident as sustained suppression of total and free IGF-1, IGF Binding Protein 3 (IGFB-3) and the acid-labile subunit of IGFB-3 in patients with acromegaly (Fig. 5). Thus by combining the pharmacodynamic and pharmacokinetic properties of PEG and GH, a potent antagonist was produced that proved effective in clinical trials and which received FDA approval in 2003 as second-line therapy in treatment of acromegaly.

PEGASYS®: Peginterferon- α 2a—A Drug “Improved” by PEGylation

FDA approval for peginterferon- α 2a (PEG-IFN- α 2a) was granted in 2001. Since that time, this drug has obtained status as first-line therapy in chronic hepatitis C infection, and is administered either alone or in combination with the broad spectrum antiviral agent ribavirin.²⁸ Chronic hepatitis C (HCV) infection affects approximately 4 million people in the US, and represents the leading cause of hepatocellular carcinoma and liver cirrhosis.²⁹ Previous treatment of HCV infection relied on interferon- α (IFN- α) monotherapy, and was succeeded by treatment with IFN- α in combination with ribavirin. The potent

Table 3. Relative Binding Affinities and Serum Half-Lives of GH Receptor Ligands Generated During the Discovery Process for Pegvisomant

Ligand	Binding Affinity (K_i) at GH Receptor nM	Fold Decrease in Binding Affinity ^a	Serum Half-Life $t_{1/2}$ (h)	Fold Increase in Serum Half-Life ($t_{1/2}$) ^b
GH	3.53 $\pm$ 0.54	1.0	0.25	1
G120K-GH	4.66 $\pm$ 0.04	1.3	0.25	1
PEG-G120K-GH	657.1 $\pm$ 0.71	186	>100	>400
B2036	3.84 $\pm$ 0.48	1.1	0.50	2
PEG-B2036	99.60 $\pm$ 6.39	28.2	>100	>400

^aDecrease in binding affinity compared with GH.

^bIncrease in $t_{1/2}$ compared with GH.

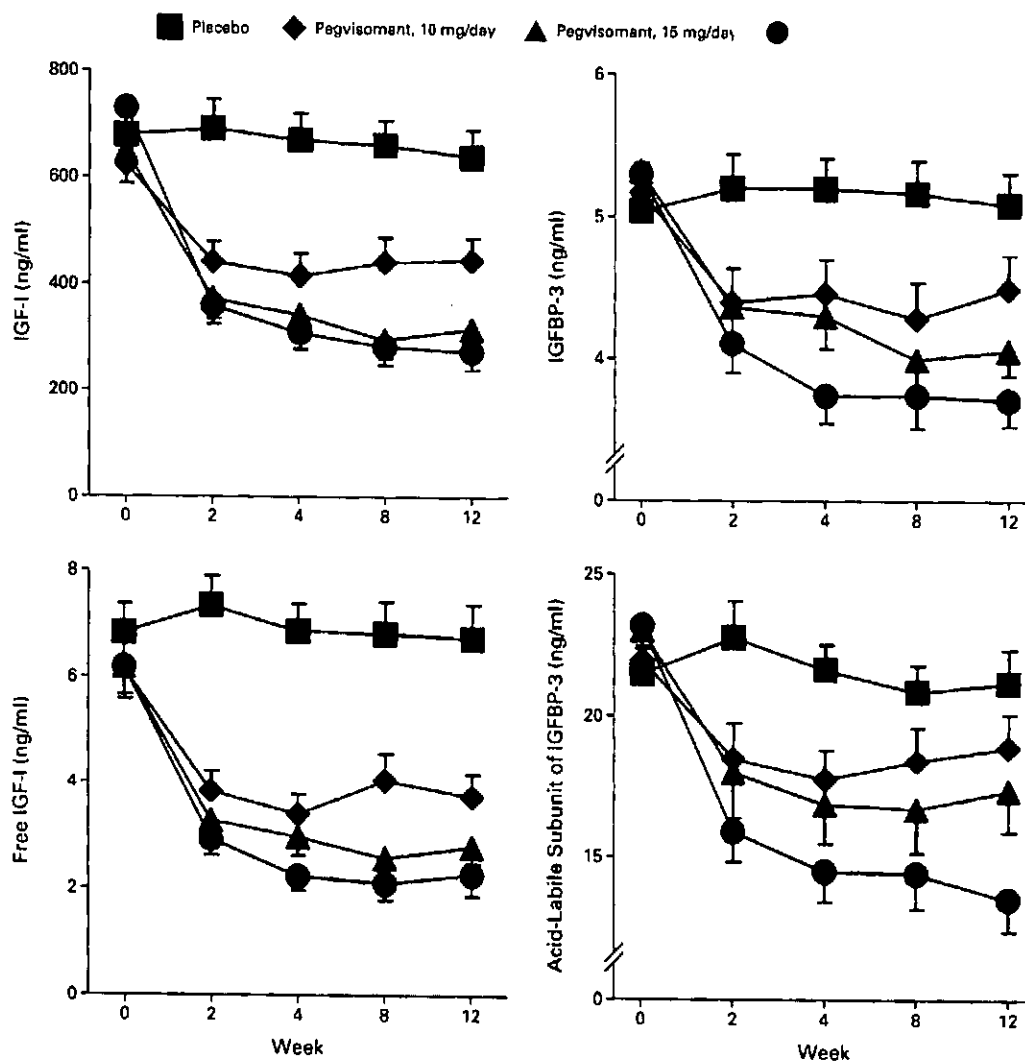
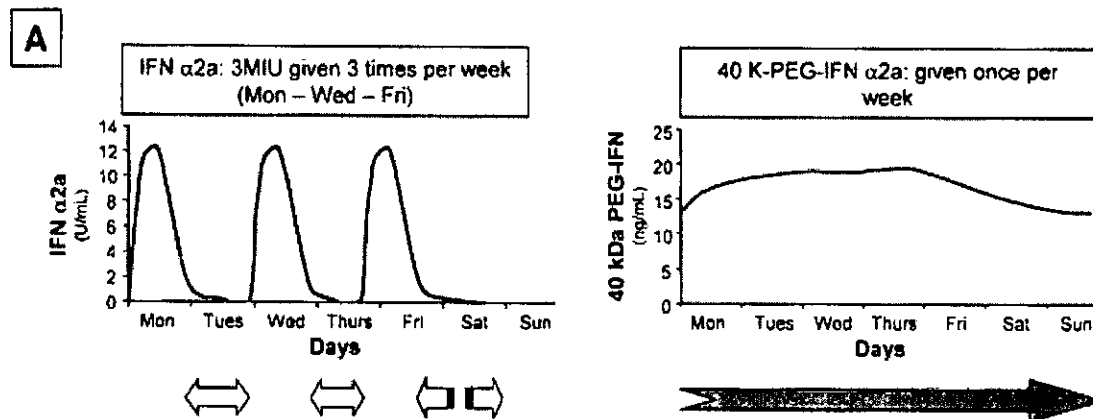


Figure 5. Clinical data show prolonged efficacy of pegvisomant. Following 12 weeks of treatment with pegvisomant, patients displayed significant, dose-dependent reductions in serum concentrations of total and free IGF-1, and the full and acid-labile subunits of IGF1-binding protein (IGF1BP-3) compared with placebo.

antiviral activity of IFN- α results from induction of interferon-stimulated genes (ISGs) via an IFN receptor-JAK/STAT mediated pathway.³⁰ Induced ISGs inhibit the translation of viral proteins and decrease the stability of the viral RNA. In addition, IFNs stimulate the innate cell-based immune response by enhancing memory T cell proliferation and natural-killer cell activation while inhibiting T cell apoptosis. The mechanism by which ribavirin augments the response to IFN is not well understood, but may involve a shift in the balance of T_H1 and T_H2 cells, inhibition of HCV RNA polymerase, mutagenesis of HCV RNA or GTP depletion.³⁰

Treatment of HCV infection with unmodified interferons produces a sustained response only in a

minority of patients however, as a result of host factors such as the viral load, viral factors such as the viral genotype, and most significantly pharmacokinetic properties of the interferons themselves.²⁸ The plasma half-life of 4–6 h results in undetectable levels of IFN- α 2a within 24 h of a subcutaneous dose, and thus requires 3 times per week dosing by subcutaneous injection.³¹ This produces continual fluctuations in plasma concentrations of IFN- α 2a, in which intervals of negligible IFN- α 2a creates periods of exposure to the virus, and opportunities for the virus to replicate and develop resistance mutations (Fig. 6A, left panel). The aim of PEGylation therefore, was to provide a means of obtaining a longer serum half-life, and a more consistent, “flatter” serum concentration—



B

	40K-PEG-IFN α 2a	IFN α 2a
No of patients	267	264
Dose, ROA, regimen	180 ug, s.c., 1/week, 48 weeks	6 MIU, s.c., 3 / week x 12 weeks, followed by 3 MIU, s.c. 3 / week x 36 weeks
Undetectable HCV RNA (< 100 copies/mL)	68%	28 %
Compliance: patients who completed course	84%	80%

Figure 6. The altered PK profile of PEG-IFN- α 2a results in a dramatic improvement in efficacy compared with unmodified IFN α 2a. (A) Serum concentration–time profiles for IFN α 2a (left panel) and PEG-IFN α 2a (right panel) demonstrating the ability of PEG to provide prolonged systemic exposure to the drug. Low serum concentrations of IFN α 2a in between doses cause periods of exposure to the virus (left panel, open arrows) which create opportunities for viral replication and development of resistance. PEG conjugation creates a prolonged continual exposure to IFN- α 2a (right panel, hatched arrow), during which serum concentrations are sufficiently high to minimize viral replication and mutation. (B) Clinical studies demonstrate that PEG-IFN α 2a, with reduced frequency of dosing, produces a significant reduction in detection of virus particles (indicated by HCV RNA copies per mL) in addition to an improvement in compliance, evident from number of patients who completed the course. HCV, hepatitis C virus; IFN, interferon; MIU, million units; ROA, route of administration; s.c., subcutaneous.

time profile with sustained levels of interferon that would generate continual anti-viral protection (Fig. 6A, right panel).

Initial attempts at PEGylation employed a 5 kDa PEG conjugated to the 19 kDa IFN- α 2a molecule, but generated an insufficient improvement over the unmodified IFN- α 2a when tested in clinical trials.¹¹ A branched 40 kDa PEG was then used to conjugate to IFN- α 2a, and produced a substantially different serum concentration–time profile (Fig. 6), displaying the desired PK behavior. PEGylation prolonged the serum half-life from 3.8 to 65 h, slowed the clearance by

more than 100-fold and reduced the volume of distribution fivefold.³² In phase III clinical trials comparing the efficacy of treatment for 48 weeks with IFN- α 2a, given subcutaneously 3 times per week, with PEG-IFN- α 2a, subcutaneously, once per week, the response rate to the treatment was more than doubled. 68% of patients who received PEG-IFN- α 2a displayed undetectable levels (<100 copies HCV RNA/mL) of virus compared with only 28% of patients who received the unmodified IFN- α 2a. In addition, compliance was improved with the PEGylated product since the proportion of patients who

finished the course rose from 60% in the IFN- α 2a cohort to 84% in the PEG-IFN- α 2a cohort (Fig. 6B).³³

Thus, by slowing renal elimination of IFN- α 2a and protecting from plasma proteases, PEG prolongs the circulation half-life of the molecule. Moreover, by reducing the tissue distribution and maintaining the drug primarily in the plasma where it acts on the virus, PEG increases the exposure of the virus to the drug and creates an improved therapeutic.

Interestingly, *in vitro* activity assays on the 40 kDa PEG-conjugated IFN- α 2a indicate that only approximately 7% of the activity of the parent molecule is retained.³⁴ By contrast, a competing product, composed of a linear 12 kDa PEG-conjugated IFN- α 2b (PEG-INTRON®), retains 28% of the parent compound anti-viral activity *in vitro*.³⁵ However, the improvement in systemic exposure offered by the 12 kDa PEG is relatively modest compared with that of the branched 40 kDa PEG, as it displays a half-life of 27–37 h, a 10-fold lower clearance and only a minor change in the volume of distribution.³² While both forms of peginterferon are effective, these data exemplify how altering the balance between PK and PD affects the overall pharmacological profile. Thus the degree to which intrinsic activity is retained is not the sole parameter dictating the efficacy of these drugs. Equally important is the overall exposure and its relationship to the pharmacodynamics of the drug.

The current commercial formulation of PEG-IFN- α 2a contains a heterogeneous mixture of monoPEGylated isomers of the IFN- α 2a molecule. The PEGylation conjugation chemistry attaches the 40 kDa PEG molecule via amide linkages to one of nine reactive lysine residues in the IFN- α 2a molecule. Recent studies have separated the mixture chromatographically and examined the activity of the individual positional isomers.^{36,37} Using VSV-infected MDBK cells and the human melanoma cell line ME15 for antiviral and antiproliferative assays respectively, Foser et al.³⁶ found that two of the isomers (K31 and K134) exhibited greater activity than the original PEG-IFN- α 2a, while the other seven isomers displayed less activity than the mixture. Variations were also observed between the isomers in the binding affinity for the extracellular portion of the interferon receptor IFNR2. The behavior of the different PEGylated positional isomers has been used to aid understanding of the structure-activity relationship of the IFN-receptor interac-

tion, and may enable the selection of a more potent isomer for development of a further improved PEG-interferon.

DIFFERENT CLASSES OF DRUGS BENEFIT FROM PEGYLATION

The benefits of PEG conjugation can be leveraged differently to improve the PK–PD balance depending on the class of drug, and on its mechanism of action. This technology has been applied to multiple classes of macromolecules, including enzymes, cytokines, polypeptide hormones, antibodies and nucleic acids, as detailed in Table 4. The molecular mechanism by which PEG improves the drug is often similar within a class, resulting in similar trends for the effects of PEG on the PK and PD parameters for drugs within those classes. Therapeutic enzymes, for example, suffer relatively little loss of activity upon PEGylation whereas hormones and cytokines, which require high affinity interactions with cell surface receptors, show significant loss of binding affinity upon PEGylation (Tab. 2). For the latter, the presence of the lipid bilayer and position of the receptor allow less room for maneuver for the ligand, and the steric hindrance created by the PEG molecule thus has a greater impact on the binding affinity. For soluble enzymes which are not tethered to cellular structures, the PEG domain is sufficiently mobile and flexible to enable access of the substrate to the catalytic site, and it can more easily be conjugated at a location which has relatively little impact on the catalytic activity.

Enzymes

Therapeutic enzymes represent a growing class of biopharmaceuticals and PEGylation has played a central role in improving or enabling several of these products.³⁸ For most enzymes examined, reduction in immunogenicity represents the principal mechanism by which PEG prolongs the circulating half life (Tab. 4). Early work on adenosine deaminase (ADA) demonstrated the ability of PEG to reduce the generation of neutralizing antibodies, extending the plasma half-life from several minutes to approximately 24 h, and led to the development of the first PEGylated enzyme therapeutic, pegademase (Adagen®), as described earlier.³⁹ Perhaps most

Table 4. Different Classes of Drugs Benefit from PEGylation by Differing Mechanisms of Action

Class	Molecule	PEG size (kDa)	PEGs per Molecule ^a	PEG Mechanism of Action	Development Status	References
Enzymes	Adenosine deaminase	5	Multiple	Proteolytic protection, reduced immunogenicity	FDA approved (<i>Adagen</i> ®)	39,40,75
	Asparaginase	5	Multiple	Reduce immunogenicity	FDA approved	42,76
	Arginine deiminase	20	22	Reduce immunogenicity	Phase I	63,77, ⁷⁸
	Catalase	1.9, 5	Multiple	Reduce immunogenicity, possibly facilitates cellular uptake	Preclinical	1,64,79
	Methioninase	5	3–7	Reduce immunogenicity	Preclinical	65,80
	Superoxide dismutase	5	10–15	Reduce immunogenicity, possibly facilitates cellular uptake	Preclinical	20,64,81
Cytokines	Uricase	20	Multiple	Reduce immunogenicity	Preclinical	44,45
	GCSF	20	1	Increase size to slow renal clearance	FDA approved (<i>Neulasta</i> ®)	46
	Interferon-α2a	40	1	Increase size to slow renal clearance	FDA approved (<i>PEGASYS</i> ®)	31,32
	Interferon-α2b	12	1	Increase size to slow renal clearance	FDA approved (<i>PEGINTRON</i> ®)	35
	GM-CSF ^b	5, 10, 20	1	Increase size to slow renal clearance	Preclinical	82
	Interferon-β1a	20	1	Increase size to slow renal clearance	Preclinical	70,83
	Interleukin-6	5	Multiple	Increase size to slow renal clearance	Preclinical	71
	TNFα ^c	5	7–8	Increase size to slow renal clearance	Preclinical	72
Hormones	GH antagonist	5	4–5	Protection from proteases	FDA approved (<i>Somavert</i> ®)	25
	Calcitonin	2	1	Protection from proteases	Preclinical	73,84
	Growth hormone	20	1	Protection from proteases	Preclinical	48,49
	GLP-1 ^d	2	1	Protection from DPPIV ^h and other peptidases	Preclinical	50
Antibodies	Fab' fragments	5, 25, 40	Multiple	Increase size to slow renal clearance	Preclinical	59
	Anti-TNFα Fab'	nd ^e	1	Increase size to slow renal clearance	Filed for approval (<i>Cimzia</i> ®)	57
	Anti-VEGF-R2 ^e Fab'	nd	1	Increase size to slow renal clearance	Phase II (<i>CDP791</i>)	56
Nucleic acids	Anti VEGF aptamer (pegaptanib)	40	1	Slow diffusion away from site of action in vitreous humor	FDA approved (<i>Macugen</i> ®)	60
	Anti PDGF ^f aptamer	40	1	Increase size to slow renal clearance	Preclinical	85

^aNumber of PEG moieties attached per molecule of parent drug.^bGM-CSF, granulocyte-macrophage colony-stimulating factor.^cTNFα, tumor necrosis factor-α.^dGLP-1, glucagon-like peptide 1.^eVEGF-R2, vascular endothelial growth factor receptor-2.^fPDGF, Platelet-derived growth factor.^hDPPIV, dipeptidylpeptidase IV.^gnd, not disclosed.

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interesting for this product is that it operates effectively despite not being able to reach the same cellular location as the endogenous enzyme which it replaces. While endogenous adenosine deaminase is primarily a cytosolic enzyme, the PEG component of pegademase limits its tissue distribution and retains it in the circulation, outside cells. ADA deficiency causes toxic accumulation of metabolites, in particular adenosine and 2'-deoxyadenosine.⁴⁰ Rapid diffusion of these nucleosides across cell membranes enables them to reach the circulation, however, where pegademase can metabolize them to inosine and 2'-deoxyinosine respectively, restoring the balance of metabolites necessary for recovery of immune function.⁴¹

Reduction in immunogenicity represents the primary benefit of PEGylation for a number of other enzymes under investigation for different chemotherapeutic applications, including pegasparaginase and PEG-uricase (Tab. 4). Pegasparaginase (Oncaspar[®]) was developed to avoid the hypersensitivity reactions which occurred in 5–20% of acute lymphoblastic leukemia (ALL) patients treated with unmodified L-asparaginase.⁴² Treatment with L-asparaginase capitalizes on the absence of asparagine synthetase in ALL tumor cells and acts to deprive them of their external supply of the nutrient.⁴³ The ability of pegasparaginase to provide this benefit without significant immunogenicity led to the FDA approval of Oncaspar[®] in 1994 for ALL patients who displayed allergic reaction to the unmodified form of the drug.⁴³ Coupled with its reduced immunogenicity, the improved PK profile which enables less frequent dosing led the FDA in 2006 to expand the use of the drug for first-line treatment of patients with acute lymphoblastic leukemia (ALL) as a component of a multi-agent chemotherapy regimen.

For the enzyme uricase, immunogenicity presents a common complication and has prompted investigation of PEG-uricase for use in conditions of hyperuricemia, such as occurs during cancer chemotherapy,⁴⁴ where toxic levels of uric acid accumulate in the blood, or in gout.⁴⁵ Since humans do not express the enzyme, recombinant uricase from other species must be employed, resulting in high antigenicity due to the species difference and the nonmammalian hyperglycosylation that occurs during manufacture in yeast. By a similar mechanism to that described for proteolytic protection, illustrated in Figure 3, PEG provides a means

of shielding carbohydrate and other antigenic groups, thereby reducing the immunogenicity of the enzyme and providing the possibility of a safer therapeutic.⁴⁴

Cytokines

Cytokines are small secreted proteins involved in the regulation of immunity, inflammation and hematopoiesis, that characteristically display short plasma circulating half-lives due to their rapid clearance by the kidneys. This has complicated attempts to develop therapeutic cytokines for boosting the immune system in conditions of immunodeficiency. Since PEG conjugation to cytokines can increase their overall hydrodynamic volume, it provides a means to reduce the rate of renal filtration and extend the plasma circulating time, and has been investigated for a number of cytokines (Tab. 4).

GCSF, granulocyte-colony stimulating factor (filgrastim: Neupogen[®]), is a cytokine involved in stimulating production of neutrophils, whose short half-life complicates its therapeutic use for treatment of chemotherapy-induced neutropenia.⁴⁶ The short half-life of GCSF results from its rapid clearance, which is mediated by two pathways: renal filtration and receptor-mediated internalization. Thus, following GCSF-mediated stimulation of neutrophil generation, GCSF binds to its cognate receptors on the upregulated neutrophils and mediates its own clearance. The development of a PEGylated version of GCSF, called pegfilgrastim (Neulasta[®]), capitalized on the ability of PEG to affect the renal route of clearance while having a nominal effect on the neutrophil-mediated pathway. This was particularly important for this cytokine, since the negative-feedback mechanism operates to limit the number of neutrophils generated. Thus PEGylated GCSF displays slowed renal clearance, providing a significant improvement in plasma half-life, while retaining sufficient biological activity to stimulate production of neutrophils.⁴⁷ The newly generated neutrophils bind the circulating PEG-GCSF and remove it from the circulation by an endocytic-lysosomal pathway, thus providing a limit to the upregulation and preventing excessive formation of neutrophils which could be harmful.

Several other cytokines display benefits from the sustained plasma levels achieved by PEG conjugation. As with interferon- α 2a, described

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previously, the altered PK profile produced by PEGylation presents opportunities not merely for convenience improvement but provides a direct benefit to the pharmacodynamics and hence efficacy of such drugs.

Polypeptide Hormones

A number of polypeptide hormones have been investigated or developed as biotherapeutics for treatment of endocrine disorders caused by either lack of, or excessive, hormone levels. In several cases, PEGylation presents an effective strategy for providing protection from proteolytic enzymes to enable the development of viable therapeutics (Tab. 4).^{24,48–50} As polypeptide hormones generally bind cell-surface receptors which are integral membrane proteins, the relatively bulky PEG moiety often interferes with high affinity binding to the target receptor and reduces the activity of the hormone. For this class in particular, therefore, considerable efforts have been targeted at selecting the most appropriate site on the molecule, or engineering appropriate sites, for conjugation of the PEG polymer. Early work on PEGylation of growth hormone, for example, employed a nonselective strategy that conjugated PEG to free amines on lysine residues, and demonstrated a direct relationship between number of PEG moieties and loss of binding affinity.⁵⁰ In this case, the increased exposure from prolonged circulation time was not sufficient to compensate for the considerable loss in activity. A more recent study, however, demonstrated that by engineering a threonine to cysteine mutation (T3C) in the GH polypeptide, a cysteine-targeted PEGylation strategy using a 20 kDa PEG could yield a monoPEGylated GH derivative that retains good binding activity and benefits from the prolonged circulation time.⁴⁹ Several other polypeptide hormones involved in metabolic disorders have been PEGylated to prolong their plasma circulating time, including calcitonin,⁵¹ GLP-1,^{50,52} insulin,^{52–54} and neuropeptide Y (NPY).⁵⁵ In the case of calcitonin and insulin, nasal and pulmonary delivery respectively were attempted to provide a prolonged delivery system that would extend the PK–PD profile and reduce the frequency of administration.^{51,54} In this approach, the large PEG moiety serves to slow absorption through the nasal or pulmonary epithelium, in addition to prolonging circulating time once in the systemic circulation. Here, PEG

retains the drug in the nasal cavity or lung, which effectively serve as reservoirs, and choice of the appropriate PEG size controls the rate at which the drug crosses the epithelium to reach the plasma compartment.

Incorporating the PEGylation strategy into the design of a polypeptide hormone during the Discovery phase of drug development, and considering the PK and PD characteristics early on in the process, presents promising new avenues for advancing the development of improved hormone biotherapeutics.

Antibodies

Two products in development, Cimzia[®] (CDP870: PEG-anti-TNF α Ab) and CDP791[®] (PEG-anti-VEGFR-2), for treatment of rheumatoid arthritis and solid tumors respectively, are Fab' antibody fragments that employ PEG conjugation to improve the drugs' PK profile while having a minimal loss on the antibody–antigen interaction.^{56,57} Studies aimed at determining the optimal site on IgG Fab' antibody fragments for conjugating PEG demonstrated that the hinge region cysteine residues can tolerate attachment of one or two PEG moieties, of up to total 40 kDa molecular weight, with little effect on antigen binding affinity. This is complemented by significant increases in plasma circulating times resulting from the reduced glomerular filtration by the kidneys and lower immunogenicity than the parent IgG,^{58,59} and suggests a mechanism for successfully PEGylating antibody Fab' fragments.

These data should aid in the design of PEGylation strategies as the field of therapeutic antibody fragments continues to develop, and further antibody targets continue to be identified.

Nucleic Acids

Pegaptanib sodium (Macugen[®]) represents the first approved RNA aptamer and was developed as an anti-VEGF directed therapeutic for treatment of age-related macular degeneration.⁶⁰ Aptamers are oligonucleotides that bind with high affinity to target molecules;⁶¹ for pegaptanib the target is VEGF, a principal mediator of the angiogenesis and increased vascular permeability that lead to this form of macular degeneration. For full efficacy this drug requires intravitreal injection to enable the aptamer to access the local VEGF that

causes neovascularization. Conjugation of the 40 kDa PEG serves to slow diffusion out of the vitreous humor thus maximizing efficacy and minimizing systemic exposure. PEG conjugation causes a fourfold drop in binding affinity,⁶² but its prolonged retention at the site of action more than compensates for this and enables sufficient VEGF inactivation to yield good clinical efficacy.

As nucleic acids provide more tools for creating drugs in the form of antisense oligonucleotides, aptamers and siRNAs, PEGylation is likely to emerge as an effective tool for improving the pharmacological profile by producing an optimal balance between the PK and PD characteristics.

SUMMARY

PEGylation of macromolecules has advanced considerably over the last two decades, and the appearance of PEGylated drugs promises to continue to accelerate as the field of biotherapeutics expands. Understanding how PEGylation affects the pharmacology of drugs at a molecular level enables incorporation of a PEGylation strategy into drug design from the early stages in the development pathway. This optimization of the PK–PD balance early in the process may provide a more efficient approach to generating viable drug candidates, and may facilitate the creation of novel drugs from multiple classes of macromolecule.

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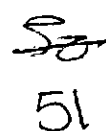
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