

OLARTEMOURE

OLARTE MOURE & ASOCIADOS LTDA.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 10-160886- -00034-0000

Fecha: 2015-07-16 16:29:53 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve 379 FASENACIONALII
Act 412 REPOSPRESRECU Folios: 63

Carlos R. Olarte
Juan G. Mour

Alexander Agudelo
Carlos A. Parr
Juan F. Acosta
Mónica Guevar
Andrés Alvarado
María C. Calderó
Mauricio Ávil
Gina Cáceres
Isabella Herrero
Zaava Ravi
Alejandro Mour
Miguel F. Porra
Catalina Amay
Laura M. Escoba
Lorena Andrad
Sergio Arboled
Catalina Jiménez
Daniela González
Maritza Sierra
Gina A. Arias
Guillermo Parr
Enika Jhisset Suárez
Liliana Galindo

Medellín
María C. Múner
Eddie Manota
Adrián Santamaría
Camilo A. Berni

Barranquilla
Gina de Echeon

Señores

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Re: Expediente N°. 10-160.886

Solicitud de Patente de Invención titulada: "COMPUESTOS DE INSULINA
MODIFICADA [B28-L-LISINA, B29-L-PROLINA] Y PEGILADA"

Solicitante: ELI LILLY AND COMPANY

Nuestra Referencia: P2010/000170

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. 28784, notificada por edicto desfijado el 2 de julio de 2015.

1. ANTECEDENTES

1.1 La solicitud de patente ventilada en este expediente fue presentada el 22 de diciembre de 2010, con un Capítulo Reivindicatorio original de 36 reivindicaciones.

1.2 La Superintendencia de Industria y Comercio (SIC) emitió el primer examen de fondo notificado por fijación en la lista No. 451 del 26 de diciembre de 2012, correspondiente al Oficio No. 23674, objetando la claridad, y el nivel inventivo de la presente invención.

1.3 En respuesta al primer examen de fondo, el 14 de mayo de 2013 presenté un Capítulo Reivindicatorio Modificado con 15 reivindicaciones y proporcioné argumentos en favor de la claridad y el nivel inventivo de la materia limitada.

1.4 La SIC por medio de la Resolución No. 47947, notificada por edicto desfijado el 20 de septiembre de 2013, negó las Reivindicaciones 1 a 15 por supuesta falta de nivel inventivo, soportada en argumentos que no se elevaron en el primer examen de fondo.

1.5 El 27 de septiembre de 2013 presenté un Recurso de Reposición, en el que argumenté que la solicitante no tuvo la oportunidad de controvertir los nuevos argumentos presentados por la SIC, por lo que la Resolución No. 47947 del 14 de agosto de 2013 violó los derechos al debido proceso y el derecho a la defensa del solicitante.

1.6 La SIC, por medio de la Resolución No. 13067, notificada por edicto desfijado el 4 de abril de 2014, devolvió la presente invención a trámite y, posteriormente, emitió un segundo examen de fondo correspondiente al Oficio No. 12946, notificado por fijación en lista 041 de fecha 14 de octubre de 2014, objetando el título, la claridad y el nivel inventivo de la presente invención.

1.7 El 23 de febrero de 2015 respondí oportunamente al segundo examen de fondo, donde aporté un Capítulo Reivindicatorio Modificado de 13 reivindicaciones, modifiqué el título de la invención y proporcioné argumentos en favor de la claridad y el nivel inventivo de la misma.

1.8 La SIC, mediante la Resolución No. 28784 notificada por edicto desfijado el 02 de julio de 2015, ahora niega nuevamente la presente invención 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 presentadas el 23 de febrero de 2015 (Radicado No. 10-160886-0029-0000), o en su defecto aquellas reivindicaciones que el Despacho considere cumplen con los requisitos de patentabilidad.

3. FUNDAMENTO DEL RECURSO

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

3.1. El Despacho insiste en que la presente invención carece de nivel inventivo a la luz del documento "*Insulin LISPRO*"¹ (D1) y las patentes US6890518 (D3) y US2001031726 (D4).

a- i) La SIC argumenta que D1 es el estado de la técnica más cercano a la presente invención tal como se reclama en la Reivindicación 1, en tanto dicho documento divulga un análogo de insulina conocido como la insulina Lispro que consiste en una cadena A y una cadena B que son idénticas a las SEQ ID NO: 1 y 3 de la presente invención, respectivamente, comprendiendo enlaces disulfuro entre las cisteínas Cys7A-Cys7B, Cys20A-Cys19B, y Cys6A-Cys11A (página 177, Figura 2). La SIC agrega que D1 y la presente invención difieren en que la proteína aquí reclamada está pegilada, mientras que en D1 no se hace una mención a la pegilación. Esta diferencia mejora las propiedades farmacocinéticas de la insulina, por lo que se puede afirmar que la presente invención pretende modificar la insulina de D1 para obtener un compuesto con propiedades farmacocinéticas mejoradas.

No obstante, el Despacho resalta que dicha modificación resulta obvia a la luz de D3, pues éste divulga compuestos pegilados de insulina modificada en los que el PEG es de alto peso molecular (15, 20 y 25 kDa), con el fin de disminuir la biodisponibilidad de la insulina y así incrementar el tiempo de vida media (párrafos 22, 85-86, y 90-91). Por consiguiente, la Reivindicación 1 y sus dependientes no son inventivas a la luz de D1 y D3.

ii) A su vez, la SIC resalta que D4 es el estado de la técnica más cercano la composición de la Reivindicación 5, en tanto este documento divulga composiciones de insulina Lispro, TRIS entre 16.5-24.7mM y un agente isotonzante como fosfato o citrato (párrafos 46-48), diferenciándose de la presente invención en la pegilación de la proteína. Esta diferencia, según el Despacho, aumenta la estabilidad de la insulina, por lo que la invención pretende modificar la composición de D4 para obtener una composición con insulina más estable.

Sin embargo, y de la misma manera que para la Reivindicación 1, la SIC argumenta que tal diferencia resulta obvia considerando que D3 enseña compuestos pegilados de insulina modificada en los que el PEG es de alto peso molecular, dando como resultado insulinas con propiedades mejoradas. A su vez, D3 divulga una composición que comprende una insulina modificada pegilada, TRIS y azúcares no reductores para acondicionar la osmolaridad de la composición (párrafos 22, 90-91, y 124-142). Así, la Reivindicación 5 y sus dependientes tampoco son revisten nivel inventivo.

¹ Holleman, F & Hoekstra, J. 1997. *Insulin LISPRO*. New England J. Med. 337(3):176-183

b- Respecto a mis argumentos presentados en respuesta al segundo examen de fondo, la SIC insiste en que los documentos D1, D3 y D4 brindan las divulgaciones y sugerencias suficientes para que la persona medianamente versada en la materia desarrolle de manera obvia la presente invención, empleando los mismos argumentos mencionados anteriormente.

Asimismo, el Despacho argumenta que, si bien los resultados de la presente invención muestran un incremento en la vida media de la insulina reclamada en comparación con la glargina, la única diferencia radica en la pegilación, que no sólo es sugerida por D3, sino que también es sabido por la persona medianamente versada en la materia que la pegilación se utiliza para mantener la eficacia terapéutica durante tiempos más prolongados. A su vez, la comparación con glargina no es adecuada, porque esta insulina comprende una modificación mínima que altera el pH del entorno, y dicha comparación debió realizarse con la insulina Lispro sin pegilar.

Finalmente, la SIC agrega que los artículos "*Steady-State pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus*"² y "*Novel PEGylated Basal Insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism*"³ (aportados junto con mis respuesta al segundo examen de fondo) no proveen resultados comparativos para determinar con certeza las ventajas de la insulina reclamada sobre la insulina lispro sin pegilar, y la actividad hepato-preferencial propia de las insulinas, por lo que es de esperar que la insulina reclamada presentara dicho comportamiento.

3.2 a- Con el ánimo de brindar mayor claridad en el análisis de nivel inventivo de la presente solicitud, me permito realizar nuevamente dicho análisis de acuerdo con la Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad de la SIC⁴ (en adelante, Guía de la SIC) en donde se señala que "Durante el examen de nivel inventivo debe hacerse un juicio de valor y un análisis objetivo de las divulgaciones previas del estado de la técnica, no influenciados por el conocimiento que ya se tiene de la Invención que se está evaluando. Por tanto, con el fin de minimizar la subjetividad y evitar que se realice un análisis en retrospectiva ("hindsight" ó "a posteriori"), el examen debe relacionar la invención con la solución de un problema técnico, mediante el Método "problema-solución" (subrayado fuera de texto).

² Sinha, V. P., et al. Steady-State pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus. Diabetes, Obesity and Metabolism 16: 344-350.2014.

³ Moore, M. C., et al. Novel PEGylated Basal Insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism. Diabetes. Vol. 63. February 2014.

⁴ Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad de la SIC, 2014, sección 2.13.5, páginas 131-134 (disponible en http://api.sic.gov.co/Documentos/Guia_Examen_Patentes.pdf)

A. Identificar el estado de la técnica cercano a la invención reivindicada.

Para el presente caso, me permito resaltar que de acuerdo con la Guía de la SIC, “*El estado de la técnica cercano es un documento que se encuentra en el mismo campo técnico que la invención o trata de solucionar el mismo problema o uno semejante. Suele ser el que tiene más características en común con la invención o presenta leves modificaciones de estructura e indica la misma función, propósito (y) En caso de que haya varios documentos relacionados, el estado de la técnica cercano es el documento que menciona una función, propósito, problema a resolver ó actividad semejante al de la invención*”⁵. Igualmente, la Guía de la SIC, respecto al uso de documentos adicionales, establece que dicho documento adicional “*contiene una enseñanza que indicaría (no sólo que podría indicar, sino que indicaría) a la persona medianamente versada en la materia, enfrentada al problema técnico, cómo modificar o adaptar el estado de la técnica más cercano para resolver el problema, de la forma reivindicada, sin realizar una actividad inventiva*”⁶ (énfasis fuera de texto).

Teniendo en cuenta los lineamientos que la misma SIC ha establecido en su Guía, consideraré a D1 como el estado de la técnica más cercano a la invención, en tanto éste describe una insulina Lispro que comprende dos cadenas de aminoácidos A y B semejantes a las secuencias de SEQ ID NO: 1 y 3 de la presente invención, y que además comprende puentes disulfuro entre las cisteínas ys7A-Cys7B, Cys20A-Cys19B, y Cys6A-Cys11A, como en la presente invención.

B. Determinar la diferencia entre la invención y el estado de la técnica más cercano.

Sin embargo, y como lo he mencionado en anteriores oportunidades, D1 corresponde a una publicación académica que realiza una revisión general de datos farmacológicos y clínicos de una clase de insulina lispro y su papel en el tratamiento de pacientes con diabetes mellitus cuando se administra en regímenes que implican dos inyecciones diarias. La insulina lispro divulgada en D1 **no está PEGilada**, mientras que la insulina reclamada en la presente invención sí está pegilada.

C. Definir el efecto técnico causado y atribuible al elemento diferencial.

⁵ Ibid., página 132.

⁶ Ibid., páginas 133-134.

Es bien sabido por la persona medianamente versada en la materia que la insulina lispro de D1 corresponde a una insulina **preprandial**, esto es, que se administra antes de que el paciente vaya a ingerir su comida, la cual, además, presenta la **acción más rápida** disponible actualmente. Así, la insulina de D1 debe administrarse antes de las comidas para controlar los incrementos de los niveles de glucosa después de comer. Sin duda alguna, D1 menciona las características ventajosas de la insulina lispro allí divulgada, como un tiempo de acción corto, un pico más alto de concentración de insulina en suero, y una aparición de este pico en un menor tiempo en comparación con la insulina normal. Particularmente, D1 enseña que la duración de la acción de la insulina lispro es 60% más corta en comparación con la insulina normal, un pico de concentración en suero dos veces mayor, y una aparición de este pico a los 42 minutos de la administración (mientras que la insulina regular tiene este pico a los 101 minutos). Por ende, resulta claro que la insulina de D1 posee ventajas indiscutibles para una **insulina preprandial de acción rápida**.

Por el contrario, la insulina de la presente invención corresponde a una insulina novedosa **basal** de **acción lenta**, la cual provee niveles estables de insulina por periodos prolongados de tiempo, incluyendo el tiempo que hay entre inyecciones prandiales de insulina. La insulina de la presente invención, de manera opuesta a la de D1, posee una vida media mayor que 24 horas, en donde, al **administrarse una vez al día**, presenta un perfil de concentración de insulina en suero relativamente llano, sin variaciones pico-valle. Más específicamente, y como se demuestra en la declaración del Dr. John Michael Beals (ANEXO 1), perteneciente al grupo de inventores de la presente invención, la invención tal y como se reclama presenta un perfil farmacocinético **totalmente diferente** a las características de la insulina lispro de D1, lo que resulta en efectos sorprendentes e inesperados, permitiendo el uso de la presente invención como una **insulina basal de acción prolongada**. Particularmente, de la declaración del Dr. Beals se puede concluir lo siguiente:

- el Ejemplo 11 y la Figura 1 de la presente invención muestran que la insulina de la presente invención, pegilada con un PEG de 20kDa y 40kDa, muestra un comportamiento de concentración en suero llano a través del tiempo, con relaciones de picos y valles de 1.1 y 1.2 (donde un comportamiento totalmente llano es 1.0), mientras que la insulina detemir presenta una relación de 11.9, para una dosis diaria del agente activo;
- la pegilación con PEGs de distintos pesos moleculares en la insulina de la presente invención tiene un efecto en el comportamiento casi invariable, y ampliamente distinto al comportamiento de la insulina detemir;

- este comportamiento se corroboró en estudios clínicos con 32 pacientes, donde se observó una relación de alrededor de 1.5, lo cual se encuentra consignado en el documento "*Steady-State pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus*"⁷, que se anexó junto con mi respuesta al segundo examen de fondo, y que se anexa nuevamente como ANEXO 2 (en adelante, Sinha et al);
- sorprendentemente, la insulina de la presente invención puede ser utilizada para dosis diarias con una acción de 24-44 horas, que en un estudio clínico posterior se determinó que esta ventana de acción va de 44.5-72.2 horas (ver Sinha et al);
- es decir, es posible utilizar la insulina de la presente invención en el tratamiento de pacientes con diabetes de manera segura, manteniendo un riesgo mínimo y buen control de hipoglucemia. Los resultados de la insulina pegilada de la presente invención **van en contravía de la opinión de expertos en el campo técnico**, pues como se evidencia en una noticia de mayo de 2009, el Dr. Abraham Abuchowsky afirmó⁸ que en sus trabajos con insulina no fue buena idea pegilar la proteína porque esto llevó irremediablemente a un choque de hipoglucemia;
- hasta la fecha, no se sabía que fuese posible obtener una insulina pegilada de larga duración que (a) pueda ser administrada a un paciente de forma segura sin riesgos significativos de hipoglucemia y (b) que tuviese un comportamiento hepato-preferencial aceptable, como se muestra en el artículo "*Novel PEGylated Basal Insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism*"⁹ que también presenté en respuesta al segundo examen de fondo, y que adjunto al presente Recurso como ANEXO 3 (en adelante, Moore et al);
- el Ejemplo 12 de la invención demuestra que la variabilidad de medidas glucodinámicas en pacientes se redujo a menos de un 30% en humanos, y un estudio clínico de fase I posterior demostró que la variabilidad del área bajo curva del perfil de glucosa en sangre se redujo en un 14% y la de glucosa en sangre en ayunas en un 26% (Sinha et al). Adicionalmente, en estudios

⁷ Sinha, V. P., et al. (2014). Steady-State pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus. *Diabetes, Obesity and Metabolism* 16, p. 344-350.

⁸ <http://scidstuff.wordpress.com/2009/05/29/biotech-pioneer-life-science-leader-may-2009/>

⁹ Moore, M. C., et al. (2014). Novel PEGylated Basal Insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism. *Diabetes*, 63, p. 494-504.

- clínicos fase II se observó una menor variabilidad de medidas glucodinámicas que al emplear la insulina glargina;
- se desarrollaron insulinas Lispro pegiladas con PEG de 5kDa, 10kDa y 12kDa, encontrándose que las dos primeras fueron excretadas a través de los riñones en roedores, pues no contaban con un tamaño hidrodinámico adecuado, y la última presentaba una relación de picos y valles de 89 en roedores y 4 en humanos, muy por encima de las relaciones logradas por la insulina aquí reclamada. De hecho, una insulina Lispro pegilada con PEG de 12kDa sería indiferenciable de productos ya disponibles como Lantus® y Levemir®;
 - la sorprendente acción hepatopreferencial de la insulina de la presente invención resulta de una reducida acción periférica, mas no de un efecto sobre el hígado en sí, y puede resultar benéfico para pacientes con una administración exógena, dado que la insulina reclamada tiene el tamaño hidrodinámico suficiente para restringir la actividad periférica mientras incorpora una actividad al receptor de insulina adecuada (resultando en actividad hepatopreferencial). Este comportamiento para una insulina basal no se conocía en el estado de la técnica antes de la presente invención;
 - La insulina reclamada presenta una superioridad en HbA1c, que fue demostrada en 6 pruebas de estudios clínicos fase III, que además resultó en una reducción significativa en hipoglucemia nocturna, más la pérdida de peso en los pacientes sobre los cuales se realizaron los estudios. Nuevamente, este resultado es opuesto a lo conocido en la técnica, donde se conocía que, para lograr HbA1c más bajo, inevitablemente se sufría de aumento de peso e hipoglucemia.

Teniendo en cuenta la evidencia técnica proporcionada, salta a la vista que, contrario a lo afirmado por el Despacho, la presente invención **no** mejora las propiedades farmacocinéticas de la insulina de D1, sino que, por el contrario, las elimina completamente, por lo que afirmar que la presente invención pretende mejorar las propiedades de la insulina de D1 (como lo afirmó el Despacho en la Resolución aquí recurrida) es infundado técnicamente. De manera radicalmente opuesta, la presente invención provee efectos que no se encuentran siquiera relacionados con el funcionamiento de la insulina de D1.

Asimismo, me permito resaltar que, contrario a la opinión del Despacho, la comparación de los efectos de la presente invención con insulinas como detemir y glargina es adecuada, en tanto éstas, al igual que la presente invención, **son insulinas basales de acción prolongada**. Por el

contario, la insulina lispro de D1 es preprandial de acción rápida, lo que no corresponde de ninguna manera con el comportamiento de la insulina aquí reclamada. De hecho, no se entiende cómo el Despacho desestimó la evidencia técnica proporcionada en los documentos Sinha et al y Moore et al, y la propia evidencia en el Capítulo Descriptivo, y además sugirió en la Resolución aquí recurrida la pertinencia de ciertos datos comparativos cuando lo que solicitó comparar es incomparable. Como expliqué, la insulina lispro de D1 no es comparable con la insulina de la presente invención, pues la primera es de administración preprandial y de acción rápida, mientras que la que aquí se reclama es basal de acción prolongada y de una única administración al día. Así, los datos y resultados proporcionados son los adecuados para evaluar las ventajas de la presente invención, pues se realizaron respecto a dos formas de insulina con los mismos objetivos: **insulinas basales de acción prolongada.**

Solicitar datos comparativos con insulina lispro es un indicador de que el efecto técnico de la presente invención no ha sido comprendido por la SIC durante todo el trámite de la presente solicitud, pues la pegilación adecuada de la insulina lispro transforma totalmente su comportamiento farmacocinético.

D. Deducir el problema técnico a solucionar.

Con base en lo anteriormente expuesto, el problema técnico de la presente invención no es simplemente la modificación de la insulina de D1 para mejorar su perfil farmacocinético, como erróneamente lo asevera el Despacho en la página 5, párrafo 4, de la Resolución no. 28784 aquí recurrida:

El problema técnico objetivo que pretende resolver esta invención se puede formular así: Cómo modificar la insulina lispro ([B28-L-lisina, B29-L-prolina]) divulgada en el documento D1 para conseguir un compuesto con propiedades farmacocinéticas mejoradas.

El planteamiento del problema técnico que hace la SIC es a todas luces errado pues no considera los efectos inesperados obtenidos por la presente invención y sus consecuencias, que consisten en la obtención de una insulina basal con una acción prolongada y eficiente, y que se puede administrar de manera segura a un paciente. De esta manera, el verdadero problema técnico que resuelve la presente invención consiste en **proveer una insulina que actúe de forma basal, prolongada, eficiente y segura.**

E. **Evaluar si la invención reivindicada, partiendo del estado de la técnica cercano y del problema técnico objetivo, habría sido obvia para la persona medianamente versada en la materia.**

i) Evidentemente, el arte previo citado por el Despacho no brinda las enseñanzas suficientes para que la persona medianamente versada en la materia desarrolle inequívocamente la presente invención. De hecho, partiendo del problema técnico mencionado anteriormente, se puede concluir que en vista que D1 hace referencia a una insulina que es conocida por su acción rápida y su administración preprandial, dicho documento no sería siquiera un buen punto de partida para resolver el problema técnico. Esto es, no existen razones por las cuales una persona medianamente versada en la materia con el objetivo de producir una insulina que actúe de forma basal y prolongada, que además sea eficiente y segura, partiría de un documento que enseña una insulina de acción rápida y administración preprandial. En este punto le **solicito comedidamente al Despacho** que, de insistir en su posición, **indique** las razones por las cuales la persona medianamente versada en la materia partiría de una insulina de administración preprandial y acción rápida en la búsqueda de producir una insulina basal de acción prolongada y una administración al día.

ii) Ahora bien, respecto al documento adicional del arte previo citado por la SIC en su examen de fondo, me permito inicialmente aclarar que D3 se refiere a derivados hidrofílicos polímero-modificados de insulina que son **útiles para entregar dicha insulina en los pulmones** con propiedades farmacocinéticas y/o farmacodinámicas mejoradas comparadas con insulinas nativas. Particularmente, los derivados pegilados de insulina de D3 muestran ejemplos de PEG que tienen pesos moleculares de 750, 2000 o 5000 Da, mientras que la presente invención reclama compuestos PEG con rangos desde 17,5 kDa hasta 25 kDa, lo cual resulta completamente alejado y diferente de lo sugerido en D3, y cuya diferencia en efectos fue demostrada anteriormente.

De hecho, D3 se encuentra en el campo técnico de la **pegilación de insulinas nativas** y los efectos relacionados con ello, más no en el campo técnico de la pegilación de insulinas lispro, como en la presente invención. De esta manera, D3 no guiaría de manera obvia a la persona medianamente versada en la materia a esperar inequívocamente que una insulina lispro pegilada como la de la presente invención fuese a obtener los resultados sorprendentes mencionados en el presente Recurso, y mucho menos cumplir con los pesos moleculares de PEG aquí reclamados, cuya influencia sobre los efectos biológicos de la insulina son significativamente determinantes.

A su vez, D3 hace referencia a insulinas nativas, estructuralmente diferentes a la insulina lispro que se pegila en la presente invención, donde esta última presenta configuraciones estructurales completamente diferentes de la insulina nativa, por lo que **no** es posible extrapolar y comparar directamente los hipotéticos resultados que tendría pegar una insulina nativa y una insulina lispro. Vale la pena recordarle al Despacho, una vez más, que las invenciones en el campo químico/biotecnológico corresponden a un arte impredecible, y por mucha información técnica que esté disponible, jamás podrán predecirse los efectos y resultados de hacer cambios estructurales en las moléculas, porque de ser así, los laboratorios de investigación no existirían, y los avances de la ciencia simplemente se harían con papel y lápiz.

iii) En vista de todo lo anterior, es claro que la persona medianamente versada en la materia, con base en D1 y D3, no encontraría sugerencia o motivación alguna a producir una insulina como en la presente invención, ni mucho menos predecir los resultados sorprendentes e inesperados elucidados por los inventores por medio de trabajo de experimentación exhaustiva. Por el contrario, y siguiendo las enseñanzas del arte previo citado por el Despacho, la persona medianamente versada en la materia habría obtenido a lo sumo, una insulina lispro como la de D1 pegilada como en D3, con PEG de bajos pesos moleculares, y habría resultado, como demostraron los inventores de la presente invención, en un producto de **poca eficiencia que se excreta por los riñones.**

iv) Ahora bien, en cuanto a D4, este documento enseña composiciones farmacéuticas de polipéptidos (y sus derivados, preferiblemente insulina monomérica) que comprenden un buffer TRIS-fosfato, zinc, un conservante fenólico y un agente de isotonicidad como glicerol. Por lo tanto, aunque D4 mencione insulina lispro, dicho documento no enseña ni mucho menos sugiere insulinas lispro pegiladas o derivadas de la misma, los pesos moleculares de PEG reclamados en la presente invención, y mucho menos los efectos sorprendentes e inesperados de (1) cambio total en el comportamiento farmacocinético en comparación con una insulina lispro, y (2) superioridad sorprendente e inesperada en comparación con otras insulinas basales de larga duración como detemir y glargina.

b- Ahora bien, vale la pena señalar que de acuerdo con el Manual Andino de Patentes (MAP), las invenciones en el campo de la química/biología tienen nivel inventivo cuando el compuesto reclamado tiene una **estructura inesperada** (no derivable por la persona medianamente versada en la materia) **o** cuando presentan un **uso o efecto sorprendente**.¹⁰ Por tales razones, es claro que en el presente caso, no sólo las características estructurales de la

¹⁰ Manual Andino de Patentes, ISBN-9978-43-855-6. Sección 10.8.1. Páginas 82.

insulina reclamada son inesperadas, sino también que los efectos técnicos logrado por la misma, lo que es una clara evidencia de nivel inventivo.

Adicionalmente, me permito señalar que no puede ser aceptado el hecho de que la presente invención no es inventiva por el simple hecho de que el arte previo (particularmente D3) mencione que es posible llevar a cabo la pegilación con moléculas de PEG de distintos pesos moleculares como señala la SIC:

El documento D3 divulga compuestos pegilados de insulina modificada, donde el PEG es de alto peso molecular de 15000Da, 20000Da, 25000Da, con el propósito de disminuir la biodisponibilidad de la insulina e incrementar el tiempo de vida media, cuando la composición es un inyectable (Pág. 3, párr. [0022]; pág. 6, párr. [0085] – [0086]; pág. 7, párr. [0090] – [0091]).

Al revisar detenidamente D3, y como lo he argumentado anteriormente, se encuentra que este documento sólo hace mención a los PEGs de alto peso molecular en el párrafo [0086], pero no brinda ninguna divulgación o enseñanza adicional que le permita a la persona medianamente versada en la materia obtener la insulina de la invención y esperar razonablemente todos los inesperados efectos ya mencionados.

Aceptar el estándar de nivel inventivo del Despacho equivaldría a desdibujar el objetivo principal del sistema de patentes, que es precisamente fomentar la investigación y desarrollo de mejoras incrementales. Nada se inventa de la nada. Necesariamente, la tecnología avanza en pasos discretos constituida por mejoras concretas a lo que se conoce. El sistema de patentes recompensa al inventor que proporciona una solución que no resultaría previsible a partir del estado de la técnica. Lo anterior además quiere decir que el resultado inventivo no podría ser desarrollado sin algún grado de experimentación o que presente resultados inesperados.

En este sentido, el Tribunal Andino de Justicia (TAJ) ha determinado que la divulgación del arte previo utilizada para desvirtuar la patentabilidad de una invención, debe ser lo suficientemente detallada para que una persona medianamente versada en la materia pueda usar dicha información para reproducir y explotar la invención sin acudir a experimentación adicional alguna¹¹.

Igualmente, me permito señalarle respetuosamente al Despacho que de acuerdo con su Guía de Patentes, *“Una vez formulada una nueva idea, a menudo se puede demostrar teóricamente el modo en el que se podría llegar a ella, a partir de algo conocido, a través de una serie de etapas aparentemente fáciles. El examinador de patentes debe evitar el uso de un análisis ex post*

¹¹ Proceso 06-IP-89 (<http://intranet.comunidadandina.org/Documentos/Procesos/209-IP-2005.doc>); y Proceso 36-IP-2004 (<http://www.notinet.com.co/serverfiles/servicios/archivos/30jul04/ca36-04.htm>)

OLARTEMOURE

OLARTE MOURE & ASOCIADOS LTDA.

facto de este tipo¹² (énfasis fuera de texto). Este análisis retrospectivo se hace a todas luces evidente, especialmente cuando el Despacho:

- (1) considera que una insulina **preprandial de acción rápida**, como la de D1, es un buen punto de partida para desarrollar una insulina **basal de acción prolongada**, como en la presente invención; y
- (2) rechaza la validez de los datos comparativos presentados con anterioridad, sin tener en cuenta que tales datos comparan las propiedades farmacocinéticas de **diferentes insulinas basales de acción prolongada** (detemir y glargina) con la presente invención (que proporciona los mismos efectos de acción prolongada).

Es decir, el Despacho omite el hecho de que el arte previo citado (D1, D3 y D4) presenta los siguientes obstáculos que de ninguna manera son superados por sus divulgaciones y enseñanzas:

- ¿por qué una persona medianamente versada en la materia, en su interés de desarrollar mejores insulinas basales de acción prolongada, tomaría como base una insulina con un comportamiento farmacocinético totalmente opuesto al deseado?
- ¿por qué esta persona esperaría que pegilar la insulina lispro de la presente invención resultaría en efectos farmacocinéticos, biológicos y clínicos sorprendentemente superiores a los de otras insulinas basales de acción prolongada?
- ¿por qué la persona medianamente versada en la materia pegilaría dicha insulina de una forma distinta a lo realmente enseñado y sugerido en el arte previo (D3)?
- ¿cómo habría esperado dicha persona que no seguir las enseñanzas claras de D3 le permitiría obtener una insulina lispro basal de acción prolongada con sorprendentes comportamientos farmacocinéticos, biológicos y clínicos?
- ¿cómo habría sabido esta persona que pegilar con PEG de bajos pesos moleculares, como realmente sugiere D3, llevaría a una insulina con propiedades farmacocinéticas indeseadas y de excreción rápida?

¹² Guía para Examen de Solicitudes de Patente de Invención y Modelo de Utilidad de la SIC , 2014, sección 2.13.6.3, página 137 (disponible en http://api.sic.gov.co/Documentos/Guia_Examen_Patentes.pdf)

Ciertamente, la única respuesta a estos cuestionamientos es que se considere que la persona medianamente versada en la materia parte del conocimiento otorgado por la presente invención, lo cual no es aceptado por los lineamientos de la misma Guía de la SIC.

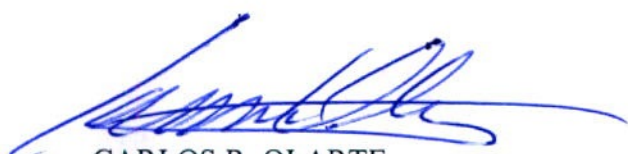
c- En conclusión, a la luz de la clara evidencia en favor del nivel inventivo de la presente invención suministrada anteriormente, la persona medianamente versada en la materia no habría encontrado sugerencia o motivación alguna que la condujera directamente a desarrollar la insulina reclamada, ni composiciones que la comprendan.

4. Para terminar, de conformidad con lo establecido en el artículo 80 del Código de Procedimiento 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 esa Superintendencia a adoptar la decisión que estime frente a la solicitud aquí formulada.

5. NOTIFICACIÓN

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:

- ANEXO 1: Copia de la Declaración jurada del Dr. John M. Beals presentada ante la Oficina de Patentes y Marcas de Estados Unidos (USPTO por sus siglas en inglés);
- ANEXO 2: Copia del artículo Sinha, V. P., et al. (2014). Steady-State pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus. *Diabetes, Obesity and Metabolism* 16, p. 344-350.

- ANEXO 3: Copia del Artículo Moore, M. C., et al. (2014). Novel PEGylated Basal Insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism. *Diabetes*, 63, p. 494-504.

Anexo I

16

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REISSUE PATENT APPLICATION

First Applicant:	John Michael BEALS et al.	Group Art Unit: 1646
Serial No.:	12/481,111	Examiner: Michael D. Pak
Application Date:	June 9, 2009	Conf No.: 4629
For:	PEGYLATED INSULIN LISPRO COMPOUNDS	
Docket No.:	X18199	

AFFIDAVIT PURSUANT TO 37 C.F.R. § 1.132

STATE OF INDIANA)
) SS:
COUNTY OF MARION)

I, John M. Beals, Ph.D., being first duly sworn, do hereby depose and say as follows:

[01] THAT, I am employed by Eli Lilly and Company, Indianapolis, Indiana, with the education and experience as described in my Curriculum Vitae appended as Attachment A which is incorporated herein by reference;

[02] THAT, I have been continuously employed since 1990 by Eli Lilly and Company in the following capacities: Sept. 1990 to Oct. 1994: Senior Research Scientist; Oct. 1994 to Oct. 1998: Principal Research Scientist; Oct. 1998 to Oct. 2000: Research Advisor; Oct. 2000 to Oct. 2004: Group Leader; Oct. 2004 to Oct. 2005: Senior Research Advisor; Oct. 2005 to Oct. 2009: Research Fellow; Oct. 2009 to Oc. 2011; Senior Research Fellow; Oct. 2011 to present: Distinguished Research Fellow;

X18199

[03] **THAT**, in these positions, I have been responsible for, among other things, technical direction of research efforts on bioproducts associated with diabetes, inflammation, cardiovascular disease, dementia, autoimmune disease, and hematopoiesis, and in particular, I have extensive experience with the development and analysis of products containing insulin, insulin analogs, and insulin derivatives as well as PEGylated biomolecules;

[04] **THAT**, upon information and belief, I am a co-inventor of subject matter described and claimed in U.S. Patent Application Serial No. 12/481,111, the application identified above (as used herein "the '111 application");

[05] **THAT**, I have authored or co-authored thirty-five publications in peer-reviewed journals and nine book chapters, and have presented my technical work both nationally and internationally at various academic and scientific venues;

[06] **THAT**, in particular, I am author or co-author on the following publications or presentations relating to insulin, insulin lispro, basal insulin peglispro, or LY2605541:

- a. Jacober SJ, Rosenstock J, Bergenstal RM, Prince MJ, Qu Y, Beals JM. (2014) "Contrasting weight changes with LY2605541, a novel long-acting insulin, and insulin glargine despite similar improved glycaemic control in T1DM and T2DM.", *Diabetes Obes Metab.* 2014 Apr;16(4):351-6.
- b. Moore MC, Smith MS, Sinha VP, Beals JM, Michael MD, Jacober SJ, Cherrington AD. (2014) "Novel PEGylated basal insulin LY2605541 has a preferential hepatic effect on glucose metabolism.", *Diabetes.* 2014 Feb;63(2):494-504
- c. Moore, M. C., Smith, M. S., Sinha, V. P., Beals, J. M., Michael, M. D., Jacober, S. J., and Cherrington, A. D. (2013) "Basal Insulin LY2605541 Has Hepato-Preferential Action Across a Range of Delivery Rates.", *Diabetes* July; 62, Supplement 1, A231.
- d. Topp, B. G., Geiser, J. S., Soon, D. K. W., Heise, T., Dodson, M. D., Jacober, S. J., Beals, J. M., Sinha, V. P. (2013) "Effects of a Novel Basal Insulin, LY2605541, on Hepatic Glucose Output and Muscle Glucose Uptake: A Physiologic Based Simulation Analysis.", *Diabetes* July; 62, Supplement 1, A231 LY2605541: Leveraging Hydrodynamic size to Develop a Novel Basal Insulin, American Diabetes Association June 2012;
- e. Hansen R.J., Cutler, G.B., Vick, A., Koester, A., Li, S., Siesky, A.M., Beals, J.M. (2012) LY2605541: Leveraging Hydrodynamic Size to Develop a Novel Basal Insulin.", *Diabetes* June vol. 61, Supplement 1, A228.
- f. Jacober, S.J., Rosenstock, J., Bergenstal, R.M., Prince, M.J., Qu, Y., Beals, J.M. (2012) "Contrasting Weight Changes With LY2605541, a Novel Long-Acting

X18199

- Insulin, and Insulin Glargine Despite Similar Improved Glycemic Control in T1D and T2D", *Diabetes* June vol. 61, Supplement 1, A262.
- g. Moore, M.C., Smith, M.S., Mace, K.F., Sinha, V.P., Michael, M.D., Jacober, S.J., Beals, J.M., Cherrington, A.D., "Novel Pegylated Basal insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism", *Diabetes* June vol. 61, Supplement 1, A417.
 - h. Owens, R.A., Lockwood, J.F., Dunbar, J.D., Zhang, C., Ruan, X., Kahl, S.D., and Beals, J.M. (2012) In Vitro Characterization of Novel Basal Insulin LY2605541: Reduced Mitogenicity and IGF-IR Binding.", *Diabetes* June vol. 61, Supplement 1, A425.
 - i. Kohn WD, Micanovic R, Myers SL, Vick AM, Kahl SD, Zhang L, Striffler BA, Li S, Shang J, Beals JM, Mayer JP, DiMarchi RD. (2007) "pI-shifted insulin analogs with extended in vivo time action and favorable receptor selectivity." *Peptides* 28(4), 935-48.
 - j. Varshavsky, A. D., Birnbaum, D. T., Beals J. M., and Saxberg, B.E.H., (2000) "Global stochastic optimization in hierarchical modeling of ligand/protein binding profiles", *Kybernetes* 29(4), 452-472.
 - k. Birnbaum, D. T., Kilcomons, M. A., DeFelippis, M. R., and Beals, J. M. (1997) "Hexameric kinetics of association and dissociation of a fast acting insulin analog: LysB28ProB29-insulin", *Pharmaceutical Research* 14, 25-36.
 - l. Bakaysa, D. L., Beals, J. M., Brader, M. L., Dodd, S. W., Edwards, S. L., Havel, H. A., Radziuk, J., and Brems, D. N. (1996) "Physicochemical basis for the rapid time-action of LysB28ProB29-insulin: Dissociation of a protein-ligand complex", *Protein Science* 5, 2521-2531.
 - m. Birnbaum, D. T., Dodd, S. W., Saxberg, B. E. H., Varshavsky, A. D., and Beals, J. M. (1996) "Hierarchical modeling of phenolic ligand binding in 2Zn-insulin hexamers", *Biochemistry* 35, 5366-5378.
 - n. Ciszak, E., Beals, J. M., Frank, B. H., Baker, J. C., Carter, N. D., and Smith, G. D. (1995) "Role of C-terminal B-chain residues in insulin assembly: The structure of hexameric LysB28ProB29-human insulin", *Structure* 3, 615-622.
 - o. Dodd, S. W., Havel, H. A., Kovach, P. M., Lakshminarayan, C., Redmon, M. P., Sargeant, C. M., Sullivan, G. R., and Beals, J. M. (1995) "Reversible adsorption of soluble hexameric insulin onto the surface of insulin crystals cocrystallized with protamine: An electrostatic interaction", *Pharmaceutical Research* 12, 60-68.
 - p. Hardaway, L. A., Brems, D. N., Beals, J. M., and McKenzie, N. E. (1994) "Amide hydrogen exchange of the central B-chain helix within the T & R states of insulin hexamers", *Biochim. Biophys. Acta* 1208, 101-103.

[07] **THAT**, upon information and belief, LY2605541, also referred to as "insulin peglispro", "basal insulin peglispro", or "BIL", corresponds to a site-specific, LysB28, covalently modified 20kDa PEG insulin lispro with the following Chemical Abstracts Index Name:

poly(oxy-1,2-ethanediyl), α -hydro- ω -methoxy-, 28^B-ester with 28^B-(N⁶-carboxy-L-lysine)-29^B-L-prolineinsulin (human)

which has the corresponding CAS registry number of 1200440-65-8, and has been assigned the United States Adopted Name (USAN) of insulin peglispro;

[08] **THAT**, upon information and belief, LY2605541 is a compound falling within the scope of the present invention;

[09] **THAT**, pegylated insulin lispro compounds, such as LY2605541, of the '111 application provide multiple advantages over previously known basal insulin compounds;

[10] **THAT**, one advantage is the flat pharmacokinetic ("PK") profile as demonstrated by low peak-to-trough ratios. Example 11 of the '111 application describes a projection of the mean flatness of the PK profile in humans with once daily dosing and Figure 1 illustrates a simulation of the flatness for a 20 kDa PEGylated insulin lispro compound or a 40 kDa PEGylated insulin lispro compound, derived from preclinical PK data, compared to the simulated PK profile for insulin detemir after a week of once daily dosing. The values shown in Figure 1 are the peak-to-trough ratios projected at steady-state, which describe the flatness of the curve or, alternatively the variability of plasma drug levels, from the peak (C_{max}) of one injection to the plasma nadir or trough prior to the next injection. The closer the peak-to-trough ratio gets to a value of one, the flatter the PK/PD profile becomes. The peak-to-trough values for the 40 kDa PEGylated insulin lispro (with its associated polydispersity), the 20 kDa PEGylated insulin lispro (with its associated polydispersity), and insulin detemir are 1.1, 1.2, and 11.9, respectively. Surprisingly, for once daily basal therapy, the peak-to-trough values for the 20 kDa and 40 kDa PEGylated insulin lispro compound were vastly improved relative to commercial insulin detemir and essentially undifferentiated from each other;

X18199

[11] **THAT**, the flatness of the PK profile has also been assessed in a clinical trial having 32 patients with type 2 diabetes mellitus completing the study, which upon information and belief was in accordance with accepted standards. After 14 days of treatment, patients treated with a LY2605541 had flat PK profiles as demonstrated by peak-to-trough ratios of ~1.5 at all dosage ranges tested, consistent with the pre-clinical simulations noted above (see, Sinha, V.P., et al. Steady-state Pharmacokinetics and Glucodynamics of the Novel, Long-acting Basal Insulin LY2605541 Dosed Once-daily in Patients with Type 2 Diabetes Mellitus. *Diabetes Obes Metab* 2014;16(4):344-50.);

[12] **THAT**, another advantage of the invention in the '111 application is that the site-specific, LysB28, covalently modified 17.5kDa to 40 KDa PEG insulin lispro compounds can be dosed via a once daily injection. As described in Example 12, page 46, lines 14-18, a clinical trial conducted in humans demonstrates that a compound of the present invention can provide an apparent half-life of at least 24 and 44 hours, which was later clinically established in a 14-day study in T2DM patients to be 44.7-75.5 hours (see, Sinha, V.P., et al. Steady-State Pharmacokinetics and Glucodynamics of the Novel, Long-Acting Basal Insulin LY2605541 Dosed Once-daily in Patients with Type 2 Diabetes Mellitus. *Diabetes Obes Metab* 2014;16(4):344-50). It was surprising that a molecule with duration of action exceeding 24hrs could be administered safely with minimal and readily controllable hypoglycemia. These results suggest that a safe and effective once daily dosing regimen using a compound of this invention, i.e., with half-life greater than 24 hrs, is possible for patients with diabetes, which is contrary to an expert's opinion regarding PEGylation of insulin (see, Cliff Mintz. *Biotech Pioneer Life Science Leader*, May 2009 at

<http://scidstuff.wordpress.com/2009/05/29/biotech-pioneer-life-science-leader-may-2009/>) wherein Dr. Abraham Abuchowski stated in an interview:

“[E]arly on, I PEGylated insulin and learned that increasing its circulation time was not such a good idea because it can easily lead to hypoglycemic shock.”

I believe what was not known at the time was (1) the ability to safely and gradually dose accumulating a basal insulin to steady-state levels with molecules having $t_{1/2} > 24$ hrs (see, Heise T, Meneghini LF. Insulin stacking versus therapeutic accumulation: understanding

the differences. *Endocr Pract.* 2014 Jan-Feb;20(1):75-83.) and (2) the hepatopreferential activity with concurrently restricted peripheral activity (see, Moore, M.C., et al. Novel PEGylated Basal insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism. *Diabetes* 2014;63:494-504; see, Henry, R.R., et al. Basal Insulin Peglispro Demonstrates Preferential Hepatic Versus Peripheral Action Relative to Insulin Glargine in Healthy Subjects. *Diabetes Care* 2014;37:2609-2616).

[13] **THAT**, low intra-patient variability observed using a compound of the present invention in the treatment of patients with diabetes suggests a further surprising advantage of the present invention. Some basal insulin preparations are known to have high within-patient variability. (see, Heise, T., et al. Lower Within-Subject Variability of Insulin Detemir in Comparison to NPH Insulin and Insulin Glargine in People with Type 1 Diabetes. *Diabetes* 2004; 53:1614-1620). As described in Example 12 of the '111 application, within-patient variability for glucodynamic measurements (PD variability), using pegylated lispro as prepared essentially as described in Example 9 of the '111 application, has been shown to be less than 30% in humans. Subsequent, analysis of the Phase 1 clinical trial results indicated that LY2605541 demonstrated the glucodynamic variability to be 14% (AUC of the blood glucose profile) and 26% (fasting blood glucose) (see, Sinha, V.P., et al. Steady-State Pharmacokinetics and Glucodynamics of the Novel, Long-Acting Basal Insulin LY2605541 Dosed Once-daily in Patients with Type 2 Diabetes Mellitus. *Diabetes Obes Metab* 2014;16(4):344-50). Moreover, in Phase 2 testing, LY265541 demonstrated that patients who were monitored by a continuous glucose monitor (CGM) experienced lower intra-day glucose variability compared to insulin glargine (see, Bastyr, E.J., et al. The Novel Long-Acting Insulin LY2605541 is Superior to Insulin Glargine in Lowering Intra-Day Glucose Variability and Hypoglycemia Event Rate from Continuous Glucose Monitoring (CGM) in Patients With Type 2 Diabetes. *Diabetes* 2012; 61(p A90): 346-OR). A basal insulin compound offering such low within-patient variability is a desirable advantage of the present invention.

[14] THAT, upon information and belief, during the development of the compounds described in the '111 application, a PEGylated insulin lispro compound having a lower molecular weight, i.e., ~12 kDa PEG, was also made and tested to determine whether a lower molecular weight PEG product could provide an equally flat PK profile relative to the data generated with PEGylated insulin lispro molecules with PEG sizes ranging from 17.5 kDa to 40 kDa. The 12 kDa PEGylated insulin lispro compound was tested in rats, using a 40 kDa PEGylated insulin lispro compound as a comparator. The peak-to-trough ratio of the 40 kDa compound in normal Sprague-Dawley rats was approximately 1.4, which translates to a predicted or simulated peak-to-trough ratio in humans, using allometric scaling, of approximately 1.1. However, the peak-to-trough ratio for the 12 kDa PEGylated lispro conjugate was predicted to be approximately 89 in rats or approximately 4 in humans. These data suggested that further study of the 12 kDa compound, as a true daily basal insulin was not warranted, as its properties were not as suitable to produce a best-in-class basal insulin, compared to the properties of the 17.5 to 40 kDa PEGylated insulin lispro compounds. Moreover, subsequent studies in the 5/6th-nephrectomized rat model revealed that PEGylated insulin lispro prepared with 5kDa or 10kDa PEG moieties (with the associated polydispersity) were subject to renal clearance; whereas, PEGylated insulin lispro prepared with 20 kDa or 40kDa PEG moieties (with the associated polydispersity) were not subject to renal clearance (see, Hansen R, Cutler G, Vick A, Koester A, Siesky A, Beals J: LY2605541: Leveraging Hydrodynamic Size to Develop a Novel Basal Insulin. 2012;61(Suppl 1):A228 [896-P]). These results illustrate that lower molecular weight PEGylated insulin species studied in the art (see, Hinds KD, Kim SW. Effects of PEG conjugation on insulin properties. Adv Drug Deliv Rev. 2002 Jun 17;54(4):505-30.) or PEG/alkane hybrids (see, Gordon Still J. Diabetes Metab Res Rev. 2002 Jan-Feb;18 Suppl 1:S29-37. Development of oral insulin: progress and current status.) would not have possessed the hydrodynamic size needed to exclude renal excretion as route clearance, which is necessary to create molecules with a peak-to-trough profile with a value of ≤ 1.5 . Surprisingly and noted earlier above, pegylated insulin lispro molecules created with PEG moieties >20kDa showed no further

X18199

improvement. The ability to utilize a single 20kDa PEG affords the generation of PEGylated insulin lispro species; wherein, the PEGylated insulin lispro degradation species can be excreted via renal and hepato-biliary routes (see, Webster R, Didier E, Harris P, Siegel N, Stadler J, Tilbury L, Smith D. PEGylated proteins: evaluation of their safety in the absence of definitive metabolism studies. *Drug Metab Dispos.* 2007 Jan;35(1):9-16. Epub 2006 Oct 4.), avoids the use of larger PEG moieties that are prone to the induction of vacuolization, and minimizes PEG load allowing for chronic administration of PEGylated insulin lispro at projected doses of 4.5 nmol/kg/day in T2DM patient (see, CHMP Safety Working Party's response to the PDCO regarding the use of PEGylated drug products in the paediatric population EMA/CHMP/SWP/647258/2012).

[15] THAT, in my opinion, a 12 kDa PEGylated insulin lispro molecule would be essentially undifferentiated in its time action profile and peak-to-trough and peripheral activity properties relative to existing therapies, such as Lantus® and Levemir®, offering no improvement in basal insulin therapy for patients;

[16] THAT, two Phase 1 studies assessing LY2605541 pharmacokinetics (PK) and glucodynamics (GD) in healthy subjects, which upon information and belief were conducted in accordance with accepted practice, surprisingly in my opinion, demonstrated a relatively flat serum concentration profile for 36 hours post-dose with $t_{1/2}$ values ranging from 24 to 75.5 hours, while in sharp contrast, serum concentrations for insulin glargine peaked at approximately 12 hours, with a $t_{1/2}$ of 12-14 hours. (see, Sinha, V.P., et al. Single-Dose Pharmacokinetics and Glucodynamics of the Novel, Long-Acting Basal Insulin LY2605541 in Healthy Subjects. *J. Clin. Pharm.* 2014;54(7):792-799);

[17] THAT, several studies evaluating endogenous glucose production and glucose disposal rate over a range of doses of LY2605541 and insulin glargine, which upon information and belief were conducted in accordance with accepted practice, reported that the hepatopreferential action of LY2605541 in anesthetized dogs (see, Moore, M.C., et al. Novel PEGylated Basal insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism. *Diabetes* 2014; 63:494-504) and subsequently in healthy subjects

X18199

(see, Henry, R.R., et al., Basal Insulin Peglispro Demonstrates Preferential Hepatic Versus Peripheral Action Relative to Insulin Glargine in Healthy Subjects. *Diabetes Care* 2014; 37:2609-2616), and that, surprisingly in my opinion, the hepatopreferential action results from a lesser peripheral action compared with insulin glargine, and not an accentuated or enhanced effect on the liver itself.

[18] **THAT**, in my opinion, this hepatopreferential action was unexpected and has been proposed that this could be beneficial to patients administering an exogenous insulin (see, Herring, R., et al. Hepatoselectivity and the Evolution of Insulin. *Diabetes Obes Metab* 2013;16:1-8). It is well accepted that exogenous insulin therapy results in hyperinsulinization of the periphery and underinsulinization of the liver relative to endogenous insulin (see, Herring, R., et al. Hepatoselectivity and the Evolution of Insulin. *Diabetes Obes Metab* 2013;16:1-8; Hordern, S.V.M., et al. Comparison of the Effects on Glucose and Lipid Metabolism of Equipotent doses of Insulin Detemir and NPH Insulin with a 16-h Euglycaemic Clamp. *Diabetologia* 2005;48:420-426). As of 2008, attempts to make a hepatopreferential insulin failed to deliver basal insulin molecules with an appropriate hepatic/peripheral activity profile with sufficient time-action. In my opinion, the only basal insulin molecule exploiting sufficient hydrodynamic size to restrict peripheral activity while concurrently incorporating appropriate insulin receptor activity to attenuate peripheral activity and thus enable a predominately hepatopreferential activity profile is LY2605541.

[19] **THAT**, basal insulin peglispro surprisingly, upon information and belief, minimizes clearance and alters absorption to create a much-needed therapeutic with (1) a sufficiently flat peak-to-trough profile when used with once daily dosing protocol, (2) an apparent $t_{1/2}$ ranging from 24-75.5 hours to reduce day-to-day within patient variability, (3) a surprisingly acceptable hypoglycemic profile, and (4) superiority in HbA1c for basal insulin peglispro in all six of the Phase III trials that were conducted against active comparators. (see, PRN Newswire, May 12, 2014, Lilly's Basal Insulin Peglispro Shows Superiority in HbA1c Reduction Compared to Insulin Glargine in Three Phase III Trials in Patients With Type 2 Diabetes.; see, PRN Newswire, September 4, 2014, Lilly's Basal Insulin Peglispro Demonstrated HbA1c Superiority against Lantus® in Phase III Trials in

Patients with Type 1 Diabetes). Moreover, upon information and belief, basal insulin peglispro demonstrated a rate of nocturnal hypoglycemia that was significantly lower in T1DM and T2DM patients taking basal insulin peglispro compared to those taking insulin glargine. Furthermore, upon information and belief, the Phase III data substantiated earlier Phase II data showing a statistically significant difference in weight in patients, with those T1DM patients taking basal insulin peglispro experiencing weight loss on the background of lower HbA1c, an indicator of better control, or those T2DM patients taking basal insulin peglispro experiencing comparable to statistically significant less weight gain compared to patients taking insulin glargine, wherein they experienced weight gain. In general, lower HbA1c necessitates more intensive insulin therapy, which with currently available insulin therapies, is associated with increased hypoglycemia and weight gain. Lastly, upon information and belief, in the Phase III T1DM trials, no statistically significant difference was observed in HbA1c between basal insulin peglispro dosed at the same time every day versus insulin peglispro dosed at variable times, something that, upon information and belief, has been impossible to show with NPH, insulin detemir, and insulin glargine, due to the half-lives being less than 24 hours; thus, making it impossible to dose-accumulate with QD dosing thereby resulting in products with large peak-to-trough values. The long plasma half-life of insulin peglispro produces a smaller peak-to-trough ratio and thus enables greater flexibility in dosing.

[20] THAT, a continuous glucose monitoring cohort from a Phase 2 study comparing LY2605541 with insulin glargine on hypoglycemia and glycemic variability in patients with type 2 diabetes, which upon information and belief was conducted in accordance with accepted practice, reported that LY2605541 treatment compared with insulin glargine resulted in fewer patients with hypoglycemic events and less time in the hypoglycemic range and was not associated with protracted hypoglycemia (see, Bergenstal, R.M., et al. Lower Glucose Variability and Hypoglycemia Measured by Continuous Glucose Monitoring With Novel Long-Acting Insulin LY2605541 Versus Insulin Glargine. *Diabetes Care* 2014; 37:659-665);

X18199

[21] **THAT**, upon information and belief, glycemic control with currently available insulins in patients with type 2 diabetes is often associated with weight gain and such weight gain can be associated with increased cardiovascular risk (see, Jansen, H.J., et al. Pronounced Weight Gain in Insulin-treated Patients with Type 2 Diabetes Mellitus is Associated with an Unfavorable Cardiometabolic Risk Profile. *J. Medicine* 2010;68(11):359-366 and Lee, M., Aronne, L. Weight Management for Type 2 Diabetes Mellitus: Global Cardiovascular Risk Reduction. *Am. J. Cardiology* 2007;99(4A): 68B-79B);

[22] **THAT**, in my opinion, all commercially available insulin products, when properly used to aggressively treat patients to target, i.e., HbA1c < 7%, are associated with insulin-induced weight gain and increased events of hypoglycemia;

[23] **THAT**, a long-acting basal insulin which does not in general produce weight gain, and that can result in weight loss or be weight neutral, is highly desired for treatment of patients suffering from diabetes;

[24] **THAT**, two independent Phase 2 studies in type 1 and type 2 diabetes evaluating weight changes with LY2605541 and insulin glargine, which upon information and belief were conducted in accordance with accepted practice, surprisingly in my opinion, reported modest weight loss and improved glycemic control with LY2605541, while modest weight gain and improved glycemic control were observed with insulin glargine. (see, Jacober, S.J., et al. Contrasting Weight Changes with LY2605541, a Novel Long-Acting Insulin, and Insulin Glargine Despite Similar Improved Glycemic Control in T1DM and T2DM. *Diabetes Obes. Metab.* 2014;16:351-356);

[25] **THAT**, a clinical study comparing effects of LY2605541 versus insulin glargine on daily mean blood glucose as part of a basal-bolus regimen for type 1 diabetes, which upon information and belief was conducted in accordance with accepted practice, surprisingly in my opinion, reported that LY2605541 had greater improvements in glycemic control, increased total hypoglycemia, and reduced nocturnal hypoglycemia, as well as reduced weight and lowered mealtime insulin doses. (see, Rosenstock, J., et al. Better Glycemic Control and Weight Loss with Novel Long-Acting Basal Insulin

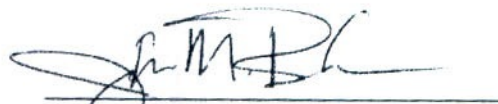
X18199

LY2605541 Compared With Insulin Glargine in Type 1 Diabetes. Diabetes Care 2013;36:522-528);

[26] **THAT**, in summary, weight loss with administration of LY2605541 as noted above, was in my opinion unexpected and surprising;

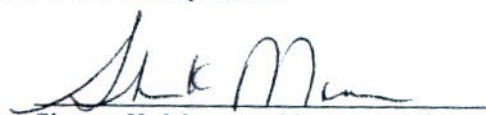
[27] **AFFIANT FURTHER STATES THAT**, in my opinion, the pegylated insulin lispro compounds claimed in the '111 application provide unexpected and surprising solutions to problems associated with known intermediate and basal insulin products as noted above, and these solutions are advantageous for patients in need of basal insulin therapy.

Further affiant sayeth not.


John M. Beals

On this 9th day of September 2014, before me personally appeared John M. Beals to me known and known to me to be the individual described in and who executed the foregoing instrument, and who thereupon acknowledged to me that he executed the same as a free act and deed for the uses and purposes therein expressed.




Sharon K. Moman - Notary Public
Resident of Marion County, Indiana

My commission expires July 10, 2020

X18199

ATTACHMENT A

Curriculum Vitae

John M. Beals, Ph.D.

Business
Lilly Research Laboratories
A Division of Eli Lilly and Company
Lilly Corporate Center
Indianapolis, IN 46285

Updated Tuesday, September 09, 2014

EDUCATIONAL RECORD:

8/1981 to 5/1987	University of Notre Dame, Notre Dame, Indiana Ph.D. Degree in Chemistry Field: Biochemistry GPA: 3.500 Advisor: Dr. Francis J. Castellino Thesis: <i>"I. Evaluation of the phosphatidylserine requirement of the intrinsic Factor X activating system. II. Homology and secondary structure in serine proteases."</i>
8/1977 to 5/1981	Loras College, Dubuque, Iowa B.S. Degrees in Biology and Chemistry GPA: 3.706, Magna Cum Laude Advisors: Dr. Edward T. Cawley and Dr. Kenneth Kraus Thesis: <i>"Water quality of the Catfish Creek watershed, 1980; An overview."</i>

EXPERIENCE:

10/2011 to Present	Distinguished Research Fellow Eli Lilly and Company, Indianapolis, IN BioTechnology Discovery Research <i>Responsible for the technical direction of a research team of ~60 scientists in the field of Diabetes. I participate in strategy groups, support the evaluation of potential in-licensing, serve on multiple committees that promote the advancement of the scientific staff and support external initiatives for the company.</i>
10/2009 to 2011	Senior Research Fellow Eli Lilly and Company, Indianapolis, IN BioTechnology Discovery Research <i>Responsible for the technical direction of a focused research team and research influence over a group of ~20 scientists in the field of</i>

X18199

diabetes. I participate in strategy group, support the evaluation of potential in-licensing opportunities and contributed to legal support of numerous patents.

10/2005 to
10/2009

Research Fellow
Eli Lilly and Company, Indianapolis, IN
BioTechnology Discovery Research: Biotherapeutics Optimization and Drug Delivery
Responsible for the technical direction of a focused research team with efforts directed at the optimization of protein therapeutics, development of novel assay systems, and evaluation of alternate bioproduct drug delivery methodologies. I was responsible for the establishment of research collaborations with Complex Biosystems and Ambrx. I actively participate on multiple strategy groups in Discovery and Development. In addition, I am supporting the evaluation of potential in-licensing opportunities and contributed to legal support of numerous patents.

10/2004 to
10/2005

Senior Research Advisor
Eli Lilly and Company, Indianapolis, IN
BioTechnology Discovery Research: Biotherapeutics Optimization and Drug Delivery
Responsible for the technical direction of a focused research team with efforts focusing on the optimization of protein therapeutics, development of novel assay systems, and evaluation of alternate bioproduct drug delivery methodologies. I played a pivotal role in the acquisition and integration of AME as well as initiated collaborative research programs at GlycoFi. I actively participated on multiple strategy groups in Discovery and Development. In addition, I am supporting the evaluation of potential in-licensing opportunities and contributed to legal support of numerous patents.

10/2000 to
10/2004

Research Advisor, Group Leader
Eli Lilly and Company, Indianapolis, Indiana
BioTechnology Discovery Research: Protein Optimization Team
Responsible for the technical direction of research efforts for a 23-member team (8 Ph.D.s and 15 associates) on internal bioproduct opportunities associated with diabetes, inflammation, cardiovascular disease, dementia, autoimmune disease, and hematopoiesis. Efforts focused on engineering properties into bioproducts that enhance the pharmaceutical attractiveness and usefulness of the bioproduct. In addition, I supported the evaluation and execution of research plans at AME, the evaluation

potential in-licensing opportunities and contributed to legal support of numerous patents. I participated in Discovery and Development Strategy Groups.

- | | |
|-----------------------|---|
| 10/1998 to
10/2000 | <p>Research Advisor
Eli Lilly and Company, Indianapolis, Indiana
bioResearch Technologies and Proteins: Protein Optimization Team
<i>Research focused on the rational development of a stable, new, non-glycosylated protein drug candidate for IND submission.</i></p> |
| 6/1997 to
10/1998 | <p>Principal Research Scientist
Eli Lilly and Company, Indianapolis, Indiana
bioResearch Technologies and Proteins: Protein Optimization Team
<i>Initial focus of my efforts was on the interviewing, hiring, and establishment of a world-class protein engineering team (25-members), referred to as the Protein Optimization Team. Research has focused on the rational development of a stable, new, non-glycosylated protein drug candidate for IND submission.</i></p> |
| 10/1994 to
6/1997 | <p>Principal Research Scientist
Eli Lilly and Company, Indianapolis, Indiana
Biopharmaceutical Product Development
<i>Research has focused on protein-protein and protein-excipient interactions in formulations for marketed product support, manufacturing process unit operations and their influence on protein formulation stability for marketed product support, structural properties of proteins for NDA submission, and pre-formulation characterization of a new protein drug candidate.</i></p> |
| 9/1990 to
10/1994 | <p>Research Scientist
Eli Lilly and Company, Indianapolis, Indiana
Parenteral Products Research and Development
<i>Research has focused on protein-protein and protein-excipient interactions as well as manufacturing process unit operations investigations to determine their influence on protein formulations.</i></p> |
| 4/1987 to
7/1990 | <p>National Institute of Health Postdoctoral Fellow
Cornell University, Ithaca, New York
Field: Biophysical Chemistry
Advisor: Dr. Harold A. Scheraga
<i>Research focused on the use of fluorescence energy transfer for the study of hydrophobic initiation sites in the protein folding in bovine RNase A.</i></p> |
| 8/1983 to
4/1987 | <p>Research Assistant, University of Notre Dame
<i>Research focused on the biophysical, structural, and kinetic characterization of the intrinsic Factor X activating system in the bovine coagulation system.</i></p> |

X18199

- 8/1981 to 8/1982 Teaching Assistant, University of Notre Dame
Responsibilities included instruction and supervision of undergraduate chemistry laboratories, administration and grading of examinations and laboratory reports.
- 1980 Summer Student Director of the 1980 Student-Oriented Studies Program, Loras College
Responsibilities included management of 10 person scientific research team and financial resources for a NSF grant to study the Catfish Creek Watershed in Dubuque Iowa.
- 1979 Summer Participant in the University of Illinois' Undergraduate Research Program in Engineering, Champaign, IL
Program provided independent research in the area of fiber reinforced concrete.

PUBLICATIONS:

1. Jacober SJ, Rosenstock J, Bergenstal RM, Prince MJ, Qu Y, Beals JM. (2014) "Contrasting weight changes with LY2605541, a novel long-acting insulin, and insulin glargine despite similar improved glycaemic control in T1DM and T2DM.", *Diabetes Obes Metab.* 2014 Apr;16(4):351-6.
2. Moore MC, Smith MS, Sinha VP, Beals JM, Michael MD, Jacober SJ, Cherrington AD. (2014) "Novel PEGylated basal insulin LY2605541 has a preferential hepatic effect on glucose metabolism.", *Diabetes.* 2014 Feb;63(2):494-504
3. Moore, M. C., Smith, M. S., Sinha, V. P., Beals, J. M., Michael, M. D., Jacober, S. J., and Cherrington, A. D. (2013) "Basal Insulin LY2605541 Has Hepato-Preferential Action Across a Range of Delivery Rates.", *Diabetes July; 62, Supplement 1, A231.*
4. Topp, B. G., Geiser, J. S., Soon, D. K. W., Heise, T., Dodson, M. D., Jacober, S. J., Beals, J. M., Sinha, V. P. (2013) "Effects of a Novel Basal Insulin, LY2605541, on Hepatic Glucose Output and Muscle Glucose Uptake: A Physiologic Based Simulation Analysis.", *Diabetes July; 62, Supplement 1, A231.*
5. Kharitonov, A., Beals, J. M., Coskun, T., Dunbar, J. D., Hansen, B.C., Bumol, T. F., and Moller, D. E. (2013) "Biological Profile of LY2405319, a Human FGF21 Variant and a Clinical Candidate", *Diabetes July; 62, Supplement 1, A262.*
6. Kharitonov A, Beals JM, Micanovic R, Striffler BA, Rathnachalam R, et al. (2013) "Rational Design of a Fibroblast Growth Factor 21-Based Clinical Candidate, LY2405319.", *PLoS ONE* 8(3): e58575. doi:10.1371/journal.pone.0058575.
7. Hansen R.J., Cutler, G.B., Vick, A., Koester, A., Li, S., Siesky, A.M., Beals, J.M. (2012) LY2605541: Leveraging Hydrodynamic Size to Develop a Novel Basal Insulin.", *Diabetes June vol. 61, Supplement 1, A228.*

X18199

8. Jacober, S.J., Rosenstock, J., Bergenstal, R.M., Prince, M.J., Qu, Y., Beals, J.M. (2012) "Contrasting Weight Changes With LY2605541, a Novel Long-Acting Insulin, and Insulin Glargine Despite Similar Improved Glycemic Control in T1D and T2D", *Diabetes* June vol. 61, Supplement 1, A262.
9. Moore, M.C., Smith, M.S., Mace, K.F., Sinha, V.P., Michael, M.D., Jacober, S.J., Beals, J.M., Cherrington, A.D., "Novel Pegylated Basal insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism", *Diabetes* June vol. 61, Supplement 1, A417.
10. Owens, R.A., Lockwood, J.F., Dunbar, J.D., Zhang, C., Ruan, X., Kahl, S.D., and Beals, J.M. (2012) In Vitro Characterization of Novel Basal Insulin LY2605541: Reduced Mitogenicity and IGF-IR Binding.", *Diabetes* June vol. 61, Supplement 1, A425.
11. Davis G.C., Beals J.M., Johnson C., Mayer M.H., Meiklejohn B.I., Mitlak B.H., Roth J.L., Towns J.K., Veenhuizen M. (2009) "Recommendations regarding technical standards for follow-on biologics: comparability, similarity, interchangeability." *Curr Med Res Opin.* 25(7) 1655-1661.
12. Kohn WD, Micanovic R, Myers SL, Vick AM, Kahl SD, Zhang L, Striffler BA, Li S, Shang J, Beals JM, Mayer JP, DiMarchi RD. (2007) "pI-shifted insulin analogs with extended in vivo time action and favorable receptor selectivity." *Peptides* 28(4) 935-48.
13. Beals J.M. and Shanafelt AB (2006) "Enhancing Exposure of Protein Therapeutics." *Drug Discov. Today Technol.* 3 (2006), pp. 87-94.
14. Huang L., Lu J., Wroblewski V.J., Beals J.M., Riggan R.M. (2005) "In Vivo Deamidation Characterization of Monoclonal Antibody by LC/MS/MS." *Anal Chem.* 77(5), 1432-1439.
15. Lu J., Witcher D.R., White M.A., Wang X., Huang L., Rathnachalam R., Beals J.M., Kuhstoss S. (2005) "IL-1beta Epitope Mapping Using Site-Directed Mutagenesis and Hydrogen-Deuterium Exchange Mass Spectrometry Analysis.", *Biochemistry* 44(33), 11106-14.
16. Darling R. J., Kuchibhotla U., Glaesner W., Micanovic R., Witcher D. R., Beals J.M. (2002) "Glycosylation of Erythropoietin Affects Receptor Binding Kinetics: Role of Electrostatic Interactions", *Biochemistry* 41(49), 14524-31.
17. Varshavsky, A. D., Birnbaum, D. T., Beals J. M., and Saxberg, B.E.H. (2000) "Global stochastic optimization in hierarchical modeling of ligand/protein binding profiles", *Kybernetes* 29(4), 452-472.
18. Zhang, F., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., Clawson, D. K., DiMarchi, R. D., Furman, T. C., Hale, J. E., Hsiung, H. M., Schoner, B. E., Smith, D. P., Zhang, X. Y., Wery, J.-P., Schevitz, R. W. (1997) "Crystal structure of the *obese* protein leptin-E100", *Nature* 387, 206-209.

X18199

19. Birnbaum, D. T., Kilcomons, M. A., DeFelippis, M. R., and Beals, J. M. (1997) "Hexameric kinetics of association and dissociation of a fast acting insulin analog: Lys^{B28}Pro^{B29}-insulin", *Pharmaceutical Research* 14, 25-36.
20. Bakaysa, D. L., Beals, J. M., Brader, M. L., Dodd, S. W., Edwards, S. L., Havel, H. A., Radziuk, J., and Brems, D. N. (1996) "Physicochemical basis for the rapid time-action of Lys^{B28}Pro^{B29}-insulin: Dissociation of a protein-ligand complex", *Protein Science* 5, 2521-2531.
21. Birnbaum, D. T., Dodd, S. W., Saxberg, B. E. H., Varshavsky, A. D., and Beals, J. M. (1996) "Hierarchical modeling of phenolic ligand binding in 2Zn-insulin hexamers", *Biochemistry* 35, 5366-5378.
22. Ciszak, E., Beals, J. M., Frank, B. H., Baker, J. C., Carter, N. D., and Smith, G. D. (1995) "Role of C-terminal B-chain residues in insulin assembly: The structure of hexameric Lys^{B28}Pro^{B29}-human insulin", *Structure* 3, 615-622.
23. Dodd, S. W., Havel, H. A., Kovach, P. M., Lakshminarayan, C., Redmon, M. P., Sargeant, C. M., Sullivan, G. R., and Beals, J. M. (1995) "Reversible adsorption of soluble hexameric insulin onto the surface of insulin crystals cocrystallized with protamine: An electrostatic interaction", *Pharmaceutical Research* 12, 60-68.
24. Hardaway, L. A., Brems, D. N., Beals, J. M., and McKenzie, N. E. (1994) "Amide hydrogen exchange of the central B-chain helix within the T & R states of insulin hexamers", *Biochim. Biophys. Acta* 1208, 101-103.
25. Beals, J. M., Haas, E., and Scheraga, H. A. (1991) "Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", *Biochemistry*, 30, 7680-7692.
26. Beals, J. M., Chibber, B. A. K., and Castellino, F. J. (1989) "The evaluation of the phosphatidylserine requirement in the factor X activating complex", *Arch. Biochem. Biophys.*, 268, 485-501.
27. Beals, J. M., Weber, J., Derwent, P., Grant, K. L., and Castellino, F. J. (1988) "Prediction of the secondary structures of bovine blood coagulation factor IX, factor X and prothrombin", *J. Protein. Chem.*, 7, 613-632.
28. Beals, J. M., and Castellino, F. J. (1988) "Circular dichroism analysis of the secondary structure of bovine blood coagulation factor IX, factor X, and prothrombin", *J. Protein. Chem.*, 7, 593-612.
29. Castellino, F. J. and Beals, J. M. (1987) "The genetic relationships between the kringle domains of human plasminogen, prothrombin, Tissue Plasminogen Activator, Urokinase and Coagulation Factor XII", *J. Mol. Evol.*, 26, 358-369.
30. Beals, J. M. and Castellino, F. J. (1986) "The interaction of bovine factor IX, its activation intermediate, factor IX α , and its activation products, factor IX $\alpha\alpha$ and factor IX $\alpha\beta$, with acidic phospholipids of varying composition", *Biochem. J.* 236, 861-869.

X18199

31. Castellino, F. J., Sator de Serrano, V., Powell, J. R., Johnson, W. R., and Beals, J. M. (1986) "Examination of the secondary structure of the kringle 4 domain of human plasminogen", *Arch. Biochem. Biophys.* 247, 312-320.
32. Powell, J. R., Beals, J. M., and Castellino, F. J. (1986) "Secondary predictions of human plasminogen and the bovine prothrombin kringle loops", *Arch. Biochem. Biophys.* 248, 390-400.
33. Neven, L. G., Duman, J. G., Beals, J. M., and Castellino, F. J. (1986). "Overwintering adaptation of the stage beetle, *Ceruchus piceus*. removal of ice nucleators in winter to promote supercooling", *J. Comp. Physiol. B.* 156, 707-716.
34. Duman, J. G., Neven, L. G., Beals, J. M., Olson, K. R., and Castellino, F. J. (1985). "Freeze-tolerance adaptations, including haemolymph protein and lipoprotein nucleators in the larvae of the crane fly, *Tipula trivittata*", *J. Insect. Physiol.* 31, 1-8.
35. Cummings, H. S., Ploplis, V. A., Beals, J. M., and Castellino, F. J. (1984). "Interspecies cross reactivity of monoclonal antibodies to various epitopes of human plasminogen", *Arch. Biochem. Biophys.* 230, 306-315.

BOOK CHAPTERS

1. Beals, J. M. "Insulin Analogues Improving the Therapy of Diabetes" (2014) in *Successful Drug Discovery*, Volume 1, Eds. Fischer, J. and Rotella, D. P., Wiley-VCH, Weinheim, Germany.
2. Beals, J. M., DeFelippis, M. R., Kovach, P. M., and Jackson, J.A. (2013) "Insulin", in *Pharmaceutical Biotechnology*, 4th Edition., Eds. Robert D. Sindelar, Bernd Meibohm, Daan J. A. Crommelin, Springer-Verlag, Berlin.
3. Beals, J. M., DeFelippis, M. R., and Kovach, P. M. (2006) "Insulin", in *Pharmaceutical Biotechnology*, 3rd Edition., Eds. J. A. Crommelin, Robert D. Sindelar, Bernd Meibohm, CRC Press, Boca Raton, Florida.
4. Beals, J. M., Brader, M. L., DeFelippis, M. R., and Kovach, P. M. (2002) "Insulin", in *Pharmaceutical Biotechnology*, 2nd Edition, Eds. Crommelin, D. J. A. and Sindelar, R. D. Informa, Gubelstrasse, Switzerland.
5. Beals, J. M. and Kovach, P. M. (1997) "Insulin" in *Pharmaceutical Biotechnology for Pharmacists and Pharmaceutical Scientists*, Eds. D. J. A. Crommelin and R. D. Sindelar. Harwood Academic Publishers gmbh, Amsterdam.
6. Scheraga, H. A., Beals, J. M., Buckler, D. R., Haas, E., and Krausz, S. (1992) "Structure and dynamics of chain-folding-initiation-sites in ribonuclease A." in *Time-Resolved Laser Spectroscopy in Biochemistry III*. Ed. J. Lackowicz. Bellingham, WA: Society of Photo-Optical Instrumentation Engineers, 1640: 672-675.
7. Castellino, F. J., Urano, T., deSerrano, V. S., and Beals, J. M. (1990) "The structure and activation of human plasminogen", in *Lipoprotein(a)*, Chapter 6, 87-101, Academic Press, Inc.

X18199

8. Beals, J. M. (1987) "I. Evaluation of the phosphatidylserine requirement of the intrinsic factor X activating system. II. Homology and secondary structure in serine proteases", Ph. D. Thesis, University of Notre Dame, Notre Dame, IN.
9. Castellino, F. J., Chibber, B. A. K., Beals, J. M., and de Serrano, V. S. (1987) "The structure and activation of human plasminogen", in *Fundamental and Clinical Fibrinolysis*, eds. Castellino, F. J., et al., Elsevier Science Publishers B. V. (Biomedical Division).

PRESENTATIONS:

"Careers in Biochemistry", Beals, J.M., July 18, 2012, Rose Hulman Institute of Technology Operation Catapult Summer Science Camp Student Presentation, Indianapolis, Indiana.

"LY2605541: Leveraging Hydrodynamic Size to Develop a Novel Basal Insulin", Hansen R.J., Cutler, G.B., Vick, A., Koester, A., Li, S., Siesky, A.M., Beals, J.M., October 1-5, 2012, 48th European Association for the Study of Diabetes Meeting, Talk, Berlin, Germany.

"Contrasting Weight Changes With LY2605541, a Novel Long-Acting Insulin, and Insulin Glargine Despite Similar Improved Glycemic Control in T1D and T2D", Jacober, S.J., Rosenstock, J., Bergenstal, R.M., Prince, M.J., Qu, Y., Beals, J.M., October 1-5, 2012, 48th European Association for the Study of Diabetes Meeting, Poster, Berlin, Germany.

"Novel Pegylated Basal insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism" Moore, M.C., Smith, M.S., Mace, K.F., Sinha, V.P., Michael, M.D., Jacober, S.J., Beals, J.M., Cherrington, A.D., October 1-5, 2012, 48th European Association for the Study of Diabetes Meeting, Talk, Berlin, Germany.

"In Vitro Characterization of Novel Basal Insulin LY2605541: Reduced Mitogenicity and IGF-IR Binding.", Owens, R.A. Lockwood, J.F., Dunbar, J.D., Zhang, C., Ruan, X., Kahl, S.D., and Beals, J.M., October 1-5, 2012, 48th European Association for the Study of Diabetes Meeting, Talk, Berlin, Germany.

"LY2605541: Leveraging Hydrodynamic Size to Develop a Novel Basal Insulin.", Hansen R.J., Cutler, G.B., Vick, A., Koester, A., Li, S., Siesky, A.M., Beals, J.M., June 9-12, 2012, 72nd American Diabetes Association Meeting, Poster 896, Philadelphia, PA.

"Contrasting Weight Changes With LY2605541, a Novel Long-Acting Insulin, and Insulin Glargine Despite Similar Improved Glycemic Control in T1D and T2D", Jacober, S.J., Rosenstock, J., Bergenstal, R.M., Prince, M.J., Qu, Y., Beals, J.M., June 9-12, 2012, 72nd American Diabetes Association Meeting, Poster 1023, Philadelphia, PA.

"Novel Pegylated Basal insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism" Moore, M.C., Smith, M.S., Mace, K.F., Sinha, V.P., Michael, M.D., Jacober, S.J., Beals, J.M., Cherrington, A.D., June 9-12, 2012, 72nd American Diabetes Association Meeting, Poster 1609, Philadelphia, PA.

"In Vitro Characterization of Novel Basal Insulin LY2605541: Reduced Mitogenicity and IGF-IR Binding.", Owens, R.A. Lockwood, J.F., Dunbar, J.D., Zhang, C., Ruan, X., Kahl, S.D., and Beals, J.M., June 9-11, 2012, 72nd American Diabetes Association Annual Meeting, Poster 1643, Philadelphia, PA.

"The Role of Protein Optimization in Improving the Pharmaceutical Properties of Biotherapeutic Candidates.", Beals, J.M., January 23, 2010, WCBP CMC Strategy Forum, Washington, D.C.

"Career Path in Biochemistry: Personal Story", Beals J.M., January 16, 2010, Gordon Research Conference, Galveston, Texas.

"Careers in Biochemistry", Beals, J.M., July 22, 2009, Rose Hulman Institute of Technology Operation Catapult Summer Science Camp Student Presentation, Indianapolis, Indiana.

"Evolution of Insulin Therapy", Beals, J.M., April 21, 2009, Loras College, Dubuque, Iowa.

"Careers in Biochemistry." Beals, J.M. October 25, 2008, National Organization of Black Chemists and Chemical Engineers, Indianapolis, Indiana.

"Biologics as a Platform for Inflammation Therapies." Beals, J.M. September 21-24, 2008, 15th International Inflammation Research Association Conference, Session Chair, Chantilly, Virginia.

"Careers in Biochemistry", Beals, J.M., July 23, 2008, Rose Hulman Institute of Technology High School Summer Program, Indianapolis, Indiana.

"The Role of Protein Engineering in Improving Pharmaceutical Properties of Biotherapeutics." Beals, J.M., February 18, 2008, Loras College, Dubuque, Iowa.

"The Role of Protein Engineering in Improving Pharmaceutical Properties of Biotherapeutics." Beals, J.M., November 7, 2007, Keynote Speaker, Rose Hulman Institute of Technology 4th Annual IRC Undergraduate Student Research, Terre Haute, Indiana

"The Role of Protein Optimization in Improving the Pharmaceutical Properties of Biotherapeutic Candidates: A Discovery-based Focus to Stability Relevant to Development and Commercialization." Beals, J.M., July 15-19, 2007, Biochemical Engineering Meeting, Quebec, CA

"The Role of Protein Optimization in Improving the Pharmaceutical Properties of Biotherapeutic Candidates: A Discovery-based Focus to Stability Relevant to Development and Commercialization" Beals, J.M., June 13, 2007, The Crossroad of Biotechnology 12th Meeting, Montreal, Canada

"Glycoengineering of FGF-21 produces potent molecule with increased half-life.", Baldwin, D.B., Beals, J.M., Coble, K.A., Dunbar, J.D., Ford, A., Huang, L., Kharitonov, A., Koester, K., Li, S., Rathnachalam, R., Wroblewski, V., December 12-14, 2006, 2nd IBC's Protein Engineering for Biotherapeutics, San Diego, California.

X18199

"Protein Engineering for Functional Attributes: Improving Pharmacodynamic Properties." Beals, J.M., Dec 12-14, 2006, Session Chair at the 2nd IBC's Protein Engineering for Biotherapeutics, San Diego, California.

"Biopharmaceuticals and Follow-on Biologics" Beals, J.M., October 19, 2006, American Intellectual Property Law Association, Washington, D.C.

"Molecular Evolution of Epo Binding to an EpoR Mutein", Beals, J.M., September 27, 2006, Biochemistry Lecture, Loras College, Dubuque, Iowa

"Optimization of Protein-Protein Interactions by Directed Evolution through Yeast Display", Chacko, B.M., Darling, R.J., and Beals, J.M., January 15-20, 2006, 2006 Gordon Conference on Reversible Associations in Structural & Molecular Biology, Ventura, California

"Optimization of Protein-Protein Interactions by Directed Evolution through Yeast Display", Chacko, B.M., Darling, R.J., and Beals, J.M., July 30, 2005, 2005 Protein Society Annual Meeting, Boston, Massachusetts

"The Role of Protein Optimization in Improving the Pharmaceutical Properties of Biotherapeutic Candidates: A Discovery-based Focus to Stability Relevant to Development and Commercialization", Beals J.M and Kuchibhotla U., July 27-29, 2005, 2nd Annual Stability-Indicating Assays and Forced Degradation Studies for Proteins, Bethesda Maryland

"Optimization of Protein and Peptide Therapeutics through PEGylation", Beals, J.M., June 9, 2005, 2005 AAPS National Biotechnology Conference - PEGylation Short Course, San Francisco, California

"Characterization of In Vivo Degradation in Fc-Fusion Proteins using Electrospray Ionization Mass Spectrometry", Beals J.M., Glaesner W., Huang, L., and Koester A. June 5-9, 2005, 53rd ASMS Conference on Mass Spectrometry and Allied Topics, San Antonio, Texas

"Optimization of Protein and Peptide Therapeutics through PEGylation", Beals, J.M., April 25, 2005, Loras College, Dubuque, Iowa

"Understanding the Role of Electrostatics in Erythropoietin Receptor/Ligand System", Darling R. J., Kuchibhotla, U., and Beals, J. M., October 11, 2004, 5th Annual Local American Chemical Society Poster Session, DePauw University, Greencastle, Indiana.

"The Impact of PEGylation on Erythropoietin/Erythropoietin Receptor Binding Kinetics and *In Vitro* Biology", Minnicus, A., Millican, R. L., Darling, R. J., and Beals, J. M., October 11, 2004, 5th Annual Local American Chemical Society Poster Session, DePauw University, Greencastle, Indiana.

"Receptor Binding Kinetics of Erythropoietin are Affected by Glycosylation: Role of Electrostatic Interactions", Darling, R. J., Kuchibhotla, U., and Beals, J. M., October 11, 2004, 5th Annual Local American Chemical Society Poster Session, DePauw University, Greencastle, Indiana.

“Biochemistry-based Careers in Bioproduct R&D”, Beals, J.M., September 9, 2004, DePauw University, Greencastle, Indiana.

“Understanding the Role of Electrostatics in Erythropoietin Receptor/Ligand System”, Beals, J.M., September 9, 2004, DePauw University, Department of Chemistry and Biochemistry, Greencastle, Indiana.

“Understanding the Role of Electrostatics in Erythropoietin Receptor/Ligand System”, Darling R. J., Kuchibhotla, U., and Beals, J. M., January 25-30, 2004, Gordon Research Conference - Reversible Associations in Structural and Molecular Biology, Ventura, California.

“The Impact of PEGylation on Erythropoietin/Erythropoietin Receptor Binding Kinetics and *In Vitro* Biology”, Minnicus, A., Millican, R. L., Darling, R. J., and Beals, J. M., January 25-30, 2004, Gordon Research Conference - Reversible Associations in Structural and Molecular Biology, Ventura, California.

“IL-1 β Epitope Mapping Using Site-directed Mutagenesis, NMR and H/D Exchange Mass Spectrometry Analysis”, Lu, J., Kuhstoss, S., Kline, A., Witcher, D., White, M., Wang, X., Rathnachalam, R., Beals, J. M., and Huang, L., January 25-30, 2004, Gordon Research Conference - Reversible Associations in Structural and Molecular Biology, Ventura, California.

“Understanding the Role of Electrostatics in Erythropoietin Receptor/Ligand System”, Beals, J. M., Darling, R. J., and Kuchibhotla, K., October 15, 2002, Loras College, Dubuque, Iowa.

“Careers in Biochemistry”, Beals, J. M., October 15, 2002, 2002 ITAG Young Scholars' Conference, Loras College, Dubuque, Iowa.

“Understanding the Role of Electrostatics in Erythropoietin Receptor/Ligand System”, Beals, J. M., Darling, R. J., and Kuchibhotla, K., June 6, 2002, Keynote Talk at the University of Notre Dame Chemistry and Biochemistry Annual Retreat, Pokagon State Park, Algona, Indiana.

“Scientific Career Opportunities at Lilly”, Beals, J. M., June 6, 2002, University of Notre Dame Chemistry and Biochemistry Annual Retreat, Pokagon State Park, Algona, Indiana.

“Understanding the Role of Electrostatics in Erythropoietin Receptor/Ligand System”, Beals, J. M., Darling, R. J., and Kuchibhotla, K., April 19, 2002, Loras College, Dubuque, Iowa.

“Scientific Career Opportunities at Lilly”, Beals, J. M., April 19, 2002, Loras College, Dubuque, Iowa.

“Receptor Binding Kinetics of Erythropoietin are Affected by Glycosylation: Role of Electrostatic Interactions”, Darling, R. J., Kuchibhotla, U., and Beals, J. M., February 10-15, 2002, Gordon Research Conference on Reversible Associations in Structural and Molecular Biology, Ventura, California.

X18199

"A Researcher's View of Statistical Design of Experiments for Assessing Biophysical Properties of Biopharmaceutical Formulations", Beals, J. M., May 19-21, 1997, Midwest Biopharmaceutical Statistics Workshop, Muncie, Indiana.

"Crystal Structure of the obese Protein Glu¹⁰⁰hOB", Zhang, F., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., Clawson, D. K., DiMarchi, R. D., Furman, T. C., Hale, J. E., Hsiung, H. M., Schoner, B. E., Smith, D. P., Zhang, X. Y., Wery, J.-P., Schevitz, R. W., July 19-25, 1997, American Crystallization Association Meeting, St. Louis, Missouri.

"Structural Studies of LisPro-insulins", Ciszak, E., Frank, B. H., Beals, J. M., Yip, C. M., DeFelippis, M. R., Smith, G. D., July 19-25, 1997, American Crystallization Association Meeting, St. Louis, Missouri.

"Formation and Disruption Kinetics of Cobalt-Insulin Hexamers: Ligand/Anion Binding and Cooperativity", Birnbaum, D. T., Kilcomons, M. A., Beals, J. M., and DeFelippis, M. R., July 8-12, 1995, 9th Symposium of The Protein Society, Boston, Massachusetts.

"Reversible Adsorption of Soluble Hexameric Insulin onto the surface of Insulin Crystals Cocrystallized with Protamine: An Electrostatic Interaction", Dodd, S. W., Havel, H. A., Kovach, P. M., Lakshminarayan, C., Redmon, M. P., Sargeant, C. M., Sullivan, G. R., and Beals, J. M., May 22, 1995, American Association of Pharmaceutical Scientists Midwest Regional Meeting, Chicago Illinois.

"The Structure of Lys^{B28}-Pro^{B29}-Insulin", Frank, B. H., Baker, J. C., Beals, J. M., Carter, N. D., Ciszak, E., Pekar, A. H., and Smith, G. D., November 6, 1994, International Diabetes Federation, Kobe Japan.

"Adsorption of Soluble Insulin Hexamer onto the Surface of Insulin Crystals Cocrystallized with Protamine", Beals, J. M., Dodd, S. W., Havel, H. A., Lakshminarayan, C., Redmon, M. P., Sargeant, C. M., Sullivan, G. R., and Kovach, P. M., August, 1994, 49th Annual Calorimetry Conference; Santa Fe, New Mexico.

"Isothermal titrating calorimetry study of phenolic ligand binding in 2Zn- and 2Co-insulin hexamers in the presence of chloride ion", Beals, J. M., Birnbaum, D. T., Dodd, S. W., Varshavsky, A. D., and Saxberg, B. E. H., August, 1994, 49th Annual Calorimetry Conference; Santa Fe, New Mexico.

"Mathematical modeling and algorithms for phenolic ligand binding to insulin hexamer: Model refinement to optimize fit for reduced dimensionality", Varshavsky, A. D., Beals, J. M., and Saxberg, B. E. H., August, 1994, 49th Annual Calorimetry Conference; Santa Fe, New Mexico.

"The structure of LysPro-insulin", Ciszak, E., Smith, G. D., Baker, J. C., Beals, J. M., Carter, N. D. and Frank, B. H., June, 1994, American Crystallographic Association, Atlanta, Georgia.

"Protein-protein and protein-ligand interactions in NPH formulations", Beals, J. M., March, 1994, Loras College, Dubuque, Iowa.

"Pharmaceutical Opportunities for Biochemists", Beals, J. M., March, 1994, Loras College, Dubuque, Iowa.

"T→R transition in 2Zn-insulin hexamer: Development of the algorithm for modeling the cooperative binding of preservative", Varshavsky, A. D., Saxberg, B. E. H., Dodd, S. W., and Beals, J. M., 48th Annual Calorimetry Conference, July, 1993, Durham, North Carolina.

"T→R Transition in 2Zn-Insulin Hexamer: Analysis of Cooperative Preservative Binding from Isothermal Calorimetry Data", Beals, J. M., Dodd, S. W., Varshavsky, A. D., and Saxberg, B. E. H., 48th Annual Calorimetry Conference, July, 1993, Durham, North Carolina.

"Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", Beals, J. M., Zymogenetics, Inc., April, 1990, Seattle, Washington.

"Pharmaceutical Opportunities for Biochemists", Beals, J. M., ACS Roadshow, Saginaw Valley State University, November, 1991, Saginaw, Michigan.

"Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", Beals, J. M., Eli Lilly and Company, January, 1990, Indianapolis, Indiana.

"Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", Beals, J. M., Kraft Foods, January, 1990, Glenview, Illinois.

"Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", Beals, J. M., University of Cincinnati, February, 1990, Cincinnati, Ohio.

"Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", Beals, J. M., Merck Pharmaceutical, Inc., March, 1990, Rahway, New Jersey.

"Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", Beals, J. M., Proctor and Gamble, March, 1990, Cincinnati, Ohio.

"Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding-initiation-site", Beals, J. M., Oregon State University, April, 1990, Corvallis, Oregon.

"The role of surface charge in the assembly of coagulation factors on phospholipid vesicles", Chibber, B. A. K., Beals, J. M., and Castellino, F. J., International Symposium on Biochemical Role of Eukaryotic Cell Surface Macromolecules, January, 1987, New Delhi, India.

"Structural homology between bovine Christmas factor and Stuart factor", Beals, J. M., Johnson, W. R., Derwent, P. F., and Castellino, F. J., Joint Meeting of the American Society of Biological Chemists and the Division of Biological Chemistry of the American Chemical Society, June, 1986, Washington, D. C.

X18199

"Interaction of bovine factor IX and its activation intermediates and products with defined membranes of varying composition", Beals, J. M. and Castellino, F. J., Meeting of the American Society of Biological Chemists, April, 1985, Anaheim, California.

ISSUED PATENTS:

US PATENT 7491697, 2009/02/17, ELI LILLY AND COMPANY, MUTEINS OF FIBROBLAST GROWTH FACTOR 21

PUBLISHED PATENTS:

US2009312236A, 2009/06/09, ELI LILLY AND COMPANY, PEGYLATED INSULIN LISPRO COMPOUNDS

US2005014679A, 2002/12/12, ELI LILLY AND COMPANY, INSULIN MOLECULE HAVING PROTRACTED TIME ACTION

US2007100133A, 1998/11/30, ELI LILLY AND COMPANY, ERYTHROPOIETIC COMPOUNDS

US2005070692A, 2003/02/20, ELI LILLY AND COMPANY, ANTI-INTERLEUKIN-1 BETA ANALOGS

US2008108560A, 2003/02/21, ELI LILLY AND COMPANY, HETEROLOGOUS G-CSF FUSION PROTEINS

US20030100502 A1, 2003/05/29, ELI LILLY AND COMPANY, TREATING MUSCULOSKELETAL DISORDERS USING LP85 AND ANALOGS THEREOF

WO9152128A, 2008/6/18, ELI LILLY AND COMPANY, PEGYLATED INSULIN LISPRO COMPOUNDS

WO5061712A, 2003/12/10, ELI LILLY AND COMPANY, MUTEINS OF FIBROBLAST GROWTH FACTOR 21

WO2003076567 A2, 2003/09/18, ELI LILLY AND COMPANY, HETEROLOGOUS G-CSF FUSION PROTEINS

WO2003073982 A2, 2003/09/12, ELI LILLY AND COMPANY, ANTI-INTERLEUKIN-1 BETA ANALOGS

WO2003053339 A2, 2003/07/03, ELI LILLY AND COMPANY, INSULIN MOLECULE HAVING PROTRACTED TIME ACTION

WO220751 A2, 2002/03/14, ELI LILLY AND COMPANY, HYPERGLYCOSYLATED POLYPEPTIDES

X18199

WO0189450 A2, 2001/11/29, ELI LILLY AND COMPANY, TREATING MUSCULOSKELETAL DISORDERS USING LP85 AND ANALOGS THEREOF

WO0032772 A2, 2000/06/08, ELI LILLY AND COMPANY, ERYTHROPOIETIC COMPOUNDS

WO9923115 A1, 1999/05/14, ELI LILLY AND COMPANY, GLYCOSYLATED OBESITY PROTEIN ANALOGS

WO9828414 A1, 1998/07/02, ELI LILLY AND COMPANY, ANTI-OBESITY PROTEINS

WO9828335 A1, 1998/07/02, ELI LILLY AND COMPANY, ANTI-OBESITY PROTEINS

WO9816545 A1, 1998/04/23, ELI LILLY AND COMPANY, THERAPEUTIC PROTEINS

WO9807446 A1, 1998/02/26, ELI LILLY AND COMPANY, PROTEIN FORMULATIONS

WO9735620 A1, 1997/10/02, ELI LILLY AND COMPANY, FORMULATIONS OF OB PROTEIN

WO9726013 A1, 1997/07/24, ELI LILLY AND COMPANY, OBESITY PROTEIN FORMULATIONS

WO9726012 A1, 1997/07/24, ELI LILLY AND COMPANY, OBESITY PROTEIN FORMULATIONS

WO9726011 A1, 1997/07/24, ELI LILLY AND COMPANY, OBESITY PROTEIN FORMULATIONS

WO9726004 A1, 1997/07/24, ELI LILLY AND COMPANY, OBESITY PROTEIN FORMULATIONS

HONORS:

Scholarship Recipient - Loras College
Member of Delta Epsilon Sigma Honor Fraternity, Alpha Chapter - Loras College
Participant in National Science Foundation's Review Board for Undergraduate Research Programs, Denver, CO - Loras College, 1977

X18199

Presiding Member at the National Science Foundation's Winter Meeting for the Student-Oriented Studies Programs, Washington, D. C. - Loras College, 1978
American Heart Association's Paul A. Nicoll Fellowship - University of Notre Dame, 1984-1985
Arthur J. Schmitt Dissertation Year Fellowship - University of Notre Dame, 1985-1986
Rohm and Haas Graduate Student of the Year in Chemistry - University of Notre Dame, 1985-1986
National Institute of Health Postdoctoral Fellowship - Cornell University, 1987-1990
2500-Day Product Development Award - Eli Lilly & Co., 1995
Lilly Research Laboratories President's Recognition Award, 1995
Lilly Research Laboratories President's Recognition Award, 1997
Lilly Change the World Award, Q3'2002
Lilly Quality Advocate Award, Q4'2004
Lilly Research Laboratories President's Recognition Award, 2007
Lilly Quality Advocate Award, Q1, 2011

SOCIETIES:

American Chemical Society (1987-Present)
American Association for the Advancement of Science (1987-Present)
New York Academy of Science (1989-1992; 1998-2005)
Protein Society (2003-2005)

POSTDOCTORAL RESEARCH SUMMARY:

The principle aim of my research at Cornell focused on the identification of potential chain-folding-initiation-sites (CFIS) and studying their contribution to the refolding of Ribonuclease A (RNase A). My work utilized a tryptic digest fragment identified as O-T-16, a C-terminal 20 amino acid peptide of RNase A. Theoretical predictions suggest that this hydrophobic region of RNase A is a potential CFIS and possibly dictates the intra-molecular interaction necessary for rapid folding of RNase A. My results indicated the potential for a compact structure in O-T-16, with limited structural distributions observed under folding conditions.

My approach to this problem employed non-radiative energy transfer (NET) to ascertain distance constraints, from 15-70 Å, in O-T-16 under folding and unfolding conditions. This approach necessitated that site specific, stoichiometric chemical modifications be made to the peptide. The labeling involved extensive use of reverse-phase and ion-exchange HPLC. The inter-probe distances were determined by measuring the effects of non-radiative energy transfer on the fluorescence of the donor using both steady-state and lifetime decay experiments. The distance distributions in the structures, determined from the initial NET results, indicated that hydrophobic bonds play significant role in the expression of structure in this peptide under folding conditions.

X18199

My primary purpose for doing postdoctoral research in the area of protein folding was to learn more about protein structure in an attempt to assimilate and apply this information in the areas of protein-protein and protein-lipid interactions.

GRADUATE THESIS RESEARCH SUMMARY:

The principle aim of my graduate research at Notre Dame was to investigate the roles that acidic phospholipid, the protein cofactor, factor VIII_a, and protein structure of factors IX_a and X play in the assembly of the bovine intrinsic tenase complex. The study was an integrated one utilizing techniques used in both lipid and protein chemistry. My results showed that 1) domains of phosphatidylserine (PS) on the lipid surface are not necessary for vitamin-K dependent binding, 2) binding of factor IX_a and its activation products are PS concentration dependent, 3) two different kinetic models are required to explain activation of factor X in the presence and absence of factor VIII_a, and 4) the identification of factors IX and X as a+b proteins with secondary structure models determined using circular dichroism and a combined predictive algorithm.

The research approach required the isolation of blood coagulation factors VIII, IX, X, and XI purified to homogeneity using protein precipitation in addition to gel-permeation, ion-exchange, and affinity chromatography. These coagulation factors were activated and stabilized for binding studies and kinetic assays of the reconstituted factor X activating complex.

Phospholipid vesicles composed of phosphatidylserine and phosphatidylcholine were used as a lipid source. Physical properties of vesicles were defined in an attempt to understand the roles these properties play in the assembly of the complex. Properties studied included vesicle size, composition, and inner/outer head group ratios utilizing techniques of quasi-elastic light scattering (QELS), high performance liquid chromatography (HPLC), and fluorescence spectroscopy, respectively.

Calcium mediated binding studies of numerous blood coagulation factors to defined vesicles were performed using dynamic light scattering. In addition, the physical size of various proteins when bound to vesicles were determined using QELS. The kinetics of the reconstituted complex were studied utilizing a continuous coupled colorimetric assay.

The secondary structures of the protease and substrate of the tenase complex were studied utilizing a two-dimensional approach involving circular dichroism and empirical calculations. This technique integrated computerized algorithms that resulted in the development of an empirical predictive program designed for both micro- and mini-computers.

Finally, work not related to the above study, involved the development and use of monoclonal antibodies to study structure and function relationships in plasminogen. In addition, the secondary structural elements of the kringle domains of plasminogen, tissue plasminogen activator, and prothrombin were

Steady-state pharmacokinetics and glucodynamics of the novel, long-acting basal insulin LY2605541 dosed once-daily in patients with type 2 diabetes mellitus

V. P. Sinha¹, D. C. Howey¹, S. L. Choi¹, K. F. Mace¹ & T. Heise²

¹Eli Lilly and Company, Indianapolis, IN, USA

²Prof. Heise, Germany

Aims: To assess the pharmacokinetics (PK) and glucodynamics (GD) of LY2605541 in patients with type 2 diabetes mellitus.

Methods: This parallel-group, open-label, dose-escalation study examined the PK and GD of basal insulin LY2605541 after single and multiple dose administration. Fixed doses of LY2605541 (0.33–1.00 U/kg) were given once-daily (OD) for 14 days to insulin-treated patients with type 2 diabetes. A 24-h euglycaemic glucose clamp was conducted on days 1 and 14.

Results: PK steady state was achieved within 7–10 days and the peak-to-trough fluctuation was <2, translating to a nearly 'peakless' glucose infusion rate at steady state and with a duration of action of at least 24 h. Across dose levels $t_{1/2}$ ranged from 44.7 to 75.5 h (~2–3 days). As steady state was achieved, there were dose-dependent reductions in the prandial insulin dose and in fasting blood glucose, which decreased to 60–100 mg/dl across dose levels. Within-patient variability was <14 and <26% for the area under the concentration versus time curve (AUC) of the 8-point blood glucose profile and fasting blood glucose, respectively. The nocturnal glucose control between 03:00 and 09:00 hours was relatively unchanged. Mild hypoglycaemia was the most common adverse event.

Conclusions: In this Phase I study of fixed LY2605541 doses without titration, LY2605541 was well-tolerated and demonstrated a flat PK and GD profile accompanied by glucose normalization, prandial insulin dose reduction and no severe hypoglycaemia.

Keywords: insulin analogues, pharmacodynamics, pharmacokinetics, type 2 diabetes

Date submitted 6 May 2013; date of first decision 28 June 2013; date of final acceptance 30 September 2013

Introduction

Basal insulin, in combination with oral antidiabetes medications, has been a successful initial therapy option for patients with type 2 diabetes mellitus [1–4]. Its success can be measured by the ability to provide several desirable characteristics, including a solution-based formulation that can readily be integrated into various delivery devices; a prolonged activity profile that supports a once-daily dosing regimen; no pronounced peaks in activity that can induce hypoglycaemia; reproducible absorption from the subcutaneous tissues with minimal variation in blood glucose-lowering activity; and a weight-gain neutral or weight-loss profile. While improvements have been made in the pharmacokinetic/pharmacodynamic (PK/PD) properties of basal insulin over the last decade, currently available basal insulins still do not meet all desired characteristics. In particular, neutral protamine Hagedorn (NPH) insulin exhibits a marked peak in its PK profile [5–7], with the basal insulin analogues, insulin glargine and insulin detemir exhibiting a smaller peak [8]. Additionally, NPH, insulin glargine and insulin detemir do not always cover 24-h insulin needs of patients with once-daily

administration [7,9,10]. PD variation is observed in particular with either NPH or glargine [11].

The novel basal insulin analogue, LY2605541, is a long-acting insulin composed of insulin lispro covalently bound, via a urethane bond, to a 20-kDa polyethylene glycol (PEG) chain. LY2605541 has a large functional size and a prolonged duration of action. This extended activity is believed to be mediated through slower absorption and reduced renal clearance [12]. Additionally, the tissue distribution of LY2605541 is believed to be similar to endogenous insulin, with restricted peripheral activity and retained hepatic activity [13].

This study assessed the PK and glucodynamic (GD) profile of LY2605541 after single dose and multiple daily doses in patients with type 2 diabetes previously treated with insulin. Secondary objectives included assessing the safety, tolerability and intra-patient variability of LY2605541 PK and GD parameters.

Patients and Methods

Patients and Study design

This Phase I, open-label, parallel, four-arm, multiple-ascending dose study was completed at a single center in Germany between April 2009 and July 2009. The sample size of eight patients per cohort was expected to provide adequate power

Correspondence to: Vikram P. Sinha, U.S. Food and Drug Administration, 10203 New Hampshire Ave, Silver Spring, MD 20903, USA.
E-mail: Vikram.Sinha@FDA.HHS.gov

for parameter estimation and is consistent with other Phase I studies evaluating safety, PK and/or GD parameters. The study was approved by an ethical review board. All study procedures were carried out in compliance with the Declaration of Helsinki and Good Clinical Practices. All subjects provided written informed consent.

Eligible patients were men or women, 18–65 years of age (inclusive), with type 2 diabetes mellitus diagnosed at least 1 year prior to enrollment, and who were treated with basal and bolus insulin for at least 3 months prior to screening. Patients were required to have a glycosylated haemoglobin (HbA1c) $\leq 10\%$ and a fasting C-peptide level of ≤ 1.0 nmol/l at screening to prevent stimulation of endogenous insulin through glucose infusion during the clamp experiments. Patients were excluded if they used a total daily dose of basal insulin that exceeded 0.8 (1)U/kg/day had taken any glucose-lowering medications other than insulin for at least 3 months before the screening visit, had >1 episode of severe hypoglycaemia, or had received chronic systemic glucocorticoid therapy (excluding topical, intra-articular and inhaled preparations) in the past year, or any glucocorticoid therapy within 30 days prior to the screening visit.

Patients were assigned to 1 of 4 treatment cohorts of ascending LY2605541 dose. LY2605541 was administered as mg/kg (nmols/kg) with dose conversion to units (U) as follows: 0.025 mg $\approx$ 1 nmol and 9 nmols = 1 U of LY2605541. Given this conversion from mg/kg to U/kg, dose levels were 0.33 U/kg (Cohort 1), 0.67 U/kg (Cohort 2), 1.00 U/kg (Cohort 3) and 0.50 U/kg (Cohort 4). The planned dose in Cohort 4 was intended to be 1.33 U/kg; however, this dose was reduced due to the number of events of mild hypoglycaemia in Cohort 3.

Study Procedures

Patients were admitted to the Clinical Research Unit on day 2 and remained resident throughout the dosing period (days 1 through 14), until discharge on day 16. The last dose of basal insulin glargine or insulin detemir on day 2 was required to be administered prior to bedtime (23:00 hours at the latest) in order to safely avoid any carryover effects of long-acting insulin preparations. Patients were allowed to subcutaneously administer short-acting insulin (Humulin[®], Eli Lilly and Company, Indianapolis, IN, USA) for mealtime coverage on day 1. LY2605541 was administered subcutaneously, once daily in the morning on days 1–14. Upon discharge, patients were followed on an outpatient basis until at least 30 days after the

last dose of LY2605541. Immunogenicity testing was conducted approximately 30 days after the last dose of study drug.

Euglycaemic glucose clamps (24-h duration) were performed on days 1 and 14. Patients were fasted (except for water) from approximately 8 h prior to dosing until completion of the 24-h clamp period. Intravenous (i.v.) insulin infusion of regular human insulin (Humulin[®]) was started at least 4 h before administration of LY2605541 to achieve a target blood glucose level of 90 mg/dl (5.0 mmol/l). The goal was to achieve this target by at least 2 h before dosing so that the Humulin[®] infusion rate could be lowered to a level where the blood glucose remained stable and at the same time no glucose infusion was required. The i.v. insulin infusion was stopped completely 15 min before trial drug administration. After the administration of LY2605541, a 20% glucose solution was infused at a variable rate (via Biostat[™], Miles Laboratories, Elkhart, IN, USA) in order to maintain blood glucose at the clamp level of 90 mg/dl. The rate of glucose delivery was adjusted by the Biostat in response to changes in blood glucose at 1-min interval until 24-h post dosing.

Fixed meals were administered to patients on all inpatient days, except days 1 and 14. The daily caloric intake was 2200 kcal/day, which was increased to 2600 kcal/day in Cohort 3 to ensure stabilization of blood glucose values. An adjustable dose of short-acting bolus insulin was administered on an individual basis to achieve pre-prandial glucose values between 70 and 180 mg/dl. In the event that blood glucose concentrations fell below 60 mg/dl (confirmed by a repeat measurement), or in the event that a patient experienced symptoms consistent with hypoglycaemia, an adequate portion (at least 10 g) of carbohydrates were administered orally and blood glucose testing was performed for every 20 min until the blood glucose level rose above 70 mg/dl.

Serum samples for PK analysis were obtained on day 1 (pre-dose, 0.5, 2, 4, 6, 12 and 24 hours), day 2 (pre-dose), day 7 (pre-dose, 4, 8 and 12 h), days 10, 13 and 14 (pre-dose, 2, 4, 6, 8 and 12 h), days 15, 17, 19, 21, 23 and 25. Samples were analyzed using a validated LY2605541-specific, enzyme-linked immunosorbent assay (ELISA) method in which the lower limit of quantification was 75 pM and the upper limit of quantification was 900 pM. Samples above the limit of quantification were diluted and reanalyzed to yield results within the calibrated range. Serum samples for GD measures were additionally obtained during the clamp procedure and 8-point glucose profiles were performed daily from day 2 through day 16.

Table 1. Demographic and baseline characteristics.

	0.33 U/kg (n = 8)	0.50 U/kg (n = 8)	0.67 U/kg (n = 8)	1.00 U/kg (n = 8)
Age (years)	55.0 (42–65)	56.5 (50–64)	58.9 (47–65)	55.3 (49–62)
Gender, male, n (%)	8 (100)	6 (75)	8 (100)	8 (100)
BMI (kg/m ²)	31.4 (29.3–36.9)	30.4 (27.5–33.3)	32.0 (28.9–36.2)	30.2 (27.0–33.7)
Body weight (kg)	97.7 (81.5–123.6)	89.9 (69.5–105.1)	103.6 (89.5–114.5)	96.8 (86.9–106.6)
Duration of T2DM (years)	13.7 (7.1–21.8)	17.9 (7.3–28.3)	13.4 (9.0–21.3)	19.8 (12.9–39.3)
HbA1c (%)	7.3 (6.3–8.5)	8.2 (6.6–9.6)	8.1 (6.2–9.9)	7.4 (6.1–8.6)

Data are mean (range), with the exception of gender which is presented as number (n) and percentage (%) of male patients. BMI, body mass index; T2DM, type 2 diabetes mellitus; HbA1c, glycosylated haemoglobin.

Table 2. Summary of LY2605541 PK parameters following multiple once-daily doses in patients with type 2 diabetes.

PK parameter	0.33 U/kg n = 8		0.50 U/kg n = 8		0.67 U/kg n = 8		1.00 U/kg n = 8	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
$C_{max,ss}$ (pmol/l)	4090 (32)	4000 (19)	7250 (25)	5460 (22)	9210 (26)	8180 (11)	16000 (28)	12800 (33)
$C_{av,ss}$ (pmol/l)	3520 (31)	3210 (28)	6400 (25)	4420 (20)	7450 ^a (27)	7110 (10)	13400 (25)	10800 (33)
$AUC_{(0-12)ss}$ (pmol·h/l)	42200 (31)	38600 (28)	76800 (25)	53000 (20)	89400 ^a (27)	85300 (10)	161000 (25)	130000 (33)
$AUC_{(0-24)ss}$ (pmol·h/l)	—	75700 (24)	—	101000 (22)	—	169000 (13)	—	242000 (30)
$AUC_{(0-24)ss}$ (pmol·h/l)	—	75600 (24)	—	101000 (22)	—	168000 (13)	—	242000 (30)
$t_{max,ss}$ (h)	—	6.0 (2.0–24.0)	—	0.006 (0.0–12.0)	—	7.0 (2.0–12.0)	—	2.0 (0.0–4.0)
$t_{1/2,ss}$ (h)	—	67 (52.7–91.8)	—	64.5 (42.4–91.5)	—	44.7 (35.4–62.5)	—	75.5 (58.8–111)
PTF	—	1.56 (12)	—	1.48 (10)	—	1.42 (13)	—	1.49 (7)
CL _{ss} /F (l/h)	—	3.73 (27)	—	3.86 (16)	—	3.54 (20)	—	3.52 (32)
R _a	—	7.48 (46)	—	7.24 (57)	—	7.52 (52)	—	12.2 (44)

$AUC_{(0-12)ss}$, area under the concentration versus time curve from time zero to time 12 h at steady state; $AUC_{(0-24)ss}$, area under the concentration versus time curve from zero to 24 h during a dosing interval at steady state; $C_{av,ss}$, average drug concentration under steady state conditions based on the equation: $AUC_{(0-12)ss}/12$; CL_{ss}/F , apparent total body clearance of drug at steady state after extra-vascular administration; $C_{max,ss}$, maximum observed drug concentration at steady state; CV%, coefficient of variation; n, number of subjects; PK, pharmacokinetic; PTF, the peak-to-trough fluctuation ratio calculated based on the equation: $C_{max,ss}/C_{min,ss}$, where C_{max} and C_{min} are the maximum and minimum drug concentrations in the dosing interval, respectively; R_a, accumulation ratio calculated from $AUC_{(0-24)ss}/t_{1/2,ss}$; $t_{1/2,ss}$, half-life associated with the terminal rate constant (λ_z) in non-compartmental analysis at steady state; $t_{max,ss}$, time of maximum observed drug concentration at steady state.

^aThe geometric mean (CV%) C_{max} values on day 1 for the 0.33, 0.5, 0.67, and 1.0 U/kg dose groups were 628 (33), 949 (49), 1530 (43) and 1310 (62) pmol/l, respectively.

^bn = 7.

^cMedian (range).

^dIn Cohort 4 (the 0.50 U/kg dose level), all patients had a $t_{max,ss}$ of 00:00 hours, except one who had a $t_{max,ss}$ of 12 h; hence, the median $t_{max,ss}$ was 00:00 hours.

^eGeometric mean (range).

Safety was assessed via monitoring of adverse events (AEs), clinical laboratory values, physical examinations and electrocardiograms (ECGs). Additional blood samples were obtained for the determination of fasting blood glucose concentration and C-peptide concentration.

Pharmacokinetic and Glucodynamic Analysis

PK parameters were analyzed by conventional non-compartmental analysis using WinNonlin Enterprise Edition® software (version 5.01, Pharsight Corp., St. Louis, MO, USA). Serum insulin concentrations were used to calculate PK parameters, including maximum observed drug concentration (C_{max}), time of maximum observed drug concentration (t_{max}) and area under the concentration versus time curve (AUC). PK parameters were individually calculated for each patient. Log-transformed C_{max} and AUC were evaluated using a model to estimate dose-normalized geometric means for day 1, day 14 and ratio of day 14 over day 1 (with corresponding 90% confidence intervals). PK parameter estimates were evaluated to delineate dose proportionality at day 14 [14]. The serum concentration of LY2605541 was compared between days 1, 7 and 14 to assess the accumulation and wash-out effect. Within- and between-patient coefficient variation (CV) in PK parameters was assessed based on steady-state data.

For GD parameters, data were determined from the euglycaemic glucose clamp procedure, where the glucose infusion rate (GIR) over time was used as a measure of insulin effect. A locally weighted scatterplot smoothing (LOESS) function (smoothing parameter of 0.2) was applied to all individual GIR versus time profiles in each treatment group and/or period using S Plus® software (version 7.0.6, Insightful Corp., Seattle, WA, USA). The mean LOESS fits

from all patients in each treatment group was used for the generation of mean GIR plots over time whereas unsmoothed data was used to calculate GD parameters. The primary GD parameters, maximum GIR (R_{max}) and total glucose infusion over the clamp duration (G_{tot}) were log-transformed and compared between day 1 and day 14. Fasting and postprandial glucose concentrations were summarized over time to assess the accumulation and wash-out effect of LY2605541 on glucose.

Results

Patient Disposition and Demographics

The demographics and baseline (day 1) characteristics of the study population are summarized in Table 1. A total of 32 patients (30 male and 2 female) with type 2 diabetes were enrolled in the study with 8 patients enrolled into each of four dose cohorts. All patients received study treatment and completed the study. Age and body mass index (BMI) were comparable across all dose cohorts. All patients were Caucasian. The average duration of type 2 diabetes was approximately 16 years (range of 7–39 years).

Pharmacokinetic Evaluations

LY2605541 PK parameters across all dose levels are summarized in Table 2. The concentration-time profile suggests a relatively flat profile in steady state with a peak-to-trough fluctuation at day 14 of <2 across all dose levels. Concentrations increased with steady state being achieved 7–10 days after dosing (figures 1, 2). Trough concentrations at day 7 were higher than those on day 14; however, the average concentrations were in the range of those observed on day 14 indicating that

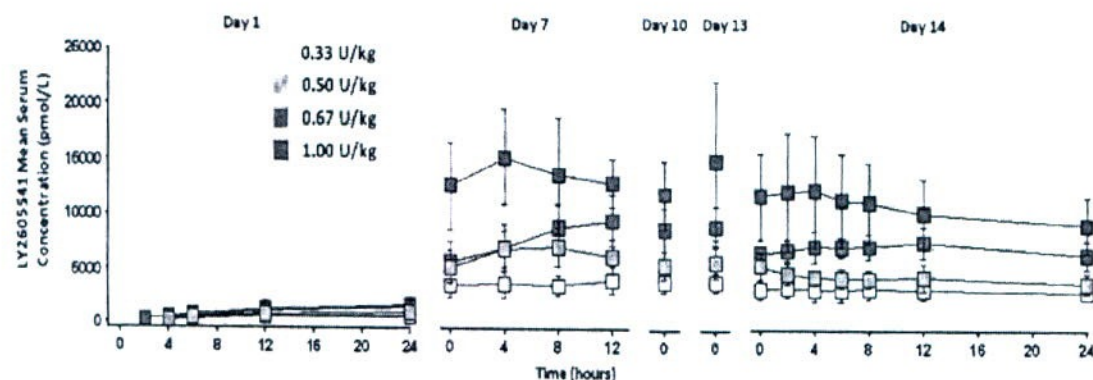


Figure 1. LY2605541 serum concentration-time profiles following single doses and multiple once-daily doses in patients with type 2 diabetes.

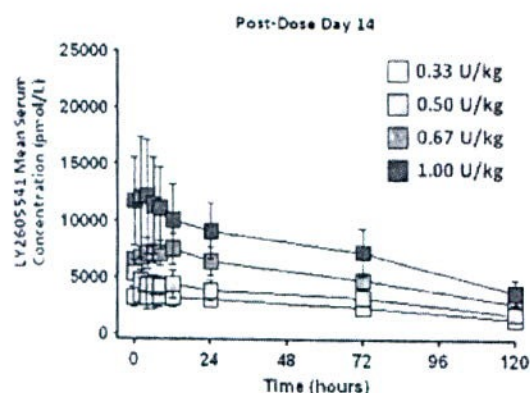


Figure 2. LY2605541 serum concentration-time profile post-dose day 14.

steady state had been reached by day 7. Monitoring of PK samples for 2 weeks postdosing showed an apparent half-life of 2–3 days (44.7–75.5 h).

A slightly more than proportional increase in PK parameter values was observed across the dose range of 0.33–1.00 U/kg as demonstrated by the ratio of dose-normalized means (R_{dnm}) for AUC [1.12 (CI 0.93, 1.36)] and C_{max} [1.09 (CI 0.91, 1.31)].

For all PK parameters, the within-patient variability was <35% [AUC_(0–24 h) of 34.32% and C_{max} of 29.35%] and the between-patient variability was <25% [AUC_(0–24 h) of 24.06% and C_{max} of 21.22%].

Glucodynamic Evaluations

LY2605541 GD parameters are shown in Table 3. Consistent with PK observations, administration of multiple once-daily dosing of LY2605541 resulted in an essentially peakless GIR profile at steady state. The glucose-lowering effect of LY2605541 was evenly distributed over the first and second 12 h after injection with AUC- G_{tot} 0–12 h/AUC- G_{tot} 12–24 h being 53.5% (8.97). There was a dose-dependent glucose effect on the GIR (figure 3). The similarity in G_{tot} between the 0.50 and 0.67 U/kg dose likely reflects the between-subject variability in drug concentrations between the two dose groups.

Dose response was demonstrated with regard to glucose infusion rate, fasting blood glucose and bolus insulin requirements. The fasting glucose declined over time with an average change from baseline (day 1) to day 14 of approximately 39 mg/dl (2.2 mmol/l) and was decreased to 60–100 mg/dl (3.3–5.6 mmol/l) across the dose range (figure 4). Further decrease in fasting glucose was prevented by oral carbohydrate intake. Nocturnal glucose control between 03:00 and 09:00 hours was relatively unchanged. The bolus insulin dose declined from study start, with no bolus administrations after day 7 for the 1.00 U/kg cohort.

Within-patient variability was <14 and <26% for the AUC of the 8-point blood glucose profile and fasting blood glucose, respectively.

Safety

Mild hypoglycaemia was the most common treatment-emergent adverse event (TEAE) [44 events (67.7%)] in 38% of patients. All hypoglycaemia events were of mild severity. No local injection site reactions were observed. No serious adverse events (SAEs) were reported. No patients discontinued the study due to an adverse event.

Clinical laboratory tests, vital signs, ECGs and antibodies to LY2605541 did not reveal clinically significant results or changes from baseline. During the course of the study, three patients had elevated alanine aminotransferase (ALT) levels; however, all observed elevations in ALT were below three times the upper limit of normal and all bilirubin levels were normal for these patients.

Discussion

In this Phase I study, LY2605541 was well-tolerated and demonstrated a flat PK and GD profile with no evidence of severe hypoglycaemia. Atypical of insulin dosing, this study was conducted as a 'fixed' dose over 14 days; patients were not titrated, nor were doses decreased within a cohort. This allowed investigating the PK and GD properties of LY2605541 without the need to account for dose adjustments or a potential delay in drug response. LY2605541 has a duration of action of > 24 h,

Table 3. Summary of LY2605541 GD parameters following single doses and multiple once-daily doses in patients with type 2 diabetes.

GD parameters	0.33 U/kg		0.50 U/kg		0.67 U/kg		1.00 U/kg	
	Day 1	Day 14	Day 1	Day 14	Day 1	Day 14	Day 1	Day 14
N	7	8	7	8	8	8	8	8
R_{max} (mg/min/kg)	0.259 (947)	1.20 (25)	0.143 (1455)	1.72 (82)	0.261 (1134)	1.60 (55)	0.299 (674)	2.98 (54)
G_{tot} (mg/kg)	122 (911)	920 (37)	54.2 (4540)	1520 (109)	107 (992)	1720 (60)	147 (1442)	3320 (60)

CV%, coefficient of variation; GD, glucodynamic; G_{tot} , total glucose infused over the clamp duration; R_{max} , maximum glucose infusion rate.

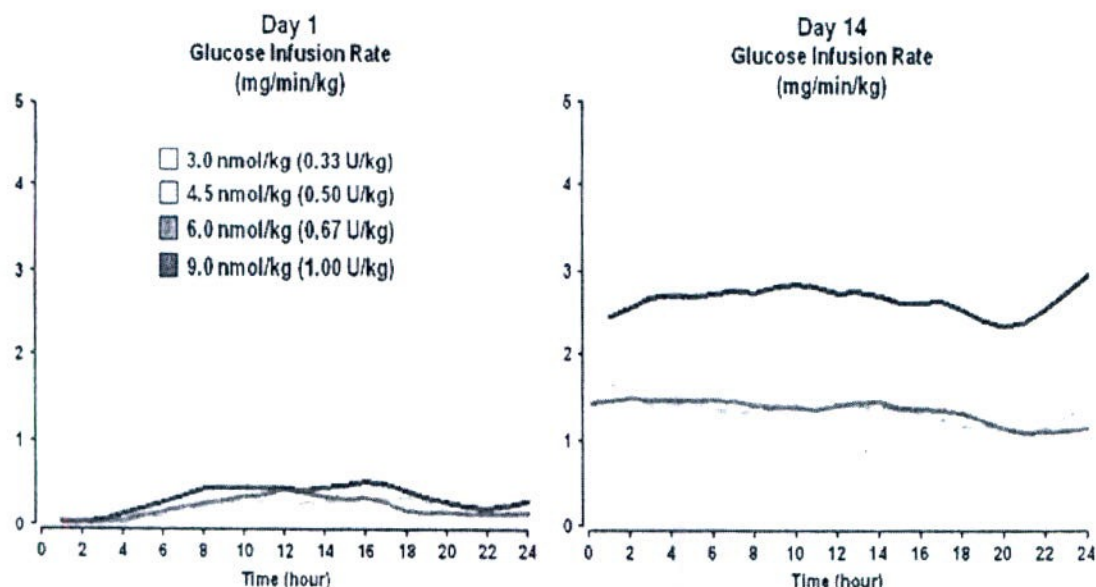


Figure 3. Mean locally weighted scatterplot smoothing (LOESS)-fitted glucose infusion rate profiles following single doses and multiple once-daily doses in patients with type 2 diabetes.

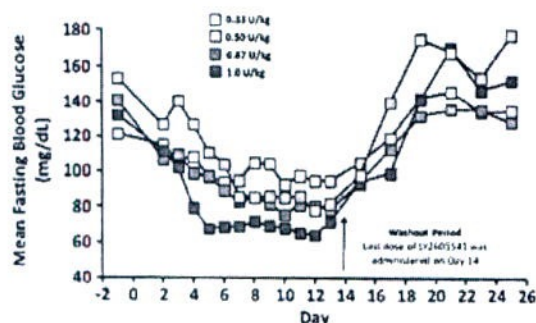


Figure 4. Mean fasting blood glucose following multiple once-daily doses in patients with type 2 diabetes.

a half-life of approximately 2–3 days, and minimal variability in patients with type 2 diabetes.

This study illustrates the favourable characteristics of LY2605541 as a long-acting basal insulin. The apparent half-life of LY2605541 (2–3 days) is considerably longer than that reported for insulin glargine (12.5 h) [15], insulin detemir (6–7 h) [16,17] and insulin degludec (25.4 h) [15]. The long

half-life relative to the dosing interval (QD) of LY2605541 explains the low fluctuation in PK levels (i.e. the low peak-to-trough fluctuation <2) in steady state. The long half-life of LY2605541 translates into a prolonged duration of action and long and flat GD profile. While all clamps were stopped after 24 h in this study (and therefore duration of action could not be fully assessed), the mean GD profiles on day 14 were very flat and showed considerably less peak-to-trough fluctuation than observed with insulin glargine or insulin detemir [6,7,18].

Trough concentrations on day 7 were higher than those on day 14. While the reason for this is not clearly understood, a dilution effect is a possibility, due to the large volume of fluid infused on day 14 during the euglycaemic clamp procedure. Further, day 14 PK levels were sampled under clamp conditions with the subjects being supine in contrast to day 7 where subjects were usually sitting for blood draws. Trough levels on days 10 and 13 were also equivalent to those on day 7, indicating that steady state was achieved on day 7.

Administration of LY2605541 produced a long, flat GD profile with small peak-to-trough fluctuations. The GIR profile at steady state mirrored the flat PK profile (concentration vs. time profiles) at steady state. On day 14, the GIR and G_{tot} are indicative of a dose-dependent response with a

GIR $\sim 1\text{--}2\text{ mg/min/kg}$ in the $0.50\text{--}0.67\text{ U/kg}$ dose range of LY2605541. In accordance with the hypothesized mechanism of action, LY2605541 may have restricted peripheral activity, but retains hepatic activity to mimic endogenous insulin distribution [13]. As such, the GIR may not reflect the full effect of LY2605541. Normalization of fasting blood glucose was in a time and dose-dependent manner.

The nocturnal glucose control between 03:00 and 09:00 hours was relatively unchanged, thus potentially providing patients with type 2 diabetes with a lower risk of nocturnal hypoglycaemia. These results are consistent with the GD properties observed in the randomized, controlled study of once-daily LY2605541 versus insulin glargine in patients with type 2 diabetes in which the rate of nocturnal hypoglycaemia was statistically significantly lower with LY2605541 [19].

The GD response for LY2605541 demonstrated low intra-subject variability which is an important consideration for insulin dosing with respect to both reliability and limiting the risk of hypoglycaemia. The intra-subject variability results in this study are consistent with results from the Phase II trial conducted by Bergenstal et al., in which the intra-day blood glucose variability was statistically significantly lower with LY2605541 versus insulin glargine [19]. In terms of PK response, the intra-subject variability (CV%) for C_{max} (21.22%) observed in this trial is lower than both NPH insulin (24%) and insulin glargine (34%), and slightly higher than that of insulin detemir (18%) reported in a randomized, double-blind study comparing the intra-subject variability of the three insulins [11].

LY2605541 was generally well tolerated in patients with type 2 diabetes when administered subcutaneously as multiple doses up to 1.00 U/kg . All events of hypoglycaemia were mild, and no events were prolonged or difficult to manage. Hypoglycaemia appeared to be dose-dependent with 30 events occurring in Cohort 3 (1.00 U/kg). A *post hoc* analysis of the distribution of hypoglycaemia by time of day did not show any trends in terms of time-related occurrence. The daily caloric intake was increased from 2200 to 2600 kcal/day in Cohort 3 to ensure stabilization of blood glucose values. No bolus insulin doses were administered to patients in Cohort 3 after day 7. This is a clear indication of the strong pharmacological effect of LY2605541 at steady state in high doses which may require a reduction in pre-meal insulin requirements at least in some patients. A similar phenomenon was observed in a clinical study in patients with type 1 diabetes who also had to reduce their average short-acting insulin dose [20].

In conclusion, the use of LY2605541 exhibits desirable characteristics of a long-acting basal insulin, including a prolonged activity profile that supports once-daily dosing, no pronounced peaks in activity and minimal variability. Taken together, these data support the clinical investigation of LY2605541 in a once-daily dosing regimen. Clinical studies assessing the glucose-lowering effects and safety and tolerability of LY2605541 in patients with type 1 and type 2 diabetes are ongoing and LY2605541 is currently in Phase III development.

Acknowledgements

The authors acknowledge the writing and editorial assistance of Stephanie Brillhart, inVentiv Health Clinical (Burlington,

MA, USA). This study was funded by Eli Lilly and Company. Dr V. K. S. is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of Interest

The institution of T. H. received research grants from the following pharmaceutical companies: Astellas, Bayer, Becton Dickinson, Biocon, Boehringer Ingelheim, Evolva, Glaxo Smith Kline, Hoffmann LaRoche, Johnson&Johnson, Eli Lilly, Novo Nordisk, Noxxon, Prosidion, Sanofi and Skye Pharma in the past 12 months. In addition, T. H. received travel grants, consulting fees and speaker honoraria from Novo Nordisk and Boehringer Ingelheim. V. P. S. and D. C. H. are former employees, and current stockholders of Eli Lilly and Company. S. L. C. and K. F. M. are employees and stockholders of Eli Lilly and Company. V. P. S., D. C. H., K. F. M. and T. H. designed this study. V. P. S., D. C. H. and K. F. M. conducted this study. V. P. S. performed statistical analysis and data analysis. V. P. S., D. C. H., S. L. C., K. F. M. and T. H. helped in preparation and writing of manuscript and approval of manuscript. S. L. C. performed PK/GD analysis. T. H. carried out data acquisition and interpretation of data.

References

- Riddle MC, Kosenstock J, Gerich J. The treat-to-target trial: randomized addition of glargine or human NPH insulin to oral therapy of type 2 diabetic patients. *Diabetes Care* 2003; **26**: 3080–3086.
- Buse JB, Wollenbuttel B, Herman WH et al. DURABILITY of basal versus lispro mix 75/25 insulin efficacy (DURABLE) trial 24-week results: safety and efficacy of insulin lispro mix 75/25 versus insulin glargine added to oral antihyperglycemic drugs in patients with type 2 diabetes. *Diabetes Care* 2009; **32**: 1007–1013.
- Nothen DM, Buse JB, Davidson MB et al. Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes. *Diabetes Care* 2009; **32**: 193–203.
- Swinen SG, Hoekstra JR, DeVries JH. Insulin therapy for type 2 diabetes. *Diabetes Care* 2009; **32**(Suppl. 2): S253–S259.
- Lepore M, Pampanelli S, Fanello C et al. Pharmacokinetics and pharmacodynamics of subcutaneous injection of long-acting human insulin analog glargine, NPH insulin, and ultralente human insulin and continuous subcutaneous infusion of insulin lispro. *Diabetes* 2000; **49**: 2142–2148.
- Klein O, Lyngø J, Endahl L, Damholt B, Nøse L, Heise T. Albumin-bound basal insulin analogues (insulin detemir and NN344): comparable time-action profiles but less variability than insulin glargine in type 2 diabetes. *Diabetes Obes Metab* 2007; **9**: 290–299.
- Heise T, Prober TR. Towards peakless, reproducible and long-acting insulins: An assessment of the basal analogues based on isoglycemic clamp studies. *Diabetes Obes Metab* 2007; **9**: 648–659.
- Hirsch IB. Insulin analogues. *N Engl J Med* 2005; **352**: 174–183.
- Ashwell SG, Amiel SA, Bilous RW et al. Improved glycaemic control with insulin glargine plus insulin lispro: a multicentre, randomized, crossover trial in people with type 1 diabetes. *Diabet Med* 2006; **23**: 285–292.
- Bartley PC, Boqvist M, Larsson J, Phylactou A. Long-term efficacy and safety of insulin detemir compared to neutral protamine Hagedorn insulin in patients with type 1 diabetes using a treat-to-target basal-bolus regimen.

- with insulin aspartat needs: a 2-year, randomized, controlled trial. *Diabet Med* 2008; **25**: 442–449.
11. Heise T, Nosek L, Rønn BB et al. Lower within-subject variability of insulin detemir in comparison to NPH insulin and insulin glargine in people with type 1 diabetes. *Diabetes* 2004; **53**: 1614–1620.
 12. Hansen RJ, Cutler GB, Vick A, Royster A, Li S, Siksky AM, Beals JM. LY2605541: leveraging hydrodynamic size to develop a novel basal insulin. Poster 896-P at the 72nd Scientific Sessions of the American Diabetes Association (ADA) 2012.
 13. Moore MJ, Smith MS, Mace KF et al. Novel PEGylated insulin LY2605541 has a metabolic preferential effect on glucose metabolism (abstract). *Diabetes* 2012; **61**(Suppl. 1): A417.
 14. Smith RP, Vandenhende TR, DeSante KA et al. Confidence interval criteria for assessment of dose proportionality. *Pharm Res* 2000; **17**: 1278–1283.
 15. Heise T, Hoveltmann U, Nosek L, Bottcher Su, Grannall K, Haahr H. Insulin degludec: two-fold longer half-life and more consistent pharmacokinetic profile than insulin glargine. *Diabetologia* 2011; **54**(suppl. 1): 1046.
 16. Doney C, Lippert K, Warrick Von SW, Hall MA. Insulin detemir is characterized by a consistent pharmacokinetic profile across age groups in children, adolescents, and adults with type 1 diabetes. *Diabetes Care* 2003; **26**: 3087–3092.
 17. Brunner GA, Sendhofer G, Wutte A et al. Pharmacokinetic and pharmacodynamic properties of long-acting insulin analogue NN104 in comparison to NPH insulin in humans. *Exp Clin Endocrinol Diabetes* 2000; **108**: 100–105.
 18. Porcellati F, Rossello P, Busciantella NR et al. Comparison of pharmacokinetics and dynamics of the long-acting insulin analogs glargine and detemir at steady state in type 1 diabetes: a double-blind, randomized, crossover study. *Diabetes Care* 2007; **30**: 2447–2452.
 19. Bergenstal RM, Rosenstock J, Arakaki RF et al. A randomized, controlled study of once daily LY2605541, a novel long-acting basal insulin, versus insulin glargine in basal insulin-treated patients with type 2 diabetes. *Diabetes Care* 2012; **35**: 2140–2147.
 20. Rosenstock J, Bergenstal RM, Blevins TE et al. Better glycaemic control and weight loss with the novel long-acting basal insulin LY2605541 compared with insulin glargine in type 1 diabetes: a randomized, crossover study. *Diabetes Care* 2013; **36**: 522–528.

Mary Courtney Moore,¹ Marta S. Smith,¹ Vikram P. Sinha,² John M. Beals,² M. Dodson Michael,² Scott J. Jacober,³ and Alan D. Cherrington¹

Novel PEGylated Basal Insulin LY2605541 Has a Preferential Hepatic Effect on Glucose Metabolism



The impact of the novel basal insulin LY2605541 (LY) on hepatic and nonhepatic glucose uptake (non-HGU) was evaluated. Conscious dogs underwent euglycemic clamps with tracer and hepatic balance measurements. Clamp period infusions were peripheral venous regular insulin ($0.1 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ [control], $n = 6$) or LY (bolus [nmol/kg], continuous [$\text{nmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$]: $0.5, 0.5$ [$n = 6$]; $0.375, 0.375$ [$n = 5$]; $0.25, 0.25$ [$n = 4$]), somatostatin, and glucose, as well as intraportal glucagon (basal). During the clamp, the dogs switched from net hepatic glucose output to uptake (rates reached $2.1 \pm 1.2, 0.9 \pm 2.1, 8.6 \pm 2.3$, and $6.0 \pm 1.1 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ within 5 h in control, LY_{0.25}, LY_{0.375}, and LY_{0.5}, respectively). Non-HGU in LY increased less than in control; the ratio of change from basal in non-HGU to change in net hepatic glucose balance, calculated when glucose infusion rates (GIRs) were $\sim 20 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in all groups, was higher in control (1.17 ± 0.38) versus LY_{0.25} (0.39 ± 0.33), LY_{0.375} (-0.01 ± 0.13), and LY_{0.5} (-0.09 ± 0.07). Likewise, the change from baseline in glucose R_d -to- R_a ratio was greatest in control (1.4 ± 0.3 vs. $0.6 \pm 0.4, 0.5 \pm 0.2$, and 0.6 ± 0.2 in LY_{0.25}, LY_{0.375}, and LY_{0.5}, respectively). In contrast to exogenously administered human insulin, LY demonstrated preferential hepatic effects, similar

to endogenously secreted insulin. Therefore, the analog might reduce complications associated with current insulin therapy.

Diabetes 2014;63:494–504 | DOI: 10.2337/db13-0826

Marked improvements have been made in insulin analogs for basal-bolus therapy over the last few years. Insulins detemir and glargine reduce the risk of hypoglycemia compared with the older NPH insulin (1) and come closer to the ideal of creating a peakless basal insulin (2). However, neither detemir nor glargine maintains 24-h basal insulinemia for all individuals on all days (3), and thus improved analogs are needed. Insulin degludec, approved for use in Europe, Japan, and Mexico, is the first of the next-generation analogs to reach the market. The $T_{1/2}$ of insulin degludec is approximately two times that of glargine (4), with the prolonged activity being achieved by the formation of soluble multihexamers upon subcutaneous injection, followed by gradual release of insulin monomers from the aggregate (5). Compared with glargine in treat-to-target trials, insulin degludec has brought about similar reduction in HbA_{1c} at lower dosages and with lower rates of nocturnal hypoglycemia (6,7). Another new basal insulin, LY2605541 (LY), is currently in phase 3 trials.

¹Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, TN

²Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN

³Lilly Diabetes, Eli Lilly and Company, Indianapolis, IN

Corresponding author: Mary Courtney Moore, genie.moore@vanderbilt.edu.

Received 23 May 2013 and accepted 23 September 2013.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db13-0826/-/DC1>.

V.P.S. is currently affiliated with the U.S. Food and Drug Administration, Silver Spring, MD.

© 2014 by the American Diabetes Association. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

See accompanying commentary, p. 390.

LY is insulin lispro with a 20-kDa polyethylene glycol moiety covalently attached to lysine B28 via a urethane bond (8,9). Its large hydrodynamic (i.e., functional) size (10) may contribute to slowing absorption and reducing clearance, resulting in prolonged duration of action. In phase I trials, the half-life of LY was between 1 and 3 days and reached steady-state concentrations within 7–10 days with ~8- to 13-fold increase in exposure at steady-state. After a single dose, the requirement for glucose infusion was at least 36 h, indicating prolonged pharmacodynamic action. At steady-state, nearly peak-less glucose infusion profiles were observed due to low peak-to-trough pharmacokinetic fluctuation (11,12). In two phase II trials in patients with type 1 and type 2 diabetes, LY was associated with a lower rate of nocturnal hypoglycemia and glucose variability compared with insulin glargine. At the same time, it resulted in similar glycemic control in the type 2 diabetic patients but better glycemic control in the type 1 diabetic patients. In the type 1 diabetic patients, there was a slightly higher rate of total hypoglycemia but a lower prandial insulin requirement (13,14). In addition, these patients, who were previously treated with other basal insulins, showed modest weight loss when switched to LY compared with modest weight gain with insulin glargine. They also exhibited modestly higher triglycerides compared with those on insulin glargine (13,14). These effects prompted an examination of the effect of LY on specific tissues, including the liver, kidney, and skeletal muscle. The chronically catheterized conscious dog was chosen as the model because it permits cannulation of the vessels required for assessing glucose balance across these organs/tissues and because of the similarity in insulin's regulation of glucose metabolism in the human and the dog.

RESEARCH DESIGN AND METHODS

Animals and Surgical Procedures

The protocol was approved by the Vanderbilt University Institutional Animal Care and Use Committee, and the animals were housed and cared for according to Association for Assessment and Accreditation of Laboratory Animal Care International guidelines. Approximately 16 days prior to study, each dog underwent surgical preparation under general anesthesia. In the initial studies (part A; $n = 12$), sampling catheters were placed in a femoral artery, the hepatic portal vein, the left common hepatic vein, a renal vein, and an iliac vein; infusion catheters were inserted in a jejunal and a splenic vein; and ultrasonic flow probes (Transonic Systems, Ithaca, NY) were placed around the hepatic artery, the portal vein, a renal artery, and an external iliac artery as previously described (15–18). In follow-up experiments (part B), the renal and iliac cannulas and flow probes were not placed, but all other aspects of the preparation were as described above. Criteria for use in a study were as previously described (17).

Experimental Protocol

On the morning of study, the proximal ends of the flow probes and catheters were exteriorized under local anesthesia. A primed, continuous infusion of [^3H]glucose was initiated via a peripheral vein, and the dog was allowed to rest quietly in a Pavlov harness throughout the period of tracer equilibration (90 min). This was followed by 30 min of baseline sampling (90–120 min) and a euglycemic clamp period lasting 5 h (from 120 to 420 min) in part A. At 120 min, infusions of somatostatin (Bachem, Torrance, CA) and insulin were begun via a peripheral vein, while glucagon was administered intraportally at a basal rate ($0.5 \text{ ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$). The dogs in part A were randomly assigned to receive LY initiated with a 0.5 nmol/kg priming dose followed by continuous infusion at $0.5 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ (LY_{0.5} group, $n = 6$) or regular human insulin (control group, $n = 6$) at $0.11 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ with no priming dose. The analog is designed for subcutaneous injection, but it was infused via peripheral vein in these experiments in order to come to near-steady-state in a reasonable period of time and to mimic the route by which subcutaneously injected insulin arrives at the liver and other insulin-sensitive tissues. There is no known modification of the molecule during the absorption from the subcutaneous tissues, and after absorption it circulates in the same manner as endogenous insulin.

The insulin infusion rates used (determined in pilot studies; data not shown) in the two groups required equivalent glucose infusion rates (GIRs) to maintain euglycemia over the time course of the experiments. Glucose (50%; Hospira, Lake Forest, IL) was infused via peripheral vein as needed to maintain euglycemia. All sampling catheters functioned with the exception of a renal catheter in one LY_{0.5} animal and a hindlimb catheter in one control dog. At the end of each experiment, the dog was anesthetized, and hepatic biopsies were freeze clamped *in situ* as previously described (19). Subsequently, the dog was killed and the hypothalamus was removed and freeze clamped (20). Tissues were stored at -80°C to await further analysis.

Part B studies were identical to those in part A, except that the euglycemic clamp lasted 7 h (from 120 to 540 min), and the dogs were randomized to either the LY_{0.375} ($n = 5$; 0.375 nmol/kg prime, $0.375 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion) or LY_{0.25} ($n = 4$; 0.25 nmol/kg prime, $0.25 \text{ nmol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ infusion) groups. The purpose of part B was to determine whether the hepato-preferential effects of LY, observed in part A, would be evident at different dosages of the analog. Additionally, the longer clamp period was instituted to determine whether lower LY infusion rates sustained the hepato-preferential effects.

Sample Analyses

Plasma glucose, [^3H]glucose, glucagon, canine and human insulin, and nonesterified fatty acid (NEFA) levels and blood lactate and glycerol concentrations were measured

using standard methods as previously described (19,21). Plasma triglycerides were assayed spectrophotometrically (TRO100; Sigma-Aldrich, St. Louis, MO).

Serum concentrations of LY were assayed using a dual-antibody ELISA specific for LY (Charles River Laboratories, Senneville, QC, Canada). The quantitation range was 20–500 pmol/L, with the standard curve range 15–1,000 pmol/L. Samples with concentrations higher than 500 pmol/L were diluted so that the resulting concentration was within the quantitation range. Standard curve and quality control samples were analyzed with each set of study samples and passed acceptance criteria (within $\pm 20\%$ except at lower limit of quantitation: within $\pm 25\%$).

Western blotting was carried out on hepatic and hypothalamic tissue from control and LY_{0.5} dogs; all analytic procedures have previously been described (22). Antibodies against total and phosphorylated Akt (Ser⁴⁷³), signal transducer and activator of transcription 3 (STAT3) (Tyr⁷⁰⁵), and glycogen synthase kinase-3 β (GSK3 β) (Ser⁹) were purchased from Cell Signaling (Danvers, MA). Protein bands were quantified using ImageJ software (<http://rsb.info.nih.gov/ij/>).

Calculations

Organ balance calculations and estimation of hepatic sinusoidal hormone concentrations and net hepatic carbon retention were carried out as previously described in detail (15–18). Tracer-determined glucose turnover was calculated with the circulatory model of Mari et al. (23). Unidirectional hepatic glucose uptake was calculated endogenous glucose R_a minus net hepatic glucose balance.

Two-way repeated-measures ANOVA (group $\times$ time) was used for statistical analysis of time course data, with post hoc analysis by Tukey test (Sigmaplot 11; Systat Software, San Jose, CA). Since part A was 2 h shorter than part B, statistical comparisons do not include the data from the last 2 h of part B. Unpaired t tests were used for comparison of tissue analytes. A P value < 0.05 was considered statistically significant.

RESULTS

Hormone Concentrations and Hepatic Blood Flow

Portal vein blood flow declined 15–25% in response to somatostatin infusion ($P < 0.05$ for Δ from baseline in all groups), while there was a slight (albeit nonsignificant) 0–10% rise in hepatic artery blood flow (Table 1).

Arterial plasma C-peptide concentrations declined to near the limits of detection in all groups during the clamp period, demonstrating that insulin secretion was fully suppressed (data not shown). Arterial and hepatic sinusoidal glucagon concentrations remained near basal throughout the clamp period and did not differ significantly among groups ($P = 0.22$) (Table 1).

Plasma insulin concentrations during the baseline period were similar in all groups (data shown for control group only [Fig. 1A]). In the control group, the arterial

TABLE 1—Hepatic blood flow and plasma glucagon concentrations

	Baseline period	Clamp period
Hepatic artery blood flow (mL $\cdot$ kg ⁻¹ $\cdot$ min ⁻¹)		
Control	6.9 $\pm$ 1.2	7.3 $\pm$ 1.1
LY _{0.5}	5.7 $\pm$ 0.7	6.3 $\pm$ 0.7
LY _{0.375}	6.8 $\pm$ 0.5	7.4 $\pm$ 0.7
LY _{0.25}	5.3 $\pm$ 0.7	5.2 $\pm$ 0.9
Portal vein blood flow (mL $\cdot$ kg ⁻¹ $\cdot$ min ⁻¹)		
Control	25.8 $\pm$ 1.5	19.5 $\pm$ 1.5
LY _{0.5}	28.0 $\pm$ 2.3	21.7 $\pm$ 1.5
LY _{0.375}	25.0 $\pm$ 1.8	21.2 $\pm$ 1.7
LY _{0.25}	22.2 $\pm$ 2.2	17.3 $\pm$ 1.3
Arterial plasma glucagon (ng/L)		
Control	50 $\pm$ 3	43 $\pm$ 2
LY _{0.5}	50 $\pm$ 6	45 $\pm$ 4
LY _{0.375}	50 $\pm$ 3	45 $\pm$ 5
LY _{0.25}	62 $\pm$ 8	55 $\pm$ 8
Hepatic sinusoidal plasma glucagon (ng/L)		
Control	58 $\pm$ 3	52 $\pm$ 2
LY _{0.5}	54 $\pm$ 6	57 $\pm$ 7
LY _{0.375}	56 $\pm$ 6	55 $\pm$ 5
LY _{0.25}	74 $\pm$ 4	67 $\pm$ 6

Data are mean $\pm$ SEM. $n = 6$ /group for control and LY_{0.5}; $n = 5$ for LY_{0.375}, and $n = 4$ for LY_{0.25}. Basal period values are the mean of two sampling times (90 and 120 min); clamp period values are the mean of the sampling points between 120 and 420 min (for control and LY_{0.5}) and between 120 and 540 min (for LY_{0.375} and LY_{0.25}). There were no significant differences among groups.

concentrations during the clamp period were twofold basal levels, but peripheral venous insulin delivery resulted in a fall in the portal vein concentrations to ~50% of basal. Consequently, hepatic sinusoidal insulin concentrations in the control group tended to decline (from 96 $\pm$ 30 pmol/L [baseline] to 68 $\pm$ 7 pmol/L [clamp period]; $P = 0.14$), while renal vein plasma insulin concentrations increased in parallel with the rise in arterial insulin. The mean arterial LY concentrations during the final hour (near-steady-state) were 11,651 $\pm$ 946, 7,589 $\pm$ 843, and 3,906 $\pm$ 858 pmol/L in LY_{0.5}, LY_{0.375}, and LY_{0.25}, respectively (Fig. 1B).

Fractional hepatic, intestinal, and renal extractions of regular insulin during the clamp were 50 $\pm$ 3%, 29 $\pm$ 2%, and 61 $\pm$ 3%, respectively. The extraction of LY by the liver could not be precisely quantified because of the high analog concentrations and relatively low tissue clearance. The extraction by the intestinal tract averaged 5 $\pm$ 3% at the highest dose, but at the lower LY infusion rates, the analog concentrations were too similar in the artery and portal vein to detect intestinal extraction. In LY_{0.5}, renal extraction of the LY was 13 $\pm$ 4% (not evaluated in LY_{0.375} and LY_{0.25}).

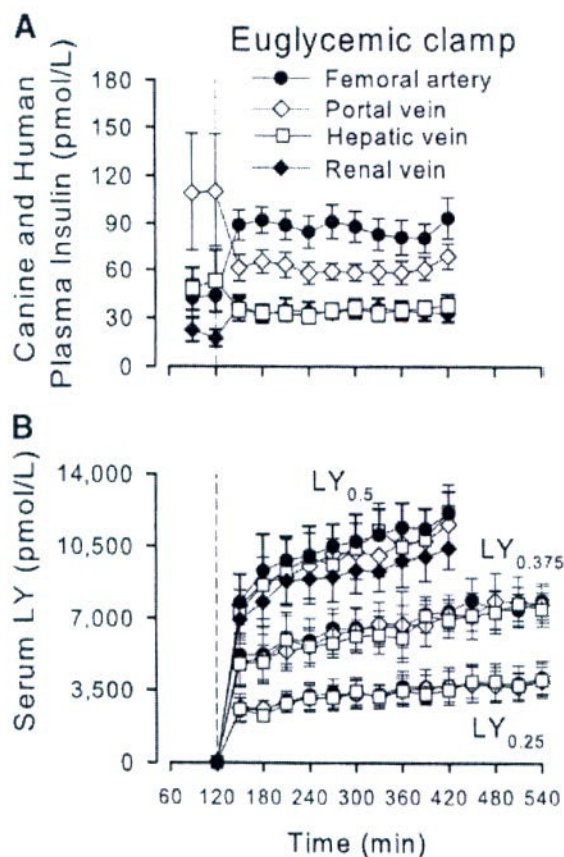


Figure 1—Plasma concentrations of endogenous insulin (basal period) and regular human insulin (clamp period) in control dogs (A) and serum concentrations of LY (B). Arterial, portal vein, hepatic vein, and renal vein (available for control and LY_{0.5} groups only) concentrations of insulin or analog. Data are mean $\pm$ SEM; $n = 6$, 6, 5, and 4 for control, LY_{0.5}, LY_{0.375}, and LY_{0.25}, respectively.

Glucose Metabolism

Arterial plasma glucose during the clamp period remained at basal concentrations [~ 6.1 mmol/L in all groups (Fig. 2A)]. The GIRs were not significantly different between control and LY_{0.5} or LY_{0.375} (Fig. 2B) but were lower ($P < 0.05$) in LY_{0.25} than in control. All groups exhibited net hepatic glucose output (NHGO) ($8\text{--}11 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) during the baseline period. In the control group, NHGO gradually declined from baseline, reaching approximately zero by 420 min ($\Delta 10.3 \pm 1.4 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) (Fig. 2C). In LY_{0.5}, on the other hand, there was a rapid and marked impact at the liver, such that there was a switch from NHGO to net hepatic glucose uptake (NHGU) within 30 min of beginning the LY_{0.5} infusion, and the change from baseline in net hepatic glucose balance reached $16.8 \pm 2.2 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ by 420 min ($P < 0.05$ between groups). The change from baseline in LY_{0.375} and LY_{0.25} averaged 18.8 ± 2.6 ($P < 0.05$ vs. control) and 9.5 ± 3.1 (NS vs. control)

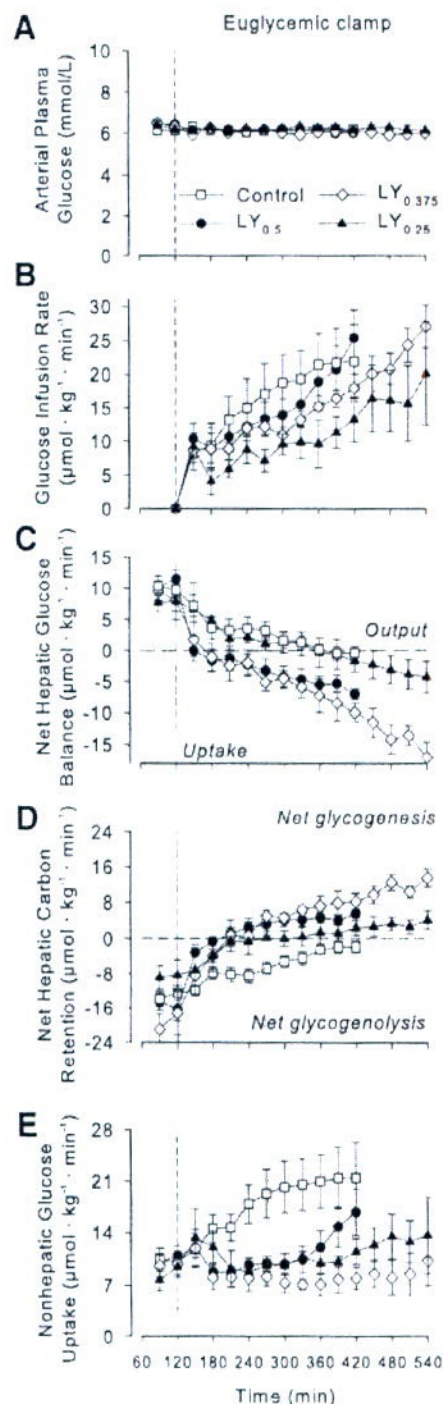


Figure 2—Arterial plasma glucose concentrations (A), GIRs (B), net hepatic glucose balance (NHGB) (C), net hepatic carbon retention (NHCR) (D), and non-HGU (E). Control ($n = 6$), LY_{0.5} ($n = 6$), LY_{0.375} ($n = 5$), and LY_{0.25} ($n = 4$). Significant differences ($P < 0.05$): LY_{0.5} and LY_{0.375} vs. control for NHGB, NHCR, and non-HGU; LY_{0.25} vs. control for GIR, NHCR, and non-HGU; LY_{0.375} vs. LY_{0.5} for non-HGU; LY_{0.25} vs. LY_{0.5} for NHGB, GIR, and NHCR; LY_{0.25} vs. LY_{0.375} for NHGB and NHCR.

$\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ by 420 min. All LY dosages were associated with significantly greater net hepatic carbon retention than exhibited by the control group (Fig. 2D). The effect of each treatment on endogenous glucose R_a was very similar to its effect on net hepatic glucose balance (Fig. 3A). In the control group, there was a gradual decline in glucose R_a , with the fall from basal totaling $8.9 \pm 1.9 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ by the final hour. When the change in endogenous glucose R_a between the basal period and 360–420 min (the final hour during part A) was calculated, it was clear that the change was significantly greater in LY_{0.5} and LY_{0.375} than in control (Fig. 3C).

Peripheral insulin infusion in the control group had an immediate impact on nonhepatic glucose uptake (non-HGU), and by the final hour non-HGU had increased $11.1 \pm 4.5 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ over the basal rate (Fig. 2E). In the LY groups, non-HGU rates initially fell below basal. In the LY_{0.5} group, it began to increase gradually after 330 min, with the increase from basal in the final hour being $4.1 \pm 1.5 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ($P = 0.14$ versus control). The LY_{0.25} group also exhibited a small increase in non-HGU during the late part of the clamp, while the rate did not rise above basal at any time in the LY_{0.375} group. In an effort to compare the control and the different LY groups under equivalent conditions, we

calculated the ratio of the change from basal in non-HGU to the change in NHGU at a time when GIRs were equivalent in all groups ($\sim 20 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$). The lower the ratio in Table 2, the more hepato-preferential the action. The ratio was much higher in control than in LY_{0.5} and LY_{0.375} ($P < 0.01$), and it tended to be higher than in LY_{0.25}. No significant differences were seen in either net renal or hindlimb glucose balance between the control and LY_{0.5} groups (data not shown).

LY_{0.375} and LY_{0.25}, but not LY_{0.5}, stimulated glucose R_d significantly less than regular insulin through 420 min (Fig. 3B). When the ratio of change from baseline in glucose R_d to change in glucose R_a was calculated at the time when GIR was $\sim 20 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in each group, the control group exhibited a ratio $>$ twofold that in the LY groups (Table 2).

Lactate Metabolism

All groups exhibited net hepatic lactate output (NHLO) during the baseline period, with the rates in LY_{0.375} and LY_{0.25} being significantly higher and lower, respectively, than in the control group (Table 3). During the clamp period, the control group exhibited a slight increase in NHLO followed by a return to the baseline rate. In all of the LY groups, NHLO during the clamp period declined to a nadir no different from $0 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ in

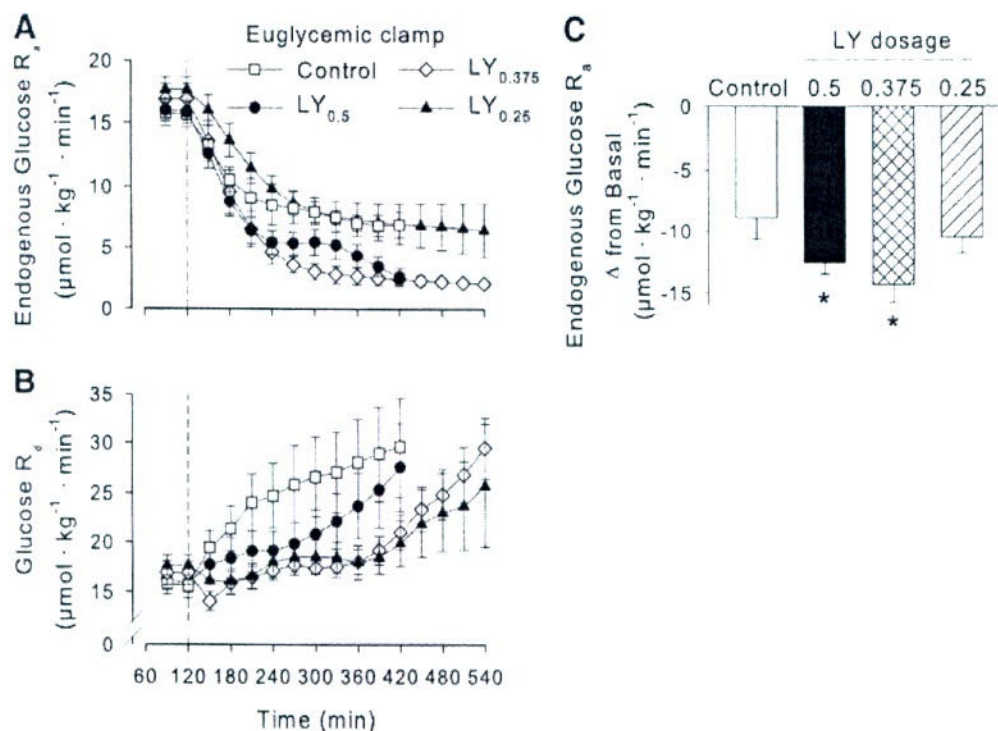


Figure 3—Tracer-determined rate of endogenous glucose R_a (A) and R_d (B). C: Magnitude of the decline in endogenous glucose R_a between the baseline period and 360–420 min (the last hour of study for the control and LY_{0.5} groups). Control ($n = 6$), LY_{0.5} ($n = 6$), LY_{0.375} ($n = 5$), and LY_{0.25} ($n = 4$). $P < 0.05$ for LY_{0.375} vs. control for both R_a and R_d . $P < 0.05$ for LY_{0.25} vs. control for R_d . * $P < 0.05$ vs. control.

TABLE 2—Ratios of change from baseline in non-HGU to change in NHGU, as well as change from basal in tracer-determined glucose R_d to the change in endogenous glucose R_a

Group	$\Delta \text{non-HGU}:\Delta \text{NHGU}$	$\Delta R_d:\Delta R_a$
Control	1.17 ± 0.38	1.37 ± 0.32
LY _{0.5}	$0.09 \pm 0.07^*$	$0.60 \pm 0.24\ddagger$
LY _{0.375}	$-0.01 \pm 0.13^*$	$0.51 \pm 0.22\ddagger$
LY _{0.25}	$0.39 \pm 0.33\ddagger$	$0.61 \pm 0.43\ddagger$

Data are mean $\pm$ SEM. $n = 6/\text{group}$ for control and LY_{0.5}; $n = 5$ for LY_{0.375}, and $n = 4$ for LY_{0.25}. Ratios were calculated at the point at which GIR in each group was $\sim 20 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (330, 360, 450, and 540 min for control, LY_{0.5}, LY_{0.375}, and LY_{0.25}, respectively). There were no significant differences among the LY groups. $^*P < 0.01$ vs. control. $\ddagger P = 0.07$ vs. control. $\ddagger P < 0.05$ vs. control.

LY_{0.5}. The LY_{0.375} and LY_{0.25} groups actually switched to net hepatic lactate uptake. NHLO resumed during the latter part of the clamp but did not reach baseline rates by the end of the experiment in any of the LY groups. As a result of these changes, blood lactate concentrations tended to fall initially and then return toward baseline levels in the LY groups.

The control group exhibited net hindlimb lactate uptake throughout the clamp, with uptake increasing significantly as NHLO peaked and then declining to near-baseline rates. Conversely, net hindlimb lactate uptake decreased in LY_{0.5} as NHLO declined, with a subsequent small increase in net limb lactate uptake corresponding to the increase in NHLO by the end of the clamp. Net renal lactate uptake did not change significantly from

baseline in either control or LY_{0.5}, and the two groups did not differ at any time point (data not shown).

Glycerol, Fatty Acid, and Triglyceride Metabolism

Arterial glycerol and NEFA concentrations in the control group declined promptly under clamp conditions in response to the peripheral hyperinsulinemia. In contrast, the glycerol and NEFA concentrations initially increased in the LY groups and then gradually declined (Fig. 4A and C). Net hepatic and renal (renal not shown) uptakes of glycerol and NEFA decreased during the clamp in the control group and initially increased in the LY groups, corresponding to the changes in the substrate concentrations (Fig. 4B and D). Net hindlimb glycerol output did not differ between the control and LY_{0.5} groups at any time (data not shown). During the clamp, arterial plasma triglyceride concentrations declined significantly in all groups (Fig. 4E). By 420 min, triglyceride concentrations had fallen $\sim 40\%$ below baseline in the control and LY_{0.5} and LY_{0.375} groups and 15% in LY_{0.25}. The concentrations were not significantly different among groups at 420 min.

Tissue Analyses

There was no significant difference between regular insulin and LY_{0.5} in phosphorylation of Akt in the hypothalamus or STAT3 protein in the liver (Fig. 5A and B; Supplementary Fig. 1). On the other hand, phosphorylation of Akt and GSK3 β in the liver was significantly enhanced with LY_{0.5} versus regular insulin (Fig. 5C and D).

DISCUSSION

Insulin secretion into the portal vein results in $\sim$ three-fold greater exposure of the liver, versus other tissues, to insulin. The increased exposure of the liver to the

TABLE 3—Lactate data

	Baseline period	Clamp period time (min)						
		180	240	300	360	420	480	540
Arterial lactate ($\mu\text{mol/L}$)								
Control	701 $\pm$ 69	624 $\pm$ 72	623 $\pm$ 105	623 $\pm$ 81	648 $\pm$ 73	650 $\pm$ 106	—	—
LY _{0.5}	771 $\pm$ 122	580 $\pm$ 83	483 $\pm$ 63	503 $\pm$ 77	548 $\pm$ 93	548 $\pm$ 117	—	—
LY _{0.375}	952 $\pm$ 150*	802 $\pm$ 176	552 $\pm$ 107	471 $\pm$ 41	534 $\pm$ 71	602 $\pm$ 62	743 $\pm$ 68	761 $\pm$ 71
LY _{0.25}	684 $\pm$ 42	551 $\pm$ 96	539 $\pm$ 73	482 $\pm$ 113	498 $\pm$ 125	542 $\pm$ 84	628 $\pm$ 125	887 $\pm$ 344
Net hepatic lactate balance ($\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$)								
Control	6.4 $\pm$ 1.9	8.4 $\pm$ 0.9	9.8 $\pm$ 0.9	7.2 $\pm$ 1.0	4.7 $\pm$ 1.2	4.6 $\pm$ 1.4	—	—
LY _{0.5}	9.7 $\pm$ 2.9	5.6 $\pm$ 1.9	0.6 $\pm$ 2.1*	1.4 $\pm$ 1.4*	3.0 $\pm$ 1.2*	3.5 $\pm$ 0.5	—	—
LY _{0.375}	20.6 $\pm$ 6.7*	9.9 $\pm$ 4.1	3.0 $\pm$ 4.2	-0.1 $\pm$ 1.7*	0.0 $\pm$ 1.5*	3.6 $\pm$ 1.7	3.4 $\pm$ 1.7	7.0 $\pm$ 0.9
LY _{0.25}	1.7 $\pm$ 2.4*	-1.8 $\pm$ 2.2*	-2.9 $\pm$ 2.2*	-2.6 $\pm$ 1.9*	-2.0 $\pm$ 1.7*	-1.3 $\pm$ 0.7*	-0.4 $\pm$ 2.0	0.2 $\pm$ 1.3

Data are mean $\pm$ SEM. $n = 6, 6, 5$, and 4 for control, LY_{0.5}, LY_{0.375}, and LY_{0.25}, respectively. Basal period values are the mean of two sampling times; the clamp period took place between 120 and 420 min (for control and LY_{0.5}) and between 120 and 540 min (for LY_{0.375} and LY_{0.25}). Positive values for balance indicate net hepatic output. There were no significant differences among the LY groups; $P = 0.1$ for basal period net balance in LY_{0.375} compared with the rates in LY_{0.5} and LY_{0.25}. $^*P < 0.05$ vs. control.

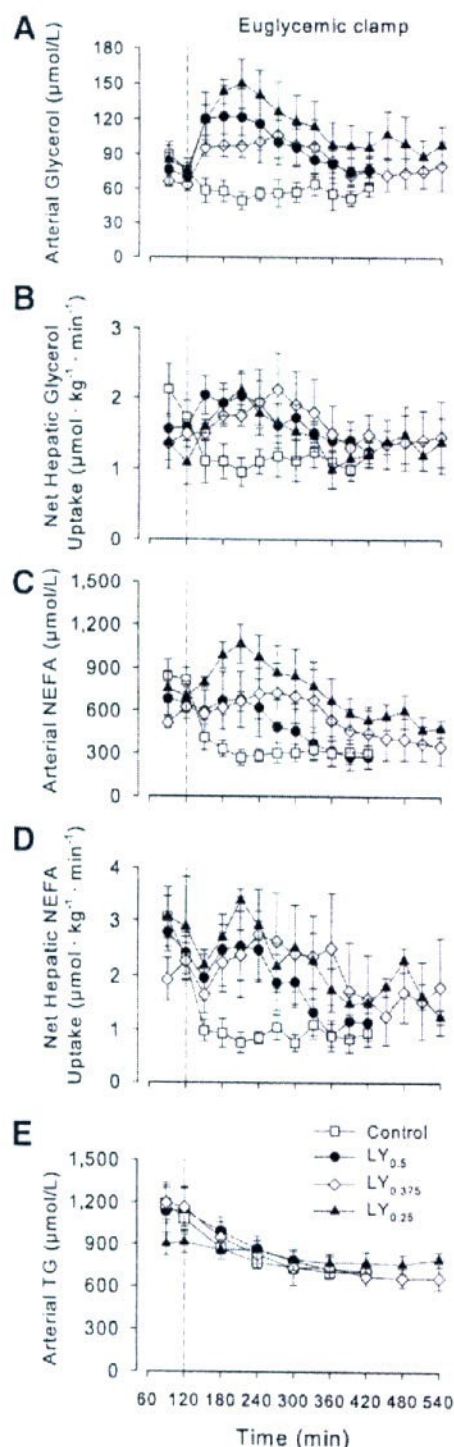


Figure 4—Arterial blood glycerol (A), plasma NEFA (C), and plasma triglyceride (TG) (E) concentrations and net hepatic uptakes of glycerol (B) and NEFA (D). Control ($n = 6$), LY_{0.5} ($n = 6$), LY_{0.375} ($n = 5$), and LY_{0.25} ($n = 4$). $P < 0.05$ for all LY groups vs. control for arterial glycerol and NEFA and for net hepatic uptake of glycerol and NEFA. There were no significant differences among groups in TG concentrations. There were no significant differences among LY groups for any parameter.

hormone results from its secretion into a limited vascular pool and the fact that the liver extracts 50–60% of the insulin to which it is exposed (24). In contrast, when insulin is administered subcutaneously, the normal portal-peripheral insulin gradient is lost so that the peripheral tissues are comparatively hyperinsulinemic while the liver is comparatively hypoinsulinemic (25,26). The portal vein, hepatic vein, and hepatic sinusoidal insulin concentrations in the control group during the clamp period were ~30–40% below basal, while the arterial concentrations were approximately two times basal. Although the arterial concentrations did not increase as much as would be anticipated after a mixed meal (sixfold basal or more), they were inappropriately high in relation to the other blood vessels sampled, which also exhibit sixfold or more increases in insulin after meal ingestion (27). Partly as a result of the relative overinsulinization of peripheral tissues, even individuals with well-controlled insulin-treated diabetes are apt to experience glycemic fluctuations, hypoglycemia, and dyslipidemia, which have been suggested to contribute to microvascular and macrovascular complications (28). Theoretically, insulin analogs with hepato-preferential actions could reduce the risk of these complications (29,30).

While it may seem counterintuitive that a hepato-preferential insulin could reduce hypoglycemia, since the liver's response is key to restoring euglycemia, relative overinsulinization of peripheral tissues contributes to hypoglycemia and impaired counterregulation. Lipolysis is an important contributor to gluconeogenesis under hypoglycemic conditions, and the relative overinsulinization of the peripheral lipid depots resulting from current peripherally administered insulin therapies would be expected to impede gluconeogenesis (31–33). Moreover, even though skeletal muscle glucose uptake decreases during hypoglycemia associated with conventional insulin therapy, it does not cease. Due to the relatively large mass of skeletal muscle, this results in significant ongoing glucose disposal, a process that would be curtailed by a hepato-preferential insulin. More importantly, a hepato-preferential insulin puts the primary burden of hypoglycemia on the organ best suited to cope because hepatic insulin signaling is markedly reduced in the presence of hypoglycemia; i.e., the insulin receptor is essentially disengaged (34). Even when overinsulinized and deprived of increases in counterregulatory hormones and central neural stimuli, the liver is capable of responding directly to hypoglycemia by increasing its glucose output (35–37).

At the doses examined, LY exhibited a clear hepato-preferential effect compared with human insulin when both products were delivered via peripheral vein. This was demonstrated by two independent measures of glucose metabolism: organ balance and tracer-determined turnover. It should be noted that a single control group was used as a comparator for all three LY dosages. In

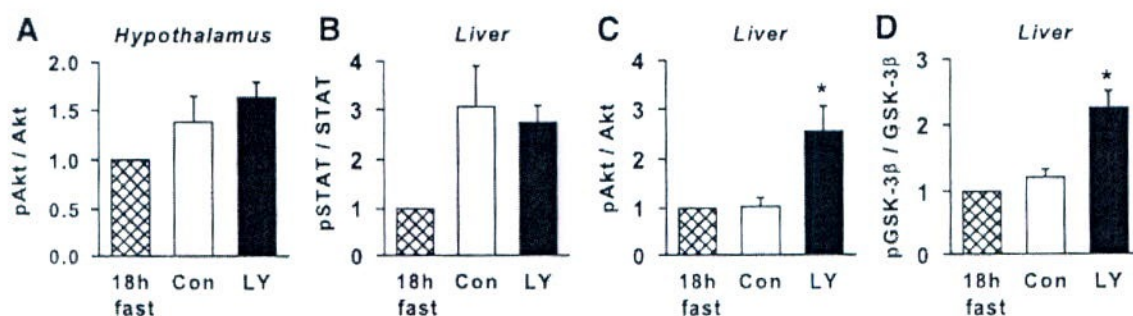


Figure 5—Molecular markers of insulin signaling in the control and LY_{0.5} groups. Hypothalamic phosphorylated Akt (pAkt), expressed relative to total Akt (A), as well as hepatic pSTAT3/STAT3 (B), pAkt/Akt (C), and pGSK3β/GSK3β (D), in control (con) (white bars) and LY_{0.5} (black bars) ($n = 6$ /group). The hatched bars are mean data from 2 additional dogs, not included in the experiments, that were fasted for 18 h and then killed to provide reference (non-insulin stimulated) tissue. See Supplementary Fig. 1 for representative blots. * $P < 0.05$ vs. control.

order for groups to be compared, in terms of glucose disposition, they must be matched for at least one parameter of interest. In that regard, the LY_{0.5} and LY_{0.375} groups were not significantly different in GIR from the control group ($P = 0.72$ and 0.35 , respectively). Both LY_{0.5} and LY_{0.375} exhibited a faster and greater effect on net hepatic glucose balance than regular insulin, quickly shifting the liver from NHGO to NHGU. Conversely, their effect on non-HGU was delayed and minimal compared with regular insulin. At the lowest LY dose, the analog's effects on net hepatic glucose balance were comparable to those in the control group throughout 420 min ($P = 0.974$), making possible a comparison of nonhepatic effects in the presence of similar rates of NHGO. The impact of LY_{0.25} on non-HGU was markedly lower than that of regular insulin. Of the three LY dosages examined, LY_{0.375} appeared to have the greatest effect at the liver concomitant with the least effect on nonhepatic tissues.

Tracer methodology yielded results consistent with hepatic glucose balance data. The two higher doses of LY caused a significantly greater fall in endogenous glucose R_d than regular insulin (even though significant differences from control in the rates were not evident as early as they were with organ balance data), while the effect of LY_{0.25} was very similar to that of regular insulin ($P = 0.34$). On the other hand, both LY_{0.375} and LY_{0.25} stimulated glucose R_d significantly less than regular insulin. Moreover, all doses of LY, compared with regular insulin, were associated with a significantly smaller change in glucose R_d relative to the impact on endogenous R_d .

In contrast to its rapid effects on the liver, as noted above, LY's effect on nonhepatic tissues was more gradual than that of regular insulin. In particular, LY's effect on fat metabolism appeared to be slow. Lipolysis is extremely sensitive to an elevation of regular insulin, and thus it was quickly suppressed in the control group, with glycerol concentrations reaching a stable nadir within 30 min of the start of the clamp. In none of the LY groups did the glycerol concentrations fall below the

baseline values at any time, suggesting a lack of lipolytic inhibition by the analog at these doses. NEFA levels, on the other hand, fell below baseline levels during the clamp in all LY groups, consistent with an increase in reesterification. Nevertheless, regular insulin lowered NEFA much more promptly than LY. The nadir of NEFA concentrations ($271 \pm 57 \mu\text{mol/L}$) occurred within 90 min after the start of the clamp period in the control group, while the nadir in LY_{0.5} ($278 \pm 92 \mu\text{mol/L}$) was not reached until 270 min after the start of the clamp, and LY_{0.375} and LY_{0.25} did not reach their lowest concentrations (353 ± 130 and $467 \pm 96 \mu\text{mol/L}$, respectively) until the last hour. An insulin-induced fall in circulating NEFA provides an indirect means for reducing hepatic glucose production (38,39), and thus the sluggish response of glycerol and NEFA concentrations to LY administration suggests that it was the direct effect of the analog that inhibited hepatic glucose production.

A decrease in NEFA levels and hepatic NEFA uptake is associated with an increase in hepatic glycolysis (39). The exact mechanism for this is unclear, but it may well result from a fall in intrahepatic citrate, one of the major inhibitors of phosphofructokinase, the first rate-determining enzyme in the glycolytic pathway. Alternatively, the fall in NEFA could alter the intrahepatic redox state, increasing the NADH-to-NAD ratio and leading to an activation of lactate dehydrogenase. (For further discussion, see Sindelar et al. [39]) Thus, when net hepatic NEFA uptake decreased in the control group, there was an increase in the release of glycolytic carbon (lactate) from the liver. Conversely, there appeared to be a prompt suppression of glycolysis (hepatic lactate release) in response to LY, likely related to the increase in lipolysis early in the experimental period. The two lower rates of LY infusion not only reduced NHLO but also actually prompted the liver to shift to net uptake of lactate, consistent with increased storage of carbon as glycogen in the liver.

The liver is a key organ in the extraction of regular insulin, but in contrast, hepatic extraction of LY

appeared to be very low. As with other slowly cleared insulin analogs, such as detemir (40), the plasma LY concentrations were high relative to those of regular insulin. However, it is evident that we achieved three distinct LY levels and near-steady-state concentrations, particularly with the longer studies in Part B. With such high circulating concentrations and low clearance, it is difficult to quantify tissue LY extraction precisely. Even though the kidney was an important site of extraction for LY, renal LY extraction was proportionally much less than with human insulin, as designed (9). When LY was tested in subjects with renal impairment (ranging from mild to end-stage renal disease), the apparent clearance and half-life of the drug did not differ from that in subjects with normal renal function (41). Thus, the pharmacokinetic properties of LY do not appear to be affected by impairment in renal function.

Because of the large hydrodynamic size of LY, it might not be expected to cross the blood-brain barrier (42). Nevertheless, hypothalamic Akt phosphorylation at the end of the experiment was not different in the control and LY-treated animals. The affinity of LY for the insulin receptor, although low (<6% of that of insulin lispro [43]), might permit some passage into the central nervous system (CNS) via saturable mechanisms. Additionally, there is a potential for LY to reach the CNS via extracellular pathways such as are used by some very large molecules, e.g., albumin and certain antibodies (44–46). The current findings are not unique or completely unexpected, since the insulin analog detemir (which is tightly bound to serum albumin [47]) has been reported not to cross the mouse blood-brain barrier (48), and yet it has a more rapid and pronounced effect on hypothalamic insulin receptor phosphorylation than human insulin when both are injected intravenously in mice (49). The similarity of phosphorylated STAT3/STAT3 in the livers of the control and LY_{0.5} groups is consistent with an effect of LY in the CNS, since hepatic STAT3 phosphorylation has been suggested to be a marker of brain insulin signaling (50,51) that can be blunted with infusion of an ATP-sensitive K⁺-channel inhibitor or a phosphatidylinositol-3 kinase inhibitor into the 3rd cerebroventricle of the dog (51). Nevertheless, hepatic STAT3 phosphorylation in response to brain insulin signaling occurs slowly also (50–52), affecting gene transcription slowly, and thus could not have been involved in bringing about the rapid response effect on liver glucose metabolism observed with LY infusion.

The significant stimulation of hepatic Akt and GSK3 β phosphorylation in the LY-treated versus control animals is in keeping with the hepato-preferential metabolic actions of the analog. Conversely, the lack of apparent stimulation of phosphorylation of these proteins by regular insulin (in view of the data from the 18-h-fasted, non-insulin-treated animals) is in keeping with the lack of increase in hepatic insulin concentrations during the clamp; indeed, the hepatic sinusoidal insulin

concentrations were below basal in the control group. GSK3 β is inactivated by phosphorylation, thus allowing glycogen synthesis to proceed. Consistent with this, net hepatic carbon retention, a good surrogate measure for hepatic glycogen synthesis (53), was enhanced in the LY-treated groups.

The physiologic basis of the hepato-preferential effect of LY is not fully understood but is likely related to its large hydrodynamic size. While this may impede passage from the capillaries into the interstitium of peripheral target tissues, i.e., muscle and adipose, the hepatic sinusoidal capillaries are fenestrated, with openings of 100–200 Å in diameter, allowing entry of larger molecules into the organ (e.g., lipoproteins), with many factors affecting hepatic clearance (54,55).

In conclusion, LY at all dosages examined exhibited a more hepato-preferential effect than regular human insulin when both products were delivered by peripheral vein. The effect on the liver was evident throughout the duration of the clamp period, with the use of both tracer and organ balance techniques. If these effects can be sustained during long-term dosing, peripherally administered LY appears to have the potential to reproduce the hepato-preferential effects of endogenously secreted insulin—an effect that might reduce complications associated with current insulin therapy.

Funding. The Vanderbilt Diabetes Research and Training Center Metabolic Physiology Shared Resource and Hormone Assay and Analytical Services Cores (SP-60-AM20593) made important contributions to this work. A.D.C. holds the Jacquelyn A. Turner and Dr. Dorothy J. Turner Chair in Diabetes Research.

Duality of Interest. These studies were funded by Eli Lilly and Company. V.P.S. is a shareholder of Eli Lilly and Company and was an employee at the time of this study. J.M.B., M.D.M., and S.J.J. are employees and shareholders of Eli Lilly and Company. A.D.C. is a consultant for Eli Lilly and Company. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. M.C.M. carried out the studies, interpreted data, and drafted the manuscript. M.S.S. carried out the studies and analyses. V.P.S., S.J.J., and A.D.C. participated in the design of the studies. Interpreted data, and reviewed and edited the manuscript. J.M.B. interpreted data and reviewed and edited the manuscript. M.D.M. participated in the design of the studies and reviewed and edited the manuscript. A.D.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 72nd Scientific Sessions of the American Diabetes Association, Philadelphia, PA, 8–12 June 2012, and at the 48th Annual Meeting of the European Association for the Study of Diabetes, Berlin, Germany, 1–5 October 2012.

References

- Little S, Shaw J, Home P. Hypoglycemia rates with basal insulin analogs. *Diabetes Technol Ther* 2011;13(Suppl. 1):S53–S64
- Bolli GB, Andreoli AM, Lucidi P. Optimizing the replacement of basal insulin in type 1 diabetes mellitus: no longer an elusive goal in the post-NPH era. *Diabetes Technol Ther* 2011;13(Suppl. 1):S43–S52

3. Simon AC, DeVries JH. The future of basal insulin supplementation. *Diabetes Technol Ther* 2011;13(Suppl. 1):S103–S108
4. Heise T, Nosek L, Bottcher SG, Hastrup H, Haahr H. Ultra-long-acting insulin degludec has a flat and stable glucose-lowering effect in type 2 diabetes. *Diabetes Obes Metab* 2012;14:944–950
5. Jonassen I, Havelund S, Hoeg-Jensen T, Steensgaard DB, Wahlund PO, Ribel U. Design of the novel protraction mechanism of insulin degludec, an ultra-long-acting basal insulin. *Pharm Res* 2012;29:2104–2114
6. Birkeland KI, Home PD, Wendisch U, et al. Insulin degludec in type 1 diabetes: a randomized controlled trial of a new-generation ultra-long-acting insulin compared with insulin glargine. *Diabetes Care* 2011;34:661–665
7. Bode BW, Buse JB, Fisher M, et al.; BEGIN® Basal-Bolus Type 1 Trial Investigators. Insulin degludec improves glycaemic control with lower nocturnal hypoglycaemia risk than insulin glargine in basal-bolus treatment with mealtime insulin aspart in Type 1 diabetes (BEGIN® Basal-Bolus Type 1): 2-year results of a randomized clinical trial. *Diabet Med* 2013;30:1293–1297
8. Pandeyarajan V, Weiss MA. Design of non-standard insulin analogs for the treatment of diabetes mellitus. *Curr Diab Rep* 2012;12:697–704
9. Beals JM, Cutler GB, Doyle B, et al. PEGylated insulin lispro compounds. International Patent Application WO 2009/1521128 A1. 17 Dec 2009
10. Hansen RJ, Cutler GB Jr, Vick A, et al. LY2605541: leveraging hydrodynamic size to develop a novel basal insulin (Abstract). *Diabetes* 2012;61 (Suppl. 1):A228
11. Sinha VP, Howey DC, Soon DKW, et al. Single-dose pharmacokinetics (PK) and glucodynamics (GD) of the novel, long-acting basal insulin LY2605541 in healthy subjects (Abstract). *Diabetes* 2012;61(Suppl. 1):A273
12. Heise T, Howey DC, Sinha VP, Choi SL, Mace KF. Steady-state pharmacokinetics (PK) and glucodynamics (GD) of the novel, long-acting basal insulin LY2605541 dosed once-daily (QD) in patients with type 2 diabetes mellitus (T2DM). *Diabetes* 2012;61(Suppl. 1):A256
13. Bergenstal RM, Rosenstock J, Arakaki RF, et al. A randomized, controlled study of once-daily LY2605541, a novel long-acting basal insulin, versus insulin glargine in basal insulin-treated patients with type 2 diabetes. *Diabetes Care* 2012;35:2140–2147
14. Rosenstock J, Bergenstal RM, Blevins TC, et al. Better glycemic control and weight loss with the novel long-acting basal insulin LY2605541 compared with insulin glargine in type 1 diabetes: a randomized, crossover study. *Diabetes Care* 2013;36:522–528
15. McGuinness OP, Fujiwara T, Murrell S, et al. Impact of chronic stress hormone infusion on hepatic carbohydrate metabolism in the conscious dog. *Am J Physiol* 1993;265:E314–E322
16. Wasserman DH, Lacy DB, Bracy D, Williams PE. Metabolic regulation in peripheral tissues and transition to increased gluconeogenic mode during prolonged exercise. *Am J Physiol* 1992;263:E345–E354
17. Winnick JJ, An Z, Moore MC, et al. A physiological increase in the hepatic glycogen level does not affect the response of net hepatic glucose uptake to insulin. *Am J Physiol Endocrinol Metab* 2009;297:E358–E366
18. Galassetti P, Chu CA, Neal DW, Reed GW, Wasserman DH, Cherrington AD. A negative arterial-portal venous glucose gradient increases net hepatic glucose uptake in euglycemic dogs. *Am J Physiol* 1999;277:E126–E134
19. Moore MC, Rossetti L, Pagliassotti MJ, et al. Neural and pancreatic influences on net hepatic glucose uptake and glycogen synthesis. *Am J Physiol* 1996;271:E215–E222
20. Ramnanan CJ, Saraswathi V, Smith MS, et al. Brain insulin action augments hepatic glycogen synthesis without suppressing glucose production or gluconeogenesis in dogs. *J Clin Invest* 2011;121:3713–3723
21. Galassetti P, Shota M, Zinker BA, Wasserman DH, Cherrington AD. A negative arterial-portal venous glucose gradient decreases skeletal muscle glucose uptake. *Am J Physiol* 1998;275:E101–E111
22. Edgerton DS, Ramnanan CJ, Grueter CA, et al. Effects of insulin on the metabolic control of hepatic gluconeogenesis in vivo. *Diabetes* 2009;58:2766–2775
23. Mari A, Stojanovska L, Proietto J, Thorburn AW. A circulatory model for calculating non-steady-state glucose fluxes. Validation and comparison with compartmental models. *Comput Methods Programs Biomed* 2003;71:269–281
24. Chap Z, Ishida T, Chou J, et al. First-pass hepatic extraction and metabolic effects of insulin and insulin analogues. *Am J Physiol* 1987;252:E209–E217
25. Sindelar DK, Balcom JH, Chu CA, Neal DW, Cherrington AD. A comparison of the effects of selective increases in peripheral or portal insulin on hepatic glucose production in the conscious dog. *Diabetes* 1996;45:1594–1604
26. Sindelar DK, Chu CA, Venson P, Donahue EP, Neal DW, Cherrington AD. Basal hepatic glucose production is regulated by the portal vein insulin concentration. *Diabetes* 1998;47:523–529
27. Coate KC, Kraft G, Lautz M, Smith M, Neal DW, Cherrington AD. A high-fat, high-fructose diet accelerates nutrient absorption and impairs net hepatic glucose uptake in response to a mixed meal in partially pancreatectomized dogs. *J Nutr* 2011;141:1643–1651
28. Sönksen PH, Russell-Jones D, Jones RH. Growth hormone and diabetes mellitus. A review of sixty-three years of medical research and a glimpse into the future? *Horm Res* 1993;40:68–79
29. Hopkins KD, Russell-Jones DL, Lehmann ED, Wheeler MJ, Sönksen PH. Intraperitoneal insulin affects insulin-like growth factor binding protein-1 in a well-controlled type I diabetic patient. *Diabetes Care* 1993;16:1404–1405
30. Shojaei-Moradie F, Powrie JK, Sundermann E, et al. Novel hepatoselective insulin analog: studies with a covalently linked thyroxyl-insulin complex in humans. *Diabetes Care* 2000;23:1124–1129
31. Avogaro A, Beltramo P, Gnudi L, et al. Alcohol intake impairs glucose counterregulation during acute insulin-induced hypoglycemia in IDDM patients. Evidence for a critical role of free fatty acids. *Diabetes* 1993;42:1626–1634
32. Fanelli C, Calderone S, Epifano L, et al. Demonstration of a critical role for free fatty acids in mediating counterregulatory stimulation of gluconeogenesis and suppression of glucose utilization in humans. *J Clin Invest* 1993;92:1617–1622
33. Lucidi P, Rossetti P, Porcellati F, et al. Mechanisms of insulin resistance after insulin-induced hypoglycemia in humans: the role of lipolysis. *Diabetes* 2010;59:1349–1357
34. Rivera N, Ramnanan CJ, An Z, et al. Insulin-induced hypoglycemia increases hepatic sensitivity to glucagon in dogs. *J Clin Invest* 2010;120:4425–4435
35. Connolly CC, Myers SR, Neal DW, Hastings JR, Cherrington AD. In the absence of counterregulatory hormones, the increase in hepatic glucose production during insulin-induced hypoglycemia in the dog is initiated in the liver rather than the brain. *Diabetes* 1996;45:1805–1813
36. Jackson PA, Cardin S, Coffey CS, et al. Effect of hepatic denervation on the counterregulatory response to insulin-induced hypoglycemia in the dog. *Am J Physiol Endocrinol Metab* 2000;279:E1249–E1257
37. Jackson PA, Pagliassotti MJ, Shota M, Neal DW, Cardin S, Cherrington AD. Effects of vagal blockade on the counterregulatory response to insulin-induced hypoglycemia in the dog. *Am J Physiol* 1997;273:E1178–E1188

38. Rebrin K, Steil GM, Mittelman SD, Bergman RN. Causal linkage between insulin suppression of lipolysis and suppression of liver glucose output in dogs. *J Clin Invest* 1996;98:741-749
39. Sindelar DK, Chu CA, Rohlie M, Neal DW, Swift LL, Cherrington AD. The role of fatty acids in mediating the effects of peripheral insulin on hepatic glucose production in the conscious dog. *Diabetes* 1997;46:187-196
40. Moore MC, Smith MS, Turney MK, Boysen S, Williams PE. Comparison of insulins detemir and glargine: effects on glucose disposal, hepatic glucose release and the central nervous system. *Diabetes Obes Metab* 2011;13:832-840
41. Linnebjerg H, Choi SL, Lam ECQ, Mace KF, Hodgson TS, Sinha VP. Pharmacokinetics (PK) of the novel, long-acting basal insulin LY2605541 in subjects with varying degrees of renal function (Abstract). *Diabetes* 2012; 61(Suppl. 1):A296
42. Banks WA. Characteristics of compounds that cross the blood-brain barrier. *BMC Neurol* 2009;9(Suppl. 1):S3
43. Owens RA, Lockwood JF, Dunbar JD, et al. In vitro characterisation of novel basal insulin LY2605541: reduced mitogenicity and IGF-IR binding. *Diabetologia* 2012;55:120
44. Banks WA. Brain meets body: the blood-brain barrier as an endocrine interface. *Endocrinology* 2012;153:4111-4119
45. Banks WA, Broadwell RD. Blood to brain and brain to blood passage of native horseradish peroxidase, wheat germ agglutinin, and albumin: pharmacokinetic and morphological assessments. *J Neurochem* 1994;62:2404-2419
46. Broadwell RD, Sofroniew MV. Serum proteins bypass the blood-brain fluid barriers for extracellular entry to the central nervous system. *Exp Neurol* 1993;120:245-263
47. Kurtzhals P, Havelund S, Jonassen I, Kiehr B, Ribel U, Markussen J. Albumin binding and time action of acylated insulins in various species. *J Pharm Sci* 1996;85:304-308
48. Banks WA, Morley JE, Lynch JL, Lynch KM, Mooradian AD. Insulin detemir is not transported across the blood-brain barrier. *Peptides* 2010;31:2284-2288
49. Hennige AM, Sartorius T, Tschritter O, et al. Tissue selectivity of insulin: cetemir action in vivo. *Diabetologia* 2006;49:1274-1282
50. Inoue H, Ogawa W, Asakawa A, et al. Role of hepatic STAT3 in brain-insulin action on hepatic glucose production. *Cell Metab* 2006;3:267-275
51. Ramnanan CJ, Kraft G, Smith MS, et al. Interaction between the central and peripheral effects of insulin in controlling hepatic glucose metabolism in the conscious dog. *Diabetes* 2013;62:74-84
52. Ramnanan CJ, Edgerton DS, Rivera N, et al. Molecular characterization of insulin-mediated suppression of hepatic glucose production in vivo. *Diabetes* 2010;59:1302-1311
53. Satake S, Moore MC, Igawa K, et al. Direct and indirect effects of insulin on glucose uptake and storage by the liver. *Diabetes* 2002;51:1663-1671
54. Baker M, Parton T. Kinetic determinants of hepatic clearance: plasma protein binding and hepatic uptake. *Xenobiotica* 2007;37:1110-1134
55. Braet F, Wisse E. Structural and functional aspects of liver sinusoidal endothelial cell fenestrae: a review. *Comp Hepatol* 2002;1:1