

JOSE LLOREDA CAMACHO & CO.

ABOGADOS

Calle 72 No. 5-83 Piso 5, Bogotá, D.C. Tel: 6069700 Fax 6069701

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-010508- -00010-0000

Fecha: 2013-04-11 17:07:36 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 412 REPOSPRESRECU Folios: 57

Señor

SUPERINTENDENTE DE INDUSTRIA Y COME
DIVISIÓN DE NUEVAS CREACIONES

E. S. D.

Re: PI.-ARRAY BIOPHARMA INC. y GENENTECH, INC.-Solicitud de Patente de
Invención en Fase Nacional PCT para "CICLOPENTA [D] PIRIMIDINAS
HIDROXILADAS Y METOXILADAS COMO INHIBIDORES DE LA
PROTEÍNA KINASA AKT".-Clase C07D 239/70.-Expediente número 09-
010.508.-

Yo, **ALICIA LLOREDA RICAURTE**, mayor, vecina de esta ciudad, identificada con la cédula de ciudadanía número 39.690.713 de Usaquén, Representante Legal en Bogotá para todos los asuntos relacionados con la Propiedad Industrial de las sociedades **ARRAY BIOPHARMA INC. y GENENTECH, INC.**, organizadas y existentes de conformidad con las leyes del Estado de Colorado, Estados Unidos de América y del Estado de California, Estados Unidos de América, domiciliadas en Boulder, Estado de Colorado, Estados Unidos de América y South San Francisco, Estado de California, Estados Unidos de América, atentamente manifiesto a usted que interpongo **RECURSO DE REPOSICIÓN** contra la Resolución Número **6084**, dictada por el señor Superintendente de Industria y Comercio el 25 de Febrero de 2013, en relación con la negación del privilegio de patente de invención para la solicitud en referencia, por una supuesta falta de **Nivel Inventivo** de dicha solicitud.

I. FUNDAMENTOS DE DERECHO

Me permito manifestar ante ese Despacho que el mencionado Recurso de Reposición encuentra fundamento en que el contenido de la Resolución Número **6084** evidencia que durante el examen técnico realizado a la solicitud denegada de ninguna manera se consideró el alcance e importancia de los compuestos derivados de ciclopenta[d]pirimidinas hidroxiladas que son objeto de la invención allí reclamada en su verdadera dimensión.

Es así que, a pesar de los argumentos proporcionados por el solicitante en el memorial de Noviembre 23 de 2012 (en respuesta al Oficio No. **15034**), en la Resolución impugnada ese Despacho mantiene las objeciones frente al Nivel Inventivo de la solicitud.

En vista de lo anterior, a continuación me permito brindar argumentos técnicos que no sólo demostrarán a ese Despacho que la presente invención sí cumple con el requisito de Nivel Inventivo exigido por el Artículo 18 de la Decisión 486 de 2000, sino que además establecerán que el análisis realizado es incorrecto, toda vez que se interpreta de manera errónea tanto la invención como el arte previo; constituyéndose por lo tanto la negación de la solicitud en una clara violación al mencionado Artículo 18 de la Decisión Andina 486 de 2000.

Adicionalmente, me permito señalar que con el fin de clarificar aún más el objeto de la invención y resaltar las diferencias que existen con respecto al arte previo es deseo del solicitante modificar el capítulo reivindicatorio actualmente presentado, tal como se expondrá a continuación.

1. PRESENTACIÓN DE SOLICITUD DIVISIONAL

(a) Inicialmente y de conformidad con el Artículo 36 de la Decisión Andina 486 de 2000, el Artículo 7 del Decreto 2591 de 13 de Diciembre de 2000 y el Artículo 11 de la Resolución Número 00210 de 15 de Enero de 2001, me permito manifestar que **la presente solicitud de patente se ha dividido en dos solicitudes**, a saber:

(i) Una solicitud original compuesta por 28 reivindicaciones, las cuales corresponden a una limitación de la materia reclamada por el capítulo reivindicatorio actualmente presentado para la solicitud de patente número **09-010.508**, modificado como se expondrá más adelante.

(ii) Una solicitud fraccionada número **09-010.508A**, la cual comprende 87 reivindicaciones que encuentran pleno sustento en el capítulo reivindicatorio presentado junto con mi memorial de 23 de Noviembre de 2012 para la solicitud número **09-010.508**.

(b) En conexión con lo anterior, a continuación especifico los documentos anexos al presente memorial y que se relacionan con cada una de las solicitudes resultantes del presente fraccionamiento:

(i) **Solicitud Original: "CICLOPENTA [D] PIRIMIDINAS HIDROXILADAS Y METOXILADAS COMO INHIBIDORES DE LA PROTEÍNA KINASA AKT"**

Capítulo reivindicatorio modificado compuesto por 28 reivindicaciones.

(ii) **Solicitud Divisional A: "CICLOPENTA [D] PIRIMIDINAS HIDROXILADAS Y METOXILADAS COMO INHIBIDORES DE LA PROTEÍNA KINASA AKT"**.

JOSE LLOREDA CAMACHO & CO.

Calle 72 No. 5-83 Piso 5, Bogotá, D.C. Tel: 6069700 Fax 6069701

- Recibo número 13-0029800, expedido por la Superintendencia de Industria y Comercio, que acredita el pago de los derechos que causa el presente fraccionamiento;
- Recibos número 13-0032355, 13-0032353, 13-0029824, 13-0029823, 13-0029832, 13-0032376, 13-0029835, 13-0029837 y 13-0029836, expedidos por la Superintendencia de Industria y Comercio, que acreditan el pago por las reivindicaciones **11 a 87**;
- Nuevo Resumen-Objeto y Finalidad de la Invención;
- Páginas **1 a 207** de la descripción, que corresponden al texto de la descripción actualmente presentado para la solicitud de patente número **09-010.508**;
- Páginas **208 a 247** que corresponden al nuevo capítulo reivindicatorio compuesto por 87 reivindicaciones que, como se expuso previamente, encuentran pleno sustento en el capítulo reivindicatorio presentado ante ese Despacho junto con mi memorial de 23 de Noviembre de 2012 para la solicitud número **09-010.508**;
- Arte Final;
- Extracto de la solicitud, en sus dos formas, que incluye el título que se encuentra de acuerdo con el objeto de la presente invención fraccionada y que incluye la reivindicación 1;
- Tarjeta de propietario;
- Copia del documento que acredita los traspasos de los derechos de la presente invención que los autores de dicho invento hacen a favor de mis representados, junto con sus Traducciones Oficiales;
- Copia de los documentos que acreditan a la suscrita ALICIA LLOREDA y/o al doctor GUSTAVO TAMAYO, y/o a la doctor IGNACIO SANTAMARÍA y/o a al doctor ENRIQUE ÁLVAREZ, en los términos del Párrafo 1º del Artículo 543 del Decreto-Ley 410 de 1971, como Representantes Legales en Bogotá para todos los asuntos relacionados con la Propiedad Industrial de las sociedades **ARRAY BIOPHARMA INC.** y **GENENTECH, INC.**, organizadas y existentes de conformidad con las leyes del Estado de Colorado, Estados Unidos de América y el Estado de California, Estados Unidos de América, domiciliadas en Boulder,

Estado de Colorado, Estados Unidos de América y South San Francisco, Estado de California, Estados Unidos de América; y

- Formato Único de Conversión, División y Fusión de Solicitudes.

(c) Como la fecha de presentación de la solicitud resultante del presente fraccionamiento es la misma --**4 de Febrero de 2009**--, atentamente solicito a usted se sirva dar a la solicitud fraccionada el mismo número de Expediente de la solicitud original, acompañada de la letra **A**, para su identificación.

(d) En cuanto a la reivindicación de la prioridad respecto de la solicitud divisional, es la misma invocada en la solicitud original, la cual fue reivindicada oportunamente en Fase Internacional de la solicitud número US 60/818,718 de 6 de Julio de 2006.

(e) Ratifico a usted que las sociedades titulares de la solicitud fraccionada en cuestión son **ARRAY BIOPHARMA INC.** y **GENENTECH, INC.**, organizadas y existentes de conformidad con las leyes del Estado de Colorado, Estados Unidos de América y el Estado de California, Estados Unidos de América, domiciliadas en Boulder, Estado de Colorado, Estados Unidos de América y South San Francisco, Estado de California, Estados Unidos de América.

(f) Así mismo, reitero a usted que el documento que acredita mi calidad de Representante Legal en dichas sociedades para todos los asuntos de la Propiedad Industrial, fueron presentados con mi petitorio de **4 de Febrero de 2009**.

(g) De conformidad con lo establecido por el Artículo 36 de la Decisión 486 de 2000, solicito a ese Despacho se sirva aceptar y estudiar la solicitud divisional presentada y conceder el privilegio de patente solicitado.

2. MODIFICACIONES AL CAPÍTULO REIVINDICATORIO

Antes de entrar a discutir los argumentos que demuestran el Nivel Inventivo que conlleva la solicitud en estudio, y en conexión con la presentación de la solicitud divisional anteriormente señalada, me permito manifestar que el solicitante, al amparo de lo estipulado por el Artículo 34 de la Decisión Andina 486 de 2000, ha modificado el capítulo reivindicatorio actualmente presentado, con el fin de delimitar más claramente la invención y hacer aún más evidentes las características que no podrían de ninguna manera ser derivadas del arte previo.

JOSE LLORED A CAMACHO & CO.Calle 72 No. 5-83 Piso 5, Bogotá, D.C. Tel: 6069700 Fax 6069701

Respecto a estas modificaciones, me permito resaltar que no implican de ninguna manera una ampliación al alcance de la solicitud, tal como fue originalmente presentada, toda vez que se está limitando el alcance de la solicitud de conformidad con las realizaciones preferidas de la invención, como se presenta a continuación:

(i) **Reivindicación 1:** Corresponde a la reivindicación 1 actualmente presentada en donde la materia reclamada ha sido restringida a las realizaciones más preferidas de la invención comprendidas por la Fórmula 1B. Sustento para esta modificación se encuentra a lo largo del texto de la solicitud, por ejemplo en el párrafo [00126] de la memoria descriptiva.

(ii) **Reivindicación 2:** Corresponde a la reivindicación 11 actualmente presentada.

(iii) **Reivindicación 3:** Esta reivindicación encuentra sustento en el párrafo [0064] del texto de la descripción actualmente presentado para la solicitud.

(iv) **Reivindicaciones 4 a 11:** Estas reivindicaciones corresponden respectivamente a las reivindicaciones 12, 15, 16, 17, 18, 19, 20 y 30 actualmente presentadas.

(v) **Reivindicación 12:** Esta reivindicación corresponde a la reivindicación 71 actualmente presentada en donde se han eliminado los compuestos que ya no hacen parte del alcance del capítulo reivindicatorio ahora modificado.

(vi) **Reivindicaciones 13 a 27:** Corresponden de manera respectiva a las reivindicaciones 73 a 87 actualmente presentadas.

(vii) **Reivindicación 28:** Corresponde a la reivindicación 72 actualmente presentada.

(viii) Adicionalmente, las dependencias de las reivindicaciones han sido ajustadas de conformidad con la numeración ahora dada al capítulo reivindicatorio modificado.

(ix) Finalmente, las reivindicaciones 2 a 10, 13, 14, 21 a 29, 31 a 70 actualmente presentadas ya no hacen parte del capítulo reivindicatorio ahora modificado.

Consecuentemente, el capítulo reivindicatorio modificado está constituido por **28 reivindicaciones** las cuales se refieren de manera particular a realizaciones preferidas de la invención, en concordancia con las enseñanzas de la descripción tal como ha sido presentada.

En vista de este hecho, y a la luz de la facultad que la Decisión Andina 486 otorga al solicitante mediante las disposiciones que establece el Artículo 34 de la misma:

"Artículo 34.- El solicitante de una patente podrá pedir que se modifique la solicitud en cualquier momento del trámite. La modificación no podrá implicar una ampliación de la protección que correspondería a la divulgación contenida en la solicitud inicial.

Del mismo modo, se podrá pedir la corrección de cualquier error material" (énfasis añadido).

Resulta claro que la presentación del mencionado capítulo reivindicatorio modificado y de la solicitud divisional antes referenciada está amparada bajo la legislación Andina, y por lo tanto muy comedidamente solicito a ese Despacho tener en consideración dicho capítulo reivindicatorio modificado en su análisis del presente Recurso y aceptar a trámite la mencionada solicitud divisional.

3. ARGUMENTOS QUE DEMUESTRAN EL NIVEL INVENTIVO DE LA INVENCION DENEGADA DE ACUERDO CON LO ESTABLECIDO EN EL ARTICULO 18 DE LA DECISION ANDINA 486 DE 2000

Por otro lado, en relación con el Nivel Inventivo de la presente solicitud me permito manifestar que ese Despacho no repara en los argumentos presentados en mi memorial de 23 de Noviembre de 2012, los cuales establecen de forma incontrovertible el Nivel Inventivo que conlleva la solicitud denegada frente al estado del arte más próximo, y por lo tanto ese Despacho afirma en la Resolución impugnada que el documento **WO 2005051304 (D1)** afecta la altura inventiva de la solicitud presentada a la luz de los siguiente argumentos:

Con relación a este documento el señor Examinador sostiene que la diferencia entre los compuestos de la reivindicación 1 de la presente invención y aquellos compuestos del documento D1 consiste en *"la variación del anillo de cinco miembros de la estructura fusionada, ya que en los compuestos de la solicitud corresponde a un anillo carbocíclico de 5 miembros de tipo ciclopentano sustituidos en la posición 7 por hidroxilo (-OH), mientras que en el documento D1 corresponde a un anillo de cinco miembros heterocíclicos como: pirrol, 5H-pirrol, o 1,3-dihidro-pirrolidin-2-ona o en su defecto a un anillo carbocíclico y aromático pero de seis miembros como fenilo"*.

Sumado a lo anterior, el señor Examinador afirma que *"el documento D1 da a conocer compuestos inhibidores de la proliferación celular inducida por el receptor AKT y las quinasas relacionadas que tiene en su estructura anillos de piperazon-pirrol[2,3-d]pirimidinas, piperazin-5H-pirrol[2,3-d]pirimidinas o piperazin-1,3-dihidro-pirrolidin-2-*

ona[2,3-b]pirimidinas, entre otros, que son similares al de (7-hidroxi-ciclopenta)[d]pirimidin-4-il)piperazina reclamado."

A lo anterior ese Despacho añade que "La comparación de Ej.s específicos de la solicitud (Ej.s 5, 82 y 130) frente a los Ej.s del documento D1 (Ej.s 73, 76, 13 y 107), evidencia que el anillo de 7-hidroxi-ciclopentano es bioisótero de: pirrolidina, 5H-pirrolina o de 1,3-dihidro-pirrolidin-2-ona. En consecuencia, una persona normalmente versada en la materia, incluiría el anillo de 7-hidroxi-ciclopentano en los compuestos que se dan a conocer en el documento D1, esperando con certeza diseñar una serie alternativa de compuestos inhibidores de proliferación celular como los de la reivindicación 1 de la solicitud.

Lo anterior, considerando que el actividad anticancerígena se debe al núcleo farmacofórico de la molécula y la modificación realizada en la estructura, que consiste en el remplazo del anillo de cinco miembros y la introducción de un sustituyente tipo -OH en la posición 7 del anillo fusionado, no ocasiona una variación importante en su actividad. Por esta razón, es posible concluir con certeza que la materia reclamada es un derivación del estado de la técnica y por lo tanto, no tiene nivel inventivo."

Para indicar la valides de sus afirmaciones el señor Examinador señala el documento de Gui-Dong Zhu y col¹ con relación al cual sostiene que "existen estudios de relación estructura actividad en los que se evalúa la selectividad de algunos compuestos y se demuestra la manera de optimizar series de inhibidores específicos del sitio alostérico de la quinasa AKT, frete a otros receptores de la familia de quinasas AGC como las de tipo PKA, mediante estrategias de remplazo de farmacóforo de isoquinolina o quinazolina por una estructura de oxi-indol (Ej. 9e) que presenta una concentración IC₅₀ de inhibición de AKT 1 de 3.1 nM (Pág 3426 Gui-Dong Zhu y col.)"

Por lo tanto, el señor Examinador manifiesta que "el estudio de relación estructura actividad demuestra que existe relación de biosistería entre las isoquinolinas y las estructura de oxi-indol, pirrolidin-2-ona[2,3-b]piridina, tieno[2,3-c]piridina, porque presentan actividad selectiva frete a la quinasa Akt-1."

A lo anterior añade el señor Examinador que por medio del estudio de Gui-Dong Zhu y col "se corrobora lo indicado en el documento D1 para los Ej.s 73, 76, 13 y 107 estructuralmente cercanos a los compuestos de (7-OH-ciclopenta)[d]pirimidin-4-il)piperazina reclamados, en lo que corresponde a la selectividad frente al receptor Akt 1, por lo que se puede concluir con certeza que no existe evidencia de efecto inesperado por el hecho de reemplazar el anillo de pirrol por el anillo de 7-OH-cilopentano ya que la regla

¹ Gui-Dong Zhu et al. "Discovery and SAR of oxindole-pyridine-based protin kinase B/Akt inhibitors for treating cancers", Bioinirganic & Medical Chemistry Letters 16, (2006), pp. 3424-3429.

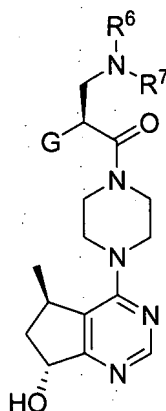
técnica le anticipaba a la persona versada en la materia la manera de sustituir los anillo en el grupo farmacofórico."

Por otro, el señor Examinador concluye que los compuestos reclamados por el capítulo reivindicatorio de la solicitud no tendrían nivel inventivo frente a las enseñanzas de D1.

Visto lo anterior y con el ánimo de demostrar que el capítulo reivindicatorio ahora modificado de la solicitud en cuestión presenta un inequívoco carácter inventivo frente a las enseñanzas del documento D1, atentamente me permito presentar las siguientes consideraciones técnicas.

3.1. La presencia de una ciclopenta [d] pirimidina hidroxilada en la estructura central de los compuestos de la presente solicitud sí genera efectos técnicos significativamente diferentes frente a los compuestos divulgados en D1

Para comenzar me permito resaltar que en virtud de las modificaciones realizadas al capítulo reivindicatorio, los compuestos que ahora son objeto del capítulo reivindicatorio modificado son específicamente los derivados de ciclopenta [d] pirimidinas hidroxiladas que tienen la fórmula 1B:



En relación a estos compuestos de fórmula 1B me permito señalar que:

- (i) En primer lugar, el párrafo [0039] de la página 11 del texto de la descripción de la presente solicitud explica que **cuando el grupo OR² (de la Formula I) es un grupo OH, tal como se reclama en los compuestos de Fórmula 1B del capítulo reivindicatorio modificado que se adjunta a este escrito, los compuestos obtenidos son al menos 50 veces más selectivos para AKT versus la proteína quinasa A (PKA), y en algunos casos son al menos 150 veces más selectivos.**

(ii) Sumado a lo anterior, el párrafo [0039] de la página 11 del texto de la descripción de la solicitud también señala que **es deseable la selectividad hacia AKT sobre la PKA "dado que la PKA está involucrada en diversos procesos celulares importantes para la función y fisiología normales de diversos tipos celulares. Adicionalmente, se cree que la inhibición de PKA no contribuye a los efectos anti-proliferativos y pro-apoptóticos de la inhibición de AKT. Por consiguiente, la inhibición de PKA podría producir efectos adversos no asociados con la inhibición de AKT sin contribuir a los beneficios modificadores de enfermedad de la inhibición de AKT"**.

En este sentido me permito dirigir la atención de ese Despacho a los estudios de Kass R. S. y Moss A. J.²; Marx S. O. y col.³; y Marks A. R.⁴, los cuales se adjuntan a este memorial, que discuten el rol de PKA en los canales cardíacos, resultando evidente para la persona versada en la materia a partir de las enseñanzas de tales documentos que la inhibición de PKA puede conducir a la cardiotoxicidad.

(iii) Además, los párrafos [00269] a [00276] de la descripción de la solicitud en cuestión describen algunos ensayos biológicos realizados para algunos de los compuestos los cuales, de conformidad con el párrafo [00276], todos tienen un valor de IC₅₀ de menos de 10µM.

(iv) En contraste con las revelaciones de la presente solicitud, de la lectura detallada del documento D1 la persona versada en la materia notará que **D1 no enseña que se desee una selectividad hacia AKT frente a PKA, de hecho, el párrafo [0008] de D1 lista PKA como un posible objetivo para terapia.**

En este sentido, me permito dirigir la atención de ese Despacho a la Tabla de Datos 2 que se adjunta al presente escrito en donde se encuentran las estructuras y resultados de actividad correspondientes de ciertos compuestos del documento D1. La persona versada en la materia reconocerá de esta Tabla que los resultados encontrados para los Ejemplos 59 y 73 de D1 establecen que estos compuestos de D1 son selectivos hacia PKA por sobre AKT.

Sumado a lo anterior vale la pena resaltar que inclusive dentro del documento D1 no se encuentran resultados de ensayos bioquímicos de inhibición de PKA o AKT, lo único que se menciona dentro de D1 a este respecto se encuentra en los párrafos [00288] a [00296] en donde se describe el protocolo para la determinación de la inhibición de AKT

² Kass R. S. y Moss A. J., "Long QT síndrome: novel insights into the mechanism of cardiac arrhythmias", The Journal of Clinical Investigation, September 2003, Vol. 112, No. 6, pp. 810- 815.

³ Marx S. O. et al., "Requirement of a Macromolecular Signaling Complex for β Adrenergic Receptor Modulation of the KCNQ1-KCNE1 Potassium Channel", Science, January 2002, Vol. 295, No. 5554, pp. 496-499.

⁴ Marks A. R., "Calcium and the heart: a question of life and death", The Journal of Clinical Investigation, March 2003, Vol. 111, No. 5, pp. 597-600.

pero ningún resultado particular es mostrado para alguno de los compuestos allí revelados.

En vista de lo anterior salta a la vista **que el documento D1 no le da a la persona del oficio normalmente versada en la materia alguna indicación que la selectividad hacia AKT por sobre PKA pueda ser conseguida junto con una inhibición potente de AKT por medio de compuestos derivados de ciclopenta [d]pirimidinas hidroxiladas, como aquellos que son reclamados por el capítulo reivindicatorio ahora modificado de la presente solicitud.**

(v) En conexión con lo anterior, me permito también dirigir la atención de ese Despacho a los resultados experimentales de la Tabla 1 que se adjunta a este memorial, en donde se muestran los valores de IC_{50} medidos de ensayos bioquímicos de AKT y PKA, y la correspondiente preferencia de selectividad hacia AKT que tienen los compuestos que caen dentro del alcance del capítulo reivindicatorio modificado de la presente solicitud.

De estos resultados se puede ver claramente que en cada caso los compuestos de la presente solicitud son potentes inhibidores de AKT y significativamente más selectivos hacia AKT que para PKA en comparación con los compuestos de D1.

En este sentido, me permito señalar que de los datos presentados en la Tabla 2 para los compuestos de D1, el Ejemplo 73 es el compuesto más activo (IC_{50} más bajo) revelado en D1, y Ejemplo 26 es el compuesto de D1 que exhibe el nivel más alto de selectividad para AKT sobre PKA.

Sin embargo, frente a estos resultados la persona versada en la materia reconocerá que aún los compuestos de D1 con la mayor selectividad hacia AKT (Ejemplo 26 con una selectividad 14.1 veces más alta hacia AKT frente a PKA) tienen una selectividad hacia AKT significativamente más baja que la selectividad mostrada por los compuestos reclamados por el capítulo reivindicatorio modificado de la presente solicitud (que en el caso del compuesto 82 que tiene la menor selectividad hacia AKT de los compuestos revelados en dichas tablas, aún así tiene una selectividad 297.5 veces más alta hacia AKT frente a PKA).

Además, salta a la vista que los compuestos de D1 con la mayor actividad inhibitoria de AKT (Ejemplo 73) tienen una preferencia para la inhibición de PKA frente a la inhibición de AKT, y para el caso del Ejemplo 59 de D1 se encuentra que este compuesto es casi 3 veces más selectivo para PKA frente a AKT.

Consecuentemente, a partir de las revelaciones de la presente solicitud y de los datos experimentales que se adjuntan a este memorial es claro que **los compuestos reclamados por el capítulo reivindicatorio ahora modificado de la presente invención son tanto (i) significativamente más selectivos hacia AKT que los compuestos de D1 y (ii) además también retienen una actividad inhibitoria alta y útil de AKT.**

Así las cosas, en vista de los argumentos anteriormente expuestos será evidente para la persona versada en la materia que las revelaciones del documento D1 no le sugieren a la persona del oficio normalmente versada en la materia que los compuestos derivados de ciclopenta[d]pirimidinas hidroxiladas reclamados por la presente invención en el capítulo reivindicatorio ahora modificado podrían tener tal selectividad alta hacia AKT al mismo tiempo que tendrían una alta actividad inhibitoria de AKT.

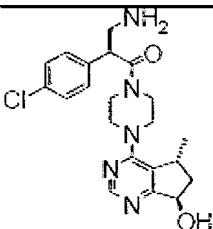
Por lo tanto, **los compuestos reclamados por la reivindicación 1 del capítulo reivindicatorio modificado son inventivos frente a las enseñanzas del documento D1 toda vez que la persona medianamente versada en la materia no podría tener una expectativa razonable de éxito en cuanto a que al seleccionar de manera específica compuestos de la formula general I de D1 pudiera obtener a partir de ellos otros que fueran potentes inhibidores de AKT y al mismo tiempo que también fueran significativamente más selectivos hacia AKT que para PKA, y esto dejando a un lado el hecho que a partir del documento D1 dicha persona tampoco encontraría ninguna indicación o sugerencia de cuáles patrones de sustitución química podrían presentar tales propiedades de actividad inhibitoria sostenida hacia AKT y de selectividad mejorada hacia AKT por sobre PKA.**

Ahora bien, en conexión con los argumentos expuestos por el señor Examinador en cuanto a que los compuestos objeto de la presente solicitud que tienen un anillo de ciclopentano sustituido por -OH en la posición 7 como parte de la estructura fusionada no generan diferencias significativas en cuanto a la actividad frente a los divulgados por D1, me permito señalar que estas afirmaciones ponen de manifiesto que el señor Examinador no ha logrado determinar de manera correcta todos los efectos técnicos que son logrados por los compuestos de la presente solicitud, **toda vez que los compuestos de la presente no solo son potentes inhibidores de AKT al igual que algunos de los compuestos revelados por D1 sino que también son significativamente más selectivos hacia la inhibición de AKT por sobre pKA en comparación con los compuestos de D1 tal como se expuso antes.**

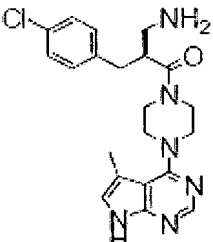
En este sentido, me permito señalar que si bien es cierto que, tal como lo señalar el señor Examinador en la página 9 de la Resolución ahora impugnada, según las Tablas de resultados 1 y 2 adjuntas a este memorial el compuesto del Ejemplo 5 de la presente

solicitud y el compuesto del Ejemplo 73 de D1 tienen actividades inhibitorias de AKT1 muy similares (de 6.9 nM y 1.67 nM) y que inclusive la del compuesto del Ejemplo 73 de D1 es menor a la del compuesto de la presente solicitud, este argumento pone de manifiesto una vez más que el señor Examinador no logra reconocer todos los efectos técnicos que tienen los compuestos de la presentes solicitud ya que estas mismas tablas de resultados revelan que el compuesto del Ejemplo 5 de la solicitud denegada no solo tiene una potente actividad inhibitoria de AKT sino que también tiene una **afinidad hacia Akt1 de 468.3 veces** por sobre PKA, mientras que el compuesto del Ejemplo 73 de D1 aunque tiene una potente actividad inhibitoria de AKT tiene una **afinidad hacia PKA de 1.1 veces** por sobre Akt1 (contrario a los compuestos de la presente solicitud). Véanse los siguientes apartes de las Tablas 1 y 2.

Compuesto de la solicitud denegada
(Aparte de la Tabla 1 adjunta a este memorial)

Example	Structure	Akt1 Average IC50 (nM)	PKA Average IC50 (nM)	Akt1 Preference
Example 5		6.9	3231	<u>468.3X</u>

Compuesto de D1 (Aparte de la Tabla 2 adjunta a este memorial)

Example	Structure	Akt1 Average IC50 (nM)	PKA Average IC50 (nM)	Akt1 Preference
Example 73		1.67	1.55	- <u>(1.1X preference for PKA)</u>

Por lo tanto, a la luz de los argumentos anteriores para la persona versada en la materia será evidente que los compuestos del capítulo reivindicatorio ahora modificado que tienen una ciclopenta [d] pirimidina hidroxilada en su estructura centra si tienen efectos técnicos significativamente diferentes frente a los compuestos divulgados por D1 ya que los compuestos ahora reclamados para la solicitud en cuestión no solo son inhibidores potentes de AKT sino que

además son más selectivos hacia AKT que para PKA (contrario a los compuestos de D1).

3.2. **Las enseñanzas del documento D1 no conducen indefectiblemente a los compuestos que son objeto de la presente solicitud.**

Ahora bien, con respecto a las enseñanzas del documento D1, el Señor Examinador afirma lo siguiente:

"considerando que la actividad anticancerígena se debe al núcleo farmacofórico de la molécula y la modificación realizada en la estructura, que consiste en el reemplazo del anillo de cinco miembros y la introducción de un sustituyente tipo -OH en la posición 7 del anillo fusionado, no ocasiona una variación importante en su actividad. Por esta razón, es posible concluir con certeza que la materia reclamada es una derivación del estado de la técnica y por lo tanto, no tiene nivel inventivo." (Página 8 de la Resolución impugnada).

A este respecto **me permito señalar que esta afirmación es una clara prueba de la incorrecta interpretación que realiza ese despacho de la invención denegada, toda vez que las enseñanzas del documento D1 no permiten identificar de manera inequívoca el grupo farmacofórico de la molécula, y, tal como se demostró en el numeral anterior del presente memorial, los compuestos de la invención sí proporcionan una variación importante en la actividad biológica de los compuestos, reflejada en la mayor selectividad de estos frente a Akt.**

En conexión con lo anterior me permito muy respetuosamente resaltar que para la persona versada en la materia resultaría evidente que un "grupo farmacofórico" no puede ser tomado simplemente como una estructura química, sino que dicho término se refiere a una reunión de una serie de características electrónicas y estéricas que permiten la unión con la molécula biológica objetivo, tal como lo señala la IUPAC⁵:

"Farmacóforo (patrón farmacofórico): Un farmacóforo es un ensamblaje de características electrónicas y estéricas que es necesario para asegurar las interacciones supramoleculares óptimas con una estructura biológica objetivo específica y para disparar (o bloquear) su respuesta biológica.

Un farmacóforo no representa una molécula real o una asociación real de grupos funcionales, sino un concepto puramente abstracto que responde a las capacidades

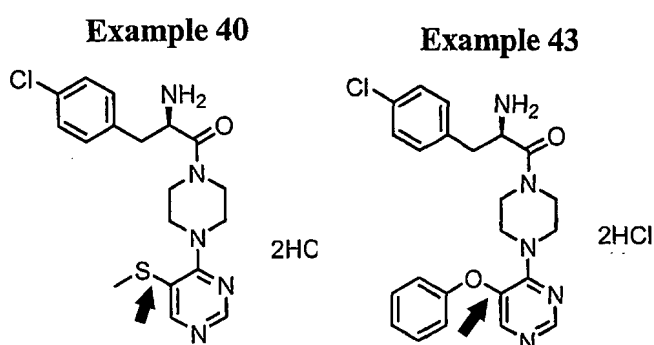
⁵ Pure & App/. Chem., Vol. 70, No. 5, pp. 1129-1143, 1998, International Union of Pure and Applied Chemistry, Chemistry and Human Health Division, Medicinal Chemistry Section, "Glossary Of Terms Used In Medicinal Chemistry" (IUPAC Recommendations 1998) Prepared for publication by C.G. Wermuth, (Chairman), C.R. Ganellin, P. Lindberg and L.A. Mitscher.

moleculares de interacción comunes de un grupo de compuestos hacia su estructura objetivo. El farmacóforo puede ser considerado como el mayor común denominador compartido por un juego de moléculas activas. Esta definición descarta el mal uso frecuentemente encontrado en la literatura de química médica el cual consiste en nombrar como farmacóforos simples funcionalidades químicas como guanidinas, sulfonamidas o dihidroimidazoles (anteriormente imidazolinas), o esqueletos estructurales típicos como flavones, fenotiazinas prostaglandinas o esteroides". (Resaltado fuera de texto).

Consecuentemente, si bien el documento D1 realiza modificaciones estructurales sobre los compuestos allí revelados, de ninguna manera se revela un estudio más detallado que permita identificar específicamente el farmacóforo con respecto a Akt.

En vista de lo anterior, cabe preguntarse ¿qué llevaría a la persona versada en la materia a incluir un anillo 3-metil ciclopentanol fusionado por una posición particular con la piridina? Es decir, de todas las posibles modificaciones estructurales que cabrían sobre las moléculas reveladas en D1, ¿qué motivaría a la persona versada en la materia a seleccionar esta modificación y no otra?

Más aún, si los Ejemplos 40 a 50 de D1 muestran moléculas en las cuales el anillo de pirimidina no está fusionado (ver figura siguiente), ¿por qué la persona versada en la materia no buscaría modificar este tipo de estructura, máxime cuando D1 no brinda resultados experimentales que conduzcan a identificar las moléculas con mayor actividad?



Sumado a lo anterior, me permito reiterar que los datos adicionales proporcionados por el solicitante han demostrado irrefutablemente que los compuestos de la invención son mucho más selectivos frente a Akt que los compuestos de D1, lo que la persona versada en la materia identificaría como una ventaja importante y una variación significativa en la actividad, ya que se elimina el riesgo de cardiotoxicidad potencial por la inhibición de PKA.

En este sentido me permito señalar que la solicitud denegada no pretende "*obtener series alternativas de los compuestos conocidos en D1 para proporcionar compuestos que inhiban de forma selectiva la proliferación celular mediante la inhibición de las quinasas AKT del subtipo 1*", como lo afirma ese Despacho, sino que la presente invención tiene como objetivo técnico obtener compuestos que inhiban selectivamente la quinsa Akt1 en relación con la inhibición de PKA, reduciendo de esta forma la cardiotoxicidad potencial por la inhibición de PKA.

En este contexto considero pertinente resaltar que la misma Resolución impugnada resalta en su página 10 que existe un alto grado de homología entre los receptores Akt y el dominio de la quinsa PKA, lo que hace aún más meritorio que los compuestos de la invención, a pesar de esta homología, se unan específica y selectivamente a Akt por sobre PKA.

A la luz de estos hechos resulta indiscutible que las enseñanzas del documento D1 no son suficientes para que la persona versada en la materia encuentre una motivación para modificar los compuestos de D1 en la manera específica que sería necesaria para obtener los compuestos de la invención, y mucho menos para que dicha persona versada en la materia pudiera tener certeza de que las moléculas resultantes de esta modificación serían más selectivas frente a Akt.

3.3. Los reemplazos bioisostéricos revelados en el documento de Zhu, et al. de ninguna manera conducen a los compuestos de la invención y mucho menos permiten predecir la selectividad mejorada que poseen dichos compuestos.

De otra parte, ese Despacho asegura que los estudios de estructura actividad de inhibidores de Akt, como aquel de Zhu, et al. (G.-D. Zhu et al. / Bioorg. Med. Chem. Lett. 16 (2006), pp. 3424-3429), brindarían herramientas suficientes para que la persona versada en la materia decidiera fijar como núcleo central de los compuestos un anillo de ciclopenta[d]pirimidina y específicamente el anillo 5-metil-7-OH-ciclopenta[d]pirimidina que tienen todos los compuestos del capítulo reivindicatorio ahora modificado esperando una alta selectividad frente a Akt.

A este respecto muy comedidamente me permito indicar que este documento no fue citado por ese Despacho previamente por lo que no se brindó al solicitante la oportunidad de argumentar contra esta objeción particular.

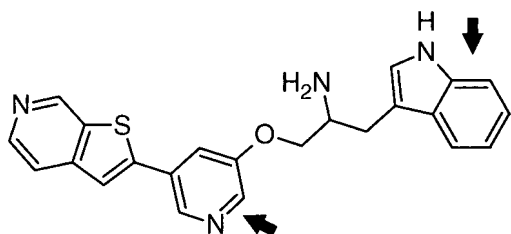
Así mismo, me permito señalar que la referencia a este documento es un indicio claro con respecto a que las enseñanzas de D1 por sí solas no llevarían inequívocamente a la persona versada en la materia a la presente invención, toda vez que para desvirtuar el nivel inventivo ese Despacho necesita basar su análisis en un documento adicional el cual,

según su modo de ver, hace predecible el efecto técnico sorprendente que poseen los compuestos de la invención.

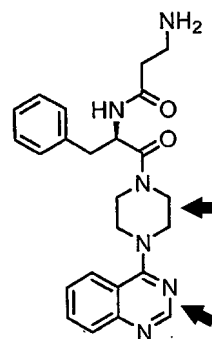
Sin embargo, me permito resaltar que aún a la luz de las enseñanzas del mencionado documento de Zhu et al. la persona versada en la materia no podría obtener de manera evidente los compuestos que son objeto de la presente solicitud y mucho menos predecir la selectividad mejorada que estos exhiben.

De esta forma, el documento de Zhu, et al. estudia cambios estructurales en compuestos que inhiben Akt, encontrando que un biciclo de isoquinolina-6-ilo puede ser sustituido por un biciclo de oxindol-5-ilo aumentando la selectividad hacia Akt con respecto a PKA. Sin embargo, la persona versada en la materia de ninguna manera tomaría esta revelación como una motivación para obtener compuestos de 5-metil-7-OH-ciclopenta[d]pirimidina como los de la presente invención, máxime cuando las moléculas de Zhu et al. son estructuralmente muy distintas a las de D1 y a aquellas que son reclamadas por la solicitud denegada.

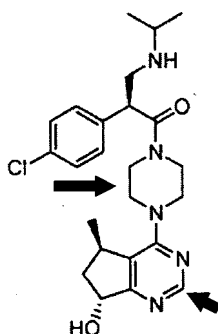
En efecto, los compuesto de Zhu et al. poseen un anillo central de piridina, unido por un puente alcoxil-amino a una anillo de indol, y en posición meta a la unión con el puente alcoxilo un sustituyente bicíclico. Contrario a esto, todos los compuestos de D1 poseen un anillo de pirimidina unido a un grupo piperazina, lo que ocurre también en las moléculas reclamadas por la solicitud denegada:



Compuesto 9a, Zhu et al.



Ejemplo 21, D1



Ejemplo 14, presente solicitud

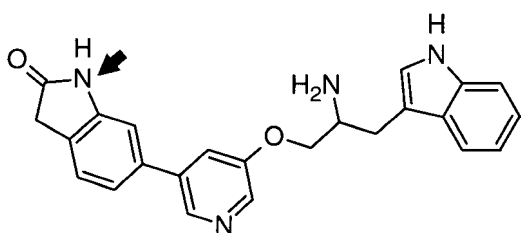
Consecuentemente, la persona versada en la materia de ninguna manera pensaría que un reemplazo que es bioisostérico para las moléculas enseñadas por Zhu, et al. podría serlo para moléculas como las de D1, toda vez que aun cuando ambos tipos de compuestos están dirigidos a la inhibición de Akt, estos son estructuralmente muy distintos, lo que hace que la interacción con la molécula biológica objetivo ocurra de forma diferente.

Con respecto a dichos reemplazos bioisostéricos revelados por Zhu, et al. la página 10 de la Resolución impugnada señala que: "El estudio de relación estructura actividad demuestra que existe relación de biosistería [sic] entre las isoquinolinas y las estructuras de oxi-indol, pirrolidin-2-ona[2,3-b]piridina, tieno[2,3-c]piridina, porque presentan actividad selectiva frente a la quinasa Akt 1".

En conexión con este señalamiento, me permito resaltar que este es una prueba de la incorrecta interpretación que realiza ese Despacho del arte previo citado, toda vez que el mismo documento de Zhu et al. se refiere de manera explícita a que el introducir una porción de oxo-indol dentro de los compuestos allí revelados puede conducir a eliminar completamente la actividad:

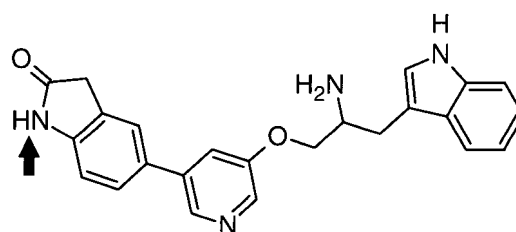
"El reemplazo del nitrógeno de la isoquinolina con un grupo carbonilo condujo a los análogos de oxindol **9d** y **9e**. Mientras que **9d** fue esencialmente inactivo, **9e** mostró un respetable IC_{50} de 3.1 contra Akt1." (Página 3426, primera columna, primer párrafo, Zhu, et al., resaltado fuera de texto).

En este sentido considero pertinente resaltar que la diferencia entre los compuestos **9d** y **9e** de Zhu, et al. es únicamente la posición en la cual el anillo de oxo-indol está unido al resto de la molécula:



Compuesto **9d**, Zhu et al.

Akt1 IC_{50} = **4400 nM**



Compuesto **9e**, Zhu et al.

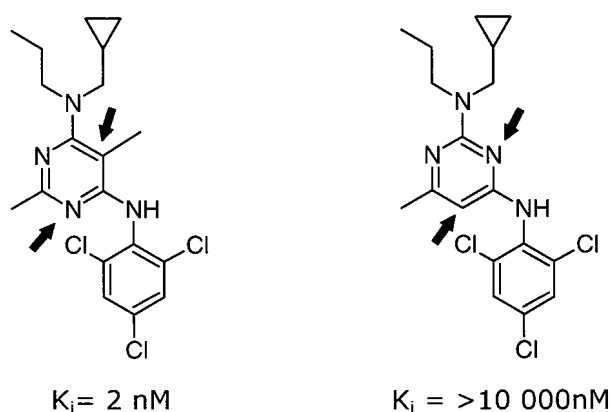
Akt1 IC_{50} = **3.2 nM**

Consecuentemente, resulta evidente que el mismo documento Zhu, et al. revela que no necesariamente el reemplazo del anillo de isoquinolina por uno de oxo-indol resultará bioisostérico porque según la posición relativa del átomo de nitrógeno se puede reducir significativamente la actividad del compuesto.

Sumado a lo anterior, aun asumiendo que el reemplazo si fuera completamente bioisostérico, me permito señalar que para la persona versada en la materia sería claro que el introducir un anillo de cinco miembros insaturado y que posee al menos un heteroátomo, no necesariamente brindará el mismo efecto que introducir un anillo carbocíclico saturado de 5 miembros como el 3-metil ciclopentanol, toda vez que el heteroátomo de los compuestos de D1 proporciona un ambiente electrónico y una diferencia de electronegatividad que pueden llegar a influenciar la actividad biológica del compuesto.

De manera similar, es claro el grupo **OH que está presente en los anillos fusionados de todos los compuestos del capítulo reivindicatorio ahora modificado, pero que está ausente en los compuestos revelados por D1, proporciona un ambiente electrónico y una diferencia de electronegatividad que influencia la actividad biológica de los compuestos toda vez que, tal como se expuso antes, son al menos 50 veces más selectivos para AKT versus la proteína quinasa A (PKA), y en algunos casos son al menos 150 veces más selectivos.**

Más aún, la persona versada en la materia reconocería que el sólo cambio de posición de un heteroátomo dentro del anillo central de un compuesto puede ser significativo dentro de su actividad, tal como se ha encontrado para ciertos Antagonistas del factor₁ de liberación de corticotropina⁶. Así, como es evidente de la siguiente figura, en este tipo de compuestos si el anillo de pirimidina central está sustituido en las posiciones 4 y 6 por los grupos amino el compuesto tiene una alta actividad inhibitoria, sin embargo si el patrón de sustitución se cambia a las posiciones 2 y 4 el compuesto disminuye 5 mil veces su actividad:



En vista de todo lo discutido hasta el momento, cabría preguntarse ¿por qué la persona versada en la materia buscaría modificar los compuestos de D1 reemplazando los anillos pirrolo[2,3-d]pirimidina, 5H-pirrolo[2,3-d]pirimidina o 1,3-dihidro-pirrolidin-2-

⁶ Chen, Chen, et al *J. Med. Chem.* 1996, 39, 4358-4360

ona[2,3-b]pirimidina no por un anillo de oxo-indol o de tieno[2,3-c]piridina, como los empleados en Zhu, et al., sino por uno de 5-metil-7-hidroxi-ciclopenta[d]pirimidina, cuando este documento de ninguna manera sugiere este tipo de reemplazo?

En este sentido me permito resaltar que el documento de Zhu, et al. asegura que: *"Varios intentos de co-cristalizar los compuestos en esta serie con Akt purificado, Akt mutado PKA y/o proteína PKA fueron infructuosos para proporcionar un cristal único con suficiente calidad para un estudio de rayos-X"* (página 3427, último párrafo). Esto implica que **el documento de Zhu, et al. no muestra una conformación de unión de los compuestos allí revelados con Akt1, y por lo tanto, dicho documento no enseña o sugiere un farmacóforo que pueda ser útil para que la persona versada en la materia identifique una forma de modificar los compuestos de D1.**

Por lo tanto, las enseñanzas del documento Zhu, et al. no brindan una motivación para que la persona versada en la materia modifique los compuestos de D1 y obtenga las moléculas que son parte de la presente invención.

3.4. Requisitos exigidos por el Artículo 18, la evaluación del Nivel Inventivo de una Solicitud de patente.

Dentro del contexto de los argumentos discutidos anteriormente, me permito señalar que el Artículo 18 de la Decisión Andina 486 de 2000 exige de forma manifiesta que la invención no debe resultar obvia ni se debe poder derivar de forma evidente de lo existente en el arte previo, y por lo tanto es en este sentido que el examinador debe encaminar su análisis para sopesar si una solicitud es o no merecedora del beneficio de patente.

Así mismo, me permito llamar la atención a ese Despacho a que el Tribunal de Justicia de la Comunidad Andina se ha pronunciado con respecto a las definiciones de "obvio" y "evidente" en la evaluación del Nivel Inventivo de una solicitud de patente⁷:

"Resulta pertinente, a los efectos del artículo 4 de la Decisión 344, definir qué se entiende por "obvio" y por "evidente" a fin de determinar qué se debe entender por Nivel Inventivo.

Lo obvio es aquello que salta a la vista de manera clara y directa; lo "que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad". Mientras que lo evidente consiste en la "certeza clara y manifiesta de la que no se puede dudar; cierto,

⁷ Tribunal de Justicia de la Comunidad Andina, PROCESO 192-IP-2005

claro, patente y sin la menor duda". De forma que algo que resulte obvio no es necesariamente evidente; empero lo que es evidente, es también obvio.

En este orden de ideas, se puede concluir que una invención goza de Nivel Inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no sea consecuencia clara y directa de dicho estado."

Con respecto a este señalamiento me permito resaltar que resulta indiscutible que para la legislación Andina, para que exista afectación del requisito de Nivel Inventivo de una solicitud de patente, es necesario que la invención allí revelada sea derivable de forma clara y directa del arte previo, y que las enseñanzas de este arte previo sin lugar a dudas conduzcan a dicha invención.

Consecuentemente, para el caso que nos compete dado que a partir de las enseñanzas del arte previo no "salta a la vista de manera clara y directa" compuestos que posean un anillo central de 5-metil-7-hidroxi-ciclopenta[d]pirimidina, y mucho menos a partir de las enseñanzas de dicho estado del arte se tiene "certeza clara y manifiesta" de que estos compuestos particulares tendrían una selectividad superior frente a Akt1 con respecto a PKA, es evidente que el arte previo no puede afectar el Nivel Inventivo de la solicitud en estudio.

En conexión con lo anterior me permito muy respetuosamente señalar que en su análisis de Nivel Inventivo de la solicitud en estudio ese Despacho incurre en lo que dentro del ámbito de las patentes se conoce como "hindsight", o análisis "Ex post facto", toda vez que conociendo de antemano la invención selecciona partes específicas de las enseñanzas contenidas dentro de los documentos citados, agrupándolas de una manera particular y modificándolas de forma que permitan obtener los compuestos reclamados por la presente solicitud, pasos que de ningún modo podrían ser evidentes para la persona versada en la materia en el momento en el cual se desarrolló la invención.

En este sentido vale la pena resaltar que al tener conocimiento de la invención es de esperarse que dichas selección, agrupación y modificaciones particulares de las enseñanzas de D1 y Zhu, et al. parezcan obvias, toda vez que ya se sabe de antemano que efectivamente estos elementos en combinación resuelven el problema planteado; sin embargo, esta información no era conocida por el inventor en el momento de desarrollar la invención, por lo que no podría tener certeza de la utilidad de realizar estos pasos.

En vista de lo anterior, dado que la única motivación que tendría la persona versada en la materia de reunir y modificar estas características particulares reveladas en el arte

previo sería la misma invención como ha sido revelada, es evidente el Nivel Inventivo que conlleva la solicitud denegada.

II. CONCLUSIONES

Con base en los argumentos técnicos mostrados previamente, me permito manifestar ante ese Despacho que la materia revelada en la solicitud de patente en cuestión, presenta un inequívoco carácter inventivo frente a las enseñanzas del arte previo citado en la Resolución Número **6084** aquí impugnada, cumpliendo así a cabalidad las disposiciones establecidas en el Artículo 18 de la Decisión Andina 486 de 2000, y por lo tanto demostrando ser merecedora del privilegio de patente requerido.

En efecto, dichos argumentos establecieron que la solicitud denegada no podría ser obtenida de manera evidente a partir de las enseñanzas de D1 máxime cuando dicho documento no sugiere la manera de mejorar la selectividad frente a Akt de los compuestos allí revelados.

En este sentido, se ha demostrado a lo largo del presente memorial que aún tomando en consideración las enseñanzas de otros documentos como el de Zhu, et al. la persona versada en la materia no obtendría de manera evidente los compuestos que son objeto de la invención denegada toda vez que ninguno de estos documentos permite evidenciar cuál sería la estructura central necesaria para tener una alta selectividad frente a Akt con respecto a PKA.

Como consecuencia de lo anterior, dado que la solicitud denegada sí se ajusta completamente a los requerimientos establecidos por la Decisión Andina 486, al negar el privilegio de patente ese Despacho claramente contraviene los Artículos 14 y 18 de dicha Decisión.

III. SOLICITUD

En vista de todo lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las argumentaciones presentadas y revocar la Resolución Número **6084** impugnada para conceder el privilegio de patente solicitado.

IV. PODER

En este punto me permito mencionar ante ese Despacho que el documento que acredita mi calidad de Representante Legal en Bogotá para asuntos relativos a la Propiedad Industrial de las sociedades peticionarias fueron presentados junto con la Solicitud de patente el 4 de Febrero de 2009.

V. ANEXOS

Anexo me permito presentar:

- Copia de la tabla de datos experimentales que fue referenciada a lo largo del texto del presente memorial, tal como fue presentada junto con el memorial de 23 de Noviembre de 2012;
- Copia del documento de Kass R. S. y Moss A. J., "Long QT síndrome: novel insights into the mechanism of cardiac arrhythmias", The Journal of Clinical Investigation, September 2003, Vol. 112, No. 6, pp. 810- 815;
- Copia del documento de Marx S. O. et al., "Requirement of a Macromolecular Signaling Complex for β Adrenergic Receptor Modulation of the KCNQ1-KCNE1 Potassium Channel", Science, January 2002, Vol. 295, No. 5554, pp. 496-499;
- Copia del documento de Marks A. R., "Calcium and the heart: a question of life and death", The Journal of Clinical Investigation, March 2003, Vol. 111, No. 5, pp. 597-600;
- Copia de las páginas 1140 y 1141 del documento *Pure & App/. Chem.*, Vol. 70, No. 5, pp. 1129-1143, 1998, International Union of Pure and Applied Chemistry, Chemistry and Human Health Division, Medicinal Chemistry Section, "Glossary Of Terms Used In Medicinal Chemistry" (IUPAC Recommendations 1998) Prepared for publication by C.G. Wermuth, (Chairman), C.R. Ganellin, P. Lindberg and L.A. Mitscher;
- Copia del documento de Chen, Chen, et al. *J. Med. Chem.* 1996, 39, 4358-4360.
- Todos los anexos correspondientes a la divisional y mencionados en el numeral 1 de este escrito.

JOSE LLOREDA CAMACHO & CO.

Calle 72 No. 5-83 Piso 5, Bogotá, D.C. Tel: 6069700 Fax 6069701

VI. DOMICILIO

Mi dirección es Calle 72 No. 5-83, Piso 6º, Bogotá D.C.

Del Señor Superintendente,



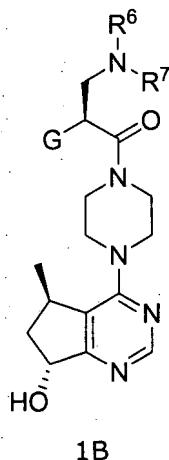
ALICIA LLOREDA RICAURTE

T.P. 53.215

Dirección para Notificaciones: **notificacionespatentes@lloredacamacho.com**

REIVINDICACIONES

1. Un compuesto de Fórmula 1B:



y enantiómeros resueltos, diastereómeros resueltos y sales del mismo, en donde:

G es fenilo opcionalmente sustituido por uno a cuatro grupos R^9 o un heteroarilo de 5-6 miembros opcionalmente sustituido por un halógeno;

R^6 y R^7 son independientemente H, OCH_3 , (cicloalquilo C_3-C_6)-(CH $_2$), (cicloalquilo C_3-C_6)-(CH $_2$ CH $_2$), V-(CH $_2$) $_{0-1}$ donde V es un heteroarilo de 5-6 miembros, W-(CH $_2$) $_{1-2}$ donde W es fenilo opcionalmente sustituido por F, Cl, Br, I, OMe, CF $_3$ o Me, cicloalquilo C_3-C_6 opcionalmente sustituido por alquilo C_1-C_3 o O(alquilo C_1-C_3), hidroxí-(cicloalquilo C_3-C_6), fluoro-(cicloalquilo C_3-C_6), CH(CH $_3$)CH(OH)fenilo, heterociclo de 4-6 miembros opcionalmente sustituido por F, OH, alquilo C_1-C_3 , ciclopropilmetilo o C(=O)(alquilo C_1-C_3), o alquilo C_1-C_6 opcionalmente sustituido por uno o más grupos independientemente seleccionados de OH, oxo, O(alquilo C_1-C_6), CN, F, NH $_2$, NH(alquilo C_1-C_6), N(alquilo C_1-C_6) $_2$, ciclopropilo, fenilo, imidazolilo, piperidinilo, pirrolidinilo, morfolinilo, tetrahidrofuranilo, oxetanilo, o tetrahidropiranilo;

o R^6 y R^7 junto con el nitrógeno al cual están unidos forman un anillo heterocíclico de 4-7 miembros opcionalmente sustituido con uno o más grupos independientemente seleccionados de OH, halógeno, oxo, CF $_3$, CH $_2$ CF $_3$, CH $_2$ CH $_2$ OH, O(alquilo C_1-C_3), C(=O)CH $_3$, NH $_2$, NHMe, N(Me) $_2$, S(O) $_2$ CH $_3$, ciclopropilmetilo y alquilo C_1-C_3 ;

cada R^9 es independientemente halógeno, alquilo C_1-C_6 , cicloalquilo C_3-C_6 , O-(alquilo C_1-C_6), CF $_3$, OCF $_3$, S(alquilo C_1-C_6), CN, OCH $_2$ fenilo, CH $_2$ O-fenilo, NH $_2$, NH-(alquilo C_1-C_6), N(alquilo C_1-C_6) $_2$, piperidina, pirrolidina, CH $_2$ F, CHF $_2$, OCH $_2$ F, OCHF $_2$, OH, SO $_2$ (alquilo C_1-C_6), C(O)NH $_2$, C(O)NH(alquilo C_1-C_6), y C(O)N(alquilo C_1-C_6) $_2$;

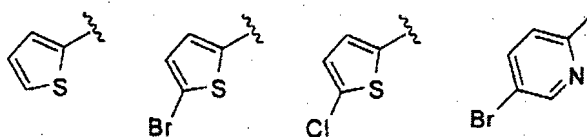
2. El compuesto de la reivindicación 1, en donde G es fenilo opcionalmente sustituido por uno o más grupos independientemente seleccionados de F, Cl, Br, I, Me, etilo, isopropilo, CN, CF $_3$, OCF $_3$, SMe, OMe y OCH $_2$ Ph.

3. El compuesto como se reivindica en cualquiera de las reivindicaciones 1 a 2, en donde G es fenilo, 2-clorofenilo, 3-clorofenilo, 4-clorofenilo, 2-fluorofenilo, 3-fluorofenilo, 4-fluorofenilo, 2-bromofenilo, 3-bromofenilo, 4-bromofenilo, 2-metilfenilo, 3-metilfenilo, 4-metilfenilo, 2-etilfenilo, 3-etilfenilo, 4-etilfenilo, 2-isopropilfenilo, 3-isopropilfenilo, 4-isopropilfenilo, 2-trifluorometilfenilo, 3-trifluorometilfenilo, 4-trifluorometilfenilo, 2-cianofenilo, 3-cianofenilo, 4-cianofenilo, 2-metoxifenilo, 3-metoxifenilo, 4-metoxifenilo, 2-etoxifenilo, 3-etoxifenilo, 4-etoxifenilo, 2-tiometilfenilo, 3-tiometilfenilo, 4-tiometilfenilo, 2-trifluorometoxifenilo, 3-trifluorometoxifenilo, 4-trifluorometoxifenilo, 2-ciclopropilfenilo, 3-ciclopropilfenilo, 4-ciclopropilfenilo, 4-cloro-3-fluorofenilo, 3,4-difluorofenilo, 4-bromo-3-fluorofenilo, 3-fluoro-4-metilfenilo, 3-fluoro-4-metoxifenilo, 3-fluoro-4-trifluorometilfenilo, 4-ciano-3-fluorofenilo, 3,4-diclorofenilo, 2,4-diclorofenilo, 2,4-difluorofenilo, 2-cloro-4-fluorofenilo, 2-fluoro-4-clorofenilo, 3,5-diclorofenilo, 3,5-difluorofenilo, 3-cloro-5-fluorofenilo, 3-cloro-4-fluorofenilo, 3-bromo-4-fluorofenilo, 3,5-difluoro-4-clorofenilo, 2,3-difluoro-4-clorofenilo, 2,5-difluoro-4-clorofenilo, 3,5-difluoro-4-bromofenilo, 2,3-difluoro-4-bromofenilo, 2,5-difluoro-4-bromofenilo y 4-(OCH₂Ph)-fenilo.

4. El compuesto como se reivindica en cualquiera de las reivindicaciones 1 a 3, en donde G es 4-clorofenilo, 2,4-diclorofenilo, 3-cloro-4-fluorofenilo, 3,4-difluorofenilo, 4-cloro-3-fluorofenilo, 3-fluoro-4-bromofenilo, 3,4-diclorofenilo, 4-metoxifenilo, 4-fluorofenilo, 4-bromofenilo, 4-cianofenilo, 4-trifluorometilfenilo, 4-tiometilfenilo, o 4-metilfenilo.

5. El compuesto de la reivindicación 1, en donde G es un tiofeno o piridina opcionalmente sustituido con un halógeno.

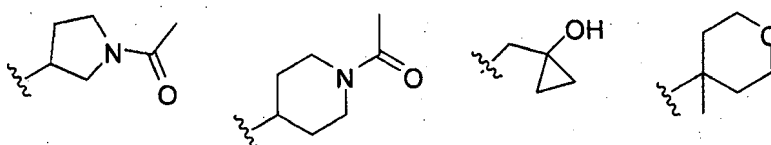
6. El compuesto como se reivindica en las reivindicaciones 1 o 5, en donde G es seleccionado de las siguientes estructuras:



7. El compuesto como se reivindica en cualquiera de las reivindicaciones 1 a 6, en donde R⁶ y R⁷ son independientemente seleccionados de H, OCH₃, (cicloalquiloC₃-C₆)-(CH₂), (cicloalquiloC₃-C₆)-(CH₂CH₂), V-(CH₂)₀₋₁ donde V es un heteroarilo de 5-6 miembros que tiene de uno a dos heteroátomos en el anillo independientemente seleccionados de N, O y S, W-(CH₂)₁₋₂ donde W es fenilo opcionalmente sustituido con F, Cl, o Me, cicloalquiloC₃-C₆ opcionalmente sustituido con OCH₃, hidroxio-(cicloalquiloC₃-C₆), fluoro-(C₃-C₆-cicloalquilo), CH(CH₃)CH(OH)fenilo, un heterociclo de 5-6 miembros opcionalmente sustituido con CH₃ o C(=O)CH₃ o alquiloC₁-C₆, opcionalmente sustituido con uno o más grupos independientemente seleccionados de

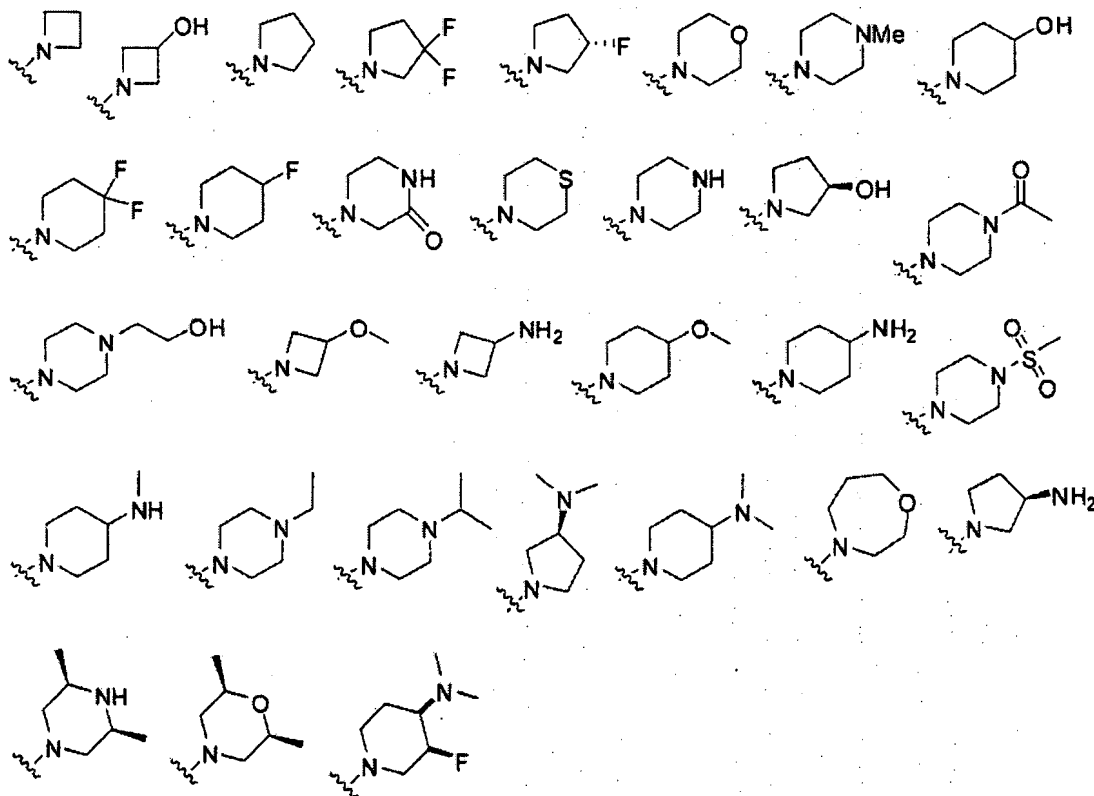
OH, oxo, O(alquiloC₁-C₆), CN, F, NH₂, NH(alquiloC₁-C₆), N(alquiloC₁-C₆)₂, fenilo, imidazolilo, piperidinilo, pirrolidinilo, morfolinilo y tetrahidropiranilo.

8. El compuesto de cualquiera de las reivindicaciones 1 a 7, en donde R⁶ y R⁷ son independientemente seleccionados de H, metilo, etilo, isopropilo, isobutilo, ter-Butilo, 3-pentilo, OCH₃, CH₂CH₂OH, CH₂CH₂OMe, CH₂H₂CF₃, CH₂CH(CH₃)OH, CH₂CH(CF₃)OH, CH₂CF₃, CH₂CH₂F, CH₂C(=O)NH₂, CH₂C(=O)NH(CH₃), CH₂C(=O)N(CH₃)₂, CH₂C(=O)NH(iPr), CH₂CH₂C(=O)NH₂, CH₂-ciclopropilo, CH₂-ciclopentilo, CH₂-tBu (neopentilo), ciclopropilo, ciclopentilo, ciclohexilo, 4-metoxiciclohexilo, 4,4-dimetilciclohexilo, 3,3-dimetilciclohexilo, CH₂-(pirid-3-ilo), 4-hidroxiciclohex-1-ilo, CH(CH₃)CH(OH)fenilo, CH(fenil)CH₂OH, CH(te-trahidropiranil)CH₂OH, CH₂CH₂CH₂(imidazolilo), CH₂CH₂(morfolinilo), CH₂(tetrahidropiranilo), CH₂CH₂(tetrahidropiranilo), pirrolidinilo, piperidinilo, tetrahidropiranilo, tetrahidrofuranilo,

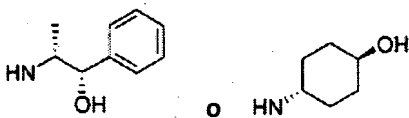


9. El compuesto como se reivindica en cualquiera de las reivindicaciones 1 a 6, en donde R⁶ y R⁷ junto con el nitrógeno al cual están unidos forman un anillo heterocíclico de 4-7 miembros, en donde dicho anillo heterocíclico está opcionalmente sustituido con uno o más grupos independientemente seleccionados de OH, halógeno, oxo, CF₃, CH₂CF₃, CH₂CH₂OH, OCH₃, C(=O)CH₃, NH₂, NHMe, N(Me)₂, S(O)₂CH₃ y alquiloC₁-C₃.

10. El compuesto de cualquiera de las reivindicaciones 1 a 6 y 9, en donde NR⁶R⁷ es seleccionado de las estructuras:



11. El compuesto como se reivindica en cualquiera de las reivindicaciones 1 a 8, en donde NR^6R^7 es NH_2 , NHMe , NHET , NHPr , $\text{NH}i\text{Pr}$, NHtBu , $\text{NH}(\text{CH}_2\text{-tBu})$, $\text{NH}(\text{CH}_2\text{-ciclopropilo})$, $\text{NH}(\text{CH}_2\text{-ciclobutilo})$, $\text{NH}(\text{ciclopentilo})$, $\text{NH}(\text{CH}_2\text{-piridilo})$, $\text{NH}(\text{ciclohexilo})$, $\text{NH}(\text{3-pentilo})$, $\text{NHCH}(\text{isopropilo})_2$, $\text{NH}(\text{CH}_2\text{CH}_2\text{OH})$, $\text{NH}(\text{CH}_2\text{CH}_2\text{CH}_2\text{OH})$, $\text{NH}(\text{CH}_2\text{CH}_2\text{OMe})$, $\text{NH}(\text{CH}_2\text{CH}_2\text{CH}_2\text{OMe})$, $\text{NH}(\text{CH}_2\text{CN})$, NMe_2 , NMeEt , NMePr , $\text{NMe}(i\text{Pr})$, $\text{NMe}(\text{CH}_2\text{-ciclopropilo})$, $\text{NMe}(\text{CH}_2\text{-ciclobutilo})$, $\text{NMe}(\text{CH}_2\text{CH}_2\text{OH})$, $\text{NMe}(\text{CH}_2\text{CH}_2\text{CH}_2\text{OH})$, $\text{NMe}(\text{CH}_2\text{CH}_2\text{OMe})$, $\text{NMe}(\text{CH}_2\text{CH}_2\text{CH}_2\text{OMe})$, NEt_2 , NEtPr , $\text{NEt}(i\text{Pr})$, $\text{NEt}(\text{CH}_2\text{-ciclopropilo})$, $\text{NEt}(\text{CH}_2\text{-ciclobutilo})$, $\text{NEt}(\text{CH}_2\text{CH}_2\text{OH})$, $\text{NEt}(\text{CH}_2\text{CH}_2\text{CH}_2\text{OH})$,



12. Un compuesto de Fórmula 1B, como se define en la reivindicación 1, seleccionado de:

(S)-3-amino-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-3-amino-2-(3,4-diclorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(metil(tetrahidro-2H-piran-4-il)amino)propan-1-ona;

(S)-2-(5-clorotiofen-2-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-clorofenil)-3-(ciclopropilmetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)-2-(4-(trifluorometoxi)fenil)propan-1-ona;

(S)-2-(4-clorofenil)-3-(ciclopropilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(3-hidroxiazetidín-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-((R)-pirrolidin-3-ilamino)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(S)-pirrolidin-3-ilamino)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-tiomorfolinopropan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-3-(4-fluoropiperidin-1-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(3-metoxiazetidina-1-il)propan-1-ona;

(S)-2-(3,4-diclorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-clorofenil)-3-(dimetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-fluoro-3-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(3-fluoro-4-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(metoxiamino)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(4-metoxipiperidin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(4-hidroxipiperidin-1-il)propan-1-ona;

(S)-3-(4-aminopiperidin-1-il)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropil(metil)amino)-propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(4-(metilamino)piperidin-1-il)propan-1-ona;

(S)-2-(4-cloro-3-(trifluorometoxi)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)-propan-1-ona;

(S)-2-(3-fluoro-4-(trifluorometoxi)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-cloro-3-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-clorofenil)-3-(4-etilpiperazin-1-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(4-isopropilpiperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-3-((S)-3-(dimetilamino)pirrolidin-1-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-((R)-tetrahidrofuran-3-ilamino)propan-1-ona;

(S)-2-(4-clorofenil)-3-(2-fluoroetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-fluoro-3-(trifluorometoxi)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(3-fluoro-4-metoxifenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-clorofenil)-3-(4-(dimetilamino)piperidin-1-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(3-cloro-5-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(3-bromo-4-metoxifenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(1,4-oxazepan-4-il)propan-1-ona;

(S)-2-(4-cloro-2-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(2-fluoro-4-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(4-clorofenil)-3-(ciclohexilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-bromofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(4-isopropilpiperazin-1-il)propan-1-ona;

(S)-2-(4-bromofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(4-hidroxipiperidin-1-il)propan-1-ona;

(S)-3-(ciclopropilmetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-2-(4-(trifluorometil)fenil)propan-1-ona;

(S)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(tetrahidro-2H-piran-4-ilamino)-2-(4-(trifluorometil)fenil)propan-1-ona;

(S)-3-(ciclopropilmetilamino)-2-(2-fluoro-4-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-3-amino-2-(4-bromo-2-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-bromofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropil(metil)amino)propan-1-ona;

(S)-2-(4-bromo-2-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-3-amino-2-(4-cloro-2-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-3-(tert-butilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-2-(4-(trifluorometil)fenil)propan-1-ona;

(S)-2-(4-cloro-2-fluorofenil)-3-(ciclopropilmetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-bromo-2-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(tetrahidro-2H-piran-4-ilamino)propan-1-ona;

(S)-2-(4-cloro-2-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(tetrahidro-2H-piran-4-ilamino)propan-1-ona;

(S)-2-(4-bromofenil)-3-(tert-butilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-cloro-3-fluorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isobutilamino)propan-1-ona;

(S)-2-(4-cloro-3-fluorofenil)-3-(ciclopentilmetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-cloro-3-fluorofenil)-3-(ciclopentilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(2-fluoro-4-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropil(metil)amino)propan-1-ona;

(S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-((2-hidroxietil)(isopropil)amino)propan-1-ona;

(S)-3-amino-2-(2-fluoro-4-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-bromofenil)-3-(4,4-dimetilciclohexilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-bromofenil)-3-(3,3-dimetilciclohexilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-3-(4,4-dimetilciclohexilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-2-(4-clorofenil)-3-(3,3-dimetilciclohexilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona;

(S)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)-2-(tiofen-2-il)propan-1-ona;

(S)-2-(S-bromotiofen-2-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(S-bromotiofen-2-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(tetrahidro-2H-piran-4-ilamino)propan-1-ona;

(R)-2-(5-bromopiridin-2-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona;

(S)-2-(5-bromotiofen-2-il)-3-(ciclopropilmetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-

ona;

(S)-2-(S-clorotiofen-2-il)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(tetrahidro-2H-piran-4-ilamino)propan-1-ona; y

(S)-2-(S-clorotiofen-2-il)-3-(ciclopropilmetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona.

13. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona y sales del mismo.

14. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(metil(tetrahidro-2H-piran-4-il)amino)propan-1-ona y sales del mismo.

15. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(3-hidroxiazetidin-1-il)propan-1-ona y sales del mismo.

16. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-((R)-pirrolidin-3-ilamino)propan-1-ona y sales del mismo.

17. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-((S)-pirrolidin-3-ilamino)propan-1-ona y sales del mismo.

18. El compuesto de la reivindicación 1 que es (S)-3-(1-acetilpiperidin-4-ilamino)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona y sales del mismo.

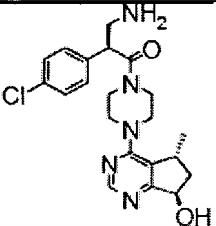
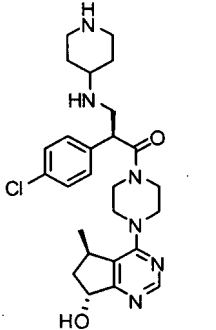
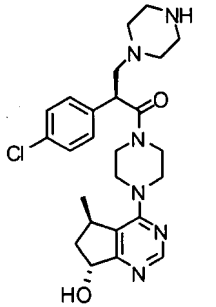
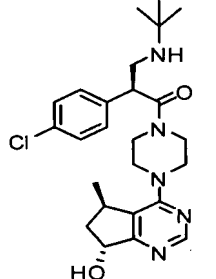
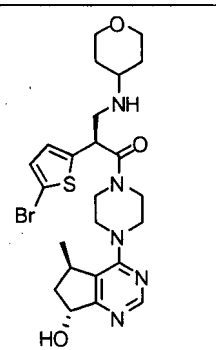
19. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(tetrahidro-2H-piran-4-ilamino)propan-1-ona y sales del mismo.

20. El compuesto de la reivindicación 1 que es (S)-2-(3,4-diclorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona y sales del mismo.

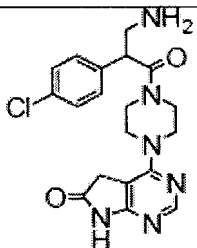
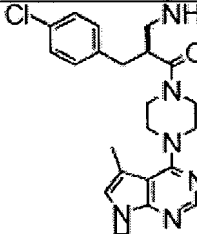
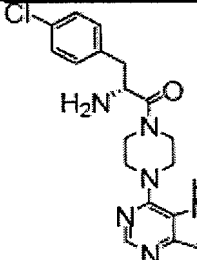
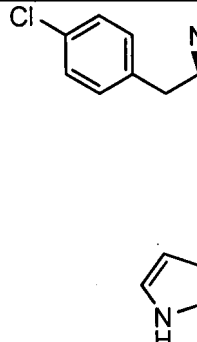
21. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-3-(dimetilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona y sales del mismo.

22. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropil(metil)amino)propan-1-ona y sales del mismo.
23. El compuesto de la reivindicación 1 que es (S)-2-(4-cloro-3-(trifluorometil)fenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(isopropilamino)propan-1-ona y sales del mismo.
24. El compuesto de la reivindicación 1 que es (S)-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(4-metoxiciclohexilamino)propan-1-ona y sales del mismo.
25. El compuesto de la reivindicación 1 que es (S)-2-(4-bromofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-3-(tetrahydro-2H-piran-4-ilamino)propan-1-ona y sales del mismo.
26. El compuesto de la reivindicación 1 que es (S)-3-amino-2-(4-clorofenil)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)propan-1-ona y sales del mismo.
27. El compuesto de la reivindicación 1 que es (S)-3-(tert-butilamino)-1-(4-((5R,7R)-7-hidroxi-5-metil-6,7-dihidro-5H-ciclopenta[d]pirimidin-4-il)piperazin-1-il)-2-(4-(trifluorometil)fenil)propan-1-ona y sales del mismo.
28. Una composición farmacéutica que comprende un compuesto como se reivindica en cualquiera de las reivindicaciones 1 a 12 y 13 a 27.

Present Application – Table 1

Example	Structure	Akt1 Average IC50 (nM)	PKA Average IC50 (nM)	Akt1 Preference
Example 5		6.9	3231	468.3X
Example 82		11.1	3302.05	297.5X
Example 130		19.15	5749.8	300.3X
Example 200		11.65	3938.3	338.1X
Example 315		8.75	2939.2	335.9X

WO 2005/051304 (D1) – Table 2

Example	Structure	Akt1 Average IC50 (nM)	PKA Average IC50 (nM)	Akt1 Preference
Example 76		41.25	100.4	2.4X
Example 73		1.67	1.55	– (1.1X preference for PKA)
Example 26		64.4	908.57	14.1X
Example 59		8.3	2.8	– (2.9X preference for PKA)

SCIENCE IN MEDICINE



Long QT syndrome: novel insights into the mechanisms of cardiac arrhythmias

Robert S. Kass¹ and Arthur J. Moss²

¹Department of Pharmacology, Columbia University College of Physicians and Surgeons, New York, New York, USA

²Department of Medicine, Heart Research Follow-up Program, University of Rochester School of Medicine and Dentistry, Rochester, New York, USA

The congenital long QT syndrome is a rare disorder in which mutation carriers are at risk for polymorphic ventricular tachycardia and/or sudden cardiac death. Discovery and analysis of gene mutations associated with variants of this disorder have provided novel insight into mechanisms of cardiac arrhythmia and have raised the possibility of mutation-specific therapeutic intervention.

J. Clin. Invest. 112:810–815 (2003). doi:10.1172/JCI200319844.

Historical perspective

In 1957, Jervell and Lange-Nielsen first described a family with long QT syndrome (LQTS) (1). The family consisted of unrelated parents and their six children, four of whom were deaf with frequent fainting attacks precipitated by acute emotional arousal and exercise. Three of the four deaf children died suddenly while playing at the ages of 4, 5, and 9 years. An ECG was never recorded for the first deceased deaf child; however, ECGs of the remaining deaf children indicated markedly prolonged QT intervals (Figure 1). Both parents and the other two children were healthy, with normal hearing and normal ECGs. In 1958, Levine and Woodworth (2) reported the sudden death of a 13-year-old deaf boy with recurrent syncope and QT prolongation. Both parents were normal with no consanguinity. This combination of congenital deafness, QT prolongation, and recurrent syncope in five children from two independent families with normal parents was initially interpreted as a condition characterized by an autosomal recessive mode of inheritance.

In 1963 and 1964, Romano et al. (3) and Ward (4) respectively, reported separate families with QT prolongation in one parent and several children, all of whom possessed normal hearing but experienced recurrent syncope and sudden death. The pattern of symptoms and ECG findings in these two families support-

ed the presence of a condition characterized by an autosomal dominant mode of inheritance.

During the 1960s and 1970s, a number of individuals and families were reported with clinical features similar to those described by Jervell and Lange-Nielsen (1) or Romano et al. (3) and Ward (4). In 1979 Moss and Schwartz established the prospective International LQTS Registry for enrollment and follow-up of proband-identified LQTS families in order to determine the clinical course and hereditary features of LQTS (5). During the past 24 years, the Registry has enrolled over 1,200 LQTS families and has provided an understanding of the diagnostic features of this disorder, the natural history of the condition, and the efficacy of various therapies. In addition, the Registry has been an invaluable resource in the identification of genetic mutations that cause this inherited disorder.

Disease diagnosis

LQTS occurs infrequently in the general population with an estimated frequency of about 1 in 5,000 people. Patients with LQTS are usually identified by QT prolongation on the ECG during clinical evaluation of unexplained syncope, as part of a family study when one family member has been identified with the syndrome, or in the investigation of patients with congenital neural deafness. Clinical criteria have been developed to determine the probability that a patient has LQTS (Table 1). The two most important diagnostic features of LQTS are prolongation of the heart rate-corrected QT interval (QTc $\geq$ 0.46 seconds) and stress-induced syncope.

The syncope that occurs in this disorder is due to a transient, rapid, polymorphic ventricular tachycardia described as torsade de pointes (twisting of the points) that is associated with the underlying delayed ventricular repolarization manifest on the ECG as QTc prolongation (Figure 1d). Sudden death occurs when a poly-

Address correspondence to: Robert S. Kass, Department of Pharmacology, Columbia University College of Physicians and Surgeons, 630 West 168th Street, P&S 7-401, New York, New York 10032, USA. Phone: (212) 305-7444; Fax: (212) 342-2703; E-mail: rsk20@columbia.edu.

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: long QT syndrome (LQTS); heart rate-corrected QT (QTc); sympathetic nervous system (SNS); protein kinase A (PKA); protein phosphatase 1 (PP1); β -adrenergic receptor (β -AR).

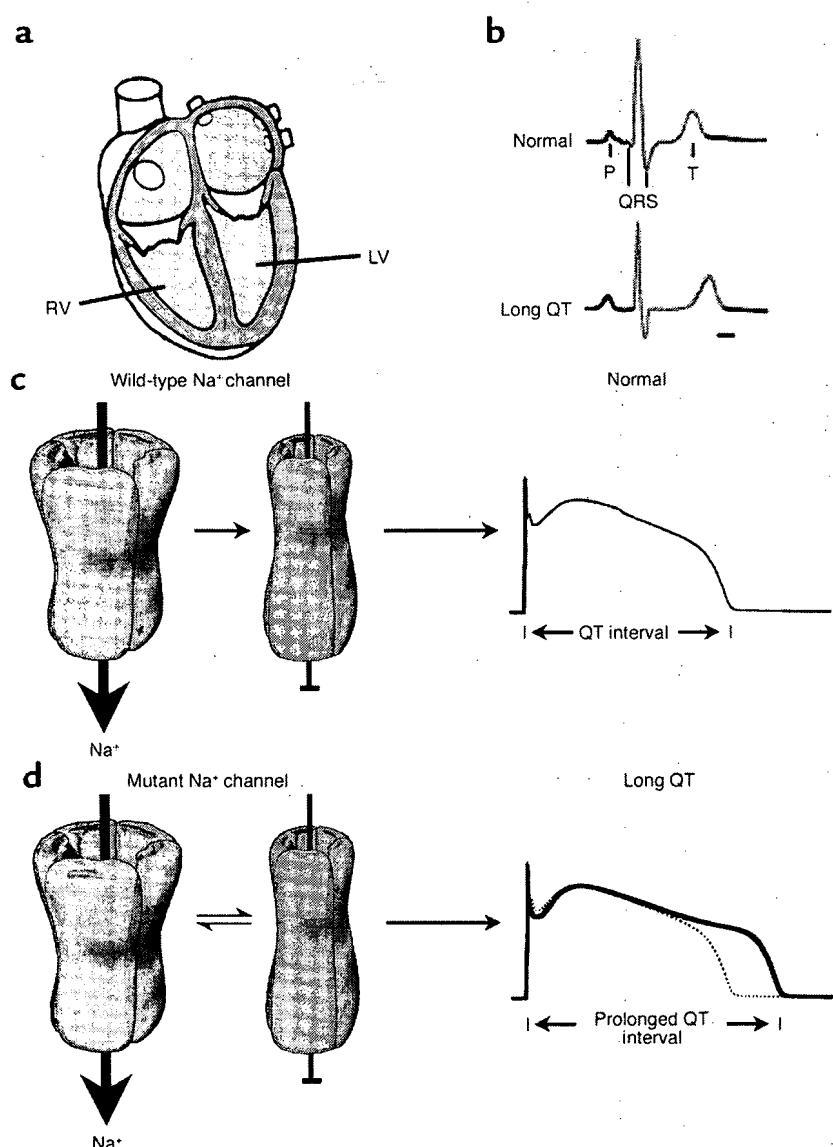


Figure 1
Mutation-altered Na⁺ channel inactivation underlies the LQT-3 phenotype. (a) Schematic view of the human heart emphasizing right ventricle (RV) and left ventricle (LV), which contain the substrates for altered electrical signaling in LQTS. (b) The clinical phenotype of LQTS is a prolonged QT interval of the ECG. (c) Gating of normal (wild-type) human heart Na⁺ channels during the action potential plateau. Channels rapidly enter an absorbing inactivated state from which they rarely reopen, and thus the QT interval (far right) is not greatly affected by wild-type Na⁺ channels. (d) Na⁺ channel gating in most forms of LQT-3 syndrome is altered. During the plateau phase, channels enter a mode of gating in which inactivation is no longer an absorbing state. A small fraction of channels open, enter a nonconducting inactivated state, and then transition back and forth between open and nonconducting states for the duration of the action potential plateau. This produces a small depolarizing current that prolongs the QT interval of the ECG of mutation carriers (far right). Modified with permission from refs. 25 and 26.

state, often referred to as the plateau phase of the ventricular action potential, is not only cardioprotective against premature excitation but is also essential to maintaining the proper timing between diastolic filling and ejection intervals. It is the plateau phase of the ventricular action potential that thus determines the QT interval of the ECG. Over 50 years ago, Silvio Weidmann (9) discovered that this crucial plateau phase is maintained by a delicate

morphic ventricular tachycardia episode deteriorates into ventricular fibrillation. Syncope and sudden death are most frequent in childhood and adolescence. The risk of cardiac events is higher in males before puberty and higher in females during adulthood (6, 7). The clinical course of patients with LQTS is quite variable and is influenced by the length of the QTc interval, gender, environmental factors, genotype, and therapy (8).

Mutation-induced ion channel dysfunction and LQTS

The ventricular action potential of the human heart is distinct in that the temporal period separating excitation of ventricular cells from relaxation, or repolarization, is very long, typically on the order of 450 milliseconds. This timing is crucial because as long as the ventricular tissue is depolarized it cannot be re-excited due to the unavailability of key voltage-gated Na⁺ channels which normally enter a nonconducting inactivated state during this period (Figure 1c). The duration of this depolarized

balance of small ionic conductances, which, though energetically favorable, predispose this period of the electrical cycle in the heart to disturbances that may be caused by otherwise harmlessly small changes in transmembrane ionic activity.

Before genetic information was successfully used to identify genes associated with congenital LQTS, various candidate ion channels (and thus channel-coding genes) were suspected to underlie the clinical defect of delayed ventricular relaxation. Molecular genetic approaches removed at least some of the uncertainty by demonstrating a clear association between mutations in genes encoding specific ion channel subunits and LQTS (reviewed in ref. 10). This work has revealed that mutations in at least five genes coding for key cardiac K⁺ channel subunits result in a loss or reduction of channel activity that can cause variants of LQTS (Table 2). Surprisingly, mutations in the principal (α) subunit of a key heart Na⁺ channel (encoded by SCN5A) that, in general, result in a gain of channel

Table 1
Diagnostic criteria for long QT syndrome

Features	Points
ECG findings ^A	
QTc	
≥0.48 s	3
0.46-0.47 s	2
0.45 s	1
Torsade de pointes ^B	2
T wave alternans	1
Notched T wave in three leads	1
Low heart rate for age ^C	0.5
Clinical History	
Syncope ^B	
With stress	2
Without stress	1
Congenital deafness	0.5
Family history	
Family members with definite LQTS ^D	1
Unexplained sudden cardiac death before age 30 among immediate family members ^D	0.5

Scoring: ≤ 1 point, low probability of LQTS; 2-3 points, intermediate probability of LQTS; ≥ 4 points, high probability of LQTS. ^AFindings in the absence of medications or disorders known to affect these ECG findings. QTc calculated by Bazett's formula, where QTc = QT/√RR. ^BMutually exclusive. ^CResting heart rate below the second percentile for age. ^DThe same family member cannot be counted for both of these criteria. Reprinted with permission from ref. 6.

function also cause yet another variant of LQTS, known as LQT-3 syndrome. These discoveries have prompted at least three new areas of research that impact not only the diagnosis and management of LQTS, but also our understanding of human cardiovascular physiology. These include (a) mutation-specific therapeutic strategies (b) identification of mutation-specific risk of cardiac events, and (c) mechanistic insight into the role of altered channel function and regulation in the control of QT intervals in the heart. The first two areas of research focus will be discussed below. Here we will focus on two areas in which fundamentally novel insight into human physiology emerged from investigation of the mechanistic basis of LQTS: the role of altered Na⁺ channel gating in control of the QT interval, and the molecular link between the sympathetic nervous system (SNS) and electrical signaling in the ventricle.

Na⁺ channels

Expression of ion channels in heterologous systems allows for investigation of inherited ion channel defects at the single-protein and cellular levels to directly identify the disease-associated alteration in ion channel function. Disease-linked mutations provide an opportunity to understand the mechanistic basis of human disease from altered molecular function to the clinical syndrome. Initial investigation of *SCN5A* mutations linked to LQT-3 syndrome revealed striking mutation-induced defects in channel behavior that were consistent with the disease

phenotype (11). Classically, Na⁺ channel activation is associated with the spread of depolarization in the heart that underlies the QRS complex of the ECG. In the case of LQT-3 syndrome, however, defects in the Na⁺ channel were linked to delay in ventricular repolarization (prolongation of the QT interval). These initial experiments clearly showed a novel mechanism that could explain this unexpected result: the inherited mutation disrupted the “inactivation” process of the channel such that, during the plateau phase of the action potential, a small number of Na⁺ channels do not inactivate (become nonconducting) but in fact reopen to provide a very small depolarizing current (Figure 1d). Exactly as Weidmann had predicted (9), even though this late current is only a fraction of the total Na⁺ channel current responsible for the QRS complex, this small disease-associated perturbation in plateau current is responsible for prolonging the QT interval in mutation carriers and raising the risk of cardiac events (12). Thus, the molecular genetic analysis of LQTS patients has led to a novel understanding of the importance of Na⁺ channel activity in controlling not only the QRS complex, but also the duration of the ventricular electrical response: the QT interval. Recently, mutations in *SCN1A*, the gene coding for the human neuronal Na⁺ channel α subunit associated with epilepsy, have been reported to cause similar defects in channel-inactivation gating (13). As yet, the cellular consequences of such epilepsy-associated mutations remain elusive, but mechanistic insights gained from investigation of cardiac defects are very likely to have widespread implications.

K⁺ channels: insight into adrenergic control of cardiac electrical activity

Investigation of the molecular basis of LQTS has led to fundamental insight into the molecular identity of key K⁺ channel subunits in the heart, notably the two key delayed rectifier currents I_{Kr} and I_{Ks}, which have been demonstrated in animal models to be crucial to control of cardiac action potential duration. In particular, mutations in *KCNQ1*, which codes for the α subunit of the I_{Ks} channel, cause LQT-1 syndrome, and mutations in *KCNE1*, the gene coding for the auxiliary β subunit of the I_{Ks} channel, cause LQT-5 syndrome (10). It had been

Table 2
Molecular and cellular mechanisms of cardiac arrhythmias

Disease	Gene (alternate name)	Protein	Reference
LQT-1	<i>KVLQT1 (KCNQ1)</i>	I _{Ks} K ⁺ channel α subunit	28
LQT-2	<i>HERG (KCNH2)</i>	I _{Kr} K ⁺ channel α subunit	29
LQT-3	<i>SCN5A</i>	I _{Na} K ⁺ channel α subunit	30
LQT-4	<i>ANKB</i>	ANKRIN-β	31
LQT-5	<i>minK (KCNE1)</i>	I _{Ks} K ⁺ channel β subunit	32
LQT-6	<i>MiRP1 (KCNE2)</i>	I _{Kr} K ⁺ channel β subunit	33
LQT-7	<i>KCNJ2</i>	I _{Kr} K ⁺ channel α subunit	34

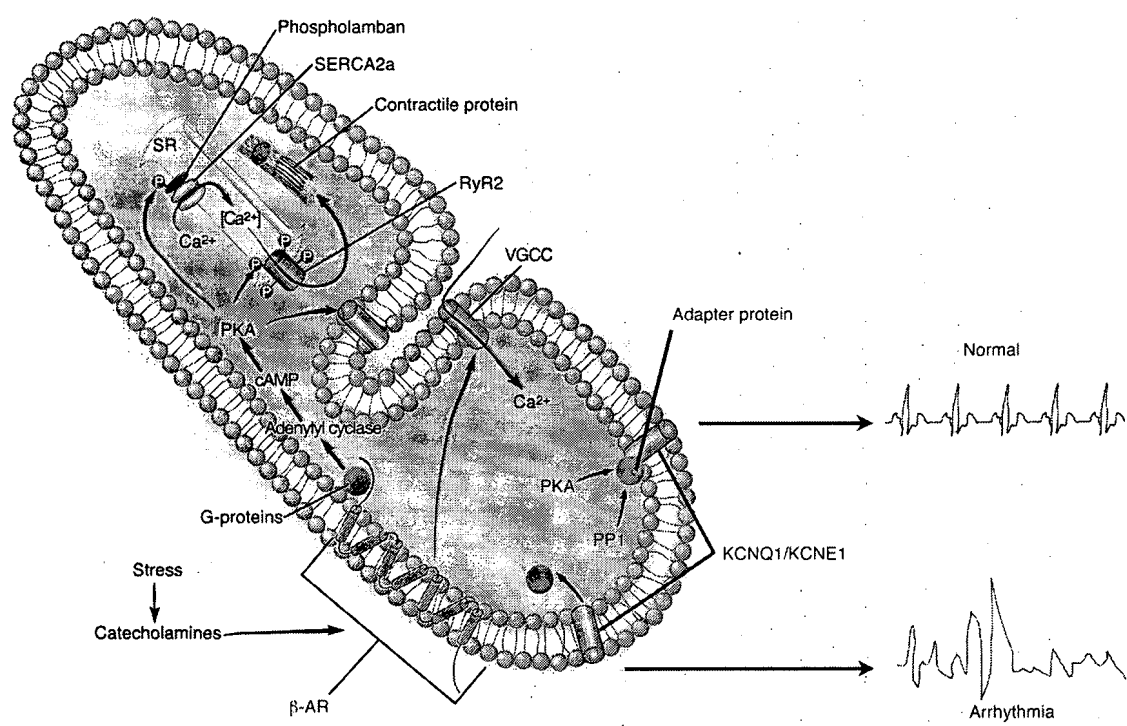


Figure 2
 Disruption of local signaling domains occurs in LQT-1 syndrome. Stimulation of β -adrenergic receptors (β -ARs) in the heart leads to PKA-dependent phosphorylation of multiple intracellular targets in cardiac myocytes. These targets include the ryanodine receptor (RyR2), L-type calcium channels, and the KCNQ1/KCNE1 K^+ channel. In response to stress in healthy patients, β -AR stimulation results in phosphorylation of all three of these targets and uniform electrical activity on the ECG (upper ECG). When the KCNQ1/KCNE1 complex is disrupted by an inherited mutation, an unbalanced cellular response occurs, which leads to dysfunctional rhythm (lower ECG). Modified with permission from ref. 27. SR, sarcoplasmic reticulum; VGCC, voltage-gated calcium channel.

well established in animal models that I_{Ks} is strongly regulated by SNS stimulation, such that SNS stimulation increases reserves of repolarizing current and contributes to the shortening of action potential that occurs in parallel with the SNS stimulation-induced increases in heart rate (14). Further, clinical data indicate that carriers of mutations in either *KCNQ1* or *KCNE1* are at increased risk of experiencing a fatal cardiac arrhythmia in the face of elevated SNS activity (15). Together, these findings motivated investigation into molecular links between the SNS and regulation of KCNQ1/KCNE1 channels in the human heart. This work resulted in the discovery that the KCNQ1/KCNE1 channel actually forms a macromolecular signaling complex: coupled to the carboxy-terminal domain of the channel is an adaptor protein, yotiao, which in turn binds to the regulatory enzymes protein kinase A (PKA) and protein phosphatase 1 (PP1) (Figure 2) (16). Thus the channel, via the adaptor protein, recruits enzymes that can upregulate (PKA) and downregulate (PP1) channel activity by phosphorylation and dephosphorylation of a serine in its amino-terminal domain (16). When this complex is disrupted, the channel is not properly regulated and there is imbalance in control of the ventricular action potential, which leads to high risk of arrhythmia. Because other targets of PKA, such as the ryanodine receptor of the sarcoplasmic reticulum, form independent macro-

molecular signaling complexes, selective disruption of the KCNQ1/KCNE1 signaling complex by inherited mutations may disrupt a micromolecular signaling domain restricted to one channel – a potentially novel mechanism that may mechanistically contribute to the genesis of cardiac arrhythmias (Figure 2) (17).

Clinical evaluation: phenotype can suggest genotype

The discovery that distinct LQTS variants were associated with genes coding for different ion channel subunits has had a major impact on the diagnosis and analysis of LQTS patients. It is clear that there are distinct risk factors associated with the different LQTS genotypes, and this must be taken into account during patient evaluation and diagnosis. The greatest difference in risk factors becomes apparent in comparing LQT-3 syndrome patients (*SCN5A* mutations) and patients with LQT-1 syndrome (*KCNQ1* mutations) or LQT-2 syndrome (*HERG* mutations). This was made clear in an extensive and collaborative study in which the risk of cardiac events was found to be greatest during rest, during bradycardia, or, in the case of LQT-3 syndrome patients, under conditions of low sympathetic nerve activity (15). In contrast, cardiac events in LQT-2 syndrome patients were associated with arousal and/or conditions in which patients were startled,

whereas LQT-1 syndrome patients were found to be at greatest risk of cardiac events during exercise, conditions of elevated heart rate, and sympathetic nerve activity (15). Clearly, identifying the genotype is essential during diagnosis, as risk factors are somewhat gene specific. Some groups have begun investigating the use of exercise or stress testing to estimate the likelihood that a patient possesses a specific genotype (18). Additionally, a major effort has been made to link ECG waveform characteristics to genotype in order to estimate the genetic basis of familial LQTS in patient candidates (19). However, ultimately, genotype identification by either direct sequencing or other technologies is necessary to unequivocally determine the genetic basis of the clinical defect.

Therapeutic strategy defined by genotype

One of the major contributions of the integration of cellular, molecular, and genetic techniques in the investigation of the causes and treatment of congenital LQTS has been the emergence of mutation-specific strategies for disease therapy. Because the functional consequences of most of the Na⁺ channel mutations that cause LQT-3 syndrome are subtle increases in channel activity during the action potential plateau, cellular experimental work suggested that conventional Na⁺ channel blockers such as mexiletine, tocainide, and lidocaine might prove useful in treating this LQTS variant (20). This has, in fact, proven to be the case, and other Na⁺ channel blockers such as flecainide have emerged with great promise as therapeutic agents specific for LQT-3 syndrome patients (21, 22). β -blocker therapy is most effective in preventing recurrence of cardiac events and lowering the death rate in LQT-1 and LQT-2 syndrome patients but is much less effective in the treatment of LQT-3 syndrome patients (15, 23). β -blocking drugs are associated with a significant reduction in cardiac events in LQTS patients, even though these drugs have minimal effect on the QTc interval (23). It is believed that the efficacy of β -blocker therapy is related to an attenuation of adrenergic-mediated trigger mechanisms in the disorder. β -blockers do not provide absolute protection against fatal cardiac arrhythmias. Recent studies indicate that the implanted cardioverter defibrillator is effective in preventing sudden cardiac death in LQTS patients (24). The combination of β -blocker drugs and an implanted defibrillator is a safe and reliable form of therapy for managing high-risk LQTS. Particularly in the case of LQT-1 syndrome patients, β -blocker administration is thought to reduce incidents of cellular imbalance in response to stimulation of β -adrenergic receptors (β -ARs) (Figure 2), which occurs when one target of β -AR stimulation, the KCNQ1/KCNE1 channel, is eliminated from the cellular response. In fact, because lower heart rates provoke cardiac events in LQT-3 mutant gene carriers, β -blockade may be pro-arrhythmic in this patient group. Thus genotype, which underlies phenotype, can dictate the most promising therapeutic approach.

What remains unknown and future directions

Investigations into the clinical aspects and basic causal mechanisms of LQTS have provided novel and important insight into the fundamental nature of the electrical activity of the human heart and how small perturbations in ion flow can have important consequences for human disease. We know that there are multiple variants of the disease that are linked to mutations in specific ion channel subunit genes, and that the unifying clinical phenotype is delay of repolarization in the ventricle. Though great progress has been made, many important questions remain. What is the cellular mechanism responsible for the triggering of fatal events in the context of the whole heart? Why does repolarization delay (QT prolongation) lead to early after-depolarizations and trigger arrhythmogenic events? Does altered cellular calcium play a role in these arrhythmias? Are mutations in other ion channel genes responsible for as yet unrecognized variants of LQTS? Finally, can we identify gene variants that may predispose individuals to arrhythmia risk if, and only if, other subtle mutations and/or polymorphisms exist in another gene in the same individual? Answers to these questions with respect to LQTS will no doubt provide further insight into the fundamental mechanisms of the incredibly robust and resilient, but ever so sensitive, electrical network of the human heart.

1. Jervell, A., and Lange-Nielsen, F. 1957. Congenital deafmutism, functional heart disease with prolongation of the Q interval, and sudden death. *Am. Heart J.* 54:59-68.
2. Levine, S.A., and Woodworth, C.R. 1958. Congenital deaf-mutism, prolonged Q-T interval, syncopal attacks and sudden death. *N. Engl. J. Med.* 259:412-417.
3. Romano, C., Gemme, G., and Pongiglione, R. 1963. Artimie cardiache rare dell'eta pediatrica. *Clin. Pediatr. (Phila.)* 45:656-683.
4. Ward, O.C. 1964. New familial cardiac syndrome in children. *J. Ir. Med. Assoc.* 54:103-106.
5. Moss, A.J., and Schwartz, P.J. 1982. Delayed repolarization (QT or QTU prolongation) and malignant ventricular arrhythmias. *Mod. Concepts Cardiovasc. Dis.* 51:85-90.
6. Schwartz, P.J., Moss, A.J., Vincent, G.M., and Crampton, R.S. 1993. Diagnostic criteria for the long QT syndrome. An update. *Circulation* 88:782-784.
7. Locati, E.H., et al. 1998. Age- and sex-related differences in clinical manifestations in patients with congenital long-QT syndrome: findings from the International LQTS Registry. *Circulation* 97:2237-2244.
8. Moss, A.J., et al. 1991. The long QT syndrome. Prospective longitudinal study of 328 families. *Circulation* 84:1136-1144.
9. Weidmann, S. 1951. Effect of current flow on the membrane potential of cardiac muscle. *J. Physiol.* 115:227-236.
10. Sanguinetti, M.C. 2000. Long QT syndrome: ionic basis and arrhythmia mechanism in long QT syndrome type 1. *J. Cardiovasc. Electrophysiol.* 11:710-712.
11. Bennett, P.B., Yazawa, K., Makita, N., and George, A.L. 1995. Molecular mechanism for an inherited cardiac arrhythmia. *Nature* 376:683-685.
12. Clancy, C.E., and Rudy, Y. 1999. Linking a genetic defect to its cellular phenotype in a cardiac arrhythmia. *Nature* 400:566-569.
13. Lossin, C., Wang, D.W., Rhodes, T.H., Vanoye, C.G., and George, A.L., Jr. 2002. Molecular basis of an inherited epilepsy. *Neuron* 34:877-884.
14. Kass, R.S., and Wiegand, S.E. 1982. The ionic basis of concentration-related effects of noradrenaline on the action potential of calf cardiac purkinje fibres. *J. Physiol.* 322:541-558.
15. Schwartz, P.J., et al. 2001. Genotype-phenotype correlation in the long-QT syndrome: gene-specific triggers for life-threatening arrhythmias. *Circulation* 103:89-95.
16. Marx, S.O., et al. 2002. Requirement of a macromolecular signaling complex for beta adrenergic receptor modulation of the KCNQ1-KCNE1 potassium channel. *Science* 295:496-499.
17. Kass, R.S., Kurokawa, J., Marx, S.O., and Marks, A.R. 2003. Leucine/isoleucine zipper coordination of ion channel macromolecular signal-

- ing complexes in the heart. Roles in inherited arrhythmias. *Trends Cardiovasc. Med.* **13**:52-56.
18. Takenaka, K., et al. 2003. Exercise stress test amplifies genotype-phenotype correlation in the LQT1 and LQT2 forms of the long-QT syndrome. *Circulation.* **107**:838-844.
19. Moss, A.J., et al. 1995. Electrocardiographic T-wave patterns in genetically distinct forms of the hereditary long QT syndrome. *Circulation.* **92**:2929-2934.
20. An, R.H., Bangalore, R., Rosero, S.Z., and Kass, R.S. 1996. Lidocaine block of LQT-3 mutant human Na⁺ channels. *Circ. Res.* **79**:103-108.
21. Benhorin, J., et al. 2000. Effects of flecainide in patients with new SCN5A mutation: mutation-specific therapy for long-QT syndrome? *Circulation.* **101**:1698-1706.
22. Windle, J.R., Geletka, R.C., Moss, A.J., Zareba, W., and Atkins, D.L. 2001. Normalization of ventricular repolarization with flecainide in long QT syndrome patients with SCN5A:DeltaKPQ mutation. *Ann. Noninvasive. Electrocardiol.* **6**:153-158.
23. Moss, A.J., et al. 2000. Effectiveness and limitations of beta-blocker therapy in congenital long-QT syndrome. *Circulation.* **101**:616-623.
24. Zareba, W., et al. 2003. Implantable cardioverter defibrillator in high-risk long QT syndrome patients. *J. Cardiovasc. Electrophysiol.* **14**:337-341.
25. Clancy, C.E., and Kass, R.E. 2002. Defective cardiac ion channels: from mutations to clinical syndromes. *J. Clin. Invest.* **110**:1075-1077. doi:10.1172/JCI200216945.
26. Keating, M.T., and Sanguinetti, M.C. 2001. Molecular and cellular mechanisms of cardiac arrhythmias. *Cell.* **104**:569-580.
27. Marks, A.R. 2003. Calcium and the heart: a question of life and death. *J. Clin. Invest.* **111**:597-600. doi:10.1172/JCI200318067.
28. Wang, Q., et al. 1996. Positional cloning of a novel potassium channel gene: KVLQT1 mutations cause cardiac arrhythmias. *Nat. Genet.* **12**:17-23.
29. Curran, M.E., et al. 1995. A molecular basis for cardiac arrhythmia: HERG mutations cause long QT syndrome. *Cell.* **80**:795-803.
30. Wang, Q., et al. 1995. SCN5A mutations associated with an inherited cardiac arrhythmia, long QT syndrome. *Cell.* **80**:805-811.
31. Mohler, P.J., et al. 2003. Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. *Nature.* **421**:634-639.
32. Splawski, I., Tristani-Firouzi, M., Lehmann, M.H., Sanguinetti, M.C., and Keating, M.T. 1997. Mutations in the hminK gene cause long QT syndrome and suppress IKs function. *Nat. Genet.* **17**:338-340.
33. Abbott, G.W., et al. 1999. MiRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmia. *Cell.* **97**:175-187.
34. Plaster, N.M., et al. 2001. Mutations in Kir2.1 cause the developmental and episodic electrical phenotypes of Andersen's syndrome. *Cell.* **105**:511-519.

REPORTS

Requirement of a Macromolecular Signaling Complex for β Adrenergic Receptor Modulation of the KCNQ1-KCNE1 Potassium Channel

Steven O. Marx,* Junko Kurokawa,* Steven Reiken,
Howard Motoike, Jeanine D'Armiento, Andrew R. Marks,
Robert S. Kass†

Sympathetic nervous system (SNS) regulation of cardiac action potential duration (APD) is mediated by β adrenergic receptor (BAR) activation, which increases the slow outward potassium ion current (I_{KS}). Mutations in two human I_{KS} channel subunits, hKCNQ1 and hKCNE1, prolong APD and cause inherited cardiac arrhythmias known as LQTS (long QT syndrome). We show that BAR modulation of I_{KS} requires targeting of adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase (PKA) and protein phosphatase 1 (PP1) to hKCNQ1 through the targeting protein yotiao. Yotiao binds to hKCNQ1 by a leucine zipper motif, which is disrupted by an LQTS mutation (hKCNQ1-G589D). Identification of the hKCNQ1 macromolecular complex provides a mechanism for SNS modulation of cardiac APD through I_{KS} .

Cardiac function is regulated by the SNS; stimulation of β ARs increases heart rate and contractility and shortens APD (1). β AR-

mediated regulation of I_{KS} is particularly important because patients with KCNQ1 or KCNE1 mutations have a form of LQTS (2, 3) in which fatal cardiac arrhythmias are precipitated by increased SNS activity associated with exercise and startling (4–6).

Because β AR signaling results in PKA activation, we sought to determine whether PKA and a phosphatase were components of an hKCNQ1 macromolecular signaling complex. In immunoprecipitations of hKCNQ1

from cardiac homogenates, we found that PKA catalytic and regulatory (RII) subunits, protein phosphatase 1 (PP1) (but not PP2A), and the PKA- and PP1-targeting protein yotiao [which also targets PKA and PP1 to *N*-methyl-D-aspartate (NMDA) receptors in the brain (7)] coimmunoprecipitated with hKCNQ1 from human cardiac homogenates (Fig. 1A). Thus, the PKA catalytic and regulatory subunits, PP1, and yotiao are components of the hKCNQ1 macromolecular complex.

Microcystin Sepharose beads (which bind to PP1) and cAMP Sepharose beads (which bind to the PKA regulatory subunit) specifically associated with hKCNQ1 when incubated with cardiac homogenates from a transgenic mouse (TG⁺) expressing an hKCNQ1-hKCNE1 fusion protein in the heart. No such association was detected in homogenates from wild-type (WT) mouse hearts, which lack I_{KS} because their heart rate (~500 beats per minute) requires K⁺ channels with faster kinetics to control APD (Fig. 1B). PKA catalytic and regulatory (RII) subunits, PP1, and yotiao were also associated with hKCNQ1-hKCNE1 from TG⁺ mouse hearts (Fig. 1C).

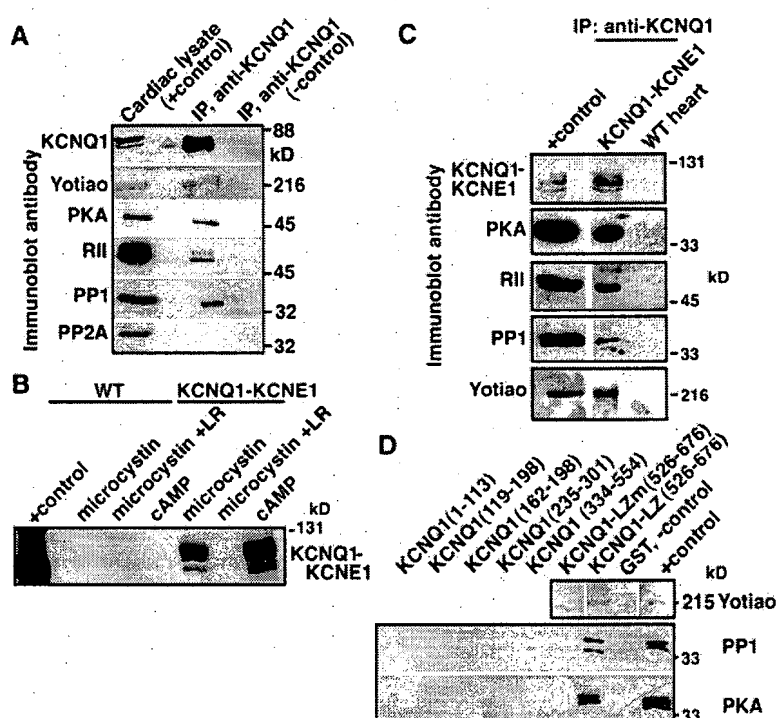
The binding of kinases and phosphatases to ion channels through targeting proteins can be mediated by leucine zipper (LZ) motifs (8). We tested whether a LZ motif in the COOH-terminus of hKCNQ1 (amino acid residues 588 to 616) functioned in targeting PKA and PP1 to hKCNQ1 through yotiao. Glutathione *S*-transferase (GST)-KCNQ1 fusion proteins were incubated with cardiac muscle

Department of Pharmacology, Center for Molecular Cardiology, Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA.

*These authors contributed equally to this report.

†To whom correspondence should be addressed. E-mail: rsk20@columbia.edu

Fig. 1. Presence of PKA, RII, PP1, and the targeting protein yotiao in the KCNQ1 macromolecular complex. (A) KCNQ1 immunoprecipitated from human cardiac homogenates using anti-KCNQ1 (22). Positive control for immunoblotting was human cardiac homogenate; negative control for immunoprecipitation was anti-KCNQ1 preabsorbed with the antigenic peptide (lane 3). (B) For pull-down assays, either microcystin- or cAMP-Sepharose beads were incubated with cardiac homogenates from wild-type (WT) or TG⁺ mice (expressing hKCNQ1-hKCNE1) (23). Bound proteins were immunoblotted with anti-hKCNQ1. Specificity of the microcystin pull-down was demonstrated using free microcystin LR. "+control" is anti-hKCNQ1 immunoblot of TG⁺ cardiac homogenate that recognizes the hKCNQ1-hKCNE1 fusion product of the transgene, which migrates at ~125 kD. (C) Proteins were immunoprecipitated from homogenates of WT or hKCNQ1-hKCNE1 TG⁺ mouse hearts with anti-hKCNQ1 and immunoblotted with anti-hKCNQ1, anti-PKA (catalytic subunit), anti-RII (PKA regulatory subunit), anti-PP1 (catalytic subunit of PP1), and anti-yotiao. "+control" is TG⁺ cardiac homogenate. (D) Proteins from cardiac homogenates (200 μ g) that associated with GST-hKCNQ1 fusion proteins (24) were immunoblotted with anti-yotiao, anti-PKA, and anti-PP1 (25). "+control" is TG⁺ cardiac homogenate. Data shown are representative of three or more experiments.



REPORTS

homogenates from human or TG⁺ (hKCNQ1-hKCNE1) mouse hearts. GST-KCNQ1-LZ, containing the LZ motif, coprecipitated PP1, PKA, and yotiao (Fig. 1D). This interaction was specific, as neither GST alone nor GST-KCNQ1 fragments from other intracellular regions without the LZ motif coprecipitated PP1, PKA, or yotiao (Fig. 1D). GST-KCNQ1-LZ did not directly bind recombinant PP1 or PKA catalytic subunits (9), indicating that the binding of PP1 and PKA to hKCNQ1 requires yotiao. Moreover, a mutant peptide (KCNQ1-LZ_m) containing alanine substitutions (Leu⁶⁰² → Ala, Ile⁶⁰⁹ → Ala) that disrupt the LZ motif did not coprecipitate PP1, PKA, or yotiao. Yotiao contains multiple coiled coils that can form LZs but has not been reported to bind to targets via LZs. Kinase and phosphatase binding to ion channels through specific targeting proteins provides a mechanism regulating phosphoryl-

ation at the subcellular level (10, 11). For example, PP1 is targeted to the type 2 ryanodine receptor-calcium release channel on the cardiac sarcoplasmic reticulum through spinophilin (8) and is now shown to interact through yotiao with hKCNQ1 in the plasma membrane.

Addition of cAMP, in the absence of exogenous PKA, induced phosphorylation of immunoprecipitated KCNQ1 (Fig. 2A). Using an alanine substitution, we identified Ser²⁷ as the unique site of PKA phosphorylation on hKCNQ1 (Fig. 2B). We did not detect AKAP 79 or AKAP 15-18 (other PKA targeting proteins) with KCNQ1. Indeed, our data show that yotiao is the only protein that targets PKA and PP1 to hKCNQ1 complex, because incubation of cardiac homogenates with a peptide containing the KCNQ1 LZ motif (which binds yotiao) prevented coimmunoprecipitation of yotiao, PKA, and PP1

with KCNQ1 (Fig. 2C). The corresponding mutant peptide containing a disrupted LZ (GST-KCNQ1-LZ_m) did not disrupt complexes of KCNQ1 with yotiao, PKA, or PP1 (Fig. 2C).

When hKCNQ1 and yotiao were coexpressed in Chinese hamster ovary (CHO) cells, PKA and PP1 were coimmunoprecipitated with hKCNQ1 (Fig. 2D). Without yotiao, PKA and PP1 did not coimmunoprecipitate with hKCNQ1 (Fig. 2D). Disrupting the hKCNQ1 LZ with alanine substitutions (KCNQ1-LZ_m) or with the LQTS-associated KCNQ1-G589D mutation (Gly⁵⁸⁹ is the first "e" position in the COOH-terminal hKCNQ1 LZ motif) prevented binding of yotiao, PKA, or PP1 to the channel (Fig. 2D). Thus, the LZ motif is required for yotiao-mediated targeting of PKA and PP1 to hKCNQ1.

The membrane-permeant cAMP analog 8 Br-cAMP (300 μ M) significantly increased hKCNQ1-hKCNE1 tail currents after +60 mV prepulses 1.8 ± 0.1 -fold ($n = 9$) (Fig. 3A) in myocytes from mice expressing the human proteins. cAMP has similar effects on native I_{KS} recorded from guinea pig ventricular myocytes (12). hKCNQ1-hKCNE1 channel activity (assayed from tail currents after +60 mV prepulses) was significantly enhanced (3.6 ± 0.7 fold, $n = 5$) (Fig. 3A) by cAMP (300 μ M) plus okadaic acid (OA, 1 μ M), a PP1 inhibitor that binds to the phosphatase catalytic site. OA alone did not significantly increase hKCNQ1-hKCNE1 currents ($n = 5$) (Fig. 3A). Thus, kinase (PKA) activity potentially regulates I_{KS} and inhibiting phosphatase (PP1) activity enhances this regulation.

Expression of yotiao was required to reconstitute cAMP-dependent regulation of the hKCNQ1-hKCNE1 channel in a heterologous expression system (CHO cells). In the absence of yotiao, there was no significant effect of intracellular cAMP (200 μ M) on hKCNQ1-hKCNE1 currents either in the absence or presence of OA (0.2 μ M, $n = 5$) (Fig. 3B). With yotiao, hKCNQ1-hKCNE1 tail current (after +60 mV conditioning pulses) was doubled by cAMP [without cAMP, 24.3 ± 7.5 pA/pF; with cAMP (200 μ M), 50.7 ± 9.2 pA/pF; $n = 5$] and OA (0.2 μ M) plus cAMP (200 μ M) increased the hKCNQ1-hKCNE1 current ~4-fold (89 ± 15.6 pA/pF, $n = 5$) (Fig. 3B).

Substituting Ala for Ser²⁷ (S27A) in KCNQ1, which is phosphorylated in response to cAMP, eliminated cAMP-dependent enhancement of hKCNQ1-hKCNE1 current ($n = 6$, Fig. 3C). Expression in CHO cells of hKCNE1 and yotiao with mutant hKCNQ1 (hKCNQ1-LZ_m) or the G589D mutant linked to LQTS (13), both of which disrupt the KCNQ1 LZ motif (Fig. 2D), ab-

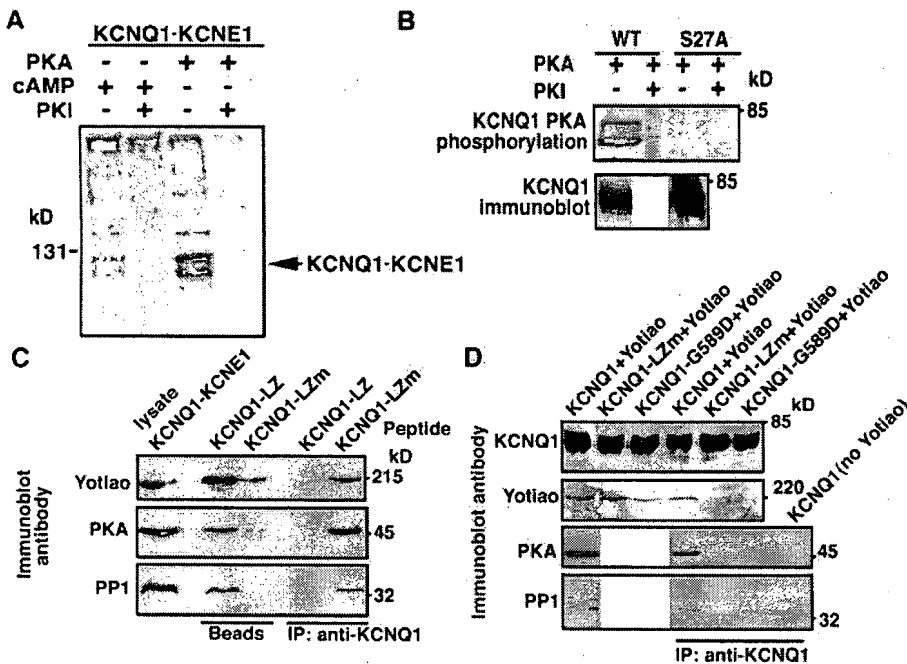


Fig. 2. Phosphorylation of Ser²⁷ on KCNQ1 by PKA requires an intact LZ motif in the COOH-terminus of KCNQ1. (A) hKCNQ1-hKCNE1 immunoprecipitated from TG⁺ mouse heart was incubated with cAMP (10 μ M) or exogenous PKA (5 units) and [γ -³²P]ATP. The immunoprecipitates were size-fractionated, followed by autoradiography. The specificity of the PKA phosphorylation was established using the PKA inhibitor PKI (500 nM). (B) Identification of Ser²⁷ as the PKA phosphorylation site on hKCNQ1 by alanine substitution (S27A). WT hKCNQ1 and hKCNQ1-S27A expressed in CHO cells were immunoprecipitated with anti-hKCNQ1 and incubated with PKA (5 units) and [γ -³²P]ATP. The immunoprecipitates were size-fractionated and phosphorylation was detected by autoradiography. Equivalent amounts of hKCNQ1 and hKCNQ1-S27A were present in immunoprecipitates, as demonstrated by immunoblotting (lower panel). Data are representative of three experiments. (C) A fusion peptide containing the COOH-terminal LZ motif of hKCNQ1 (hKCNQ1-LZ) competes off yotiao, PKA, and PP1 from the hKCNQ1-hKCNE1 complex. TG⁺ cardiac homogenates (200 μ g) were incubated with glutathione beads containing hKCNQ1-LZ or hKCNQ1-LZ_m. Supernatants from these reactions were immunoprecipitated with anti-hKCNQ1 and the precipitates were blotted for yotiao, PKA, and PP1. The "+" control is TG⁺ cardiac homogenate. (D) WT hKCNQ1, mutant channels with disrupted LZ motifs, hKCNQ1-LZ_m, or hKCNQ1-G589D were coexpressed with yotiao in CHO cells. Equal amounts of WT and mutant hKCNQ1, yotiao, PKA, and PP1 were shown by immunoblotting (first three lanes). Proteins were immunoprecipitated from lysates with anti-KCNQ1 and immunoblotted. Immunoprecipitated hKCNQ1 from CHO cells without yotiao is shown in the last lane. Data shown are representative of three or more experiments.

REPORTS

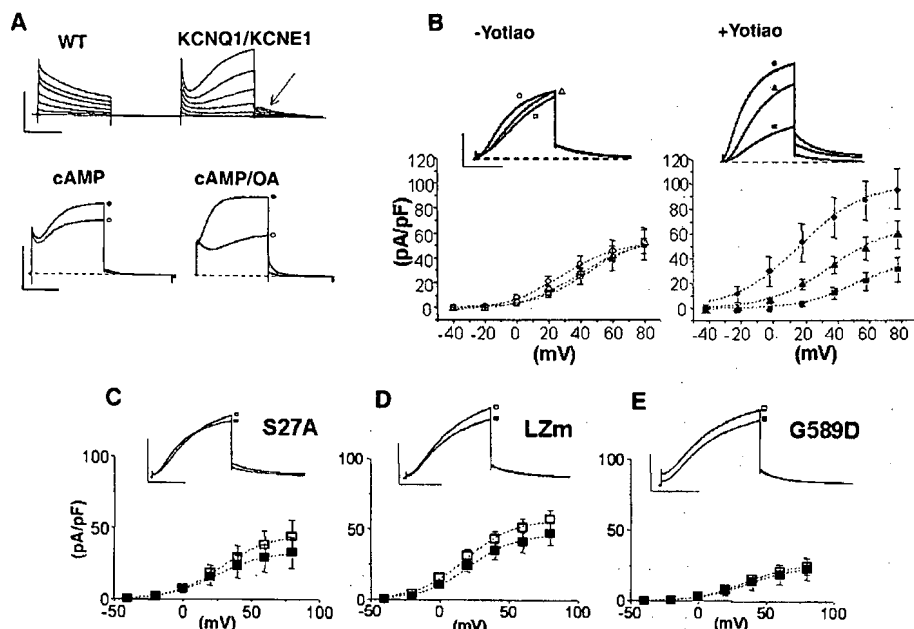


Fig. 3. Requirement of Ser²⁷, the KCNQ1 LZ motif, and yotiao for PKA-dependent regulation of I_{KS} . **(A)** Whole-cell patch clamp currents recorded from WT and TG⁺ (hKCNQ1-hKCNE1) murine cardiomyocytes (26). Tail current after +60 mV pulses was significantly increased by cAMP ($P < 0.01$, Student's t test, pre-cAMP versus cAMP, $n = 9$) and by cAMP plus OA ($P = 0.01$, Student's t test, pre-cAMP + OA versus cAMP + OA, $n = 5$). Currents shown in upper traces were activated by a series of 2-s voltage pulses from -40 mV to +80 mV (20-mV increments, 0.067 Hz) from a -65 mV holding potential and deactivated by returning to -40 mV. hKCNQ1-hKCNE1 channel current is evident as slowly activating outward current during pulses and deactivating current "tails" upon repolarization detectable in TG⁺ (arrow) but not TG⁻ cells. Myocytes were treated with cAMP and OA (bottom two traces) and hKCNQ1-hKCNE1 currents were activated by depolarization (+60 mV). Currents recorded before (open circles) and after (solid circles) a 5-min external application of 8-Br-cAMP (300 μ M) or 8-Br-cAMP (300 μ M) plus OA (1.0 μ M). Scale: 20 pA/pF and 1 s. **(B)** Requirement of yotiao for cAMP and OA dependent modulation of hKCNQ1-hKCNE1 channels in CHO cells. Shown are mean currents ($n = 5$) (+60 mV pulse, -40 mV return) as well as plots of mean tail current $\pm$ SEM vs. pulse voltage. Tail currents (after +60 mV) were significantly different by ANOVA $P < 0.05$. CHO cells were transfected with hKCNQ1-hKCNE1 without (left) or with (right) yotiao. Internal solutions without cAMP (control); with 200 μ M cAMP (cAMP); or with 200 μ M cAMP plus 0.2 μ M OA (cAMP/OA) were dialyzed at room temperature for 13 min before measurements were made. Open symbols, no yotiao; solid symbols, with yotiao. Squares, control; triangles, cAMP; circles, cAMP/OA. Scale: 100 pA/pF, 1 s. **(C)** Substituting Ala for Ser²⁷ in KCNQ1. I_{KS} was measured in CHO cells cotransfected with yotiao, hKCNE1, and hKCNQ1-S27A ($n = 6$). Scale: 100 pA/pF, 1 s. **(D)** Disruption of the KCNQ1 LZ motif by Ala substitution. CHO cells were transfected with yotiao, hKCNE1, and hKCNQ1-LZm ($n = 5$). Scale: 100 pA/pF, 1 s. **(E)** Disruption of the KCNQ1 LZ motif with the LQTS mutation (G589D). CHO cells were transfected with yotiao, hKCNE1, and hKCNQ1-G589D ($n = 5$). Scale: 50 pA/pF, 1 s. In (C) to (E), cells were dialyzed with cAMP/OA-containing (solid squares) or control (open squares) internal solutions. Currents and plots as in (B).

lated cAMP-dependent regulation of I_{KS} ($n = 5$) (Fig. 3, D and E). Thus, assembly of the macromolecular complex via LZ-mediated binding of yotiao to hKCNQ1, as well as PKA phosphorylation of Ser²⁷, are necessary to reconstitute PKA- and PP1-dependent regulation of hKCNQ1-hKCNE1 channels.

The finding that the hKCNQ1-G589D mutation prevents cAMP-dependent regulation of I_{KS} suggests that these mutant channels may not respond to β AR-mediated signaling in patients. Along with the reduced expression of I_{KS} in carriers of the hKCNQ1-G589D mutation (13), uncoupling of the channel from SNS modulation may exacerbate the defect in APD shortening and further increase the risk of exer-

cise-induced ventricular tachyarrhythmias that cause sudden cardiac death (SCD) in some patients (6). Indeed, SCD is associated with SNS stimulation in 81% of individuals with the KCNQ1-G589D mutation in whom a trigger event can be identified (13), in contrast to other K⁺ channel mutations in which only ~10% of SCD cases are triggered by SNS activation (4).

Taken together, our data show that the regulation of hKCNQ1 by PKA-dependent phosphorylation requires a macromolecular complex that includes PKA, PP1, and the targeting protein yotiao and provide a mechanistic link between the sympathetic nervous system and modulation of the cardiac APD through I_{KS} .

References and Notes

1. R. S. Kass, W. Wang, in *Cardiac Electrophysiology from Cell to Bedside*, D. P. Zipes, J. Jalife, Eds. (Saunders, Philadelphia, 2000), chap. 11.
2. M. C. Sanguinetti et al., *Nature* **384**, 80 (1996).
3. J. Barhanin et al., *Nature* **384**, 78 (1996).
4. P. J. Schwartz et al., *Circulation* **103**, 89 (2001).
5. J. A. Towbin, Z. Wang, H. Li, *Drug Metab. Dispos.* **29**, 574 (2001).
6. K. J. Paavonen et al., *Heart* **86**, 39 (2001).
7. J. W. Lin et al., *J. Neurosci.* **18**, 2017 (1998).
8. S. O. Marx et al., *J. Cell Biol.* **153**, 699 (2001).
9. S. O. Marx, S. Reiken, A. R. Marks, unpublished data.
10. P. C. Gray, J. D. Scott, W. A. Catterall, *Curr. Opin. Neurobiol.* **8**, 330 (1998).
11. J. D. Scott, T. Pawson, *Sci. Am.* **282**, 72 (June 2000).
12. K. B. Walsh, R. S. Kass, *Science* **242**, 67 (1988).
13. K. Piippo et al., *J. Am. Coll. Cardiol.* **37**, 562 (2001).
14. A. Felicello et al., *FEBS Lett.* **464**, 174 (1999).
15. W. Wang, J. Xia, R. S. Kass, *J. Biol. Chem.* **273**, 34069 (1998).
16. J. Gulick, A. Subramaniam, J. Neumann, J. Robbins, *J. Biol. Chem.* **266**, 9180 (1991).
17. Supplemental material on methods used for GST pull-down assays, membrane targeting, and subunit assembly of KCNQ1-G589D is available on Science Online at www.sciencemag.org/cgi/content/full/295/5554/496/DC1.
18. R. Mitra, M. Morad, *Am. J. Physiol.* **249**, H1056 (1985).
19. O. P. Hamill, A. Marty, E. Neher, B. Sakmann, F. J. Sigworth, *Pflügers Arch.* **391**, 85 (1981).
20. R. S. Kass, J. P. Arena, *J. Gen. Physiol.* **93**, 1109 (1989).
21. R. An et al., *J. Physiol. (London)* **516**, 19 (1999).
22. Cardiac homogenates (500 μ g) were suspended in 0.5 ml of RIPA buffer and incubated with antibody to hKCNQ1 (Santa Cruz) for 2 hours at 4°C. Protein G-Sepharose beads were added, incubated at 4°C for 1 hour, washed with RIPA buffer, and resuspended in SDS sample buffer. Microcystin-sepharose (35 μ l; Upstate Biotechnology Inc.) and cAMP-Sepharose (40 μ l; Sigma) were incubated with cardiac membrane preparations (200 μ g) at 4°C for 1 hour and then washed. Samples were heated to 95°C and size-fractionated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membranes, immunoblotted with the primary antibody, and detected by enhanced chemiluminescence (ECL, Amersham). Primary antibodies: anti-PKA catalytic subunit (1:1000), anti-PP1 (1:1000) (Transduction Laboratories, Lexington, KY), anti-hKCNQ1 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-yotiao [gift from M. Sheng (7) and from M. E. Gottesman (74)]. For preparation of CHO lysates, cells were washed twice 48 hours after transfection, scraped into phosphate-buffered saline, centrifuged at 4°C, and resuspended in 0.5 ml of 20 mM Hepes-NaOH (pH 7.5) containing 1% Triton X-100 and EDTA-free protease inhibitors (Boehringer Mannheim). Lysates were centrifuged at 10,000g for 15 min, supernatants were collected, and protein concentration was determined by Bradford assay.
23. hKCNQ1-hKCNE1 (2.6 kb) cDNA (15) under the control of the α -MHC-promoter fragment for cardiac-specific expression (16) was injected into the pronuclei of fertilized mouse eggs by the Transgenic/Chimeric Mouse Facility. Genotyping of hKCNQ1-hKCNE1 transgenic mice was by the polymerase chain reaction (PCR). Transgenic positive (TG⁺) mice did not show distinctive phenotypes.
24. hKCNQ1 cDNA template was amplified by PCR (Stratagene) and cloned into pGEX-4T1, -4T2 or -4T3 (Amersham Pharmacia Biotech) for expression as GST fusion proteins in either DH5 α or BL21 cells (Stratagene) and affinity-purified with glutathione 4B sepharose (Amersham Pharmacia Biotech). All cDNA constructs were confirmed by sequencing (17).
25. hKCNQ1 was immunoprecipitated from TG⁺ mouse heart homogenates and hKCNQ1-transfected CHO cells. Immunoprecipitates were washed with 1 $\times$ phosphorylation buffer [8 mM MgCl₂, 10 mM EGTA, and 50 mM tris/piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.8], resuspended in 10 μ l of a 1.5 $\times$ phosphorylation buffer containing either PKA catalytic

REPORTS

subunit (5 units) or cAMP (10 μ M; Sigma) with or without a PKA inhibitor (PKI₅₋₂₄, 500 nM, Calbiochem). Phosphorylation at room temperature was initiated with 33 μ M Mg-adenosine triphosphate (ATP) containing 10% [γ -³²P]ATP (NEN Life Sciences, Boston) and terminated after 5 min at room temperature with 5 μ l of stop solution (4% SDS and 0.25 M dithiothreitol). Samples were size-fractionated by SDS-PAGE (10% gel).

26. Adult murine cardiomyocytes were isolated (18). Cells were plated in petri dishes placed on the stage of an inverted microscope (IMT-2, Olympus), and

currents (myocytes and CHO cells) were recorded using the whole-cell patch-clamp technique (19) with local perfusion (20) and solutions previously described for CHO cells (15) and murine myocytes (21). Voltage pulse protocols are described in figure legends. Statistical significance was assessed with Student's *t* test for simple comparisons and analysis of variance (ANOVA) for multiple comparisons; differences at *P* < 0.05 were considered to be significant.

27. We thank W. A. Catterall for anti-AKAP 15; M. Sheng for anti-yotiao; M. Gottesman for anti-yotiao; J. D. Scott for yotiao cDNA; M. Beggs for help

with statistical analysis; S. A. Siegelbaum for critical review of the manuscript and helpful discussions. Supported by NIH grants RO1-HL44365-07 and RO1-HL56810-05 (R.S.K.), RO1-HL61503, RO1-HL56180, and RO1-A139794 (A.R.M.), PO1HL67849-01 (A.R.M., R.S.K.), and RO1-HL68093 (S.O.M.); the American Heart Association (A.R.M., J.K., and S.O.M.); and the Whitaker Foundation (S.R.). A.R.M. is a Doris Duke Charitable Foundation Distinguished Clinical Scientist.

4 October 2001; accepted 29 November 2001

Calcium, Calmodulin, and CaMKII Requirement for Initiation of Centrosome Duplication in *Xenopus* Egg Extracts

Yutaka Matsumoto and James L. Maller*

Aberrant centrosome duplication is observed in many tumor cells and may contribute to genomic instability through the formation of multipolar mitotic spindles. Cyclin-dependent kinase 2 (Cdk2) is required for multiple rounds of centrosome duplication in *Xenopus* egg extracts but not for the initial round of replication. Egg extracts undergo periodic oscillations in the level of free calcium. We show here that chelation of calcium in egg extracts or specific inactivation of calcium/calmodulin-dependent protein kinase II (CaMKII) blocks even initial centrosome duplication, whereas inactivation of Cdk2 does not. Duplication can be restored to inhibited extracts by addition of CaMKII and calmodulin. These results indicate that calcium, calmodulin, and CaMKII are required for an essential step in initiation of centrosome duplication. Our data suggest that calcium oscillations in the cell cycle may be linked to centrosome duplication.

Centrosome duplication occurs once and only once during the cell cycle, ensuring the formation of bipolar spindles that distribute replicated chromosomes equally to daughter cells. Overduplication of centrosomes causes multipolar spindles that are thought to lead to genomic instability. More than two centrosomes have been observed in many different types of tumor cells (1), and the absence of the tumor suppressor protein p53 correlates with the presence of multiple centrosomes in the cell (2). Thus, the process of centrosome duplication is likely to be important in the etiology of cancer.

There are links between the cell cycle and the centrosome duplication cycle. Centrosome duplication generally occurs at the G₁-S transition (3), and if S phase is prolonged in mammalian cells and in embryonic systems including cycling *Xenopus* egg extracts, centrosomes can reproduce multiple times. Cdk2 is identified as a direct link between the cell cycle and centrosome duplication (4–7). The

cyclin E–Cdk2 complex regulates DNA replication, and its activity is greatest at the G₁ to S phase transition. Cdk2 is also required for multiple rounds of centrosome duplication in *Xenopus* egg extracts and embryos (4, 5) and in somatic cells (6, 7). Nucleophosmin (also called B23) and the mouse Mps1p-like kinase (mMps1p) were recently identified as possible centrosomal substrates for cyclin E–Cdk2 (8, 9).

Calcium and calmodulin (CaM) are required for cells to traverse the G₁-S, G₂-M, and metaphase-anaphase boundaries of the cell cycle (10). Calcium/calmodulin-dependent protein kinase II (CaMKII), which has a wide tissue distribution (11), has been suggested to be a target of calcium and calmodulin at the G₂-M transition in various cell types (10). In addition, periodic calcium oscillations occur in each cell cycle in dividing *Xenopus* embryos (12) and in cycling egg extracts (13). In particular, calcium oscillations occur at the G₁-S boundary and near the G₂-M transition. These times correlate with centrosome duplication at the G₁-S boundary and centrosome separation at the G₂-M transition. Moreover, calcium-modulated proteins, including CaMKII and centrin [a homolog of Cdc31p, a protein essential for du-

plication of yeast centrosomes (14)], are localized in the centrosome (15, 16). Thus, we examined the role of calcium in centrosome duplication using *Xenopus* egg extracts arrested in S phase by aphidicolin in which centrosome duplication starts after addition of sperm nuclei (4).

Addition of the calcium chelator BAPTA [1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid] (17) caused depolymerization of microtubules in the extracts (18) and prevented detection of centrosomes by polarization microscopy. Therefore, centrosome duplication in the presence of BAPTA was visualized by immunofluorescence with an antibody to γ -tubulin, a well-characterized component of the centrosome (19). Extracts incubated with sperm nuclei were subjected to microtubule depolymerization with nocodazole, and the centrosomes were centrifuged onto a cover slip for staining (20, 21). The number of γ -tubulin-staining foci increased during a 6-hour incubation (Fig. 1A), and staining with antibody to either α -tubulin or centrin, a component of the centriole (14), confirmed that each γ -tubulin-staining focus (>90%) corresponded to one centriole (Fig. 1B). Because each centrosome contains two centrioles, the number of centrosomes is half the number of γ -tubulin-staining foci. A fourfold increase in centrosomes was detected during the 6-hour incubation (Fig. 1C, control), indicating that centrosomes duplicate twice in these extracts in approximately 6 hours.

In the presence of 2 mM BAPTA, no increase in centrosomes was evident during the 6-hour incubation (Fig. 1C), even though 20 mM EGTA had no effect on centrosome duplication (18). Considering that BAPTA chelates calcium more rapidly than does EGTA (17), this suggests that rapid oscillations rather than prolonged changes in the concentration of free calcium are necessary for centrosome duplication. Inositol 1,4,5-trisphosphate (IP₃) is a second messenger that releases calcium from intracellular stores (22). To determine whether calcium oscillations are mediated by the IP₃ receptor, we used heparin, an IP₃ receptor antagonist (23) that blocks calcium oscillations in *Xenopus* egg extracts (13). Heparin effectively inhibited centrosome duplication in the extracts (Fig. 1C). Moreover, after centrosomes had

Howard Hughes Medical Institute and Department of Pharmacology, University of Colorado School of Medicine, Denver, CO 80262, USA.

*To whom correspondence should be addressed. E-mail: jim.maller@uchsc.edu

against cytotoxic effector cells. *Blood*. 96:594-600.

9. Medema, J.P., et al. 2001. Blockade of the granzyme B/perforin pathway through overexpression of the serine protease inhibitor PI-9/SPI-6 constitutes a mechanism for immune escape by tumors. *Proc. Natl. Acad. Sci. U. S. A.* 98:11515-11520.

10. Djerbi, M., et al. 1999. The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. *J. Exp. Med.* 190:1025-1032.

11. Dunn, G.P., et al. 2002. Cancer immunoediting: from immune surveillance to tumor escape. *Nat. Immunol.* 3:991-998.

12. Hanahan, D., and Weinberg, R.A. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

13. Chouaib, S., Asselin-Paturel, C., Mami-Chouaib, F., Caignard, A., and Blay, J.Y. 1997. The host-tumor immune conflict: from immunosuppression to resistance and destruction. *Immunol. Today*. 10:493-497.

See the related article beginning on page 617.

Calcium and the heart: a question of life and death

Andrew R. Marks

Center for Molecular Cardiology, Departments of Medicine and Pharmacology, Columbia University College of Physicians and Surgeons, New York, New York, USA
J. Clin. Invest. 111:597-600 (2003). doi:10.1172/JCI200318067.

The importance of calcium-dependent signaling in the heart has been appreciated for decades. For example, it is well accepted that intracellular calcium release from the sarcoplasmic reticulum (SR) is required for cardiac muscle contraction. Indeed, with each heart beat the calcium concentration in the cytosol of cardiac myocytes is elevated approximately 10-fold from a resting level of ~100 nM to ~1 μ M.

Presumably, a defect in signaling that prevents effective elevation of cytosolic calcium would impair contractility as the contraction of heart muscle is directly determined by the level of calcium elevation during systole. Similarly, a defect in the removal of calcium from the cytosol during diastole would

impair cardiac relaxation, which is critically important in that it allows the heart chambers to refill with blood in preparation for the next beat.

Calcium and heart failure

Indeed, an attractive hypothesis for the mechanism underlying cardiac muscle dysfunction during heart failure, the leading cause of mortality in the developed world, is that impaired calcium release causes decreased muscle contraction (systolic dysfunction) and defective calcium removal hampers relaxation (diastolic dysfunction). Given that the measurement of cellular calcium is relatively straightforward, the obvious experiment required to address this important issue is to measure calcium in heart muscle cells from failing hearts. Such measurements have been done in isolated cardiomyocytes and, though there is a fair amount of variability in the published reports, the data tend to support the concept of a decrease in SR calcium release and a defect in the termination of release. These results imply that there are presumably defects in SR calcium release in vivo. However, there are no data showing that calcium levels are chronically elevated in heart muscle in failing hearts. Such studies await the development of reliable techniques using

calcium indicators with adequate signal to noise ratios and detection systems that will permit measurements of intracellular calcium in the living heart in intact organisms.

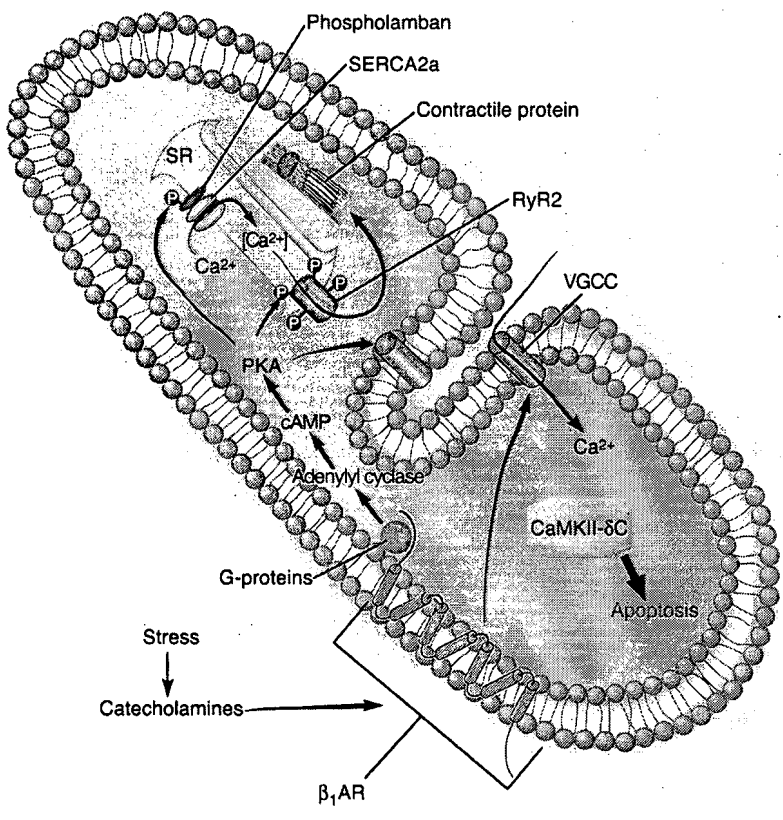
Calcium and cardiac hypertrophy

Another disease in which perturbations of calcium signaling have been alluded to is cardiac hypertrophy. Indeed, calcium elevation has been proposed as the trigger for cardiac hypertrophic signaling via the calcium-activated phosphatase calcineurin. Much attention has been focused on this possibility as a potential therapeutic target. Indeed, the initial studies identifying a role for calcineurin in hypertrophic signaling in the heart represented a tour de force, combining beautifully designed in vitro and in vivo studies that clearly demonstrated a physiologically important signaling system (1). An intriguing question is whether or not there are any clinical conditions in which one would actually want to treat (i.e. prevent) cardiac hypertrophy. Indeed, outside the cardiology community, cardiac hypertrophy is often lumped together with heart failure as though they are synonymous. This approach seems to add some excitement to the quest for a cure for cardiac hypertrophy by linking it to heart failure which, as mentioned above, is a leading cause of mortality (over 500,000 deaths per year in the US alone). Cardiac hypertrophy, on the other hand, rarely kills anybody and when it does, death is usually due to cardiac arrhythmias, not the hypertrophy per se. Indeed, most deaths linked to cardiac hypertrophy occur in individuals with inherited forms of the disease most often associated with mutations in one of the contractile proteins. These individuals exhibit abnormal pathology, including disorder in the usually well-ordered arrays

Address correspondence to: Andrew R. Marks, Center for Molecular Cardiology, Box 65, Columbia University College of Physicians and Surgeons, Room 9-401, 630 West 168th Street, New York, New York 10032, USA. Phone: (212) 305-0270; Fax: (212) 305-3690; E-mail: arm42@columbia.edu.

Conflict of interest: The author has declared that no conflict of interest exists.

Nonstandard abbreviations used: sarcoplasmic reticulum (SR); nuclear factor of activated T cells (NFAT); protein kinase A (PKA); ryanodine receptor (RyR2); Ca²⁺/calmodulin-dependent protein kinase II (CaMKII); cardiac isoform of CaMKII (CaMKII- δ C).



by Ken Beauchamp *J. Clin. Invest.*

Figure 1
The β_1 -adrenergic receptor/calcium connection. β_1 -adrenergic receptor (β_1 AR) stimulation is known to activate cardiac excitation-contraction (EC) coupling via PKA phosphorylation of the following: (I) the trigger for cardiac EC coupling, the voltage-gated calcium channel (VGCC); (II) the SR calcium release channel RyR2; and (III) the calcium uptake pathway (via PKA phosphorylation of phospholamban, which reduces inhibition of the calcium ATPase SERCA2a). Activation of these proteins by PKA phosphorylation increases systolic SR calcium release which causes both increased cardiac contraction and output. This system results in a maladaptive chronic hyperadrenergic state in heart failure because the damaged heart cannot respond adequately to the adrenergic stimulation and the sympathetic nervous system remains in a futile activated state. This results in PKA-hyperphosphorylated RyR2 channels that can cause a diastolic SR calcium leak (4), which, along with reduced SERCA2a-dependent SR calcium uptake, depletes SR calcium and contributes to contractile dysfunction. Zhou et al. (5) have now added an additional component to this model based on observations that chronic β_1 AR stimulation can activate CaMKII (possibly via increased calcium influx through the VGCC) and cause apoptosis.

of cardiac muscle fibers, suggesting that there may be a substrate for electrical instability. However, the molecular mechanisms underlying the hypertrophy and the arrhythmias in both the inherited and acquired forms of cardiac hypertrophy remain to be elucidated. Moreover, it is quite clear that under certain circumstances some forms of cardiac hypertrophy are an entirely normal physiological response, such as that seen in well-trained athletes or during pregnancy. Other forms of hypertrophy are pathologic in the sense that they ultimately deteriorate into heart failure, but at their initiation

they represent adaptive responses to hypertension (most commonly) or other causes of increased stress (afterload) on the heart such as valve disease. In these cases, it is entirely likely that preventing the hypertrophic response could be deadly as the heart would not be able to generate sufficient cardiac output in the face of increased stress.

Meaningful calcium changes

This brings us back to calcium and the heart. In systems that are well understood, calcium-dependent signals generally require a 10-fold elevation of cal-

cium to activate calcium-dependent enzymes. A good example is that of antigen-dependent T cell activation mediated via the T cell receptor, which results in intracellular calcium release through the inositol 1,4,5-trisphosphate receptor. This calcium release from the ER elevates calcium from ~100 nM to ~1 μ M resulting in the activation of calcineurin, which subsequently dephosphorylates the nuclear factor of activated T cells (NFAT). Subsequent passage of NFAT into the nucleus activates IL-2 transcription that is required for expansion of the T cell population to fight off infection. There is no solid evidence that small elevations of calcium in the sub-100 nM range are sufficient to activate any of the known calcium-dependent enzymes. Therefore, it is very difficult to understand how a calcium-activated enzyme can play a key regulatory (i.e. on/off) role in heart muscle where global cellular calcium is obligated to rise 10-fold with every heart beat. In most of us, that means a 10-fold rise of calcium every second or so, and in mice that would equate to one such elevation every 100 ms given that the mouse heart beats at a rate about 10 times higher than that of humans.

Despite these powerful arguments against a regulatory role for calcium in anything other than contraction in the heart, there remains considerable interest in pursuing the possibility that calcium-dependent signaling pathways are indeed controlling pathologic conditions. Perhaps this interest stems from earlier, unproven, claims that calcium is elevated in heart failure and cardiac hypertrophy. Although, as discussed earlier, no such elevation has ever been documented, the calcium elevation theory regarding heart disease remains in the public eye and offers an attractive therapeutic target — if elevated calcium is causing disease, we'll just have to lower it! How one would go about doing so in heart muscle without disturbing other important functions, such as contraction of the heart muscle, remains to be explained.

Concomitant with the calcium theory of heart disease has been the apoptosis theory of heart failure. The two are linked because it has been proposed that the calcium problem contributes to the apoptosis problem. The apoptosis theory of heart failure is elegantly simple. Something bad hap-

pens to the heart and triggers programmed death pathways that result in a loss of cardiomyocytes in sufficient numbers to cause failure of the heart muscle to contract adequately. While there is ample evidence that apoptosis can contribute to cardiac dysfunction in various animal models of heart failure, it remains to be proven that this process plays an important role in causing human heart failure.

Hyperadrenergic state of heart failure

While these more recent models for heart failure remain to be sorted out, there is a much older story that has been lurking in the background of our understanding of heart failure for over 40 years. This is the story about how hyperadrenergic signaling drives the progressive downhill course of heart failure (2). It has been known for over 40 years that heart failure patients live in a continuous hyperadrenergic state with high levels of circulating catecholamines (3). Many studies have shown that one consequence of this chronic hyperadrenergic state is the downregulation and desensitization of the β_1 -adrenergic receptors in the heart. The data showing this effect are strong and have compelled investigators for many years to assume that this meant that the cardiac muscle cell was living in a state of reduced β_1 -adrenergic receptor-mediated stimulation. Since the β_1 -adrenergic receptors are linked downstream to activation of cAMP-dependent protein kinase A (PKA), it was assumed that the targets of this kinase would be under-phosphorylated in failing hearts (Figure 1). Recent studies have shown that this is not the case. Indeed, it turns out that the major SR calcium release channel in the heart, the ryanodine receptor (RyR2), is PKA hyperphosphorylated in failure hearts. This results in leaky channels that may help explain the reduced calcium signal observed in heart failure (possibly due to depletion of SR calcium pools via the leaky channel) (4).

Linking β_1 -adrenergic signaling to CaMKII

In this issue of the *JCI*, Zhu et al. (5) now provide a novel and intriguing possibility that could explain additional adverse effects of the chronic hyper-

adrenergic state of heart failure. Moreover, their surprising findings implicate that β_1 -adrenergic receptors mediate signaling not via PKA, as would be expected, but through another kinase not previously thought to be downstream of β_1 -adrenergic receptors, the Ca^{2+} /calmodulin-dependent protein kinase (CaMKII). What is exciting about this study is that it links CaMKII to β_1 -adrenergic receptors for the first time. Whether or not the activation of apoptotic pathways demonstrated in vitro by Zhu et al. turns out to be an important contributor to heart failure in humans, this initial description of a novel and unexpected connection between two signaling pathways is a significant addition to the continuously expanding repertoire of cross-talk between signals in biological systems. This finding represents yet another example of why purely pharmacologic-based studies need to be viewed with skepticism, especially when claims are made about drug specificity. Indeed, even if the drugs are specific, unrecognized cross-talk downstream of the receptor may confound interpretation of the data. In the present case, despite β_1 -adrenergic receptor activation, the downstream signaling via CaMKII is not inhibited by PKA inhibitors! Indeed, this is not the first example of non-PKA signaling downstream of β_1 -adrenergic receptors. Elegant studies by Lefkowitz and colleagues have shown that β_2 -adrenergic receptor complexes can participate in nonreceptor protein tyrosine kinase signaling (6).

The Zhu et al. (5) study proposes that chronic stimulation of β_1 -adrenergic receptors (this would mimic one aspect of the chronic hyperadrenergic state that afflicts heart failure patients) results in cardiomyocyte cell death due to activation of CaMKII signaling via up regulation of calcium. They provide support for this hypothesis using a variety of in vitro manipulations including pharmacologic studies where CaMKII inhibitors, but not PKA inhibitors, were shown to block the induction of apoptosis by isoproterenol, and overexpression of the cardiac isoform of CaMKII (CaMKII- δC) was demonstrated to enhance the pro-apoptotic effect of isoproterenol.

It should be clear from the first part of this commentary that an important

unexplained question is how would CaMKII activity be increased in vivo? Put another way, how does CaMKII distinguish between the obligatory 10-fold elevations of calcium required for heart muscle contraction and the pathologic signals elicited by the chronic hyperadrenergic state? In this sense, the CaMKII story proposed by Zhu et al. (5) shares a common feature with the calcineurin story alluded to earlier (1). Both suggest that pathologic processes — cell death in the first case and cellular hypertrophy in the second — are triggered by the same signal, increased cellular calcium. It is entirely possible that both stories are correct in identifying novel signaling pathways in the heart but that neither actually requires perturbations of calcium signaling as the explanation for how these signals are initiated. In the case of calcineurin, an alternative explanation for the link between an increase in calcineurin activity and hypertrophic signaling is that the levels of calcineurin are elevated as part of growth factor-stimulated remodeling of the heart. There may also be a reduction in the activity of a kinase(s) paired with calcineurin. Both of these could occur in a calcium-independent manner.

Similarly, an alternative hypothesis to that of Zhu et al. (5) is that CaMKII-dependent apoptotic signals are indeed mediated via β_1 -adrenergic receptors but they do not involve calcium. For example, increased CaMKII-mediated effects could reflect a downregulation of the protein phosphatase that is paired with CaMKII, or an increase in CaMKII protein levels due to transcriptional effects. If one accepts that it would be incompatible with life to have a sustained elevation of calcium in the heart at levels sufficient to activate CaMKII (the result would be a “stone heart” or rigor mortis), then another possibility is that localized signaling complexes could be exposed to chronic elevations of calcium (e.g. through a “leaky” channel) that would not be reflected in global elevations of calcium. Improved imaging techniques are now capable of measuring such microdomains of calcium (7). However, such studies will be limited to ex vivo measurements in isolated cardiomyocytes. Another important task is to identify the role of β_1 -adrenergic receptor-mediated activation of CaMKII in normal physiology.

Notwithstanding these important unresolved questions, the Zhu et al. (5) study should open up a new area of investigation as additional studies probe the connections between β_1 -adrenergic receptor-mediated signaling and CaMKII-dependent effects. If future studies support the proposal by Zhu et al., that one of the consequences of prolonged β_1 -adrenergic receptor stimulation is cardiomyocyte cell death via a CaMKII-mediated pathway, it will

indeed be an important contribution to our understanding of the pathogenesis of heart failure that will suggest novel therapeutic targets.

1. Molkenkin, J.D., et al. 1998. A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell*. **93**:215-228.
2. Marks, A.R., Reiken, S., and Marx, S.O. 2002. Progression of heart failure: is protein kinase a hyperphosphorylation of the ryanodine receptor a contributing factor? *Circulation*. **105**:272-275.
3. Chidsey, C.A., Harrison, D.C., and Braunwald, E. 1962. Augmentation of plasma norepinephrine response to exercise in patients with congestive

- heart failure. *N. Engl. J. Med.* **267**:650.
4. Marx, S.O., et al. 2000. PKA phosphorylation dissociates FKBP12.6 from the calcium release channel (ryanodine receptor): defective regulation in failing hearts. *Cell*. **101**:365-376.
5. Zhu, W.-Z., et al. 2003. Linkage of β_1 -adrenergic stimulation to apoptotic heart cell death through protein kinase A-independent activation of Ca^{2+} /calmodulin kinase II. *J. Clin. Invest.* **111**:617-625. doi:10.1172/JCI200316326.
6. Luttrell, L.M., et al. 1999. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science*. **283**:655-661.
7. Zaccolo, M., Magalhaes, P., and Pozzan, T. 2002. Compartmentalisation of cAMP and Ca^{2+} signals. *Curr. Opin. Cell. Biol.* **14**:160-166.

See the related articles beginning on pages 649 and 707.

Soluble VEGF receptor Flt1: the elusive preeclampsia factor discovered?

Aernout Luttun and Peter Carmeliet

The Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Katholieke Universiteit Leuven, Leuven, Belgium
J. Clin. Invest. **111**:600-602 (2003). doi:10.1172/JCI200318015.

The occurrence of seizures (eclampsia, from the Greek "eklampsia," sudden flashing) has been a long-known and feared complication of pregnancy, often killing both mother and child. Preeclampsia, or the condition preceding full-blown eclampsia, affects up to 5% of pregnant women and is diagnosed by the onset of hypertension and proteinuria in the second trimester (1). Preeclampsia may eventually progress to glomerular malfunction, thrombocytopenia, liver and brain edema, and associated life-threatening seizures (2) (Figure 1). Preeclampsia has been sometimes

termed the "disease of theories," as several models for its pathogenesis have been proposed. But, as of today, no satisfactory unifying hypothesis has emerged (1). The restricted occurrence of preeclampsia to humans and primates and the lack of a suitable animal model have hampered the understanding of its pathogenesis (3). In this issue of the *JCI*, S.E. Maynard et al. (4) report the novel insight that circulating levels of two angiogenic growth factors, VEGF and placental growth factor (PlGF), may play a more important role than previously believed. In particular, the authors propose that, in pregnant women with preeclampsia, the placenta produces elevated levels of the soluble fms-like tyrosine kinase 1 (sFlt1) receptor, which captures free VEGF and PlGF. As a result, the normal vasculature in the kidney, brain, lungs, and other organs is deprived of essential survival and maintenance signals and becomes dysfunctional (Figure 1). As the authors show in their rodent model, this may lead to the development of hypertension and renal dis-

ease, reminiscent of preeclampsia in humans. In another study in this issue, V. Eremina et al. (5) provide additional evidence for a critical role of VEGF in renal disease during preeclampsia. These authors demonstrate that mice lacking one *VEGF* allele in renal podocytes develop the typical renal pathology found in pregnant women with preeclampsia. These studies therefore shed unprecedented light on the pathogenesis of preeclampsia and offer novel therapeutic opportunities for this disease.

sFlt1: a likely candidate preeclampsia factor

For the fetus to develop normally, it must receive sufficient oxygen and nutrients (6). These are supplied via the maternal spiral arteries in the uterus. During normal pregnancy, cytotrophoblasts convert from an epithelial to an endothelial phenotype (a process termed pseudo-vasculogenesis) and invade maternal spiral arteries. This vascular remodeling increases the bulk flow and the supply of nutrients and oxygen to the fetus by the end of the first trimester (7, 8) (Figure 1). Vascular factors such as VEGF, angiopoietins, and ephrins have been implicated in this process (7). In preeclampsia, pseudo-vasculogenesis is defective, and the resultant placental ischemia has been proposed to trigger the release of unknown placenta-derived factors. The latter would induce systemic endothelial dysfunction and thereby contribute to the renal, cardiovascular, and neurological defects of preeclampsia (Figure 1). Despite intensive efforts, the precise nature of the placenta-derived

Address correspondence to: Peter Carmeliet, Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Katholieke Universiteit Leuven, Campus Gasthuisberg, Herestraat 49, B-3000, Leuven, Belgium.
 Phone: 32-16-34-57-72; Fax: 32-16-34-59-90;
 E-mail: peter.carmeliet@med.kuleuven.ac.be.
Conflict of interest: The authors have declared that no conflict of interest exists.
Nonstandard abbreviations used: placental growth factor (PlGF); fms-like tyrosine kinase 1 (Flt1); soluble Flt1 (sFlt1).

Pure & Appl. Chem., Vol. 70, No. 5, pp. 1129–1143, 1998.
 Printed in Great Britain.
 © 1998 IUPAC

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY

CHEMISTRY AND HUMAN HEALTH DIVISION
 MEDICINAL CHEMISTRY SECTION

GLOSSARY OF TERMS USED IN MEDICINAL CHEMISTRY

(IUPAC Recommendations 1998)

Prepared for publication by

C. G. WERMUTH¹ (CHAIRMAN),
 C. R. GANELLIN², P. LINDBERG³ AND L. A. MITSCHER⁴

¹Faculté de Pharmacie, Université Louis Pasteur, Strasbourg, France

²University College London, London, UK

³Astra Hässle AB, Mölndal, Sweden

⁴School of Pharmacy, University of Kansas, Lawrence, Kansas, USA

Membership of the Section during the period (1992–1995) when this report was prepared was as follows:

President: J. G. Topliss (USA); *Vice-President:* N. Koga (Japan); *Past-President:* C. G. Wermuth (France); *Secretary:* W. D. Busse (Germany); *Titular members:* C. R. Ganellin (U.K.); L. A. Mitscher (USA); *Co-opted members:* P. Anderson (USA); P. R. Andrews (Australia); W. A. Denny (New Zealand); W. Granick (Russia); Y. Guindon (Canada); C. A. G. Haasnoot (The Netherlands); J. Ide (Japan); R. Imhof (Switzerland); P. Lindberg (Sweden); G. Tarzia (Italy); R. S. Xu (China); *National Representatives:* A. O. M. Stoppani (Argentina); E. J. Barreiro (Brazil); A. Aguin (Bulgaria); J. Krepelka (Czechoslovakia); E. K. Pohjala (Finland); A. Monge Vega (Spain).

Republication or reproduction of this report or its storage and/or dissemination by electronic means is permitted without the need for formal IUPAC permission on condition that an acknowledgement, with full reference to the source along with use of the copyright symbol ©, the name IUPAC and the year of publication are prominently visible. Publication of a translation into another language is subject to the additional condition of prior approval from the relevant IUPAC National Adhering Organization.

Glossary of terms used in medicinal chemistry (IUPAC Recommendations 1998)

Abstract: The objective of the glossary is to provide in a single document a consistent terminology and concise definitions of terms covering the various aspects of medicinal chemistry. This was felt necessary with regard to the rapid changes occurring in medicinal chemistry and also by the need to establish international definition standards. Effectively the possibility exists that in different countries certain terms may not have the same meaning, in such a case the creation of an internationally accepted definition is particularly justified.

A Working Party belonging to the IUPAC Section on Medicinal Chemistry has therefore been assembled which prepared the present glossary. Concise but sufficiently explanatory definitions have been formulated for about one hundred commonly employed terms which can be considered of particular interest to the medicinal chemistry community. The glossary has been compiled in part from definitions proposed by the Working Party in part from earlier IUPAC glossaries and in part from well-accepted definitions taken from the literature but which were sometimes published in journals or books that may not be readily accessible.

ALPHABETICAL ORDERED ENTRIES

The glossary has been compiled in part from definitions proposed by the Working Party and in part from well-accepted definitions taken from the literature. In most cases, definitions given here are for specific areas of medicinal chemistry. Some definitions taken from the Glossary for Chemists of Terms Used in Biotechnology (*Pure Appl. Chem.*, 1992, 64, 143–168) were also included, eventually in a slightly modified form; they are identified by an asterisk*. Others, which appear in the Glossary on Computational Drug Design (*Pure Appl. Chem.*, 1997, 69, 1137–1152) and in Glossary for Chemists of terms used in Toxicology (*Pure Appl. Chem.* 1993, 65, 2003–2122), are identified by a double** and a triple*** asterisk respectively.

Active transport*

Active transport is the carriage of a solute across a biological membrane from low to high concentration that requires the expenditure of (metabolic) energy.

Address-message concept

Address-message concept refers to compounds in which part of the molecule is required for binding (address) and part for the biological action (message).

ADME

Abbreviation for **Absorption, Distribution, Metabolism, Excretion**. (See also **Pharmacokinetics**; **Drug disposition**).

Affinity

Affinity is the tendency of a molecule to associate with another. The **affinity** of a **drug** is its ability to bind to its biological target (**receptor**, **enzyme**, transport system, etc.) For pharmacological **receptors** it can be thought of as the frequency with which the **drug**, when brought into the proximity of a **receptor** by diffusion, will reside at a position of minimum free energy within the force field of that **receptor**.

For an **agonist** (or for an **antagonist**) the numerical representation of **affinity** is the reciprocal of the equilibrium dissociation constant of the ligand-receptor complex denoted K_A , calculated as the rate constant for offset (k_{-1}) divided by the rate constant for onset (k_1).

Agonist***

An **agonist** is an endogenous substance or a **drug** that can interact with a **receptor** and initiate a physiological or a pharmacological response characteristic of that **receptor** (contraction, relaxation, secretion, **enzyme** activation, etc.).

Allosteric binding sites

Allosteric binding sites are contained in many **enzymes** and **receptors**. As a consequence of the binding to **allosteric binding sites**, the interaction with the normal ligand may be either enhanced or reduced.

Allosteric enzyme*

An **allosteric enzyme** is an **enzyme** that contains a region to which small, regulatory molecules ("effectors") may bind in addition to and separate from the substrate binding site and thereby affect the catalytic activity.

On binding the effector, the catalytic activity of the **enzyme** towards the substrate may be enhanced, in which case the effector is an activator, or reduced, in which case it is a de-activator or inhibitor.

Allosteric regulation

Allosteric regulation is the regulation of the activity of **allosteric enzymes**. (See also **Allosteric binding sites**; **Allosteric enzymes**).

Analog

An **analog** is a **drug** whose structure is related to that of another **drug** but whose chemical and biological properties may be quite different. (See also **Congener**).

Antagonist***

An **antagonist** is a **drug** or a compound that opposes the physiological effects of another. At the **receptor** level, it is a chemical entity that opposes the **receptor**-associated responses normally induced by another bioactive agent.

Antimetabolite***

An **antimetabolite** is a structural **analog** of an intermediate (substrate or **coenzyme**) in a physiologically occurring metabolic pathway that acts by replacing the natural substrate thus blocking or diverting the biosynthesis of physiologically important substances.

Antisense molecule

An **antisense molecule** is an **oligonucleotide** or **analog** thereof that is complementary to a segment of RNA (ribonucleic acid) or DNA (deoxyribonucleic acid) and that binds to it and inhibits its normal function.

Autacoid

An **autacoid** is a biological substance secreted by various cells whose physiological activity is restricted to the vicinity of its release; it is often referred to as local **hormone**.

Autoreceptor

An **autoreceptor**, present at a nerve ending, is a **receptor** that regulates, via positive or negative feedback processes, the synthesis and/or release of its own physiological ligand. (See also **Heteroreceptor**).

Bioassay***

A **bioassay** is a procedure for determining the concentration, purity, and/or biological activity of a substance (e.g., vitamin, **hormone**, plant growth factor, antibiotic, **enzyme**) by measuring its effect on an organism, tissue, cell, **enzyme** or **receptor** preparation compared to a standard preparation.

Bioisostere

A **bioisostere** is a compound resulting from the exchange of an atom or of a group of atoms with another, broadly similar, atom or group of atoms. The objective of a bioisosteric replacement is to create a new compound with similar biological properties to the parent compound. The bioisosteric replacement may be physicochemically or topologically based. (See also **Isostere**)

Bioprecursor prodrug

A **bioprecursor prodrug** is a **prodrug** that does not imply the linkage to a carrier group, but results from a molecular modification of the active principle itself. This modification generates a new compound, able to be transformed metabolically or chemically, the resulting compound being the active principle.

Biotransformation

Biotransformation is the chemical conversion of substances by living organisms or **enzyme** preparations.

CADD

See Computer-assisted drug design

Carrier-linked prodrug (Carrier prodrug)

A **carrier-linked prodrug** is a **prodrug** that contains a temporary linkage of a given active substance with a transient carrier group that produces improved physicochemical or pharmacokinetic properties and that can be easily removed *in vivo*, usually by a hydrolytic cleavage.

Cascade prodrug

A **cascade prodrug** is a **prodrug** for which the cleavage of the carrier group becomes effective only after unmasking an activating group.

Catabolism***

Catabolism consists of reactions involving endogenous organic substrates to provide chemically available energy (e.g., ATP) and/or to generate metabolic intermediates used in subsequent anabolic reactions.

Catabolite

A **catabolite** is a naturally occurring **metabolite**.

Clone*

A **clone** is a population of genetically identical cells produced from a common ancestor. Sometimes, "**clone**" is also used for a number of recombinant DNA (deoxyribonucleic acid) molecules all carrying the same inserted sequence.

Codon*

A **codon** is the sequence of three consecutive **nucleotides** that occurs in mRNA which directs the incorporation of a specific amino acid into a protein or represents the starting or termination signals of protein synthesis.

Coenzyme

A **coenzyme** is a dissociable, low-molecular weight, non-proteinaceous organic compound (often **nucleotide**) participating in enzymatic reactions as acceptor or donor of chemical groups or electrons.

Combinatorial synthesis

Combinatorial synthesis is a process to prepare large sets of organic compounds by combining sets of building blocks.

Combinatorial library

A **combinatorial library** is a set of compounds prepared by combinatorial synthesis.

CoMFA

See **Comparative Molecular Field Analysis**

Comparative Molecular Field Analysis (CoMFA)**

Comparative molecular field analysis (CoMFA) is a **3D-QSAR** method that uses statistical correlation techniques for the analysis of the quantitative relationship between the biological activity of a set of compounds with a specified alignment, and their three-dimensional electronic and steric properties. Other properties such as hydrophobicity and hydrogen bonding can also be incorporated into the analysis. (See also **Three-dimensional Quantitative Structure-Activity Relationship [3D-QSAR]**).

Computational chemistry**

Computational chemistry is a discipline using mathematical methods for the calculation of molecular properties or for the simulation of molecular behaviour.

Computer-assisted drug design (CADD)**

Computer-assisted drug design involves all computer-assisted techniques used to discover, design and optimize biologically active compounds with a putative use as **drugs**.

Congener***

A **congener** is a substance literally *con-* (with) *generated* or synthesized by essentially the same synthetic chemical reactions and the same procedures. **Analogs** are substances that are analogous in some respect to the prototype agent in chemical structure.

Clearly **congeners** may be **analogs** or vice versa but not necessarily. The term **congener**, while most often a synonym for homologue, has become somewhat more diffuse in meaning so that the terms **congener** and **analog** are frequently used interchangeably in the literature.

Cooperativity

Cooperativity is the interaction process by which binding of a ligand to one site on a macromolecule (**enzyme**, **receptor**, etc.) influences binding at a second site, e.g. between the substrate binding sites of an **allosteric enzyme**. Cooperative **enzymes** typically display a sigmoid (S-shaped) plot of the reaction rate against substrate concentration. (See also **Allosteric binding sites**).

3D-QSAR

See **Three-dimensional Quantitative Structure-Activity Relationship**

De novo design**

De novo design is the design of bioactive compounds by incremental construction of a ligand model within a model of the **receptor** or **enzyme** active site, the structure of which is known from X-ray or nuclear magnetic resonance (NMR) data.

Disposition

See **Drug disposition**

Distomer

A **distomer** is the enantiomer of a chiral compound that is the less potent for a particular action. This definition does not exclude the possibility of other effect or side effect of the **distomer** (See also **Eutomer**).

Docking studies

Docking studies are molecular modeling studies aiming at finding a proper fit between a ligand and its binding site.

Double-blind study

A **double-blind study** is a clinical study of potential and marketed **drugs**, where neither the investigators nor the subjects know which subjects will be treated with the active principle and which ones will receive a placebo.

Double prodrug (or pro-prodrug)

A **double prodrug** is a biologically inactive molecule which is transformed *in vivo* in two steps (enzymatically and/or chemically) to the active species.

Drug***

A **drug** is any substance presented for treating, curing or preventing disease in human beings or in animals. A **drug** may also be used for making a medical diagnosis or for restoring, correcting, or modifying physiological functions (e.g., the contraceptive pill).

Drug disposition

Drug disposition refers to all processes involved in the absorption, distribution **metabolism** and excretion of **drugs** in a living organism.

Drug latention

Drug latention is the chemical modification of a biologically active compound to form a new compound, which *in vivo* will liberate the parent compound. **Drug latention** is synonymous with **prodrug** design.

Drug targeting

Drug targeting is a strategy aiming at the delivery of a compound to a particular tissue of the body.

Dual action drug

A **dual action drug** is a compound which combines two desired different pharmacological actions at a similarly efficacious dose.

Efficacy

Efficacy describes the relative intensity with which **agonists** vary in the response they produce even when they occupy the same number of **receptors** and with the same **affinity**. **Efficacy** is *not* synonymous to **Intrinsic activity**.

Efficacy is the property that enables **drugs** to produce responses. It is convenient to differentiate the properties of **drugs** into two groups, those which cause them to associate with the **receptors** (**affinity**) and those that produce stimulus (**efficacy**). This term is often used to characterize the level of maximal responses induced by **agonists**. In fact, not all **agonists** of a **receptor** are capable of inducing identical levels of maximal responses. Maximal response depends on the efficiency of **receptor** coupling, i.e., from the cascade of events, which, from the binding of the **drug** to the **receptor**, leads to the observed biological effect.

Elimination

Elimination is the process achieving the reduction of of the concentration of a **xenobiotic** including its **metabolism**.

Enzyme*

An **enzyme** is a macromolecule, usually a protein, that functions as a (bio) catalyst by increasing the reaction rate.

In general, an **enzyme** catalyzes only one reaction type (reaction selectivity) and operates on only one type of substrate (substrate selectivity). Substrate molecules are transformed at the same site (regioselectivity) and only one or preferentially one of chiral a substrate or of a racemate is transformed (enantioselectivity[special form of stereoselectivity]).

Enzyme induction*

Enzyme induction is the process whereby an (inducible) **enzyme** is synthesized in response to a specific inducer molecule. The inducer molecule (often a substrate that needs the catalytic activity of the inducible **enzyme** for its **metabolism**) combines with a repressor and thereby prevents the blocking of an operator by the repressor leading to the translation of the gene for the **enzyme**.

Enzyme repression*

Enzyme repression is the mode by which the synthesis of an **enzyme** is prevented by repressor molecules.

In many cases, the end product of a synthesis chain (e.g., an amino acid) acts as a feed-back corepressor by combining with an intracellular aporepressor protein, so that this complex is able to block the function of an operator. As a result, the whole operation is prevented from being transcribed into mRNA, and the expression of all **enzymes** necessary for the synthesis of the end product **enzyme** is abolished.

Eudismic ratio

Eudismic ratio is the potency of the **eutomer** relative to that of the **distomer**.

Eutomer

The **Eutomer** is the enantiomer of a chiral compound that is the more potent for a particular action (See also **Distomer**).

Genome*

A **genome** is the complete set of chromosomal and extrachromosomal genes of an organism, a cell, an organelle or a virus; the complete DNA (deoxyribonucleic acid) component of an organism.

Hansch analysis**

Hansch analysis is the investigation of the quantitative relationship between the biological activity of a series of compounds and their physicochemical substituent or global parameters representing hydrophobic, electronic, steric and other effects using multiple regression correlation methodology.

Hapten***

A **hapten** is a low molecular weight molecule that contains an antigenic determinant but which is not itself antigenic unless combined with an antigenic carrier.

Hard drug

A **hard drug** is a nonmetabolizable compound, characterized either by high lipid solubility and accumulation in adipose tissues and organelles, or by high water solubility.

In the lay press the term "**Hard Drug**" refers to a powerful **drug** of abuse such as cocaine or heroin.

Heteroreceptor

A **heteroreceptor** is a **receptor** regulating the synthesis and/or the release of mediators other than its own ligand (See also **Autoreceptor**).

Homologue

The term **homologue** is used to describe a compound belonging to a series of compounds differing from each other by a repeating unit, such as a methylene group, a peptide residue, etc.

Hormone***

A **hormone** is a substance produced by endocrine glands, released in very low concentration into the bloodstream, and which exerts regulatory effects on specific organs or tissues distant from the site of secretion.

Hydrophilicity**

Hydrophilicity is the tendency of a molecule to be solvated by water.

Hydrophobicity**

Hydrophobicity is the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non polar molecules. (See also **Lipophilicity**).

IND

Abbreviation for **Investigational New Drug**.

Intrinsic activity

Intrinsic activity is the maximal stimulatory response induced by a compound in relation to that of a given reference compound (See also **Partial agonist**)

This term has evolved with common usage. It was introduced by Ariëns as a proportionality factor between tissue response and receptor occupancy. The numerical value of **intrinsic activity** (α) could range from unity (for full agonists, i.e., agonist inducing the tissue maximal response) to zero (for antagonists), the fractional values within this range denoting partial agonists. Ariëns' original definition equates the molecular nature of α to maximal response only when response is a linear function of receptor occupancy. This function has been verified. Thus, **intrinsic activity**, which is a drug and tissue parameter, cannot be used as a characteristic drug parameter for classification of drugs or drug receptors. For this purpose, a proportionality factor derived by null methods, namely, relative efficacy, should be used. Finally, "intrinsic activity" should not be used instead of "intrinsic efficacy". A "partial agonist" should be termed "agonist with intermediate intrinsic efficacy" in a given tissue.

Inverse agonist

An **inverse agonist** is a drug which acts at the same receptor as that of an agonist, yet produces an opposite effect. Also called **negative antagonists**.

Isosteres

Isosteres are molecules or ions of similar size containing the same number of atoms and valence electrons, e.g., O_2^- , F^- , Ne (See also **Bioisostere**).

Latentiated drug

See **Drug Latentiation**.

Lead discovery

Lead discovery is the process of identifying active new chemical entities, which by subsequent modification may be transformed into a clinically useful **drug**.

Lead generation

Lead generation is the term applied to strategies developed to identify compounds which possess a desired but non-optimized biological activity.

Lead optimization

Lead optimization is the synthetic modification of a biologically active compound, to fulfill all stereoelectronic, physicochemical, pharmacokinetic and toxicologic requirements for clinical usefulness.

Lipophilicity**

Lipophilicity represents the **affinity** of a molecule or a moiety for a lipophilic environment. It is commonly measured by its distribution behaviour in a biphasic system, either liquid-liquid (e.g., partition coefficient in octan-1-ol/water) or solid/liquid (retention on reversed-phase high performance liquid chromatography (RP-HPLC) or thin-layer chromatography (TLC) system). (See also **Hydrophobicity**).

Medicinal chemistry

Medicinal chemistry is a chemistry-based discipline, also involving aspects of biological, medical and pharmaceutical sciences. It is concerned with the invention, discovery, design, identification and preparation of biologically active compounds, the study of their **metabolism**, the interpretation of their mode of action at the molecular level and the construction of **structure-activity relationships**.

Metabolism*

The term **metabolism** comprises the entire physical and chemical processes involved in the maintenance and reproduction of life in which nutrients are broken down to generate energy and to give simpler molecules (**catabolism**) which by themselves may be used to form more complex molecules (anabolism).

In case of heterotrophic organisms, the energy evolving from catabolic processes is made available for use by the organism.

In **medicinal chemistry** the term **metabolism** refers to the **biotransformation** of **xenobiotics** and particularly **drugs**. (See also **Biotransformation**; **Xenobiotic**).

Metabolite

A **metabolite** is any intermediate or product resulting from **metabolism**.

Me-too drug

A **me-too drug** is a compound that is structurally very similar to already known **drugs**, with only minor pharmacological differences.

Molecular graphics**

Molecular graphics is the visualization and manipulation of three-dimensional representations of molecules on a graphical display device.

Molecular modeling**

Molecular modeling is a technique for the investigation of molecular structures and properties using computational chemistry and graphical visualization techniques in order to provide a plausible three-dimensional representation under a given set of circumstances.

Mutagen***

A **mutagen** is an agent that causes a permanent heritable change (i.e., a mutation) into the DNA (deoxyribonucleic acid) of an organism.

Mutual prodrug

A **mutual prodrug** is the association in a unique molecule of two, usually synergistic, **drugs** attached to each other, one **drug** being the carrier for the other and vice versa.

NCE

See New Chemical Entity.

NDA

Abbreviation for New Drug Application.

New Chemical Entity.

A **new chemical entity (NCE)** is a compound not previously described in the literature.

Non-classical isostere

Same meaning as **Bioisostere**.

Nucleic acid*

A **nucleic acid** is a macromolecule composed of linear sequences of nucleotides that perform several functions in living cells, e.g., the storage of genetic information and its transfer from one generation to the next DNA (deoxyribonucleic acid), the expression of this information in protein synthesis (mRNA, tRNA) and may act as functional components of subcellular units such as ribosomes (rRNA).

RNA (ribonucleic acid) contains D-ribose, DNA contains 2-deoxy-D-ribose as the sugar component.

Nucleoside*

A **nucleoside** is a compound in which a purine or pyrimidine base is bound via a N-atom to C-1 replacing the hydroxy group of either 2-deoxy-D-ribose or of D-ribose, but without any phosphate groups. (See also **nucleotide**).

The common **nucleosides** in biological systems are adenosine, guanosine, cytidine, and uridine (which contain ribose) and deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine (which contain deoxyribose).

Nucleotide

A **nucleotide** is a **nucleoside** in which the primary hydroxy group of either 2-deoxy-D-ribose or of D-ribose is esterified by orthophosphoric acid. (See also **nucleoside**).

Oligonucleotide

An **oligonucleotide** is an oligomer resulting from a linear sequences of nucleotides.

Oncogene***

An **oncogene** is a normal cellular gene which, when inappropriately expressed or mutated, can transform eukaryotic cells into tumour cells.

Orphan drug

An **orphan drug** is a **drug** for the treatment of a rare disease for which reasonable recovery of the sponsoring firm's research and development expenditure is not expected within a reasonable time. The term is also used to describe substances intended for such uses.

Partial agonist

A **partial agonist** is an **agonist** which is unable to induce maximal activation of a **receptor** population, regardless of the amount of **drug** applied (See also **Intrinsic activity**).

Pattern recognition**

Pattern recognition is the identification of patterns in large data sets using appropriate mathematical methodologies.

Peptidomimetic

A **peptidomimetic** is a compound containing non-peptidic structural elements that is capable of mimicking or antagonizing the biological action(s) of a natural parent peptide. A peptidomimetic does no longer have classical peptide characteristics such as enzymatically scissile peptidic bonds. (See also **peptoids**).

Peptoid

A **peptoid** is a **peptidomimetic** that results from the oligomeric assembly of N-substituted glycines.

Pfeiffer's rule

Pfeiffer's rule states that in a series of chiral compounds the **eudismic ratio** increases with increasing potency of the **eutomer**.

Pharmacokinetics***

Pharmacokinetics refers to the study of absorption, distribution, **metabolism** and excretion (**ADME**) of bioactive compounds in a higher organism. (See also **Drug disposition**).

Pharmacophore (pharmacophoric pattern)

A **pharmacophore** is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response.

A **pharmacophore** does not represent a real molecule or a real association of functional groups, but a purely abstract concept that accounts for the common molecular interaction capacities of a group of compounds towards their target structure. The **pharmacophore** can be considered as the largest common denominator shared by a set of active molecules. This definition

discards a misuse often found in the medicinal chemistry literature which consists of naming as **pharmacophores** simple chemical functionalities such as guanidines, sulfonamides or dihydroimidazoles (formerly imidazolines), or typical structural skeletons such as flavones, phenothiazines, prostaglandins or steroids.

Pharmacophoric descriptors

Pharmacophoric descriptors are used to define a **pharmacophore**, including H-bonding, hydrophobic and electrostatic interaction sites, defined by atoms, ring centers and virtual points.

Placebo

A **placebo** is an inert substance or dosage form which is identical in appearance, flavor and odour to the active substance or dosage form. It is used as a negative control in a **bioassay** or in a clinical study.

Potency***

Potency is the dose of **drug** required to produce a specific effect of given intensity as compared to a standard reference.

Potency is a comparative rather than an absolute expression of **drug** activity. **Drug** potency depends on both **affinity** and **efficacy**. Thus, two agonists can be equipotent, but have different intrinsic efficacies with compensating differences in affinity.

Prodrug

A **prodrug** is any compound that undergoes **biotransformation** before exhibiting its pharmacological effects. **Prodrugs** can thus be viewed as **drugs** containing specialized non-toxic protective groups used in a transient manner to alter or to eliminate undesirable properties in the parent molecule. (See also **Double prodrug**).

QSAR

See **Quantitative Structure-Activity Relationships**

Quantitative Structure-Activity Relationships (QSAR)**

Quantitative structure-activity relationships are mathematical relationships linking chemical structure and pharmacological activity in a quantitative manner for a series of compounds. Methods which can be used in **QSAR** include various regression and **pattern recognition** techniques.

Receptor*

A **receptor** is a molecule or a polymeric structure in or on a cell that specifically recognizes and binds a compound acting as a molecular messenger (neurotransmitter, **hormone**, lymphokine, lectin, **drug**, etc.).

Receptor mapping**

Receptor mapping is the technique used to describe the geometric and/or electronic features of a binding site when insufficient structural data for this **receptor** or **enzyme** are available. Generally the active site cavity is defined by comparing the superposition of active to that of inactive molecules.

Second messenger

A **second messenger** is an intracellular **metabolite** or ion increasing or decreasing as a response to the stimulation of **receptors** by **agonists**, considered as the "first messenger". This generic term usually does not prejudice the rank order of intracellular biochemical events.

Site-specific delivery

Site-specific delivery is an approach to target a **drug** to a specific tissue, using **prodrugs** or antibody recognition systems.

Soft drug

A **soft drug** is a compound that is degraded *in vivo* to predictable non-toxic and inactive **metabolites**, after having achieved its therapeutic role.

SPC

See **Structure-property correlations**

Structure-activity relationship (SAR)

Structure-activity relationship is the relationship between chemical structure and pharmacological activity for a series of compounds.

Structure-based design**

Structure-based design is a drug design strategy based on the 3D structure of the target obtained by X-ray or NMR.

Structure-property correlations (SPC)**

Structure-property correlations refers to all statistical mathematical methods used to correlate any structural property to any other property (intrinsic, chemical or biological), using statistical regression and pattern recognition techniques.

Systemic***

Systemic means relating to or affecting the whole body.

Teratogen***

A **teratogen** is a substance that produces a malformation in a foetus.

Three-dimensional Quantitative Structure-Activity Relationship (3D-QSAR)

A **three-dimensional quantitative structure-activity relationship** is the analysis of the quantitative relationship between the biological activity of a set of compounds and their spatial properties using statistical methods.

Topliss tree**

A **Topliss tree** is an operational scheme for **analog** design.

Transition-state analog

A **transition-state analog** is a compound that mimics the transition state of a substrate bound to an enzyme.

Xenobiotic***

A **xenobiotic** is a compound foreign to an organism (xenos [greek] = foreign).

Journal of
Medicinal Chemistry

Subscriber access provided by UNIV NAC DE COLOMBIA UNAL

Letter

**Design and Synthesis of a Series of Non-Peptide High-Affinity
Human Corticotropin-Releasing Factor Receptor Antagonists**

Chen, Raymond Dagnino, Errol B. De Souza, Dimitri E. Grigoriadis, Charles Q. Huang, Kyung-II Kim, Zhengyu Liu, Terry Moran, Thomas R. Webb, Jeffrey P. Whitten, Yun Feng Xie, and James R. McCarthy

J. Med. Chem., **1996**, 39 (22), 4358-4360 • DOI: 10.1021/jm960149e • Publication Date (Web): 25 October 1996

Downloaded from <http://pubs.acs.org> on March 12, 2009

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 5 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

[View the Full Text HTML](#)

Design and Synthesis of a Series of Non-Peptide High-Affinity Human Corticotropin-Releasing Factor₁ Receptor Antagonists

Chen Chen,* Raymond Dagnino, Jr.,
Errol B. De Souza, Dimitri E. Grigoriadis,
Charles Q. Huang, Kyung-Il Kim, Zhengyu Liu,
Terry Moran, Thomas R. Webb, Jeffrey P. Whitten,
Yun Feng Xie, and James R. McCarthy*

Neurocrine Biosciences, 3050 Science Park Road,
San Diego, California 92121

Received May 20, 1996

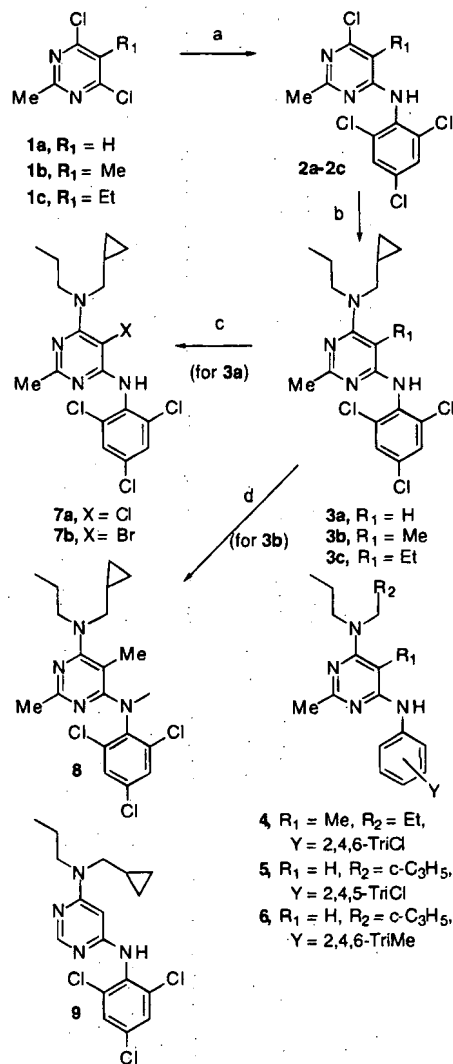
Corticotropin-releasing factor (CRF), a 41 amino acid peptide of hypothalamic origin, plays a major role in coordinating the endocrine, behavioral, and autonomic responses to stressful stimuli.¹⁻³ The actions of CRF are mediated through high-affinity receptors which are part of the superfamily of G-protein-coupled seven transmembrane proteins.⁴ Recently two members of the CRF receptor family have been cloned expressed and characterized. These receptors demonstrate a different sequence, pharmacology, and regional distribution within the central nervous system and the periphery. The CRF₁ receptor demonstrates high affinity for CRF and its related mammalian analogs,⁴⁻⁷ as well as for non-mammalian related peptides including sauvagine (frog) and urotensin I (sucker fish).⁸ The CRF₂ family of receptors, while sharing sequence homology with the CRF₁ receptor subtype, demonstrates low affinity for CRF itself but retains its high affinity for the nonmammalian forms of the peptide.^{8,9}

While there is very little known about the physiology and function of the CRF₂ subfamily of receptors, a substantial amount of preclinical and clinical data exists that suggests that CRF and its high-affinity receptor play a major role in mediating various neurological and psychological disorders, including major depression, anxiety, and a variety of stress-related disorders (for reviews, see refs 2, 3, and 10). Treatments for these disorders have been hampered by the fact that ligands for these receptors have thus far been large peptides.¹¹ We report the design and synthesis of high affinity and selective non-peptide CRF₁ receptor antagonists, 4-anilino-6-aminopyrimidines (illustrated by **3b**), targeted for the treatment of CRF-mediated disorders.

The reaction sequence for the synthesis of **3a-c**, **4**, **5**, **6**, and **9** is illustrated with **3a-c** in Scheme 1. N-Methylation of the anilino nitrogen on **3b** with excess methyl iodide and 1 equiv of sodium hydride in THF gave **8**. Pyrimidines **11** and **12** (see Chart 1 and Supporting Information for Scheme 2 with synthesis) were prepared by a similar sequence as that used for the synthesis of **3a-c** starting with commercially available 2,6-dichloro-6-methylpyrimidine (**10**) and separating the anilino pyrimidines **11** and **12** by flash chromatography.

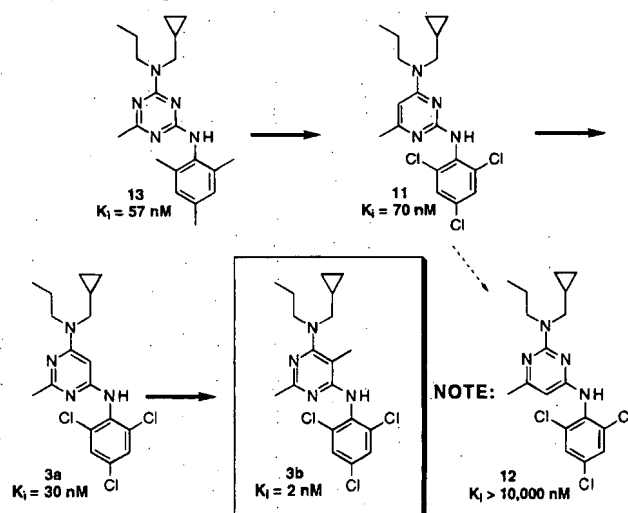
The inhibition of [¹²⁵I]CRF binding to cells expressing the human CRF₁ receptor was used to identify specific receptor antagonists in a radioligand binding assay.¹² The CRF receptors expressed in these cell lines demonstrated reversible, saturable, high-affinity binding to CRF with the pharmacological and functional charac-

Scheme 1^a



^a (a) 2,4,6-Cl₃C₆H₂NH₂, NaH, THF, RT or reflux; (b) *c*-C₃H₅CH₂NHCH₂CH₂CH₃, 100–190 °C; (c) NBS or NCS, CHCl₃, reflux; (d) NaH, MeI, THF, room temperature.

Chart 1. Sequence Followed in the Design of CRF₁ Receptor Antagonists



teristics comparable to those found in a variety of animal or human tissues.¹² CRF receptor binding assays were carried out essentially as previously described.¹³

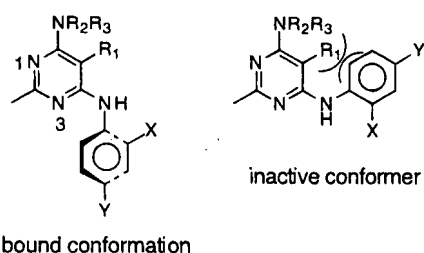


Figure 1. Proposed conformation for anilinopyrimidines to bind to the CRF₁ receptor and comparison with an inactive conformer.

Table 1. Inhibition (K_i) Values for Receptor Antagonists in Cells Stably Transfected with Human CRF₁ Receptors^a

compd	K_i (nM)	compd	K_i (nM)
3a	30	7a	1.7
3b	2.3	7b	2.0
3c	3.8	8	150
4	2.5	9	> 10000
5	253	D-PheCRF(12–41)	20
6	1390		

^a Compounds were tested at 6–12 doses for their ability to inhibit [¹²⁵I]CRF binding as described in text. Data are representative of duplicate determinations with the experiments repeated two or three times.

The design of **3b** starting from triazine **13**¹⁴ and the structure–activity relationship of related pyrimidines **11**, **3a**, and **12** with regard to CRF₁ receptor binding activity is outlined in Chart 1. Removal of the 1- or 5-nitrogen from triazine **13** (K_i = 57 nM), optimized by rapid microscale synthesis (RMS),¹⁴ gave pyrimidine **11** (K_i = 70 nM) or **3a** (K_i = 30 nM), respectively. However, changing the position of the 3-nitrogen (i.e. **12**) resulted in complete loss of activity. This CRF₁ receptor binding data led to a proposed pharmacophore model for binding to the CRF₁ receptor illustrated in Figure 1. In this model the bound conformation of the molecule requires the anilino group to be orthogonal to and below the pyrimidine (or triazine) ring. In addition, the nitrogen at the 3-position of **3a**, **3b**, **11**, and **13**, but absent in **12**, provides a critical hydrogen-bonding site. Consistent with the model, addition of a substituent at the 5-position of the pyrimidine ring resulted in a 30–50-fold increase in activity. Thus, addition of a methyl group (**3b**) or halogen (chloro, **7a**, or bromo, **7b**) provided compounds with K_i values in the 2 nM range (see Table 1). 2,4,6-Trichloroanilinopyrimidines were synthesized (**3a–c**, **4**, **6**, **8**, and **9**) on the basis of the 2,4,6-trisubstituted aryl group required for optimal activity for triazine **13** (Chart 1), the preference for trichloro over other substituents in related series (see Chart 1 and **3a** vs **6**), and the preference for an orthogonal relationship between the phenyl and pyrimidine rings with this substitution pattern. 2,4,5-Trisubstituted aryl compound **5** was over 10-fold less active than the corresponding 2,4,6-trisubstituted aryl compound **3a**. Interestingly, addition of a methyl group on the anilino nitrogen (**8**) resulted in over 100-fold loss in activity. As in the triazine **13**,¹⁴ *N*-propyl-*N*-cyclopropylmethyl was optimal for the N6 amino group, but unlike the triazine series *N,N*-dipropyl was equally active (**4**).

While direct inhibition of binding activity gives a valid measure of the potency of a compound at a specific receptor, functional tests must be employed in order to determine whether the compounds can act as agonists or antagonists at these receptors. Using membrane

preparations of the stable cell lines transfected with the CRF₁ receptor, the CRF₁ antagonist activity of the series was demonstrated by inhibition of CRF-stimulated cAMP production with **3a**, **4**, and **7a** using D-PheCRF-(12–41) as the standard (IC_{50} = 3700, 250, 100, and 200 nM, respectively). Inhibition of CRF-stimulated adenylate cyclase activity was performed as previously described.¹⁵ None of the compounds demonstrated any effects on basal cAMP production (that is in the absence of CRF or other stimulator), indicating that these compounds are devoid of agonist activity at this receptor subtype. In addition, these compounds could inhibit CRF-stimulated ACTH release from primary rat anterior pituitary cell cultures (data not shown), further demonstrating antagonist activity at CRF₁ receptors. In order to test for CRF receptor selectivity, the compounds synthesized in this series (Table 1) were assessed for inhibition of cAMP production in cells transfected with the human CRF_{2a} receptor and were completely devoid of activity (data not shown).

In conclusion, we have demonstrated the design and synthesis of selective, high-affinity non-peptide CRF₁ receptor antagonists **3b**, **3c**, **4**, **7a**, and **7b** with K_i values in the low nanomolar range. In addition, these compounds demonstrate *in vitro* inhibition of CRF-stimulated cAMP production in stable lines transfected with the human CRF₁ subtype. These compounds, and further modifications, will be of particular significance in establishing the utility and potential of CRF₁ receptor antagonists in the treatment of depression and anxiety-related disorders.

Supporting Information Available: A brief description of the biological assays, synthetic procedures for new compounds, and analytical data (8 pages). Ordering information is given on any current masthead page.

References

- (1) De Souza, E. B.; Nemeroff, C. B. *Corticotropin-releasing factor: Basic and clinical studies of a neuropeptide*; CRC Press, Inc.: Boca Raton, FL, 1990.
- (2) Dunn, A. J.; Berridge, C. W. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety of stress responses? *Brain Res. Rev.* **1990**, *15*, 71–100.
- (3) Owens, M. J.; Nemeroff, C. B. Physiology and pharmacology of corticotropin-releasing factor. *Pharmacol. Rev.* **1991**, *43*, 425–473.
- (4) Chen, R.; Lewis, K. A.; Perrin, M. H.; Vale, W. W. Expression cloning of a human corticotropin-releasing-factor receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8967–8971.
- (5) Chang, C. P.; Pearce, R. I.; O'Connell, S.; Rosenfeld, M. G. Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. *Neuron* **1993**, *11*, 1187–1195.
- (6) Perrin, M. H.; Donaldson, C. J.; Chen, R.; Lewis, K. A.; Vale, W. W. Cloning and functional expression of a rat brain corticotropin releasing factor (CRF) receptor. *Endocrinology* **1993**, *133*, 3058–3061.
- (7) Vita, N.; Laurent, P.; Lefort, S.; Chalon, P.; Lelias, J. M.; Kaghad, M.; Le, F. G.; Caput, D.; Ferrara, P. Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors. *FEBS Lett.* **1993**, *335*, 1–5.
- (8) Lovenberg, T. W.; Liaw, C. W.; Grigoriadis, D. E.; Clevenger, W.; Chalmers, D. T.; DeSouza, E. B.; Oltersdorf, T. Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 836–840.
- (9) Liaw, C. W.; Lovenberg, T. W.; Barry, G.; Oltersdorf, T.; Grigoriadis, D. E.; De Souza, E. B. Cloning and characterization of the human CRF2 receptor gene and cDNA. *Endocrinology* **1996**, *137*, 72–77.

52

- (10) De Souza, E. B.; Grigoriadis, D. E. Corticotropin-releasing factor: Physiology, pharmacology and role in central nervous system and immune disorders. In *Psychopharmacology: The Fourth Generation of Progress*; Bloom, F. E., Kupfer, D. J., Eds.; Raven Press: New York, 1994; pp 505–517.
- (11) De Souza, E. B.; Lovenberg, T. W.; Chalmers, D. T.; Grigoriadis, D. E.; Liaw, C. W.; Behan, D. P.; McCarthy, J. R. Heterogeneity of corticotropin-releasing factor receptors: Multiple targets for the treatment of CNS and inflammatory disorders. *Annu. Rep. Med. Chem.* **1995**, *30*, 21–30.
- (12) Grigoriadis, D. E.; Liaw, C. W.; Oltersdorf, T.; De Souza, E. B. Characterization of stable expression of cloned corticotropin-releasing factor receptors. *Soc. Neurosci. Abstr.* **1994**, *20*, 1345.
- (13) De Souza, E. B. Corticotropin-releasing factor receptors in the rat central nervous system: Characterization and regional distribution. *J. Neurosci.* **1987**, *7*, 88–100.
- (14) Whitten, J. P.; Xie, Y. F.; Erickson, P. E.; Webb, T. R.; De Souza, E. B.; Grigoriadis, D. E.; McCarthy, J. R. Rapid microscale synthesis (RMS), a new method for lead optimization using robotics and solution phase chemistry: Application to the synthesis and optimization of corticotropin releasing factor₁ (CRF₁) antagonists. *J. Med. Chem.* **1996**, *39*, 4354–4357.
- (15) Battaglia, G.; Webster, E. L.; De Souza, E. B. Characterization of corticotropin-releasing factor receptor-mediated adenylate cyclase activity in the rat central nervous system. *Synapse* **1987**, *1*, 572–581.

JM960149E

DIVISIONAL
ARRAY BIOPHARMA INC. Y
GENENTECH INC.
EXPEDIENTE No. 09-010.508 A