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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **03-039.889**
Solicitud de patente a nombre de **Novartis AG**

Yo, JIMENA ESCOBAR URIBE, identificada como aparece al pie de mi firma, domiciliada en la carrera 11 A No. 86-53, piso 6, obrando como apoderada de la sociedad **Novartis AG**, domiciliada en Basilea, Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. 13759 notificado por fijación en lista el 31 de octubre de 2008, relacionado con el concepto técnico de fondo No. 2221, me permito informar lo siguiente:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio en donde se realizaron las siguientes modificaciones:

1.1 Se introdujo parte de la materia caracterizante de la antigua reivindicación 3 dentro de la nueva reivindicación 1, es decir que se especificaron dentro de esta cláusula que el antagonista del receptor AT1 es valsartan o una sal farmacéuticamente aceptable del mismo, que el inhibidor de HMG-Co-A reductasa es fluvastatina, pitavastatina ó atorvastatina o una sal farmacéuticamente aceptable de las mismas; que el inhibidor de la enzima convertidora de angiotensina (ACE) es benazepril o enalapril, o una sal farmacéuticamente aceptable de los mismos, que el bloqueador del canal de calcio es amlodipina o verapamil ó una sal farmacéuticamente aceptable de los mismos; que el inhibidor de aldosterona-sintasa es fadrozol o la sal farmacéuticamente aceptable del mismo y que el diurético es clorothiazida, hidroclorotiazida, metilclorotiazida ó clorotadilon o una sal farmacéuticamente aceptable de los mismos.

El sustento para esta modificación se encuentra en el antiguo capítulo reivindicatorio y en las páginas 5 a 12 de la memoria descriptiva de la solicitud en estudio.

1.2 Dentro de la reivindicación 1 se cancelaron los literales (vi), (vii) y (vii), que hacían referencia al antagonista de aldosterona, el inhibidor doble de la enzima convertidora de angiotensina/endopeptidasa neutra (ACE/NEP) y al antagonista de endotelina, respectivamente.

Para este efecto, me permito acompañar el recibo No. 09-8,521 del 3 de febrero de 2009, con el cual queda cubierta la tasa correspondiente a la modificación que presentamos junto con este memorial.

2. Claridad de la solicitud

2.1 Descripción.

En cuanto a la objeción presentada por el señor examinador, atentamente nos permitimos manifestar que es deseo del solicitante poner en consideración de ese despacho datos comparativos adicionales a manera de prueba con los cuales se demuestra la altura inventiva de las combinaciones ahora reivindicadas en la cláusula 1 modificada.

2.2 Reivindicaciones

Con relación a la claridad del capítulo reivindicatorio, atentamente nos permitimos manifestar que el término “sal farmacéuticamente aceptable” para el ingrediente fadrozol es comprensible para una persona versada en la materia que lea la totalidad de la solicitud, pues entenderá que dicha expresión hace referencia a la sal enunciada especialmente enunciada para dicho ingrediente activo, es decir, el enantiómero (+) del clorhidrato de fadrozol. Sin embargo, con el ánimo de brindar claridad a lo escrito en la reivindicación 1, se especificó la sal para este compuesto.

De otra parte, el señor examinador podrá notar que con la restricción realzada en la cláusula 1 la cancelar los literales (vi), (vii) y (vii), que hacían referencia al antagonista de aldosterona, el inhibidor doble de la enzima convertidora de angiotensina/endopeptidasa neutra (ACE/NEP) y al antagonista de endotelina, respectivamente, ya se pueden examinar las combinaciones.

3. Novedad

El señor examinador en su concepto técnico No. 2221 argumenta que:

“D1 enseña composiciones y métodos para mejorar el efecto terapéutico de la baja presión arterial y la insuficiencia cardíaca congestiva en mamíferos. Más específicamente este invento enseña composiciones sinérgicas comprendidas por cantidades de al menos dos agentes terapéuticos seleccionados de un grupo que consiste en un inhibidor de renina, un inhibidor de la enzima convertidora de angiotensina I y un antagonista de angiotensina II. Se incluyen además

también métodos para mejorar los efectos sinérgicos. Ver folio 110 abstract, folio 111 reivindicación 1.

D2 enseña composiciones farmacéuticas que comprenden un vehículo farmacéuticamente aceptable con un antagonista del receptor de angiotensina II combinado con otros agentes como diuréticos, bloqueadores del canal del calcio, bloqueadores β adrenoreceptores, inhibidores de renina o un inhibidor de la enzima convertidora de angiotensina y un método para tratar la hipertensión. (Ver folio 112).

Las características esenciales de la composición de las reivindicaciones 1 - 5 es decir **un inhibidor de renina** de la fórmula I o una sal farmacéuticamente aceptable del mismo y **otro agente terapéutico i) Un antagonista del receptor ATi (o antagonistas de angiotensina II) y iii) y un inhibidor de la enzima convertidora de angiotensina**, habían sido divulgadas previamente en D1 (folios 110 - 112), por lo cual la materia reivindicada para las combinaciones mencionadas no es nueva.

Sobre este particular, atentamente nos permitimos decir que gracias a la cancelación de los antiguos literales (vi), (vii) y (vii) dentro de la reivindicación 1, que hacían referencia al antagonista de aldosterona, el inhibidor doble de la enzima convertidora de angiotensina/endopeptidasa neutra (ACE/NEP) y al antagonista de endotelina, respectivamente, quedan cabalmente superadas las objeciones de novedad expuestas por el señor examinador.

En este orden de ideas, también es importante señalar que la cancelación de estos literales hace que el alcance de la reivindicación 1 sea drásticamente restringido, con lo cual se supera la objeción de novedad relacionada con la existencia de multiplicidad de composiciones y con combinaciones señaladas en forma genérica, presentada por el señor examinador en su respuesta a los argumentos presentados en nuestro memorial anterior.

4. NIVEL INVENTIVO

Con relación a este requisito el señor examinador argumenta en su concepto técnico que: "El estado de la técnica más cercano es:

D1 que enseña enseñar composiciones sinérgicas comprendidas por al menos dos agentes terapéuticos seleccionados de un grupo que consiste en un inhibidor de renina, un inhibidor de la enzima convertidora de angiotensina I y un antagonista de angiotensina II. Se incluyen además también métodos para mejorar los efectos sinérgicos. Ver folio 110 abstract, folio 111 reivindicación 1.

D2 que enseña composiciones farmacéuticas que comprenden un vehículo farmacéuticamente aceptable con un antagonista del receptor de angiotensina II combinado con otros agentes como diuréticos, bloqueadores del canal del calcio, bloqueadores β adrenoreceptores, inhibidores de renina o un inhibidor de la enzima convertidora de angiotensina y un método para tratar la hipertensión. Ver abstract folio 112.

(Ver Tabla 149)

La única diferencia de esta solicitud con D1 es que esta solicitud aporta al estado de la técnica las combinaciones del inhibidor de renina con los compuestos ii) y iv) a viii) pero esta diferencia no parece conceder un efecto técnico inesperado porque la necesidad de una composición sinérgica para tratar afecciones cardiovasculares se había logrado previamente tanto en el documento D1 como en D2 por lo tanto sería obvio para un experto en la materia tomar las enseñanzas de ambos documentos para proponer las combinaciones reivindicadas.”

En este sentido, ese despacho puede constatar que ciertamente en ninguna parte de los documentos citados como anterioridades relevantes D1 y D2 se halla un indicio que llevara de manera evidente a la persona versada en la materia a las combinaciones específicas mostradas en la nueva reivindicación 1 modificada.

De hecho, el documento WO 91/17771 (D1) señala que puede haber composiciones sinérgicas comprendidas por cantidades de por lo menos dos agentes activos seleccionados de un inhibidor de renina, un inhibidor de la enzima convertidora de angiotensina I y un antagonista de angiotensina II y el documento WO 92/10097 (D2) sugiere composiciones constituidas por un antagonista del receptor de angiotensina II en un vehículo junto con diuréticos y bloqueadores del canal de calcio.

Así las cosas, con relación a las combinaciones especificadas dentro de la nueva reivindicación 1 que contienen el compuesto de la fórmula (I) con al menos uno de los componentes determinados, no habrían sido evidentes para un persona medianamente versada en la técnica, toda vez que nada en los documentos D1 y D2 sugiere que combinaciones de este estilo y que todas ellas pudieran presentar un efecto de sinergia cuando se mezclan.

En este orden de ideas, es deseo del solicitante suministrar información adicional para sustentar el hecho que todas las combinaciones ahora enunciadas en la reivindicación 1, presentan ventajas favorables para el tratamiento de enfermedades cardíacas y que las composiciones no son el producto de simples combinaciones azarosas de componentes activos.

4.1. Combinación con Bloqueador de canal de calcio (compañero de combinación (iv) de la reivindicación 1).

En este caso en particular, el solicitante desea poner en consideración de ese despacho al artículo titulado “Antihypertensive Efficacy of the Oral Direct Renin Inhibidote Aliskiren as Add-On Therapy in Patients Not Responding to Amlodipine Monotherapy” de Drummond y colaboradores publicado en el Journal of Clinical Hipertensión y el Póster titulado “Aliskiren as Add-on to Amlodipine Provides significant additional blood pressure lowering without increased oedema associated with doubling the Amlodipine dose” de Munger y colaboradores, presentado en el Congreso Mundial de Cardiología.

Como se puede observar, la combinación de Aliskiren y bloqueadores de calcio, en particular el bloqueador de calcio Amlodipina presenta ventajas terapéuticas que no podrían haber sido alcanzadas antes con ningún otro agente activo tomado de manera independiente. Como se pone de manifiesto en dichos documentos, a menudo no se alcanza un control adecuado de la presión sanguínea con la monoterapia utilizando 5 mg de amlodipina, lo cual, de manera sorprendente se puede mejorar gracias a la acción combinada de estos dos ingredientes activos, como se muestra a continuación en la figura 3 ilustrada en la página 747 del artículo en mención.

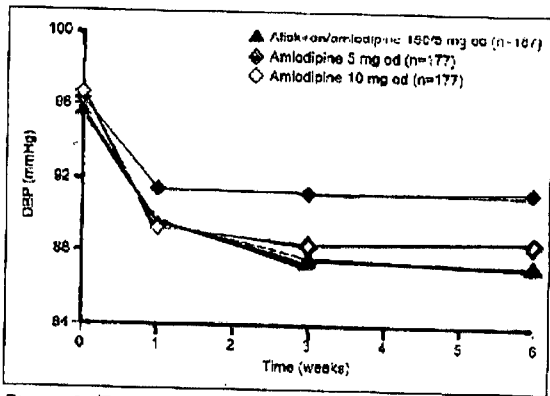


Figure 3. Time course of mean sitting diastolic blood pressure (DBP) with once-daily aliskiren 150 mg/amlodipine 5 mg, amlodipine 5 mg, and amlodipine 10 mg in hypertensive patients not responding to amlodipine 5 mg monotherapy (intent-to-treat population).

Otro aspecto importante a tener en cuenta es el hecho que el uso de un fármaco en altas dosis, como se muestra en la gráfica doblar la dosis de la Amlodipina a 10 mg, puede incrementar el riesgo de eventos laterales adversos. En este sentido, se encontró que en pacientes que no responden a la monoterapia de la dosis estándar de 5 mg de Amlodipina, la terapia combinada de Aliskiren y Amlodipina muestra una ventaja en cuanto a que la presión sanguínea puede ser controlada de la misma manera como se realiza con la terapia de dosis alta con amlodipina (10 mg), pero con un resultado mucho más importante y es que no se observó con dicha terapia combinada, la alta ocurrencia de edema periférico que sucede con la terapia de dosis elevada de Amlodipina (11.2%), toda vez que la acción combinada del Aliskiren y la Amlodipina mostró tan solo un 2.1% de dicho efecto indeseable, lo cual fue incluso más bajo que la ocurrencia de edema periférico observado con terapias con 5 miligramos de amlodipina que reporta un 3.45%.

Lo anterior, también tiene una gran importancia desde el punto de vista clínico, toda vez que significa que será posible tratar pacientes que no respondían a terapia con 5 miligramos de Amlodipina y alcanzar el control de la presión sanguínea sin tener alto riesgo de sufrir efectos colaterales adversos, principalmente de edema periférico como el observado con terapia de dosis

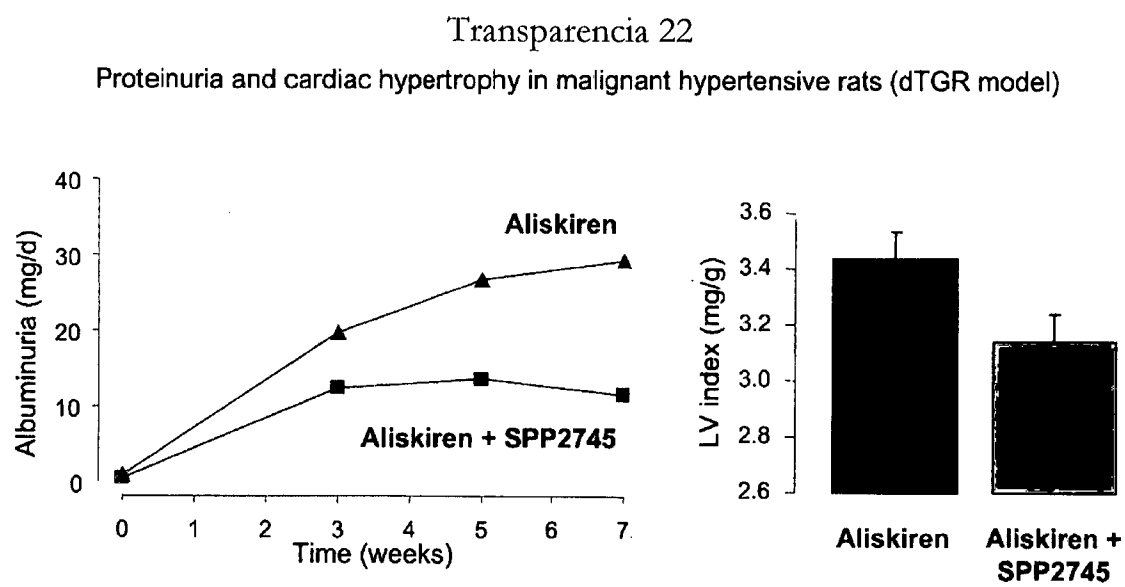
incrementada con Amlodipina, la cual era la medida más obvia para brindarle a los pacientes que no respondían a la terapia de 5 miligramos de amlodipina un control de la presión sanguínea. Por lo tanto, la combinación de aliskiren y el bloqueador del canal de calcio, se encuentra basada en un concepto inventivo.

4.2 Combinación con un inhibidor de aldosterona-sintasa (compañero de combinación (v) de la reivindicación 1).

En este caso, el solicitante desea dirigir la atención del señor examinador a la exposición adjunta (Anexo 2) de Speedel, presentada en el Día R & D en Marzo de 2008, en particular a las transparencias No. 22, 23 y 28 y el último punto (significado de POC “prueba de concepto”).

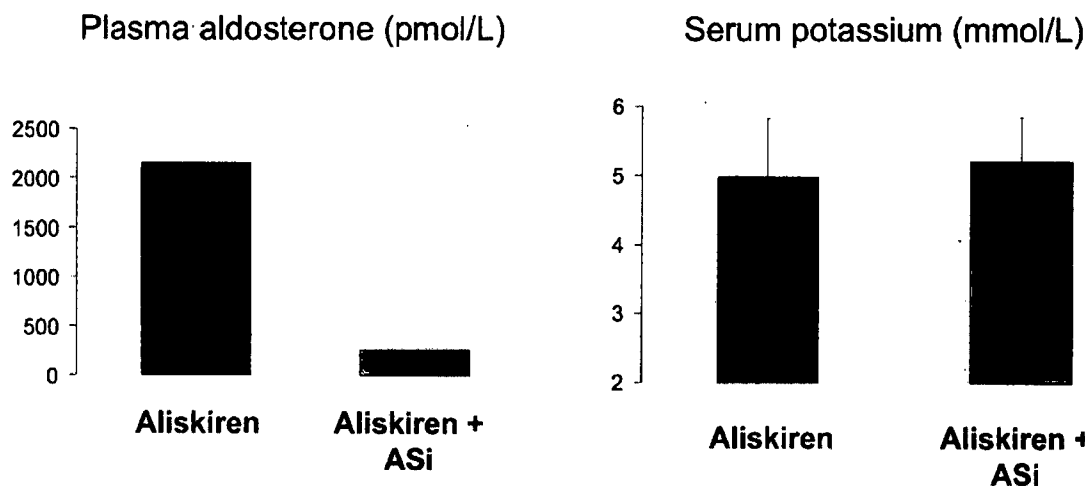
Como se puede observar en esas transparencias, también la terapia combinada de Aliskiren con un inhibidor de aldosterona-sintasa produce unas ventajas terapéuticas inesperadas que no habían podido ser alcanzadas por los agentes activos respectivos empleados en terapias independientes.

En ese orden de ideas, ese despacho puede corroborar que la presentación versa principalmente en el progreso realizado con un nuevo inhibidor de aldosterona-sintasa SPP2745. En la sección de “Key Data Sets” que comienza en la transparencia 15 se describen los resultados de este inhibidor de aldosterona-sintasa y en particular, en las trasparencias 22 y 23, se ha mostrado que cuando se aplica en combinación con Aliskiren, se puede reducir de manera significativa la proteinuria y la hipertrofia cardíaca en ratas con la terapia combinada:



Transparencia 23

Plasma aldosterone and potassium in malignant hypertensive rats (dTGR)



Además, la adición de un inhibidor de aldosterona-sintasa al aliskiren, suprime de manera efectiva la aldosterona en la sangre y al mismo tiempo, evita el desarrollo de hipercalemia debido a los niveles de potasio en suero.

Estas ventajas demuestran que la terapia combinada puede ser adecuada para el tratamiento de enfermedades cardiovasculares y además que puede estar dirigida a mejorar los resultados obtenidos con la monoterapia. Es decir, que los hallazgos realizados por el solicitante en este sentido, son muy importantes y no podrían ser susceptibles de derivación aparente, o previstas a partir de las monoterapias tomadas de manera independiente.

Por lo tanto, la combinación de Aliskiren y el inhibidor de aldosterona-sintasa se encuentra basada en un concepto inventivo.

4.3 Combinación con un inhibidor de reductasa HMG-Co-A (compañero de combinación (ii) de la reivindicación 1).

Para esta combinación, el solicitante desea poner en consideración de ese despacho el artículo titulado “*Renin inhibition reduces hypercholesterolemia-induced atherosclerosis in mice*” de Lu y colaboradores, publicado en el Journal of Clinical Investigation, como un apoyo y soporte de la utilidad del aliskiren, donde se muestra que dicho agente activo es particularmente útil en combinación con un inhibidor de HMG-Co-A reductasa también conocido como estatina.

Las estatinas son bien conocidas por reducir los niveles de colesterol y por lo tanto son empleadas en la prevención y en el tratamiento de la arterosclerosis. Recientemente se ha encontrado de manera sorprendente que la inhibición de la

renina causada por aliskiren conlleva a una reducción significativa de la arterosclerosis en ratones como así se expone en la página 2, columna 1 del artículo. Por consiguiente, el tratamiento combinado de aliskiren con una estatina resulta ser efectivo para tratar la arterosclerosis mediante el empleo de dos rutas para combatir el trastorno, una mediante la inhibición de la reductasa HMG-Co-A y la otra por la inhibición de la renina. Esto no habría sido sugerido en ninguna parte ni en ningún momento antes de la presentación de la solicitud en estudio. Esta terapia combinada está en marcha mediante experimentos con ratones para hacer hincapié adicional en este hallazgo.

La combinación de Aliskiren y un inhibidor de reductasa HMG-Co-A también se encuentra por lo tanto basada en un concepto inventivo toda vez que ofrece un nuevo camino para tratamientos terapéuticos que no habían sido conocidos ni sugeridos en la técnica anterior.

Así las cosas, nos permitimos manifestar que la información adicional anterior demuestra que las composiciones ahora reclamadas son el producto de la actividad inventiva del solicitante.

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *"habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares"* y *"(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso"*, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,


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Anexo 1

Artículo "Antihypertensive Efficacy of the Oral Direct Renin Inhibitor Aliskiren as Add-On Therapy in Patients Not Responding to Amlodipine Monotherapy" de Drummond y colaboradores publicado en el Journal of Clinical Hipertensión y el Póster titulado "Aliskiren as Add-on to Amlodipine Provides significant additional blood pressure lowering without increased oedema associated with doubling the Amlodipine dose" de Munger y colaboradores, presentado en el Congreso Mundial de Cardiología

Antihypertensive Efficacy of the Oral Direct Renin Inhibitor Aliskiren as Add-On Therapy in Patients Not Responding to Amlodipine Monotherapy

Waymon Drummond, MD;¹ Mark A. Munger, PharmD;² Mohammed Rafique Essop, MBBCh;³ Mojdeh Maboudian, PharmD, MS;⁴ Mahmudul Khan, PhD;⁴ Deborah L. Keefe, MD, MPH⁴

This study investigated the addition of the direct renin inhibitor aliskiren to amlodipine in patients with mild to moderate hypertension that was inadequately controlled with amlodipine alone. Following once-daily treatment with amlodipine 5 mg for 4 weeks, patients whose hypertension responded inadequately to therapy (mean sitting diastolic blood pressure [DBP] 90–109 mm Hg) ($n=545$) were randomized to 6 weeks of double-blind treatment with amlodipine 5 mg plus aliskiren 150 mg, amlodipine 5 mg, or amlodipine 10 mg. At the study's end, mean systolic blood pressure and DBP reductions with the combination of aliskiren 150 mg and amlodipine 5 mg (11.0/8.5 mm Hg) were significantly greater ($P<.0001$) than with amlodipine 5 mg (5.0/4.8 mm Hg)—the comparator group—but similar to amlodipine 10 mg (9.6/8.0 mm Hg). All treatments were well tolerated. Edema occurred more

frequently with amlodipine 10 mg (11.2%) than with combination therapy (2.1%) or amlodipine 5 mg (3.4%). In conclusion, aliskiren 150 mg plus amlodipine 5 mg shows similar but not better blood pressure-lowering efficacy when compared with amlodipine 10 mg in patients not completely responsive to amlodipine 5 mg; less edema was noted with combination therapy. (*J Clin Hypertens.* 2007;9:742–750) ©2007 Le Jacq

Monotherapy with antihypertensive agents is insufficient to control blood pressure (BP) in many patients with hypertension (HTN).^{1,2} Most guidelines for the management of HTN recognize that patients, especially those with more severe degrees of HTN or evidence of target organ involvement, will require therapy with at least 2 antihypertensive drugs for effective BP control.^{1,2} It has been shown that even in the controlled setting of a clinical trial, combination therapy with 2 or 3 different antihypertensive agents is often required to achieve BP level targets.³ Despite the availability of a range of antihypertensive therapies, approximately 50% to 60% of patients with HTN have BP levels that are not at goal.⁴

For patients in whom BP targets are not achieved with antihypertensive monotherapy, physicians have the choice of titration of the single agent to a higher dose or addition of a second antihypertensive medication to improve treatment response. Guidelines for the treatment of HTN recommend the use of antihypertensive agents with differing mechanisms of action,^{2,5} as this increases the

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likelihood of achieving BP control.² Thus, combining an agent that blocks the activity of the renin-angiotensin system (RAS) (an angiotensin-converting enzyme inhibitor [ACEI] or angiotensin receptor blocker [ARB]) with either a calcium channel blocker (CCB) or a diuretic would be expected to provide additional BP reductions. Both CCBs and diuretics reduce BP independent of the RAS; CCBs reduce BP by directly inducing vasodilation, while diuretics are also effective as vasodilators over the long term.

Further rationale for using CCBs with RAS blockers comes from their complementary effects on the autonomic system, RAS, and endothelium. The CCB amlodipine, as well as other CCBs, has been shown to increase sympathetic activity and decrease parasympathetic activity, whereas ACEIs and ARBs enhance parasympathetic activity but have little effect on sympathetic activity.^{6,7} This suggests that the autonomic effects of amlodipine are likely to be mitigated by concomitant administration of medications that affect the RAS.⁶ CCBs also cause reflex activation of the RAS, so use of these with a medication that blocks the activity of the RAS is a logical choice.⁸ CCBs, ACEIs, and ARBs may also exert beneficial effects on the endothelium, such as improved nitric oxide availability. It has been suggested that these agents may have complementary effects on the vascular wall.⁹⁻¹³

These complementary actions may help explain the beneficial effects of a CCB combined with an ACEI that were recently reported in the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT).¹⁴ Although the primary end points of coronary heart disease events were no different than in a β -blocker-based group, there was a lower incidence of overall cardiovascular events and all-cause mortality with the CCB/ACEI combination. Previous studies have demonstrated that therapy with CCBs and ACEIs provides significantly greater BP reductions when compared with CCB or ACEI monotherapy, with a significantly lower incidence of edema than with amlodipine alone.¹⁵⁻¹⁷ When combined with an ACEI or ARB, diuretics have also been found to be as effective in reducing BP as an CCB/ACEI combination.¹⁸

Aliskiren is the first in a new class of orally effective direct renin inhibitors approved for the treatment of HTN and is a potent inhibitor of human renin.^{19,20} The elimination half-life of aliskiren is approximately 40 hours,^{21,22} making it suitable for once-daily dosing. Aliskiren monotherapy has been shown to provide effective BP reductions in short-term placebo-controlled studies,²³⁻²⁶ providing

comparable reductions to the ARB irbesartan²³ and the diuretic hydrochlorothiazide (HCTZ).²⁶ Unlike most other antihypertensive agents, aliskiren reduces plasma renin activity (PRA) from baseline, both as monotherapy and when combined with antihypertensive agents that elevate PRA.^{24,26} Aliskiren has been shown to have a good safety profile, with adverse event rates similar to those observed with placebo at doses of ≤ 300 mg.^{23,26}

Given that adequate BP control is not achieved in many hypertensive patients with a single antihypertensive agent, it is interesting to evaluate the effects of the addition of aliskiren in cases that involve an inadequate response to antihypertensive monotherapy. Based on the rationale of combining treatments that have complementary mechanisms of action, a logical approach might be to add aliskiren to a CCB in patients not responding to CCB monotherapy. The objective of the present study was to evaluate the effect on BP when once-daily aliskiren 150 mg was added to once-daily amlodipine 5 mg in patients with mild to moderate HTN that responded only partially to once-daily amlodipine 5 mg monotherapy. The safety and efficacy of administering this combination was compared with maintaining patients on once-daily amlodipine 5 mg or increasing the amlodipine dosage to 10 mg once daily. In addition, the effects of each treatment on PRA were assessed in a subset of patients.

METHODS

Patients

Eligible patients were men and women aged 18 years or older with mild to moderate HTN (mean sitting diastolic BP [DBP] level ≥ 95 to < 110 mm Hg at baseline if untreated or DBP level < 110 mm Hg if treated). Patient exclusion criteria included the following: severe HTN (DBP level ≥ 110 mm Hg and/or mean sitting systolic BP [SBP] level ≥ 180 mm Hg); a history or evidence of secondary HTN; hypertensive retinopathy (Keith-Wagener grade III or IV); a history of hypertensive encephalopathy or cerebrovascular accident; a transient ischemic cerebral attack in the previous 12 months; heart failure (New York Heart Association classes II-IV); a history of myocardial infarction, coronary bypass surgery, or percutaneous coronary intervention in the previous 12 months; angina pectoris requiring pharmacotherapy; second- or third-degree heart block without a pacemaker; life-threatening or symptomatic arrhythmia; clinically significant valvular heart disease or severe aortic stenosis; type I or type II diabetes mellitus with poor

glycemic control (hemoglobin A_{1c} >9%); upper arm circumference >42 cm; serum sodium less than normal and serum potassium <3.5 mmol/L or ≥5.5 mmol/L; evidence of renal impairment (serum creatinine level >1.7 mg/dL in women or >2.0 mg/dL in men at screening or a history of dialysis or of nephrotic syndrome) or hepatic disease (serum glutamic oxaloacetic transaminase or serum glutamic pyruvic transaminase values >3 times the upper limit of normal at screening, or a history of hepatic encephalopathy, esophageal varices, or portocaval shunt); and any condition that may affect the evaluation of efficacy or safety data or alter the absorption, distribution, metabolism, or excretion of study drugs. Patients were therefore relatively healthy except for the presence of HTN.

Patients were recruited at 81 centers in Denmark, Germany, Greece, Korea, Malaysia, Slovakia, South Africa, and the United States. All patients provided written informed consent. The study design was approved by the appropriate local ethical review boards and conducted in accordance with the principles of the 1996 Declaration of Helsinki of the World Medical Association.

Study Design

This was a randomized, double-blind, active-controlled, parallel-group multicenter study comparing the efficacy and safety of once-daily aliskiren 150 mg/amlodipine 5 mg combination therapy with continuation of once-daily amlodipine 5 mg or dose titration to once-daily amlodipine 10 mg in patients whose HTN did not adequately respond to initial treatment with amlodipine 5 mg. The study was designed to reflect the situation faced by many physicians in clinical practice: examining alternative approaches for improving treatment response (increasing the dose of current medication or adding a second antihypertensive agent) in patients in whom specific BP targets have not been achieved with antihypertensive monotherapy. Continued treatment with once-daily amlodipine 5 mg provided a control comparator group.

Newly diagnosed or currently treated hypertensive patients provided a complete medical history and list of concomitant medications and underwent a physical examination, BP and electrocardiographic (ECG) recordings, and evaluation of laboratory safety parameters (blood chemistry, hematology, and urinalysis). Premenopausal women underwent serum and urine pregnancy tests. Following a 2-week washout of all antihypertensive medications, patients with a DBP level ≥95 mm Hg and <110 mm Hg entered a

4-week, single-blind run-in period during which they received once-daily oral amlodipine 5 mg. At the end of the run-in period, screening assessments were repeated. Patients whose HTN had not adequately responded to once-daily amlodipine 5 mg (defined as those with a DBP level ≥90 mm Hg and <110 mm Hg) were randomized to receive 6 weeks of once-daily oral treatment with amlodipine 5 mg, amlodipine 10 mg, or aliskiren 150 mg/amlodipine 5 mg (using a 1:1:1 allocation ratio). The efficacy and safety of treatment were assessed in patients at 1, 3, and 6 weeks after randomization, as described below. Patients whose SBP level was ≥180 mm Hg and/or DBP was ≥110 mm Hg at any time were withdrawn from the study and appropriate therapy was instituted.

Assessments

Efficacy. The primary efficacy variable was the change in DBP from baseline (the end of the 4-week run-in period with once-daily amlodipine 5 mg) to the end of 6 weeks of double-blind treatment. The primary objective of the study was to compare once-daily aliskiren 150 mg/amlodipine 5 mg with once-daily amlodipine 5 mg monotherapy. Secondary efficacy measures included the change from baseline in SBP, comparison of reductions in DBP and SBP between the combination therapy and amlodipine 10 mg monotherapy, the proportion of patients in each treatment group who responded to treatment (defined as a DBP level <90 mm Hg at week 6 and/or a reduction of ≥10 mm Hg from baseline), and the proportion of patients in each treatment group in whom BP control was achieved (BP level <140/90 mm Hg).

Sitting and standing BP levels were measured at baseline and after 1, 3, and 6 weeks of the double-blind period of the trial. All BP measurements were obtained at trough (24±3 hours after drug dose) using an automated BP monitor (Omron HEM-705CP; Omron Corporation, Tokyo, Japan) in accordance with the Guidelines for Management of HTN: Report of the Fourth Working Party of the British HTN Society, 2004 (BHS IV).⁵ Three BP measurements were obtained at 1- to 2-minute intervals and averaged to calculate mean DBP and SBP for that visit. Pulse rate was measured for 30 seconds immediately before measurement of sitting BP and standing BP at each visit.

In a subset of approximately 150 patients, PRA was measured by DiaSorin assay (DiaSorin, Saluggia, Vercelli, Italy) at baseline and after 6 weeks of treatment, and the change in PRA from baseline after 6 weeks' treatment was assessed.

Safety and Tolerability. Safety assessments consisted of monitoring and recording all adverse events (AEs) and serious adverse events (SAEs). Each AE was classified as mild, moderate, or severe; the investigator assessed any possible relationship with the study drug. SAEs were defined as any event that was fatal or life-threatening, resulted in persistent or significant disability, constituted a congenital abnormality, required in-patient hospitalization or prolonged hospitalization, or was considered in some other way medically significant.

Laboratory safety evaluations were performed at baseline and after 6 weeks' treatment (or at the last study visit) and included hematology, blood chemistry (urea, creatinine, glucose, bilirubin, aspartate aminotransferase, alanine aminotransferase, lactic dehydrogenase, alkaline phosphatase, electrolytes, total protein, albumin, and uric acid levels), and urinalysis. Physical examinations and ECG studies were performed at the screening visit before the washout period and at the start and the end of the double-blind treatment period.

Statistical Analyses

A sample size of at least 168 patients per group (total, 504 patients) was chosen to ensure that the study was powered (90%) to detect a statistically significant difference in DBP of at least 3 mm Hg between the once-daily aliskiren 150-mg/amlodipine 5-mg group and the once-daily amlodipine 5-mg group, assuming a 10% dropout rate and a standard deviation of 8 mm Hg. Based on previous clinical trials, an estimated 60% of patients were expected to have poorly controlled BP with amlodipine monotherapy (and were therefore expected to be eligible for the double-blind period of this study). As a result, a sample size of 840 patients was planned for entry into the single-blind run-in period.

Efficacy analyses were performed on the intent-to-treat (ITT) population (all randomized participants who received ≥ 1 dose of the study drug, had a baseline assessment, and had ≥ 1 postbaseline assessment of efficacy). For each patient, the last postbaseline measurement for each variable assessed during the double-blind treatment period was carried forward and used as the week 6 end point measurement in the analyses. Changes in the primary efficacy variable were assessed using a 2-way analysis of covariance model with treatment and region as factors and baseline DBP values as a covariate. The null hypothesis was that there was no difference in the primary efficacy variable between once-daily aliskiren 150 mg/amlodipine 5 mg and once-daily amlodipine 5 mg; the alternative

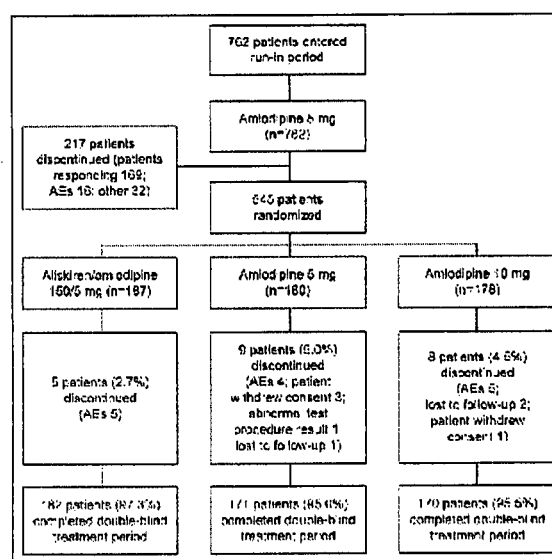


Figure 1. Patient flow. AEs indicates adverse events.

hypothesis was superiority of the combination over amlodipine 5 mg.

The secondary efficacy variables of change in SBP from baseline and comparison of SBP and DBP in the combination therapy group vs the once-daily amlodipine 10-mg group were analyzed in a way similar to the primary variable. The proportion of responders in each group was compared using a logistic regression model with treatment and region as factors and baseline DBP as a covariate. Pairwise comparisons of each treatment were made at a 2-sided significance level of $<.05$. The proportion of patients in whom BP control was achieved was analyzed in the same manner as the proportion of responders.

Safety and tolerability assessments were performed on the safety population, which was defined as all patients who received ≥ 1 dose of the double-blind study drug and had ≥ 1 postbaseline safety assessment.

RESULTS

Patients

A total of 762 patients were enrolled in the study. Of these, 169 responded to initial therapy with once-daily amlodipine 5 mg (DBP level <90 mm Hg) and 48 were not randomized for other reasons. The remaining 545 patients were randomized to double-blind treatment (187 received once-daily aliskiren 150 mg/amlodipine 5 mg, 180 received once-daily amlodipine 5 mg, and 178 received once-daily amlodipine 10 mg). In total, 541 patients were included in the ITT population ($n=187$, $n=177$, and $n=177$ for the aliskiren

Table I. Demographic and Baseline Clinical Characteristics of All Randomized Patients

CHARACTERISTIC	ONCE-DAILY ALISKIREN 150 MG/ AMLODIPINE 5 MG (N=187)	ONCE-DAILY AMLODIPINE 5 MG (N=180)	ONCE-DAILY AMLODIPINE 10 MG (N=178)
Male sex, No. (%)	105 (56.1)	95 (52.8)	92 (51.7)
Age, y	52.7 (11.9)	53.7 (10.9)	54.0 (10.6)
Age 65 years or older, No. (%)	31 (16.6)	36 (20.0)	30 (16.9)
Race, No. (%)			
Caucasian	128 (68.4)	128 (71.1)	122 (68.5)
Black	34 (18.2)	33 (18.3)	29 (16.3)
Asian	21 (11.2)	18 (10.0)	22 (12.4)
Other	4 (2.1)	1 (0.6)	5 (2.8)
Duration of hypertension, y	8.5 (7.6)	8.0 (6.8)	8.2 (7.5)
DBP, mm Hg	95.7 (4.4)	96.2 (4.8)	96.5 (4.5)
SBP, mm Hg	150.5 (11.1)	150.5 (13.2)	150.8 (12.0)
BMI, kg/m ²	30.2 (5.5)	30.2 (5.7)	30.4 (5.7)
PRA, ng/mL/h ^a	1.0 (1.8)	1.2 (3.7)	0.7 (0.7)
Metabolic syndrome, No. (%)	80 (42.8)	71 (39.4)	74 (41.6)
Diabetes, No. (%)	26 (13.9)	26 (14.4)	27 (15.2)

Data are mean (SD) unless otherwise stated. ^aEvaluated in a subset of patients: amlodipine 150 mg/aliskiren 5 mg, n=56; amlodipine 5 mg, n=48; amlodipine 10 mg, n=49. Abbreviations: BMI, body mass index; DBP, mean sitting diastolic blood pressure; PRA, plasma renin activity; SBP, mean sitting systolic blood pressure.

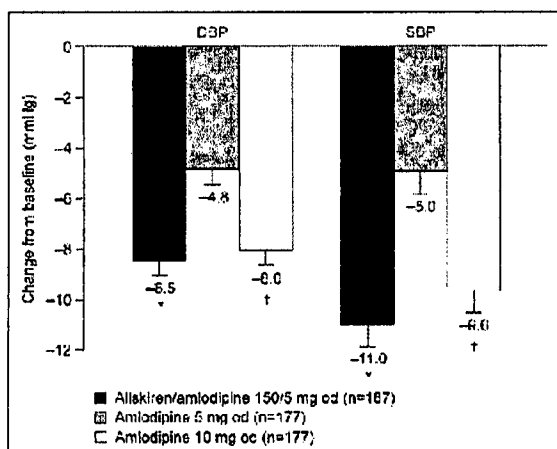


Figure 2. Change from baseline in mean sitting diastolic blood pressure (DBP) and mean sitting systolic blood pressure (SBP) at study end with once-daily aliskiren 150 mg/amlodipine 5 mg, amlodipine 5 mg, and amlodipine 10 mg in hypertensive patients not responding (DBP ≥ 90 mm Hg and < 110 mm Hg) to amlodipine 5 mg monotherapy (intent-to-treat population). ^a $P < 0.001$ for aliskiren 150 mg/amlodipine 5 mg vs amlodipine 5 mg. ^b $P = 0.002$ for amlodipine 10 mg vs amlodipine 5 mg (calculated retrospectively for completeness). Error bars indicate standard error of the mean.

150-mg/amlodipine 5-mg, amlodipine 5-mg, and amlodipine 10-mg groups, respectively). A high proportion of patients (523; 96.0%) completed the double-blind treatment period (Figure 1).

Baseline and demographic characteristics were comparable among the 3 treatment groups (Table I). The mean age of patients was 53.4 years, with 17.8% aged 65 years or older, and the mean

duration of HTN was 8 years. Most patients were Caucasian (69.4%) and 53.6% were men.

Efficacy

At study end, DBP level decreased from baseline by 8.5 ± 0.60 mm Hg (least squares mean $\pm$ standard error of the mean) in the group receiving once-daily aliskiren 150 mg/amlodipine 5 mg combination therapy compared with 4.8 ± 0.62 mm Hg in those continuing once-daily amlodipine 5 mg monotherapy (Figure 2). SBP level decreased by 11.0 ± 0.88 mm Hg with combination therapy compared with 5.0 ± 0.90 mm Hg in those receiving amlodipine 5 mg alone. The differences of 3.7 mm Hg in DBP level and 6.0 mm Hg in SBP level between the treatment groups were significant ($P < 0.001$). Patients receiving amlodipine 10 mg had a DBP reduction of 8.0 ± 0.62 mm Hg and a SBP reduction of 9.6 ± 0.90 mm Hg from baseline, which was not statistically significantly different from the reductions achieved with combination therapy ($P = .6167$ and $P = .2666$, respectively) (Figure 2).

BP reductions compared with baseline were evident after 1 week of randomized treatment in all groups (Figure 3). The mean DBP level was below the recommended target of < 90 mm Hg after 1 week of treatment in both the combination therapy group and the amlodipine 10-mg group. With combination therapy, mean SBP level was below the recommended target of < 140 mm Hg by week 3 and remained below this threshold at study end; mean SBP level was > 140 mm Hg at all time points

Table II. Overall Incidence of Adverse Events (AEs), Discontinuations Due to AEs, and AEs Occurring in $\geq 2\%$ of Patients During the Double-Blind Treatment Period (Safety Population)

ADVERSE EVENTS, NO. (%)	ONCE-DAILY ALISKIREN 150 MG/ AMLODIPINE 5 MG (N=187)	ONCE-DAILY AMLODIPINE 5 MG (N=179)	ONCE-DAILY AMLODIPINE 10 MG (N=178)
All-cause AEs	59 (31.6)	51 (28.5)	55 (30.9)
Treatment-related AEs	14 (7.5)	17 (9.5)	27 (15.2)
Discontinuation due to AEs	5 (2.7)	3 (1.7)	5 (2.8)
All-cause AEs occurring in $\geq 2\%$ of patients in any group			
Dizziness	2 (1.1)	0	5 (2.8)
Peripheral edema	4 (2.1)	6 (3.4)	20 (11.2)
Headache	5 (2.7)	9 (5.0)	3 (1.7)

in both the amlodipine 5-mg and 10-mg groups. DBP and SBP reductions from baseline were significantly greater by week 3 in the combination therapy group compared with the amlodipine 5-mg group ($P < .0001$ for both DBP and SBP), but not with the amlodipine 10-mg group.

The proportion of patients responding (defined as DBP level < 90 mm Hg at study end and/or a reduction of ≥ 10 mm Hg from baseline) was significantly higher with combination therapy than with amlodipine 5 mg (64.2% compared with 45.2%; $P = .0005$). The difference in response rate between the aliskiren 150-mg/amlodipine 5-mg group and the amlodipine 10-mg group was not significant ($P = .6373$; 59.9% of patients responded in the amlodipine 10-mg group).

The proportion of patients in each group in whom both systolic and diastolic BP control (BP level $< 140/90$ mm Hg) was achieved was significantly greater in the once-daily aliskiren 150-mg/amlodipine 5-mg group than in the once-daily amlodipine 5-mg group (42.8% vs 22.6%; $P < .0001$) but not significant when compared with the once-daily amlodipine 10-mg group ($P = .5229$; BP control was achieved in 37.9% of patients in the once-daily amlodipine 10-mg group).

In an exploratory analysis of a subgroup of patients in whom PRA was assessed ($n = 55$), combination therapy produced a 74.4% reduction in PRA at study end compared with baseline, a finding consistent with the known pharmacology of aliskiren. Amlodipine 10 mg ($n = 48$) increased PRA level by 58.0%, while in the amlodipine 5-mg group ($n = 48$), PRA level remained similar to baseline (9.9% decrease from baseline measurement followed 4 weeks of initial treatment with amlodipine 5 mg) (Figure 4).

Safety and Tolerability

During the double-blind treatment period, the number of patients experiencing new or worsened

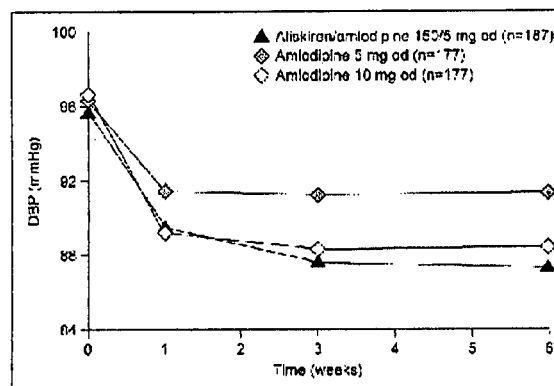


Figure 3. Time course of mean sitting diastolic blood pressure (DBP) with once-daily aliskiren 150 mg/amlodipine 5 mg, amlodipine 5 mg, and amlodipine 10 mg in hypertensive patients not responding to amlodipine 5 mg monotherapy (intent-to-treat population).

AEs was similar among groups receiving aliskiren 150 mg/amlodipine 5 mg (31.6%), amlodipine 5 mg (28.5%), and amlodipine 10 mg (30.9%) (Table II). Overall, a higher proportion of patients receiving amlodipine 10 mg experienced AEs that were suspected to be study drug-related than those receiving aliskiren 150 mg/amlodipine 5 mg or amlodipine 5 mg (15.2%, 7.5%, and 9.5%, respectively). Peripheral edema was the most frequently reported AE, occurring in a higher proportion of patients receiving amlodipine 10 mg than in those receiving combination therapy or amlodipine 5 mg (11.2%, 2.1%, and 3.4%, respectively), although the study was not powered to compare AE rates between the treatment groups (Table II). There were no marked differences among groups with regard to other AEs.

AEs classified as gastrointestinal disorders were slightly more frequent in the combination therapy group (5.9%) than in the amlodipine 5-mg (4.5%) and 10-mg (2.8%) groups, although most individual gastrointestinal AE terms occurred in only 1 or 2 patients per group and showed a similar

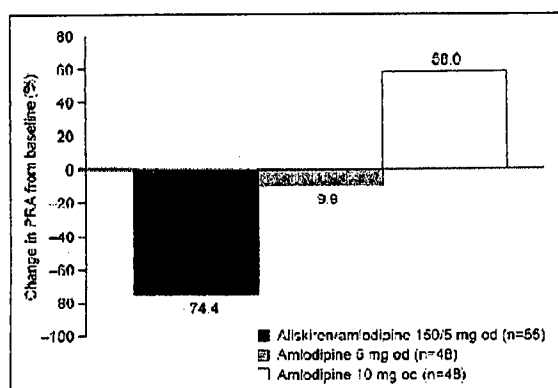


Figure 4. Change from baseline in plasma renin activity (PRA) at study end with once-daily aliskiren 150 mg/amlodipine 5 mg, amlodipine 5 mg, and amlodipine 10 mg in hypertensive patients not responding to amlodipine 5 mg monotherapy (preselected subsection of study population).

distribution across the 3 treatments. Infections and infestations were also more frequent in the combination group (7.5%) than in either amlodipine group (5 mg, 3.4%; 10 mg, 4.5%), although only upper respiratory tract infection (aliskiren 150 mg/amlodipine 5 mg, $n=2$; amlodipine 5 mg, $n=2$; amlodipine 10 mg, $n=1$) and sinusitis (aliskiren 150 mg/amlodipine 5 mg, $n=2$; amlodipine 5 mg, $n=0$; amlodipine 10 mg, $n=1$) were reported by more than 1 patient in any group. Overall, there were few discontinuations due to AEs, with a similar frequency in all 3 groups (Table II). The only AE to cause more than 1 patient to withdraw was peripheral edema, which occurred in the amlodipine 10-mg group ($n=3$).

There were 5 SAEs (1.1% [$n=2$], 0.6% [$n=1$], and 1.1% [$n=2$] of patients in the aliskiren 150 mg/amlodipine 5 mg, amlodipine 5 mg, and amlodipine 10 mg groups, respectively). These were diabetic hyperglycemic coma, diverticulitis, eye hemorrhage, myocardial infarction, and myocardial ischemia, none of which were considered to be related to study medication. No deaths were recorded.

Most laboratory parameters did not show meaningful changes during the study. Low potassium level (<3.5 mmol/L) was reported in 5 (2.7%), 13 (7.3%), and 10 patients (5.6%) receiving aliskiren 150 mg/amlodipine 5 mg, amlodipine 5 mg, and amlodipine 10 mg, respectively. None of the low potassium values were reported as AEs. Only one case of hyperlipidemia in the amlodipine 10-mg group and 1 case of hypokalemia in the amlodipine 5-mg group were suspected as being related to the study drug.

DISCUSSION

As shown in this study, aliskiren is effective in combination with amlodipine in patients with mild to moderate HTN in whom adequate BP control is not achieved with low-dose amlodipine monotherapy, but no more effective than 10 mg of amlodipine. The addition of aliskiren 150 mg to amlodipine 5 mg provided further reductions in BP level over those seen with amlodipine 5 mg monotherapy. The effect of adding aliskiren was the attainment of BP control (BP level $<140/90$ mm Hg) in almost twice as many patients compared with continued amlodipine 5 mg monotherapy. In approximately 64% of patients in the combination therapy group, goal DBP level (<90 mm Hg) was reached or at least a 10-mm Hg reduction in DBP level was observed at the end of the study, which according to findings of numerous prospective studies, has been reported to reduce the risk of cardiovascular mortality.²⁷ As noted, BP level changes with combination therapy were not significantly different from those with amlodipine 10 mg.

Higher doses of amlodipine have been associated with increased peripheral edema.²⁸ The current study findings are consistent with this observation, with the incidence of peripheral edema increasing by more than 3-fold in patients taking amlodipine 10 mg compared with those taking the 5-mg dose (11% compared with 3%); the study was not, however, designed to compare adverse event rates among the treatment groups. The occurrence of peripheral edema may require CCB therapy to be withdrawn,²⁹ but it may not prove to be a major problem in many patients: 3 patients in this study discontinued amlodipine therapy because of peripheral edema. Aliskiren in combination with amlodipine was well tolerated, consistent with previous observations of good tolerability at doses of up to 300 mg with aliskiren monotherapy.^{23,26}

Aliskiren monotherapy has previously been shown to reduce both DBP and SBP levels in patients with mild to moderate HTN; aliskiren 150 mg was comparable with irbesartan 150 mg.²³ In addition, in a 4-week study in patients with mild to moderate HTN, once-daily aliskiren 75 to 300 mg reduced BP level significantly compared with baseline and similarly to losartan 100 mg.²⁴ In an 8-week comparison study with HCTZ, aliskiren 150 and 300 mg produced significant BP reductions from baseline similar to those observed with HCTZ 12.5 and 25 mg, respectively.²⁶ To date, no study has compared the BP-lowering effects of aliskiren and CCB monotherapy.

Aliskiren has previously been investigated in combination with the diuretic HCTZ²⁶ and in combination with the ACEI ramipril.³⁰ Combination therapy with aliskiren and HCTZ or ramipril has provided significant additional reductions in BP level from baseline when compared with component monotherapies.^{26,30} The current study demonstrates that combination therapy with aliskiren and a CCB provides additional BP reductions from baseline over those seen with low-dose CCB monotherapy.

Aliskiren has been shown to suppress PRA when given as monotherapy,^{24,26} and this study confirms that this effect also occurs when aliskiren is given in combination with amlodipine. The results of this study also showed that the addition of aliskiren to amlodipine avoids the increase in PRA level observed with increasing the dose of amlodipine, while providing comparable improvements in BP level. While increasing the dose of amlodipine provided comparable BP reductions to aliskiren/amlodipine combination therapy, contrasting effects on PRA level were observed, with a 58% increase in PRA level in the amlodipine 10-mg group. Whether this is of clinical significance is still debatable. As noted, the use of agents such as ACEIs and ARBs increases renin levels but reduces BP levels and decreases cardiovascular morbidity and mortality.

CONCLUSIONS

The present study demonstrates that aliskiren, an orally active direct renin inhibitor, is effective in combination with amlodipine 5 mg in patients with mild to moderate HTN in whom sufficient BP control has not been achieved with amlodipine 5 mg monotherapy. The BP-lowering efficacy of combining these 2 agents that have complementary mechanisms of action is equivalent to that of the 10-mg dose of amlodipine, with some evidence of better tolerability. Additional studies to determine the outcome benefits of decreasing renin activity in hypertensive patients are under way.

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ALISKIREN AS ADD-ON TO AMLODIPINE PROVIDES SIGNIFICANT ADDITIONAL BLOOD PRESSURE LOWERING WITHOUT INCREASED OEDEMA ASSOCIATED WITH DOUBLING THE AMLODIPINE DOSE

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INTRODUCTION

- Calcium channel blockers (CCBs), such as amlodipine, are widely used in the treatment of hypertension.¹
- Combining CCBs with agents that suppress the renin system can provide treatment benefits – amlodipine in combination with angiotensin-converting enzyme (ACE) inhibitors showed improved blood pressure (BP) lowering over either therapy alone, with a lower incidence of oedema compared with increasing the CCB dose.²⁻⁴
- Aliskiren will be the first in a new class of orally effective direct renin inhibitors and is a potent inhibitor of human renin.⁵ In clinical studies, aliskiren monotherapy has been shown to achieve significant BP lowering in patients with mild-to-moderate hypertension,^{6,7} producing comparable BP reductions to the angiotensin receptor blocker (ARB) irbesartan⁸ and providing sustained BP lowering throughout the 24-hour dosing interval.⁹ Aliskiren has shown good safety and tolerability in clinical trials.^{6,7,9}
- Most patients do not respond adequately to initial antihypertensive therapy and will therefore require treatment with more than one agent in order to achieve adequate BP control.¹⁰ This study therefore addressed the practical issue for clinicians of the relative efficacy and safety of increasing the dose of the initial drug (amlodipine) in these patients, compared with adding a second drug (aliskiren).
- This study investigated the effect of adding aliskiren to amlodipine therapy in patients with hypertension who did not achieve full BP control with amlodipine. The study compared the efficacy, safety and tolerability of aliskiren 150 mg/amlodipine 5 mg combination therapy with continued treatment with amlodipine 5 mg or use of a higher (10 mg) amlodipine dose.

METHODS

Patients

Patients aged ≥ 18 years with mild-to-moderate hypertension (mean sitting diastolic BP [msDBP] 95–109 mmHg if untreated, or msDBP < 110 mmHg if treated) were enrolled in the study.

Study design

The study design is shown in Figure 1. Patients not responding to amlodipine 5 mg treatment (msDBP 90–109 mmHg) at the end of the run-in period were randomized equally to one of the three active treatments.

Figure 1. Study design



Efficacy assessments

The primary efficacy measure was the change in msDBP from baseline to study endpoint (week 6). Sitting BP was measured at trough using an automated BP monitor at 24 $\pm$ 3 hours post-dose.

Secondary efficacy measures included:

- change from baseline to endpoint in msSBP
- the proportion of patients responding to treatment (msDBP < 90 mmHg and/or ≥ 10 mmHg reduction in msDBP from baseline)
- the proportion of patients achieving BP control (BP $< 140/90$ mmHg).

Efficacy analyses were performed on the intent-to-treat population. Changes in BP were assessed using analysis of covariance (ANCOVA) models; BP control and responder rates were assessed using logistic regression models.

Safety and tolerability assessments

Safety assessments included monitoring and recording adverse events (AEs), laboratory evaluations, physical examinations and ECGs.

RESULTS

Patient characteristics

Of the 762 patients enrolled in the study, 169 responded to initial therapy with amlodipine 5 mg and 48 were not randomized for other reasons. The remaining 545 patients were randomized to aliskiren 150 mg/amlodipine 5 mg ($n = 187$), amlodipine 5 mg ($n = 180$) or amlodipine 10 mg ($n = 178$). In all, 523 patients (66%) completed the study.

Baseline characteristics were comparable between the treatment groups (Table 1).

Table 1. Patient demographic and baseline clinical characteristics (randomized patients)

Characteristic	Aliskiren 150 mg/ amlodipine 5 mg (n = 187)	Amlodipine 5 mg (n = 180)	Amlodipine 10 mg (n = 178)
Age, years	52.7 (11.9)	53.7 (10.9)	54.0 (10.6)
Female, n (%)	82 (43.9)	85 (47.2)	86 (48.3)
Race, n (%)			
Caucasian	128 (68.4)	128 (71.1)	122 (68.5)
Black	34 (18.2)	33 (18.3)	29 (16.3)
Asian	21 (11.2)	18 (10.0)	22 (12.4)
Other	4 (2.1)	1 (0.6)	5 (2.8)
Duration of hypertension, years	8.5 (7.6)	8.0 (6.8)	8.2 (7.5)
msDBP, mmHg	95.7 (4.4)	96.2 (4.8)	96.5 (4.5)
msSBP, mmHg	150.5 (11.1)	150.5 (13.2)	150.8 (12.0)
BMI, kg/m ²	30.2 (5.5)	30.2 (5.7)	30.4 (5.7)
Metabolic syndrome,* n (%)	80 (42.8)	71 (39.4)	74 (41.6)
Diabetes, n (%)	26 (13.9)	28 (14.4)	27 (15.2)

Values are mean (SD), unless otherwise indicated.

*Metabolic syndrome is defined by the presence of three or more of the following: 1. Waist circumference > 102 cm for men or > 88 cm for women; 2. Triglycerides ≥ 150 mg/dL (≥ 1.69 mmol/L); 3. HDL cholesterol < 40 mg/dL (< 1.04 mmol/L) for men or < 50 mg/dL (< 1.29 mmol/L) for women; 4. SBP ≥ 130 mmHg or DBP ≥ 85 mmHg; 5. Fasting glucose ≥ 110 mg/dL (≥ 6.1 mmol/L).

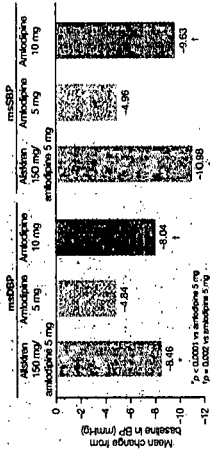
BMI, body mass index; HDL, high-density lipoprotein; msDBP, mean sitting diastolic blood pressure; msSBP, mean sitting systolic blood pressure.

Efficacy

Aliskiren 150 mg/amlodipine 5 mg produced statistically superior reductions in msDBP compared with amlodipine 5 mg ($p < 0.0001$; Figure 2) and comparable reductions to those observed with amlodipine 10 mg ($p = 0.9167$).

Aliskiren 150 mg/amlodipine 5 mg produced statistically superior reductions in msSBP compared with amlodipine 5 mg ($p < 0.0001$; Figure 2) and numerically greater reductions compared with amlodipine 10 mg ($p = 0.2866$).

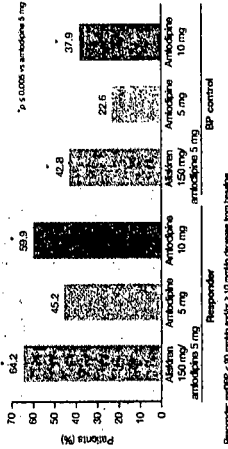
Figure 2. Mean changes in msDBP and msSBP from baseline to endpoint (intent-to-treat population)



After 1 week of treatment, BP reductions were evident in all three groups and mean msDBP values were < 90 mmHg in both the aliskiren 150 mg/amlodipine 5 mg combination and amlodipine 10 mg groups. At week 3, reductions in msDBP and msSBP were significantly ($p < 0.0001$) greater with aliskiren 150 mg/amlodipine 5 mg compared with amlodipine 5 mg.

Responder rates and the proportion of patients achieving BP control are shown in Figure 3.

Figure 3. Responder rates and BP control rates at endpoint (intent-to-treat population)



Safety and tolerability

The overall incidence of AEs was similar across all three groups, although treatment-related AEs were more frequent with amlodipine 10 mg than with amlodipine 5 mg or aliskiren 150 mg/amlodipine 5 mg (Table 2).

Peripheral oedema was reported more frequently with amlodipine 10 mg (11.2%) than with amlodipine 5 mg (3.4%) or combination therapy (2.1%). There were few discontinuations due to AEs (Table 2). The only AE leading to more than one withdrawal was peripheral oedema in the amlodipine 10 mg group ($n = 3$).

Table 2. Summary of adverse event data during double-blind treatment

Adverse event	Aliskiren 150 mg/ amlodipine 5 mg (n = 187)	Amlodipine 5 mg (n = 178)	Amlodipine 10 mg (n = 178)
Any AE, n (%)	59 (31.6)	51 (28.5)	55 (30.9)
Any treatment-related AE, n (%)	14 (7.5)	17 (9.5)	27 (15.2)
Discontinuation due to AEs, n (%)	5 (2.7)	3 (1.7)	5 (2.8)
Serious AEs, n (%)	2 (1.1)	1 (0.6)	2 (1.1)
AEs occurring in $\geq 2\%$ of patients in any group, n (%)			
Dizziness	2 (1.1)	0 (0.0)	5 (2.8)
Headache	5 (2.7)	9 (5.0)	3 (1.7)
Peripheral oedema	4 (2.1)	6 (3.4)	20 (11.2)

CONCLUSIONS

- The addition of aliskiren 150 mg to amlodipine 5 mg provided effective BP control in patients with mild-to-moderate hypertension who had not achieved BP control with amlodipine 5 mg alone.
- Aliskiren 150 mg/amlodipine 5 mg produced comparable BP reductions to amlodipine 10 mg. The addition of aliskiren 150 mg to amlodipine 5 mg provided a clinically relevant additional msSBP/msDBP reduction of 6.0/3.6 mmHg over amlodipine 5 mg monotherapy.
- Responder and BP control rates with combination therapy were significantly higher than with amlodipine 5 mg, and comparable to those with amlodipine 10 mg.
- Although amlodipine 10 mg showed similar efficacy to aliskiren 150 mg/amlodipine 5 mg, amlodipine 10 mg was associated with an increased incidence of peripheral oedema (11.2%), which was not observed with aliskiren 150 mg/amlodipine 5 mg (2.1%). Three patients (1.7%) discontinued treatment with amlodipine 10 mg due to oedema.
- Aliskiren 150 mg/amlodipine 5 mg combination therapy was well tolerated, with a similar tolerability profile to amlodipine 5 mg alone. This is consistent with the placebo-like tolerability exhibited by aliskiren monotherapy in previous clinical trials.^{6,9}

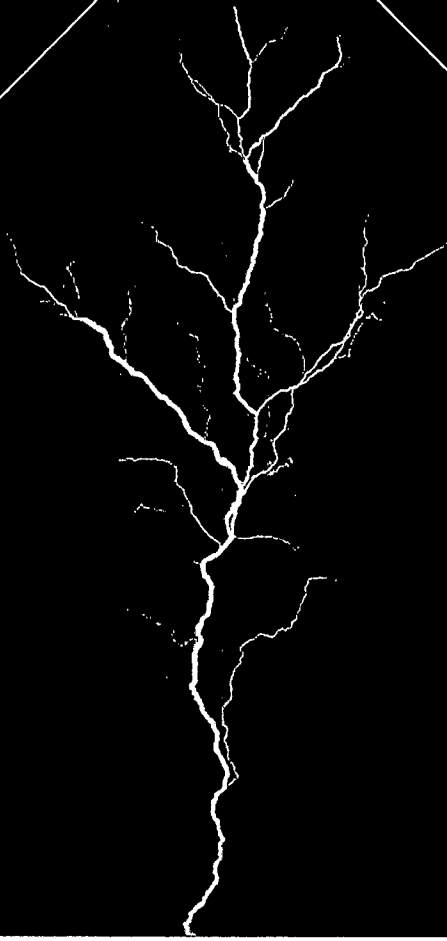
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7. Oh H-H, et al. Aliskiren, an oral renin inhibitor, provides dose-dependent efficacy and placebo-like tolerability in patients with hypertension. *J Am Coll Cardiol* 2004;43:123-30.
8. Aliskiren Combination Study Group. Aliskiren, a novel orally active renin inhibitor, provides superior blood pressure control in patients with hypertension. *J Clin Hypertens* 2005;9:293-299.
9. Vlahakis A, et al. The novel oral renin inhibitor aliskiren provides effective blood pressure control in patients with hypertension when used alone or in combination with hydrochlorothiazide. *J Clin Hypertens* 2005;9:200-208.
10. Guidelines for the Management of High Blood Pressure. Hypertension 2003;42:1258-52.

Poster 736 presented at the World Congress of Cardiology 2005, 8-8 September, Barcelona, Spain. The study was funded by Novartis Pharmaceuticals AG.

Anexo 2

Exposición de Speedel, presentada en el Día R & D en Marzo de 2008



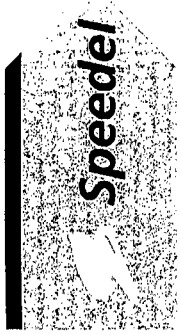
**Aldosterone Synthase Inhibitors
SPP2000**

**Dr. Christoph Schumacher
Senior Scientific Officer**

at the heart of value creation™

- **A New Aldosterone Therapy**
Rationale & Expectations
- **The SPP2000 Drug Discovery Program**
Rationale & Achievements
- **The Profile of SPP2745**
Key Data Sets
- **The Profile of SPP2745**
Expectations versus Achievements
- **SPP2745 – Next Steps**
Clinical Plans





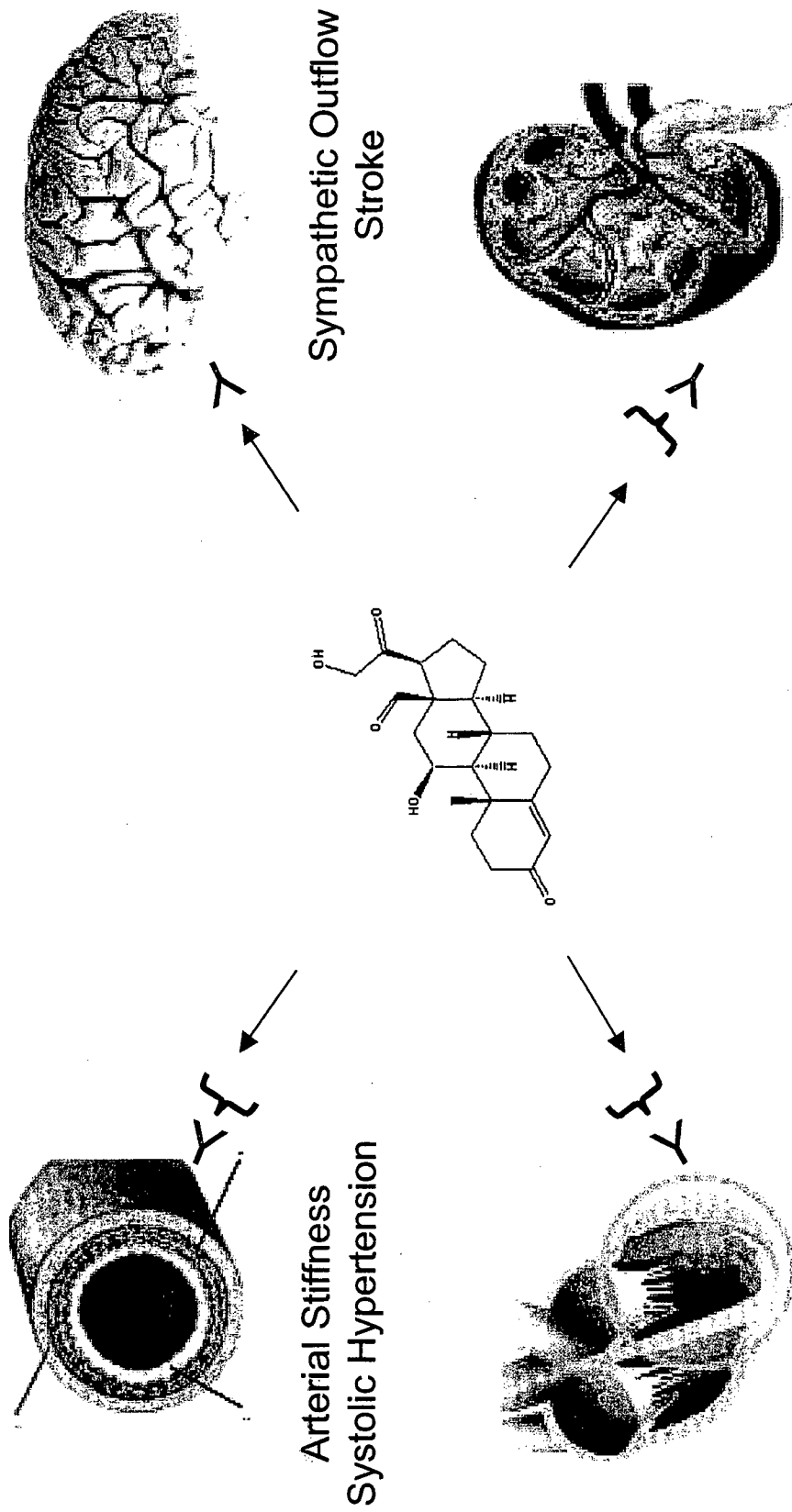
A New Aldosterone Therapy

Rationale & Expectations

SPP2000: Christoph Schumacher, I R&D Day , 31 March 2008
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Speedel Benefits of Aldosterone Blockade in CV Disease



Speedel/ Current Mineralocorticoid Receptor Blocking Therapies

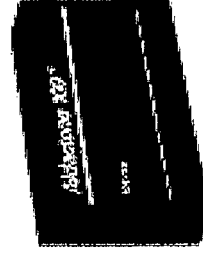
Spironolactone – Aldactone ®

Indication:

- Essential Hypertension
- Hyperaldosteronism
- Hypokalemia
- Oedema due to heart failure
- Liver cirrhosis and Ascites
- Nephrotic Syndrome

Adverse Reactions:

- Gynecomastia
- Impotence
- Hyperkalemia
- Agranulocytosis
- Diarrhea, cramps
- Decreased Libido
- Postmenopausal bleeding



Eplerenone – Inspra ®

Indication:

- Congestive Heart Failure post MI
- Hypertension (US only)

Warning:

- Hyperkalemia

Adverse Reactions:

- Gynecomastia
- Mastodynia
- Vaginal bleeding

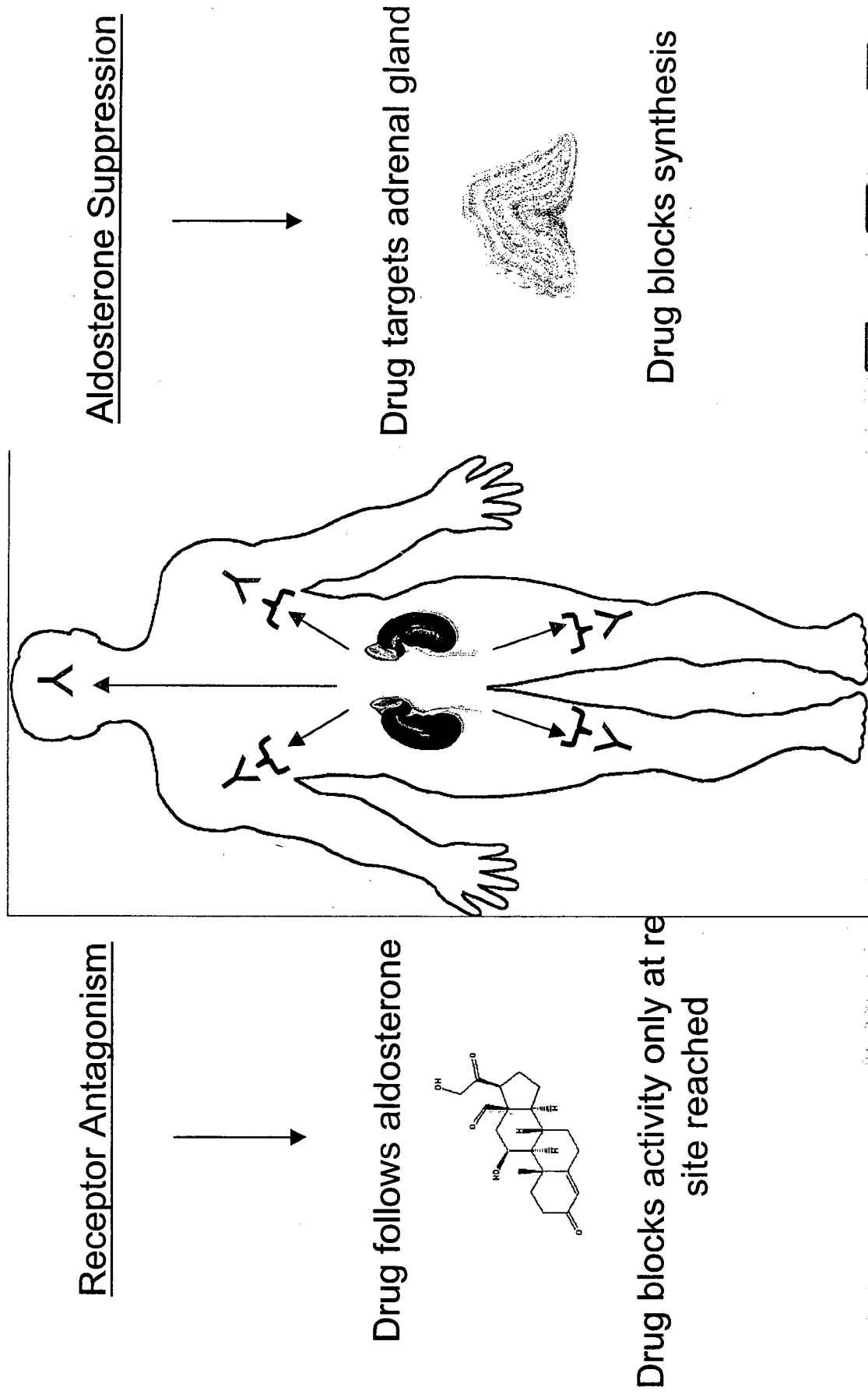


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Ref: PDR, Arzneimittelkompendium



Speedel/ „Get rid of Aldosterone or block its effects ? “



Speedel

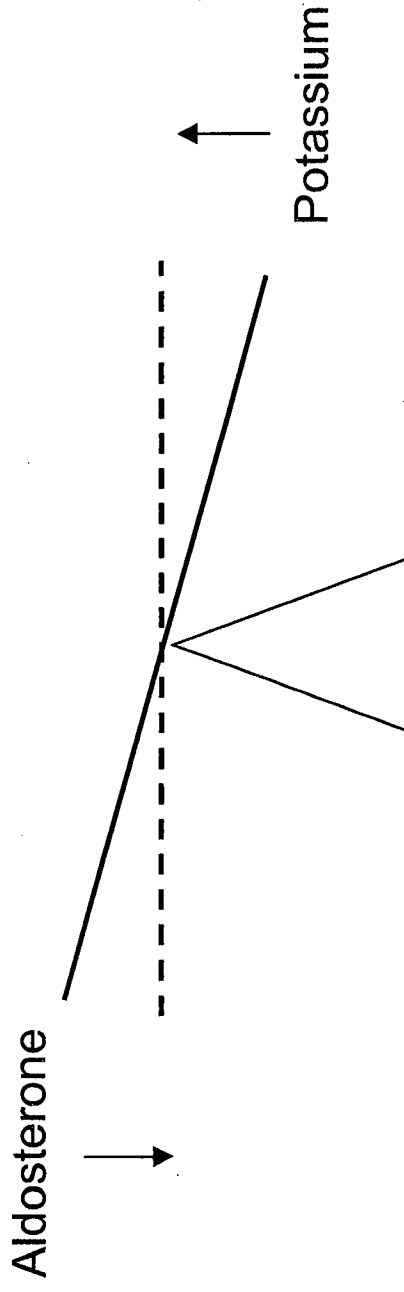
Normalize the Aldosterone / Potassium Balance

Causes of Hyperkalemia (> 5.5 mM):

- Renal impairment
- Renal tubular acidosis
- Insulin deficiency
- Aldosterone deficiency

Causes of Hypokalemia (< 3.5 mM):

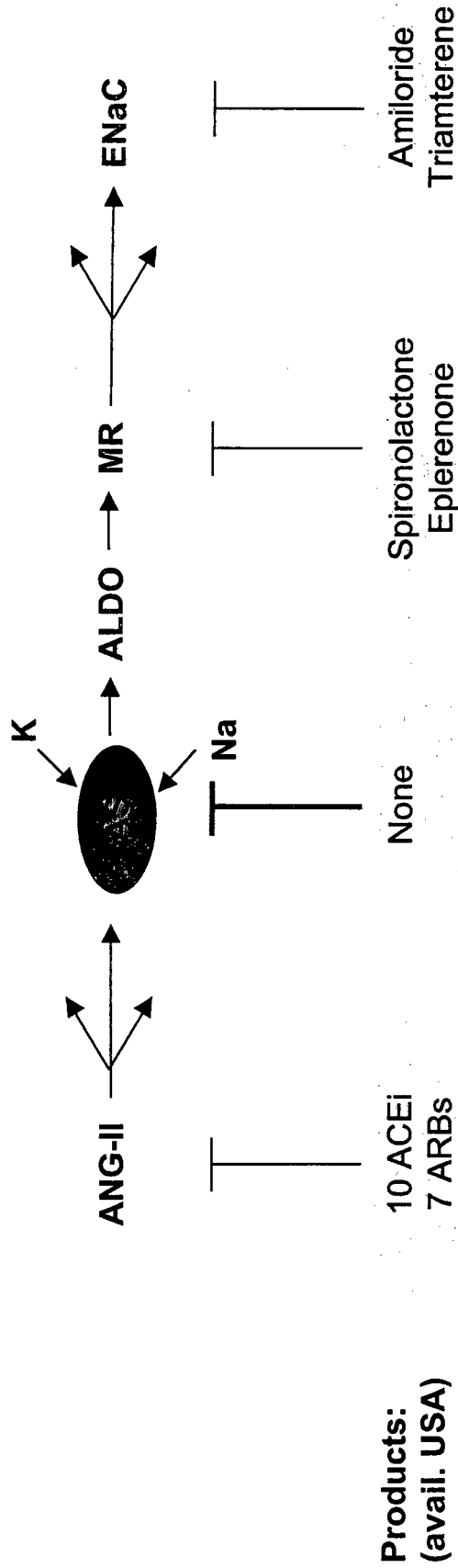
- Potassium depletion
- Increased sodium intake
- Metabolic alkalosis
- Aldosterone excess



Aldosterone / Potassium Balance



Competitive Landscape for Aldosterone Therapies



Products: (avail. USA)	10 ACEi 7 ARBs	None	Spironolactone Eplerenone	Amiloride Triamterene
Limitations:	Responsiveness	?	Gynecomastia Hyperkalemia	Hyperkalemia Rash
Sales (\$):	17 bn ²	-	0.34 bn ¹	0.58 bn ¹
R&D Projects:	TAK491 (III) SPP (II) MK8141 (II)	LCI699 (II) Worch (PC)	?	-

¹ IMS Health for 2006

² Datamonitor Report DMHC2086 for 2004



Speedel Why Aldosterone Synthase Inhibitor Therapy?

- The awareness of aldosterone's pathophysiology is expanding
- The diagnosis of hyperaldosteronism is increasing
- Current aldosterone blocking agents are not satisfactory
- A medical need for alternative aldosterone therapies is apparent
- An aldosterone-suppressing agent should offer new benefits
- Strategic fit with Speedel's pipeline and expertise



Speedel Expectations for an Ideal Aldosterone-Suppression Therapy

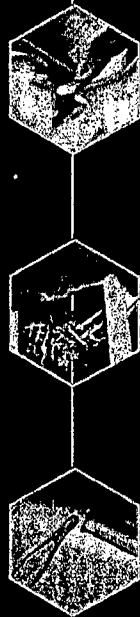
- Aldosterone suppression without endocrine adverse events
- No impairment of stress response
- Potent yet controlled aldosterone / potassium normalization
- Protective of kidney (PU), heart (LVH), vasculature (Stiffness)
- Ability to combine with an ACEi, ARB or Renin inhibitor

° elaborated by the SPP2000 clinical advisory panel consisting of G. Williams, J. Funder, B. Pitt, N. Madias, F. Luft, P. Mulatero, M. deGasparo and R. Steele
in Basel, October 8, 2007



SPP2000 Drug Discovery Program

Rationale & Achievements

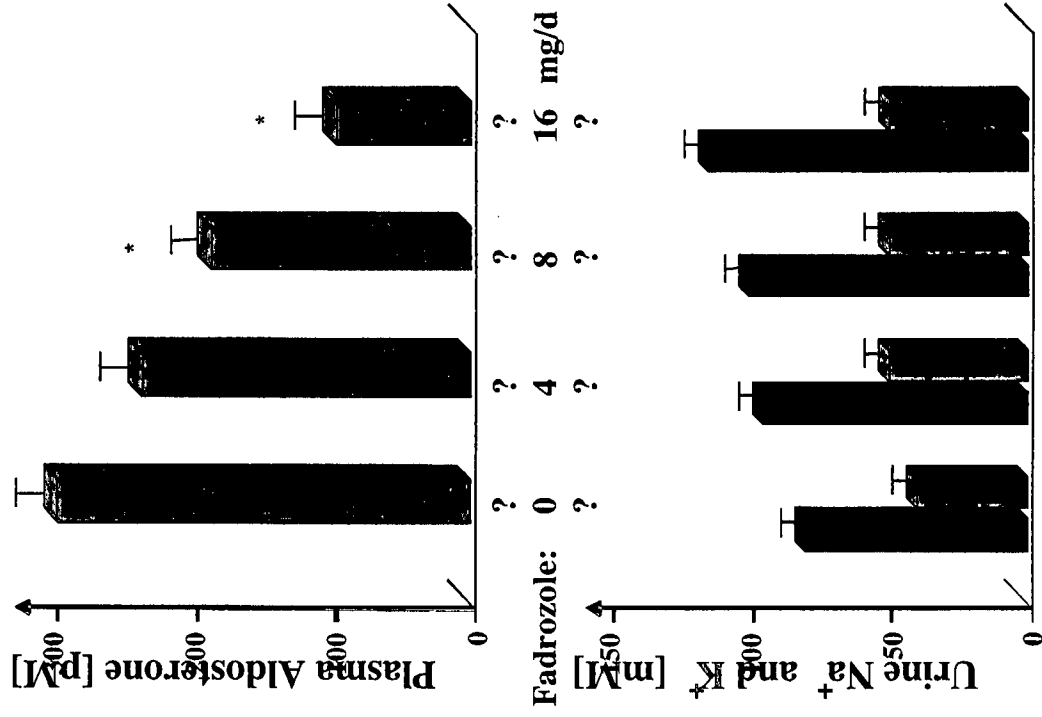


Speedel/

Aldosterone Synthase as a Drug Target

- Rate-limiting enzyme of aldosterone biosynthesis
- Genetically linked to cardiovascular disease
- Small non-steroidal compounds available that target the catalytic site of the enzyme
- No species-specificity in the rat, dog and human
- Clinical proof of the pharmacological principle shown
- Target represents a new therapeutic mode of action





Richard Santen: JCEM 73, 99-106, 1991
CGS16949 Dose-Escalation Study
in postmenopausal _ with breast cancer



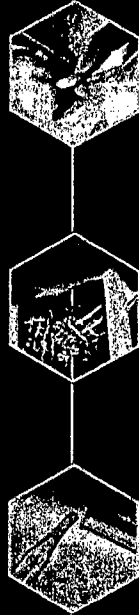
Speedel/ The SPP2000 Program: Achievements to Date

- Defined 8 patent series as discovery platform
- 13 substance patent families in public domain
- Drug discovery flowchart with 14 go/no go decision points
- Focus on 4 lead series for optimization
- 6 compounds accomplished entire flowchart to date
- First lead compound selected for regulatory development



The Profile of SPP2745

Key Data Sets



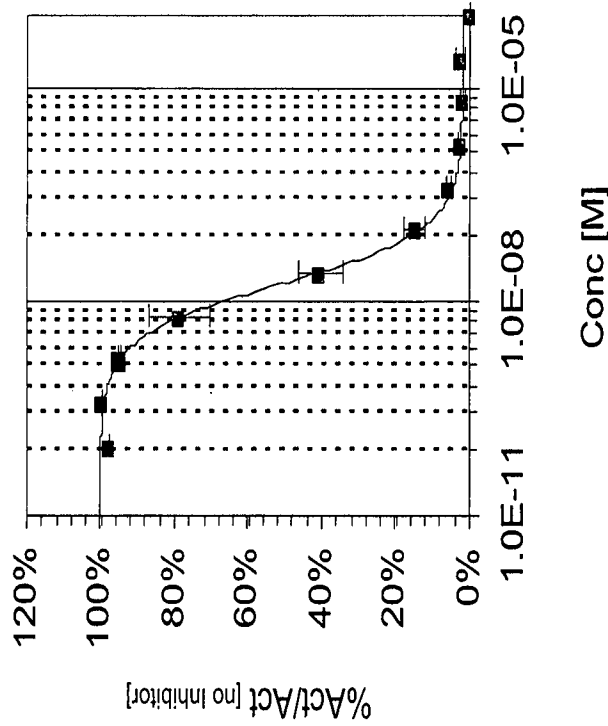
- Potent aldosterone suppression *in vitro* and *in vivo*
- High target selectivity
- Promising pharmacokinetic-pharmacodynamic properties
- Long-term efficacy in cardiovascular disease models
- Opportunity for fixed-dose combinations with RAS inhibitors
- Promising endocrine safety in exploratory toxicity studies



Speedel/ SPP2745: Potent Aldosterone Suppression

In vitro Aldosterone Synthase Inhibition

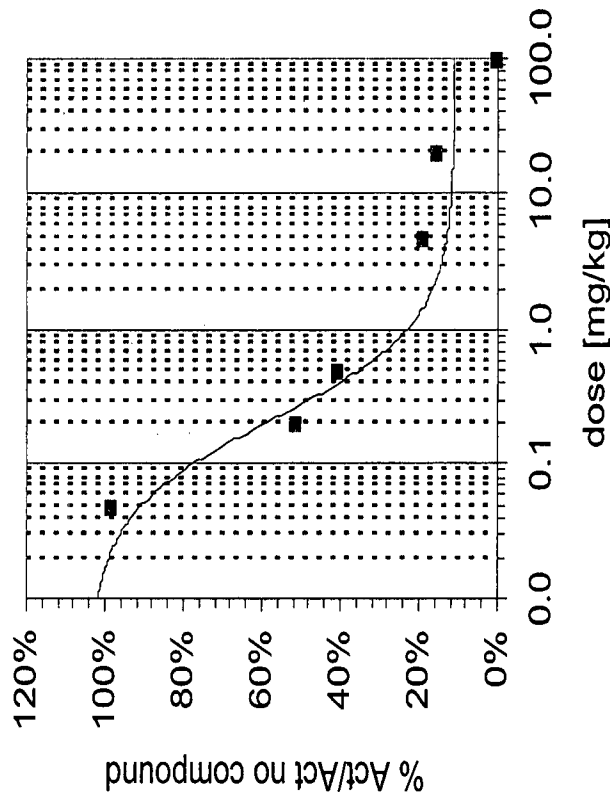
(H295R cell assay)



$IC_{50} = 20 \text{ nM}$

In vivo Plasma Aldosterone Suppression

(ACTH prestimulated rat model)



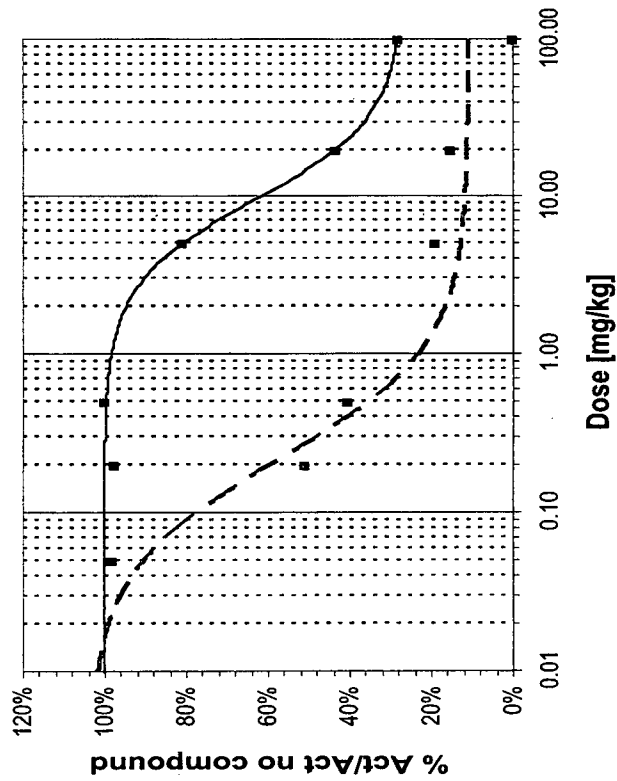
$ED_{50} = 0.2 \text{ mg/kg}$
 $ED_{80} = 0.7 \text{ mg/kg}$



Speedel SPP2745: High Target Selectivity – Stress Hormones

In vivo Plasma Corticosterone Suppression

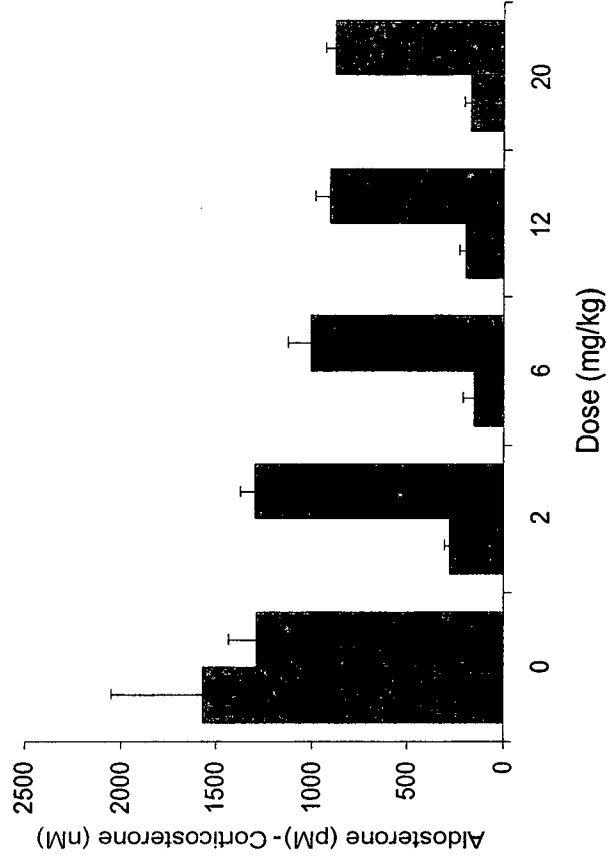
(ACTH-prestimulated rat model)



ED₅₀ = 10 mg/kg
Selectivity = 50-fold

In vivo Adrenocorticotropin-Stress Test

(ACTH-stressed rat model)

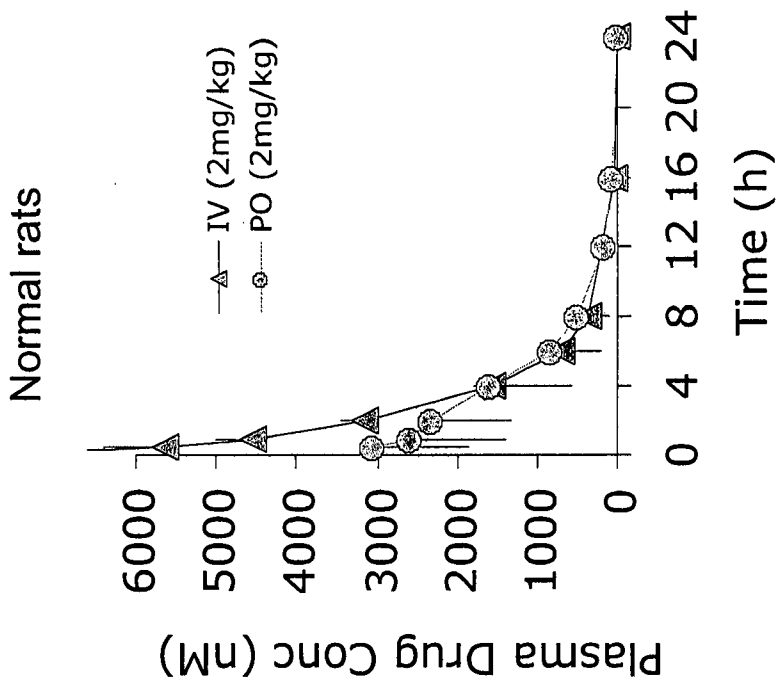


No blunting of stress response

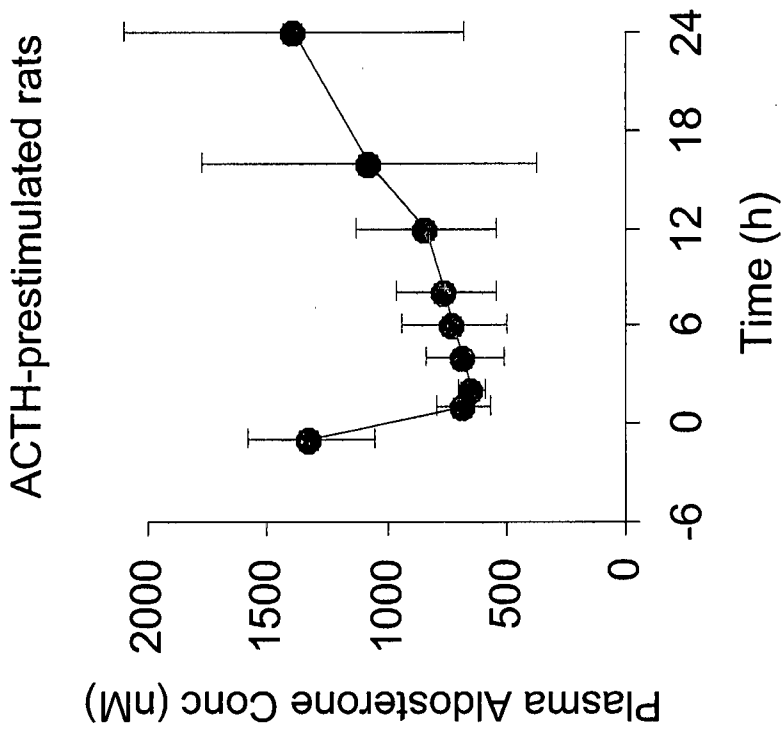


Speedel

SPP2745: Pharmacokinetics - Pharmacodynamics



BAV = 76 %

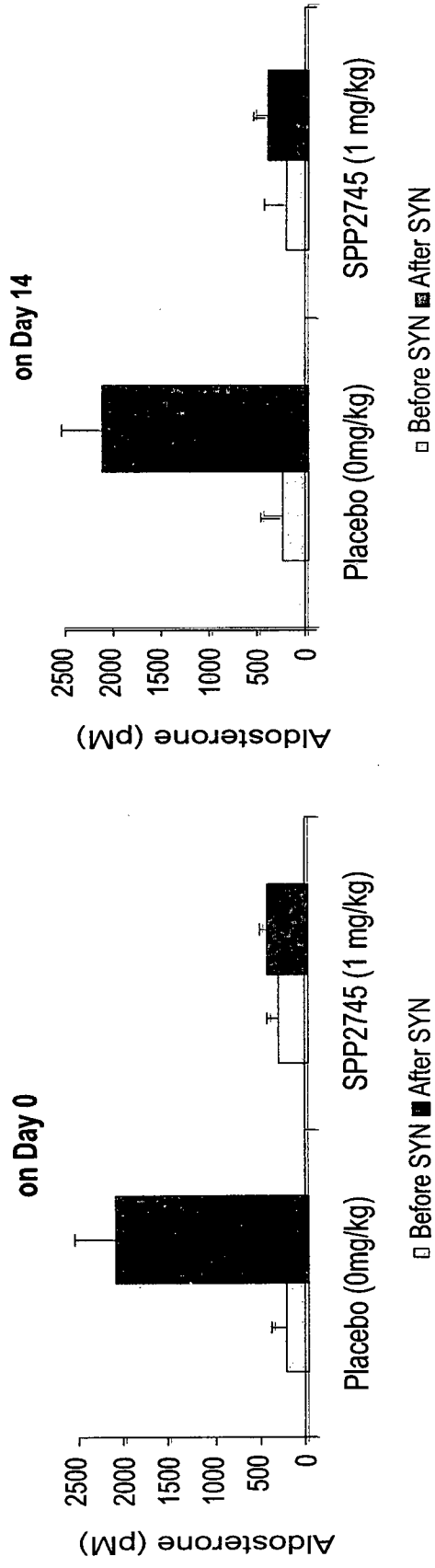


Long duration
of action



Speedel/ SPP2745: Consistent Duration of Action

Subpressor angiotensin II-infused rat model



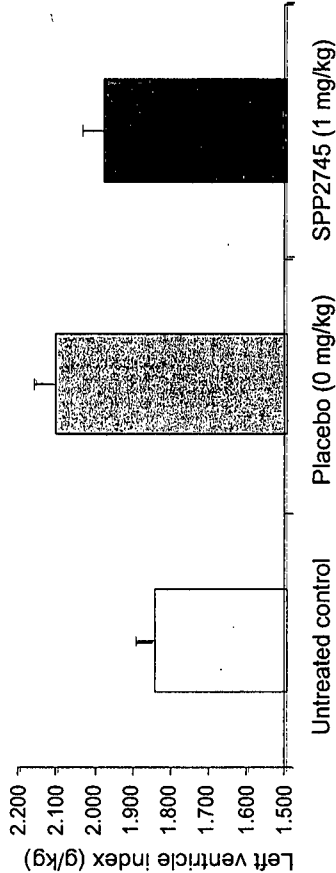
No development of tachyphylaxis



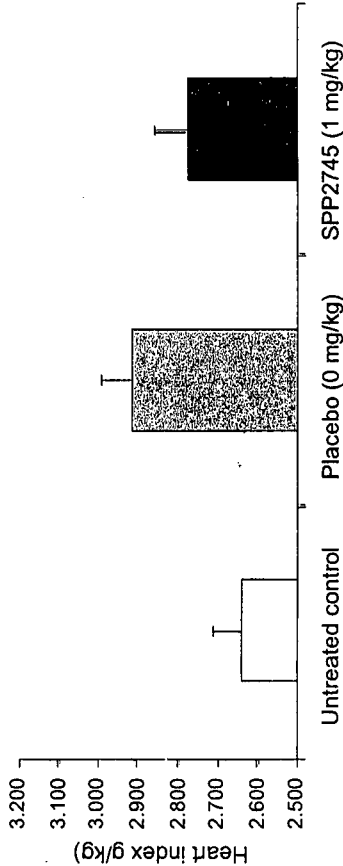
Speedel

SPP2745: Long-term Efficacy in Heart Disease Model

Subpressor angiotensin II-infused rat model



Reduced left ventricular hypertrophy

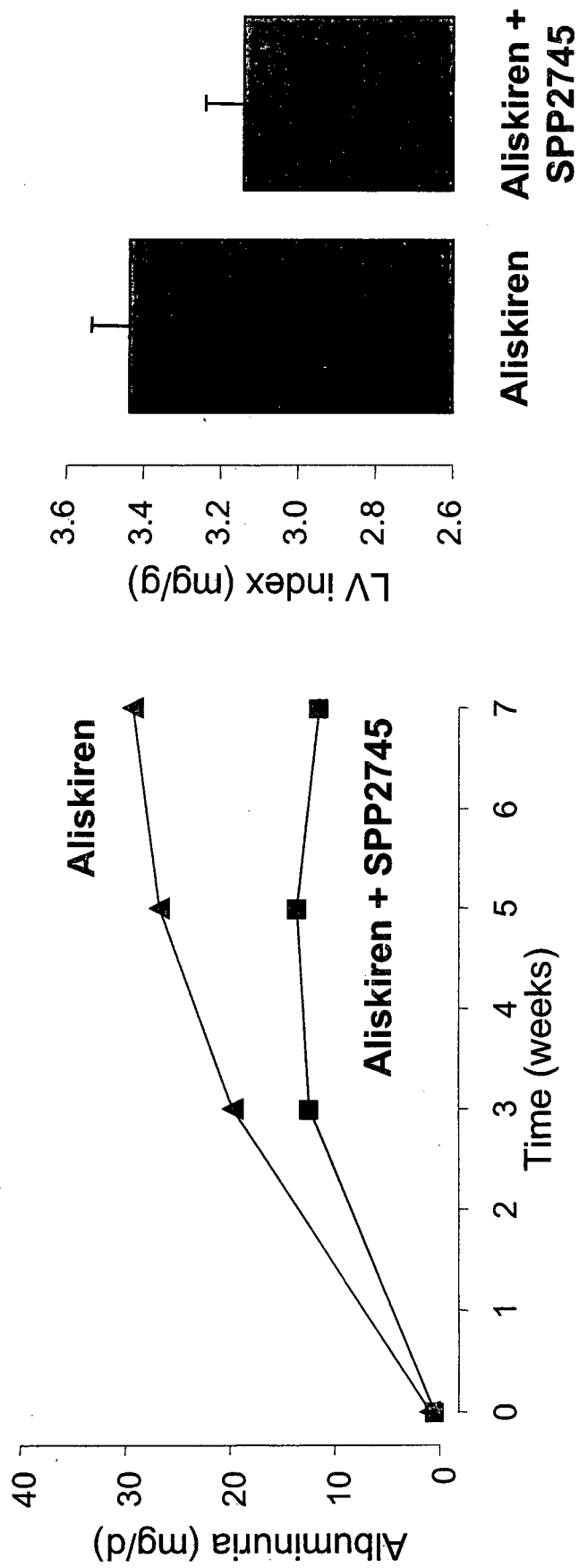


Reduced cardiac hypertrophy



Speedel/ SPP2745: Combination with Renin Inhibitors

Proteinuria and cardiac hypertrophy in malignant hypertensive rats (dTGR model)

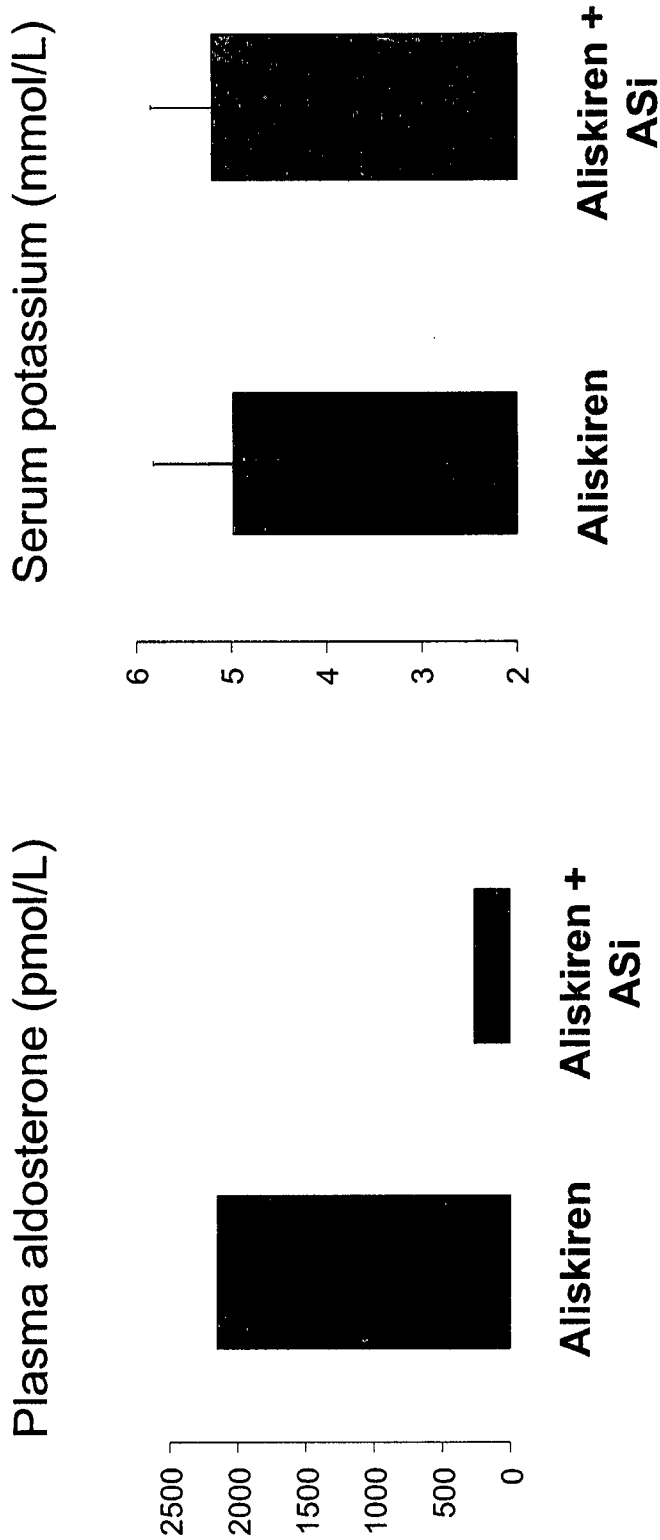


Opportunity for Fixed Dose Combinations with RASi



Speedel Plasma Aldosterone Suppression & Potassium Changes

Plasma aldosterone and potassium in malignant hypertensive rats (dTGR)



No apparent development of hyperkalemia in rodents upon RASi



No clinically relevant interactions with:

- Spectrumscreen™ (binding to 169 enzymes and receptors)
- Cytochrom P450 Enzymes (drug-drug interactions)
- hERG channel (cardiac safety)
- AMES test (mutagenicity/cytotoxicity)



Two week exploratory toxicity testing in rats

General safety:

Tolerability: Body weight, liver, heart, thyroid gland

Endocrine safety:

Stress hormone axis: Pituitary gland, adrenal gland, kidney

Sex hormone axis: Prostate, testis, seminal vesicles,
uterus, ovary, muscle

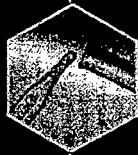
Good *in vitro* selectivity profile confirmed *in vivo*

Good drug tolerability upon sub-chronic administration



The Profile of SPP2745

Expectations versus Achievements



 **Speedel**

Speedel Expectations for an Ideal Aldosterone-Suppression Therapy

- Aldosterone suppression without endocrine adverse events
- No impairment of stress response
- Potent yet controlled aldosterone / potassium normalization
- Protective of kidney (PU), heart (LVH), vasculature (Stiffness)
- Ability to combine with an ACEi, ARB or Renin inhibitor

° elaborated by the SPP2000 clinical advisory panel consisting of G. Williams, J. Funder, B. Pitt, N. Madias, F. Luft, P. Mulatero, M. deGasparo and R. Steele
in Basel, October 8, 2007



Speede!

To date SPP2745 demonstrates:

Potent suppression of plasma aldosterone levels via new MoA

Aldosterone / Potassium Normalization

No impairment of the stress-response

Good endocrine selectivity

Efficacious in animal models of cardiovascular disease

Kidney – Decrease in proteinuria

Heart – Decrease in LVH

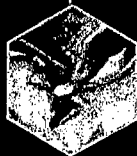
Vasculature – Improvement of stiffness

POC for fixed-dose combinations with ACEi, ARBs or RIs



SPP2745 – Next Steps

Clinical Plans



Speedel

SPP2745: The Next Steps

Regulatory Toxicology Program

CTA/IND

Single & Multiple Dosing in Healthy Volunteers

Phase I

Proof of Concept Studies in Various Indications

Phase II



Mode of Action allows establishment of therapeutic proof of principle:

○ Surrogate markers of efficacy

Plasma aldosterone, potassium, 18-hydroxy corticosterone

Urinary aldosterone, tetrahydroaldosterone, sodium, potassium

○ Surrogate markers of safety/tolerability

Plasma potassium

Orthostatic hypotension, nausea, dizziness



Speedel/ SPP2745: Phase II – Indication Development

<u>Plasma Parameters</u>	<u>Indication</u>	<u>Market (US)</u>
 Aldosterone ↑  Renin activity ↓	Primary Aldosteronism	Prevalence: 1'435'000 Diagnosed: 143'000 ¹
 Aldosterone ↑  Renin activity ↓	Treatment-resistant Hypertension	Prevalence: 3-30% Diagnosed: 10% ^{2,3}
 Aldosterone —  Renin activity ↓	Hypertension & Complications	

¹ Smartanalyst Report for Speedel 2008
² Comprehensive Hypertension - Mosby
³ Datamonitor Report DMHC1786 2002



Speedel

“We believe that
aldosterone synthesis inhibition can
be of great benefit to cardiovascular
health by normalizing the
aldosterone / Na^+ - K^+ balance”

Speedel Aldo Team 2008

SPP2000: Christoph Schumacher, I R&D Day , 31 March 2008
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37

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Anexo 3

Artículo "*Renin inhibition reduces hipercolesterolemia-induces atherosclerosis in mice*" de Lu y colaboradores, publicado en el Journal of Clinical Investigation



Renin inhibition reduces hypercholesterolemia-induced atherosclerosis in mice

Hong Lu,¹ Debra L. Rateri,¹ David L. Feldman,² Richard J. Charnigo Jr.,³ Akiyoshi Fukamizu,⁴ Junji Ishida,⁴ Elizabeth G. Oesterling,⁵ Lisa A. Cassis,^{1,6} and Alan Daugherty^{1,6}

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The role of the renin angiotensin system (RAS) in atherosclerosis is complex because of the involvement of multiple peptides and receptors. Renin is the rate-limiting enzyme in the production of all angiotensin peptides. To determine the effects of renin inhibition on atherosclerosis, we administered the novel renin inhibitor aliskiren over a broad dose range to fat-fed LDL receptor-deficient (*Ldlr*^{-/-}) mice. Renin inhibition resulted in striking reductions of atherosclerotic lesion size in both the aortic arch and the root. Subsequent studies demonstrated that cultured macrophages expressed all components of the RAS. To determine the role of macrophage-derived angiotensin in the development of atherosclerosis, we transplanted renin-deficient bone marrow to irradiated *Ldlr*^{-/-} mice and observed a profound decrease in the size of atherosclerotic lesions. In similar experiments, transplantation of bone marrow deficient for angiotensin II type 1a receptors failed to influence lesion development. We conclude that renin-dependent angiotensin production in macrophages does not act in an autocrine/paracrine manner. Furthermore, *in vitro* studies demonstrated that coculture with renin-expressing macrophages augmented monocyte adhesion to endothelial cells. Therefore, although previous work suggests that angiotensin peptides have conflicting effects on atherogenesis, we found that renin inhibition profoundly decreased lesion development in mice.

Introduction

There is evolving complexity in the renin angiotensin system (RAS) because of the identification of a spectrum of bioactive angiotensin peptides including AngII, AngIII, AngIV, and Ang1-7 (1-3). There is also an increasing number of receptors that have been recognized to interact with these angiotensin peptides including AngII type 1 (AT1) receptors, AT2 receptors, AT4 receptors, and *mas* receptors (4, 5).

AngII has long been considered the major bioactive effector molecule of the RAS and has been recognized as a major contributor to hypercholesterolemia-induced atherosclerosis (6, 7). Chronic infusion of AngII augments the size of atherosclerotic lesions in LDL receptor-deficient (*Ldlr*^{-/-}) and *apoE*^{-/-} mice (8-11). Conversely, pharmacological antagonists of specific components of the RAS have decreased the extent of atherosclerosis. Many studies have demonstrated that angiotensin-converting enzyme (ACE) inhibitors and AT1 receptor antagonists reduce atherosclerotic lesion size (7, 12-15). However, a mechanistic basis for the beneficial effects observed with ACE inhibitors and AT1 receptor antagonists has not been defined. It is currently unclear whether the efficacy is due to inhibition of AngII production, the effect of AngII on AT2 receptors, or the continued presence of other bioactive angiotensin peptides that interact with receptors other than AT1 receptors.

Renin is not only the rate-limiting enzyme in AngII formation, but also shows remarkable substrate specificity for angiotensinogen, making it an attractive target for therapeutic inhibition of all angiotensin peptides. Thus, renin inhibition may provide a unique approach to define the role of angiotensin peptides in the development of atherosclerosis.

In addition to the systemic RAS, it has now been recognized that several tissues express components of the RAS and possess the ability to synthesize AngII (16-20). With regard to atherosclerosis, all components of the classical RAS pathway have been identified in lesions and are associated predominantly with macrophages (6, 21-23). The presence of mRNA and protein for RAS components has also been detected in cultured macrophages (24-26). Although there are several potential sources of angiotensin peptides, the relative contribution to lesion formation of systemic versus local sources has not been defined.

Recent studies have demonstrated that AT1a receptors have a profound effect on atherosclerosis generated by hypercholesterolemia alone (6, 7) or in combination with AngII infusion (11). Angiotensin peptides, particularly AngII, have well-defined effects on several cell types present in atherosclerosis. These effects are particularly prominent for smooth muscle cells and endothelium, with less prominent effects on macrophages (11, 27, 28). To date, the contribution of AT1a receptors on specific cell types to the atherogenic process has not been determined unequivocally.

The purpose of the current study was to determine whether whole-body inhibition of renin decreases atherosclerotic lesion size. Furthermore, we determined whether macrophage expression of

Nonstandard abbreviations used: ACE, angiotensin-converting enzyme; AT1 receptor, AngII type 1 receptor; HPF, high-power field; LDLR, LDL receptor; RAS, renin angiotensin system.

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Table 1
Effects of renin inhibition in *Ldlr*^{-/-} mice fed a fat-supplemented diet for 12 weeks

	Vehicle	Aliskiren (2.5 mg/kg/d)	Aliskiren (25 mg/kg/d)	Aliskiren (50 mg/kg/d)
<i>n</i>	15	14	16	6
Body wt (g)	32.3 ± 1.3	28.9 ± 1.4	29.3 ± 0.7	29.9 ± 0.7
Plasma cholesterol (mg/dl)	1,185 ± 93	1,288 ± 109	1,225 ± 129	1,201 ± 102
Plasma aliskiren (μM)	0	0.03 ± 0.005 ^A	0.71 ± 0.23 ^{A,B}	0.73 ± 0.09 ^{A,B}
Plasma renin (ng/ml/30 min)	13.8 ± 2.7	31.7 ± 4.5 ^A	31.9 ± 2.9 ^A	33.0 ± 2.9 ^A
Plasma aldosterone (pg/ml)	260 ± 22	330 ± 55	229 ± 24	ND

Values are mean ± SEM. ND, not determined. ^A*P* < 0.0001 versus vehicle. ^B*P* < 0.0001 versus 2.5 mg/kg/d aliskiren.

renin contributed to the development of atherosclerosis by generating chimeric mice with renin deficiency in bone marrow-derived cells. Finally, we also defined autocrine/paracrine effects of locally synthesized angiotensin peptides on AT1a receptors in the development of atherosclerosis.

Results

Systemic renin inhibition strikingly reduced atherosclerosis. Aliskiren, an octanamide, is the first representative of a new class of nonpeptide, low-molecular weight renin inhibitors. Its action decreases plasma concentrations of AngII, which causes depression of negative feedback of renin secretion from the kidney and results in increased plasma renin concentrations (29–31). In this study, aliskiren was administered at 3 different doses to produce increasing systemic inhibition of renin. Aliskiren produced no changes in body weight, plasma cholesterol concentrations, plasma aldosterone concentrations (Table 1), or lipoprotein-cholesterol distribution (data not shown) in *Ldlr*^{-/-} mice. The lowest dose of aliskiren, 2.5 mg/kg/d, gave plasma concentrations that were 5-fold higher than the IC₅₀ against mouse renin (6.22 ± 2.25 nM), while the higher doses of 25 and 50 mg/kg/d gave similar plasma concentrations that were 100-fold above the IC₅₀ (Table 1). All doses of aliskiren increased plasma renin concentrations. Renal renin mRNA increased in a dose-dependent manner (*P* < 0.0001; Figure 1). Conversely, mRNA abundances of angiotensinogen, ACE, ACE2, AT1a receptors, and AT2 receptors did not change in renal tissue (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI32970DS1). Renin inhibition was further demonstrated by the decreased plasma concentrations of angiotensin peptides (Figure 2). Infusion of 2.5 mg/kg/d aliskiren, the lowest dose tested, reduced plasma AngII and the sum of angiotensin peptide concentrations, while the infusion of 25 mg/kg/d led to near ablation of all peptides in plasma (Figure 2).

There was a sustained decrease in systolic blood pressure during aliskiren infusion at 25 and 50 mg/kg/d (*P* < 0.0001; Figure 3). The administration of 2.5 mg/kg/d aliskiren did not produce an overall statistically significant sustained reduction in systolic blood

pressure, although there was a decrease at 1 interval measured. Because we have previously demonstrated that hypercholesterolemia caused a mild increase in blood pressure associated with activation of the RAS (6), we also sought to determine the effect of the 50-mg/kg/d aliskiren dose on systolic blood pressure in wild-type C57BL/6 male mice fed normal laboratory diet. The basal systolic blood pressure in these normolipidemic mice was less than that in strain-, age-, and gender-matched fat-fed *Ldlr*^{-/-} mice. Administration of the highest dose of aliskiren did not change systolic blood pressures in normolipidemic C57BL/6 mice, whereas both the 25- and 50-mg/kg/d doses reduced systolic blood pressures in hypercholesterolemic *Ldlr*^{-/-} mice to the level observed in normolipidemic C57BL/6 mice (Figure 3).

Atherosclerotic lesion size was measured in both the aortic intima by the en face technique and in sections of aortic root. Reductions in atherosclerosis were profound in both regions, with significant reductions in atherosclerotic lesion size observed in mice administered the 2.5-mg/kg/d dose of aliskiren. The 25- and 50-mg/kg/d doses produced similar results, with near ablation of

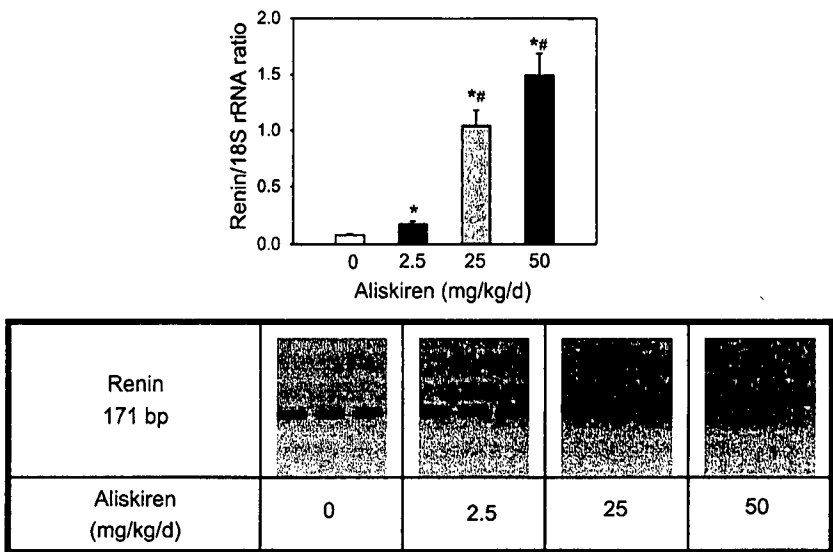


Figure 1
Renin inhibition dose-dependently increased renal renin mRNA abundance. Renin mRNA abundance (renin/18S rRNA ratio) was quantified by real-time PCR (*n* = 5 per group). mRNA bands by gel electrophoresis are also shown; the lanes were run on the same gel but were noncontiguous. Data are mean ± SEM. ^{*}*P* < 0.0001 versus vehicle; ^{**}*P* < 0.0001 versus 2.5 mg/kg/d aliskiren.

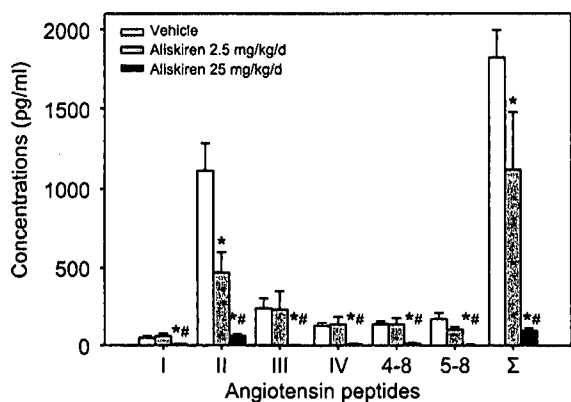


Figure 2
Renin inhibition dose-dependently decreased angiotensin peptide concentrations in mouse plasma. Angiotensin peptides in mouse plasma were resolved by HPLC and quantified with radioimmunoassay ($n = 4$ per group). Data are mean $\pm$ SEM. * $P < 0.05$ versus vehicle; # $P < 0.05$ versus 2.5 mg/kg/d aliskiren.

lesion formation in the aortic arch (Figure 4A). The administration of aliskiren also profoundly reduced atherosclerotic lesion size throughout the aortic root (vehicle, 0.16 ± 0.02 mm²; 2.5 mg/kg/d, 0.09 ± 0.01 mm²; 25 mg/kg/d, 0.07 ± 0.01 mm²; 50 mg/kg/d, 0.05 ± 0.01 mm²; $P < 0.0001$; Figure 4B). All doses of aliskiren produced similar reductions in lesion size in this region.

Increased blood pressure is a consistent risk factor for the development of atherosclerotic diseases in humans (32). Thus, we investigated the association between systolic blood pressure and atherosclerotic lesion size in both aortic arch and root in mice administered different doses of aliskiren. First, we calculated Pearson correlations between systolic blood pressure and (log-transformed) atherosclerotic lesion size within each of the 4 study groups. There were no significant correlations between systolic blood pressure and atherosclerotic lesion size in either aortic arch or root (data not shown). We also used linear regression to express atherosclerotic lesion size in terms of study group assignment and systolic blood pressure (Supplemental Figure 2). Even with adjustment for differences in systolic blood pressure, all doses of aliskiren still significantly reduced atherosclerotic lesion size in both the aortic arch and root ($P < 0.001$). On the other hand, systolic blood pressure did not predict atherosclerotic lesion size once we controlled for aliskiren dosage.

Immunostaining of atherosclerotic tissue in aortic roots was performed to determine whether reductions in lesion size during renin inhibition were accompanied by changes in cell composition. Immunostaining of lesions demonstrated that the predominance of macrophages in vehicle-infused mice was similar in aliskiren-infused mice (Figure 4C).

Systemic renin inhibition did not affect specific oxidant mechanisms. AngII has previously been shown to promote macrophage expression of 12/15 lipoxygenase protein, which promotes atherosclerosis in *Ldlr*^{-/-} mice (33, 34). The abundance of 12/15 lipoxygenase protein was determined in mouse peritoneal macrophages by Western blotting. However, we were unable to demonstrate that aliskiren administration had any effect on macrophage 12/15 lipoxygenase expression (data not shown). We also measured serum titers of autoantibodies against malondialdehyde-LDL

(MDA-LDL), which has previously been used as an index of atherosclerosis-related oxidant mechanisms (35). However, serum titers of MDA-LDL autoantibodies were unchanged by aliskiren administration (data not shown).

Cultured macrophages expressed all components of RAS and synthesized the unique precursor angiotensinogen. To determine whether macrophages express all components of the classic pathway for angiotensin peptide production, RT-PCR was used with primers that have annealing sites across intronic boundaries for each pair (36). mRNA expression of angiotensinogen, renin, and ACE was detected in mouse macrophages (Supplemental Figure 3A).

The ability of macrophages to secrete angiotensinogen was investigated by Western blotting (Supplemental Figure 3B). Mouse plasma protein had 2 specific bands of angiotensinogen at 52 and 64 kDa, whereas peritoneal macrophage culture medium presented a single band at 64 kDa that was highly glycosylated, as reported previously (36). We have been unable to develop an antibody to detect the presence of secreted renin in a Western blotting format.

The deficiency of renin has the potential to upregulate other enzymes with similar properties, such as cathepsin D (17). However, renin deficiency had no effect on cathepsin D mRNA abundance in macrophages (Supplemental Figure 3C).

Renin deficiency in bone marrow-derived cells reduced atherosclerotic lesion size unrelated to their AT1a receptors. Because whole-body inhibition of renin profoundly decreased atherosclerosis, and because isolated macrophages contain all the components for angiotensin peptide synthesis including renin, we examined the effect of renin originating from bone marrow-derived cells. We used the strategy of irradiating *Ldlr*^{-/-} mice and repopulating with bone marrow-derived cells from *renin*^{+/+} or *renin*^{-/-} mice. Although bone marrow has the potential to differentiate into many cell types, this procedure predominantly repopulates macrophages in atherosclerotic lesions (37).

In *Ldlr*^{-/-} recipient mice, the renin genotype of bone marrow-derived cells used to repopulate mice had no influence on body weight, wbc counts, plasma cholesterol concentrations, plasma concentrations of aldosterone and renin, or systolic blood pressure (Table 2). The chimeric nature of the mice was confirmed at the termination of the study by genotyping the bone marrow

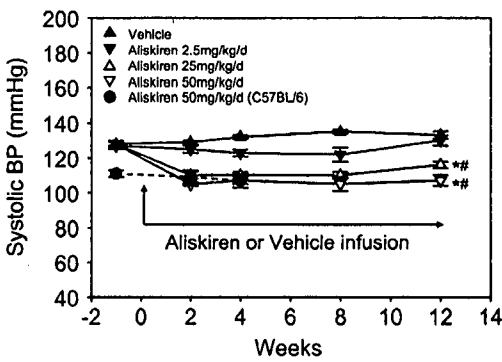


Figure 3
Renin inhibition decreased systolic blood pressure in *Ldlr*^{-/-} mice. Systolic blood pressure was measured in *Ldlr*^{-/-} mice 1 week prior to pump implantation and during 2, 4, 8, and 12 weeks of drug administration at the indicated doses. Aliskiren was also administered at a dose of 50 mg/kg/d to normal diet-fed *Ldlr*^{+/+} C57BL/6 mice (dotted line). Points represent the mean of weekly observations; error bars denote SEM. * $P < 0.0001$ versus vehicle; # $P < 0.0001$ versus 2.5 mg/kg/d aliskiren.

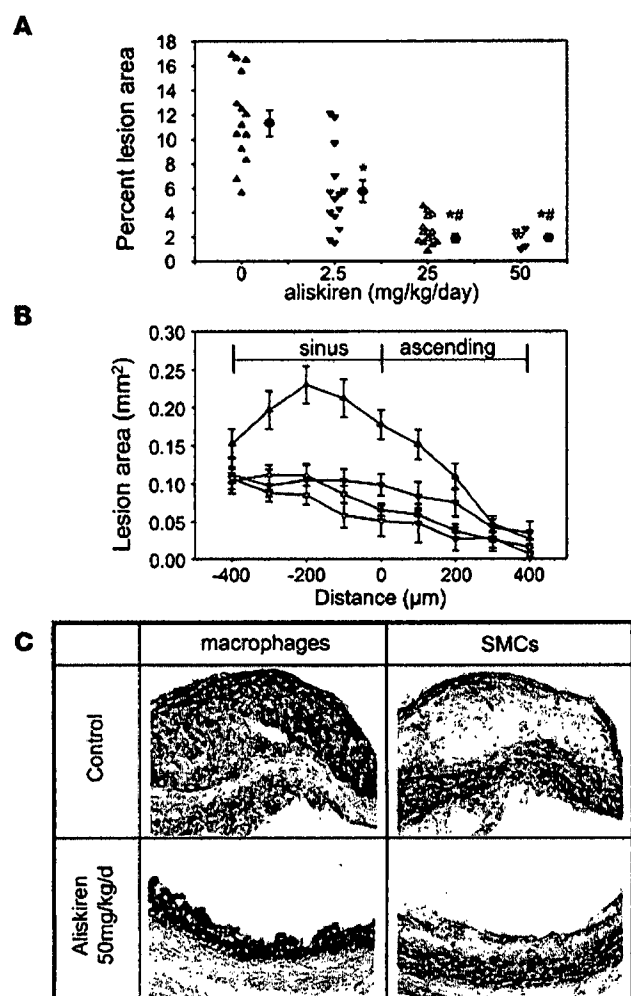


Figure 4

Renin inhibition reduced atherosclerotic lesion development without overtly altering cellular composition. (A) Atherosclerotic lesion size was measured on the intimal surface of aortic arches. Triangles represent individual mice; circles represent the mean; error bars denote SEM. * $P < 0.0001$ versus vehicle; # $P < 0.01$ versus 2.5 mg/kg/d aliskiren. (B) Atherosclerotic lesion size was measured in aortic roots. Data are represented throughout the aorta root, with transitions between the sinus and ascending arch depicted as 0. Filled triangles, PBS vehicle ($n = 14$); filled inverted triangles, 2.5 mg/kg/d aliskiren ($n = 14$); open triangles, 25 mg/kg/d aliskiren ($n = 15$); open inverted triangles, 50 mg/kg/d aliskiren ($n = 6$). Values are mean $\pm$ SEM. (C) Examples of atherosclerotic lesions immunostained for macrophages and smooth muscle cells. Aortic roots were immunostained using rat anti-mouse CD68 (1:200, left), or a rabbit anti- α -SMA (1:200, right). Original magnification, $\times 200$.

We subsequently defined whether angiotensin peptides generated by macrophages act primarily in an autocrine/paracrine manner to promote lesion formation. Of the AT1 receptor subtypes, only AT1a receptor was detected in macrophages (Figure 6A). Therefore, we performed a bone marrow transplantation study in which we irradiated $Ldlr^{-/-}$ mice and repopulated them with bone marrow-derived cells from AT1a receptor $^{+/+}$ or AT1a receptor $^{-/-}$ mice. The parameters described for the renin bone marrow transplantation studies were also unaffected by the AT1a receptor genotype of the bone marrow cells (Table 3). The genotype of the peritoneal macrophages at the end of the study confirmed the chimeric nature of the mice (Figure 6B). Furthermore, in contrast to the profound effects of renin deficiency, we failed to detect any significant effect of AT1a receptor genotype of bone marrow-derived cells on the size of atherosclerotic lesions (Figure 6C).

Macrophage renin deficiency reduced monocyte adhesion to endothelial cells. Because renin deficiency in bone marrow-derived cells decreased atherosclerosis unrelated to autocrine effects on macrophage AT1a receptors, we studied whether renin expression in macrophages would affect adhesion of monocytes to endothelial cells, a pivotal process in the initiation and progression of atherosclerosis. Thus, we examined THP-1 monocyte adhesion to HUVECs. Initial studies demonstrated that AngII (10^{-7} M for 24 hours) promoted monocyte adhesion to endothelial cells (Figure 7A). AngII-induced monocyte adhesion to HUVECs was not associated with increased endothelial VCAM-1 expression. Although AngII failed to increase VCAM-1 expression, the ability of HUVECs to express this adhesion molecule was confirmed by incubation with TNF- α (Supplemental Figure 5). Antagonism of AngII-induced monocyte adhesion by 10^{-6} M losartan demonstrated the role of AT1 receptors (Figure 7B). Having defined the role of AngII to promote adhesion, we coincubated confluent endothelial monolayers with peritoneal macrophages harvested from either renin $^{+/+}$ or renin $^{-/-}$ mice that were cultured in lower chambers of Transwell plates. Monocyte adhesion was significantly increased when HUVECs were coincubated with macrophages from renin $^{+/+}$ mice compared with coincubation with macrophages from renin $^{-/-}$ mice (Figure 7C).

Discussion

Aliskiren effectively inhibits mouse renin and profoundly reduces atherosclerosis. The present study demonstrated that renin inhibition reduced the size of atherosclerotic lesions in a dose-dependent manner in $Ldlr^{-/-}$ mice fed a fat-enriched diet. Renin inhibition was achieved by continuous subcutaneous infusion of the novel inhibitor aliskiren. Aliskiren is a potent inhibitor of human

(Figure 5A). Despite the lack of systemic changes, mice repopulated with cells derived from renin $^{-/-}$ mice had a large reduction in atherosclerotic lesion size (Figure 5B). Immunostaining of lesions demonstrated that both groups had predominantly macrophage-rich lesions. Immunostaining for renin was more apparent in $Ldlr^{-/-}$ mice repopulated with cells from renin $^{+/+}$ mice compared with cells from renin $^{-/-}$ mice (Figure 5C).

To study a potential oxidant mechanism of macrophage renin on atherogenesis, we determined macrophage 12/15 lipoxygenase expression. As with the aliskiren studies, we were unable to detect an effect of renin deficiency on macrophage 12/15 lipoxygenase expression (data not shown). We also determined whether renin deficiency in macrophages altered lipid accumulation. Cultured peritoneal macrophages from renin $^{+/+}$ or renin $^{-/-}$ mice were incubated in serum-free DMEM in the presence of acetylated LDL (20 μ g/ml) for 24 hours. Staining with Oil Red O showed intracellular accumulation of neutral lipid in both renin $^{+/+}$ and renin $^{-/-}$ macrophages incubated with acetylated LDL. To quantify the cholesterol content in macrophages incubated with acetylated LDL, we measured unesterified and esterified cholesterol by gas chromatography-mass spectrometric analysis (GC-MS). Renin deficiency in mouse macrophages did not result in any change in acetylated LDL-induced increases in intracellular unesterified and esterified cholesterol (Supplemental Figure 4).

Table 2
Effects of renin deficiency in bone marrow–derived cells in *Ldlr*^{−/−} mice fed a fat-supplemented diet for 12 weeks

	Renin ^{+/+}	Renin ^{−/−}
<i>n</i>	12	10
Body wt (g)	32.5 ± 0.7	34.6 ± 0.9
wbc count (10 ³ /μl)	8.2 ± 1.0	7.5 ± 0.6
Plasma cholesterol (mg/dl)	1,294 ± 46	1,357 ± 54
Plasma renin (ng/ml/30 min)	12.5 ± 1.8	12.0 ± 1.8
Plasma aldosterone (pg/ml)	274.3 ± 67.5	302.2 ± 91.9
Systolic BP (mmHg)	139 ± 4	137 ± 3

Values are mean ± SEM.

renin and displays species specificity for various isoforms of renin (30). The IC₅₀ for aliskiren against mouse renin is 1 order of magnitude less effective than on the human enzyme (0.6 nM), but greater than its potency against the rat enzyme (80 nM). The plasma aliskiren concentrations achieved with doses of drug in this study were all above the mouse IC₅₀. Moreover, the functional inhibitory effect of aliskiren against mouse renin was demonstrated by reductions in plasma angiotensin peptide concentrations, with almost complete ablation of angiotensin peptides in plasma at higher drug doses.

Because renin conversion of angiotensinogen to AngI is the rate-limiting step in AngII formation, renin has long been considered the optimal target for RAS inhibition. Renin inhibition prevents formation of all angiotensin peptides. In contrast, ACE inhibitors do not influence non-ACE pathways such as chymase, while AT1 receptor inhibition increases circulating concentrations of angio-

tensin peptides. In this study, we found that renin inhibition by aliskiren strikingly reduced atherosclerosis in mice. This contrasts with the effects of AT1a receptor deficiency, which were also profound, but not as efficacious as renin inhibition (6). Also, a survey of the literature for both ACE inhibition and AT1 receptor antagonists shows consistency with regard to reduced atherosclerotic lesion size, but not to the level attained with renin inhibition (7, 12, 14, 15, 38, 39). However, these differences need to be substantiated by comparing the different modes of RAS inhibition within a single study that uses multiple doses.

We have demonstrated previously that hypercholesterolemia was associated with increased RAS activity, as indicated by large increases in plasma concentrations of angiotensinogen and angiotensin peptides (6). In the present study, hypercholesterolemia was associated with increased systolic blood pressure. The role of angiotensin peptides in this elevation was demonstrated by renin inhibition reducing systolic blood pressure to a level observed in normolipidemic mice. Thus, there are several indications that renin inhibition was achieved with aliskiren to a level that affected physiological processes. Effective blockade of the RAS is associated with increased plasma renin concentrations, as demonstrated in the current study. This systemic increase results from release of renin in renal juxtaglomerular cells from AngII-induced negative feedback inhibition. The present results demonstrated that such increases in plasma renin concentration during aliskiren administration did not modulate the vasculoprotective effect of aliskiren. With increased systemic renin concentrations, there is the potential to interact with the recently cloned renin/prorenin receptor to activate fibrotic pathways (40). However, the relevance of high circulating concentrations of renin, and this receptor, to atherogenesis is unknown.

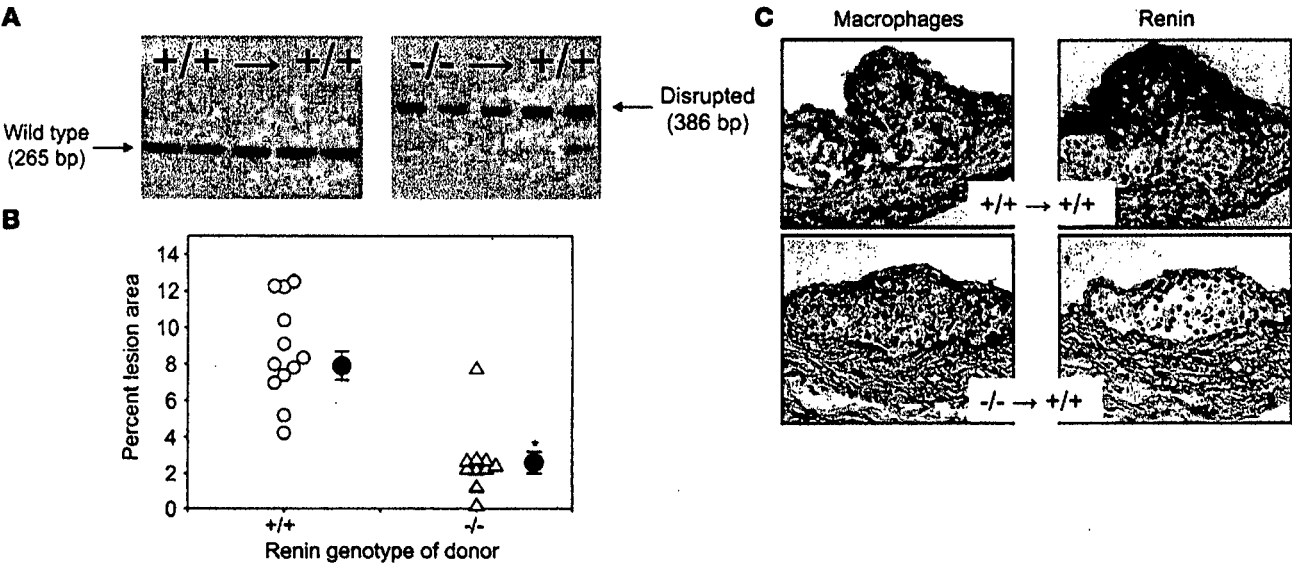


Figure 5
Renin deficiency in bone marrow–derived cells decreased atherosclerosis in *Ldlr*^{−/−} recipient mice fed a high-fat diet. (A) PCR confirmed the presence of the disrupted renin gene in bone marrow cells from recipient mice. PCR on bone marrow–derived DNA yielded an amplicon of 265 bp for the wild-type renin allele and 386 bp for the disrupted allele. The lanes were run on the same gel but were noncontiguous. (B) Atherosclerotic lesion size was measured on aortic arch intimal surfaces. Individual *Ldlr*^{−/−} recipients are represented by open symbols. Means of each group are represented by circles; error bars denote SEM. **P* < 0.001 renin^{+/+} donor (*n* = 12) versus renin^{−/−} donor (*n* = 10). (C) Renin deficiency in bone marrow–derived cells ablated renin expression in macrophages localized to atherosclerotic lesions in the aortic root. Immunostaining was performed in the atherosclerotic lesions of aortic root using rabbit anti-mouse macrophage antisera and a chicken anti-mouse renin IgY. Original magnification, ×200.

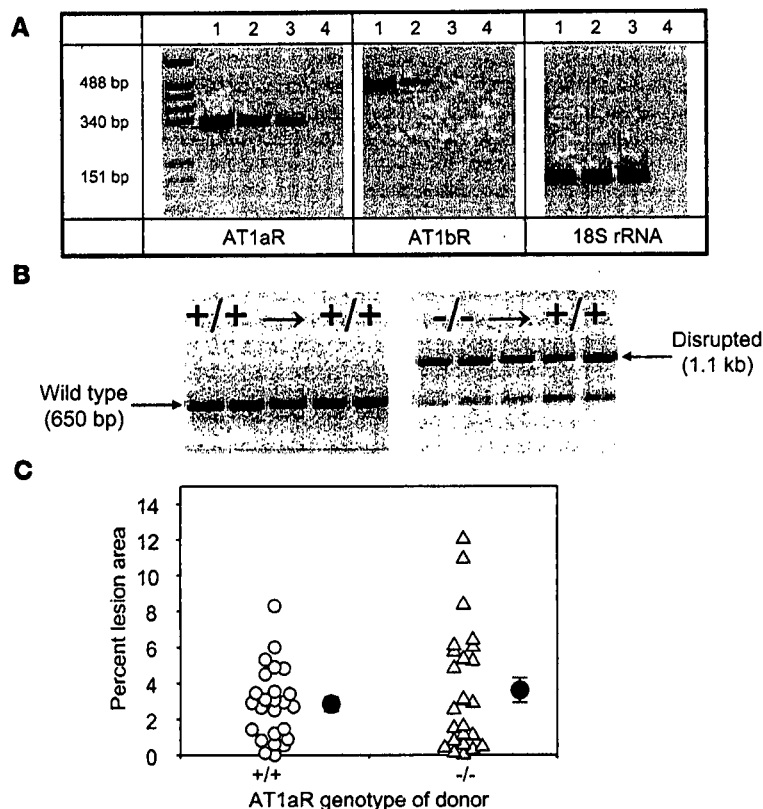


Figure 6
AT1a receptor deficiency in bone marrow-derived cells did not alter atherosclerotic lesion size. (A) RT-PCR confirmed that macrophages expressed mRNA of AT1a receptors (AT1aR), but not of AT1b receptors. Examples of positive controls (adrenal glands and aorta) and macrophages for AT1a receptors, AT1b receptors, and 18S rRNA. Lane 1, adrenal gland; lane 2, aorta; lane 3, macrophages; lane 4, non-RT negative control in adrenal glands. The lanes were run on the same gel but were noncontiguous. (B) PCR confirmed the presence of the disrupted AT1a receptor gene in mouse peritoneal macrophages from recipient mice. PCR on macrophage-derived DNA yielded an amplicon of 650 bp for wild-type AT1a receptor and 1.1 kb for the disrupted allele. (C) Atherosclerotic lesion size was measured on aortic arch intimal surfaces. Individual symbols represent *Ldlr*^{-/-} recipients. Means of each group are represented by circles; error bars denote SEM. *n* = 25 (AT1a receptor^{+/+} donor); 24 (AT1a receptor^{-/-} donor).

Reductions in atherosclerosis following aliskiren administration were profound in both vascular regions in which it was measured, with aortic root lesions having greater reductions than those in aortic arches. While higher doses of aliskiren reduced systolic blood pressure, reductions in atherosclerosis were achieved at doses of aliskiren that showed no persistent changes in blood pressure. In addition, we did not find any significant correlation between systolic blood pressure and atherosclerotic lesion size after we controlled for aliskiren dosage. Previously published studies have also demonstrated that AngII-augmented atherosclerosis is not a direct consequence of its ability to increase blood pressure. For example, while both AngII and norepinephrine increased blood pressure to the same extent in apoE^{-/-} mice, only AngII accelerated atherosclerosis (10). Furthermore, the profound decreases in atherosclerotic lesion size during administration of AT1 receptor antagonists were not observed with either hydralazine or amlodipine when administered at doses resulting in equivalent reductions in systolic blood pressure (7, 41). Thus, while increased systolic blood pressure is frequently associated with atherosclerosis, the stimulus for the hypertension appears to be the major determinant of atherogenesis rather than pressure per se (42).

AngII is well known to augment specific oxidant pathways (43). This has been particularly well documented for the NADPH system in smooth muscle cells (43). AngII stimulation of this oxidant system has been consistently demonstrated to promote smooth muscle cell hypertrophy and hyperplasia (43, 44). However, NADPH oxidase has a poorly defined role in the development of atherosclerosis. Deficiency of p47phox has previously been noted to decrease atherosclerotic lesion size in the aortic

arch, but not the aortic root (45), while no effects were determined in another study (46). Furthermore, gp91phox deficiency had no effect on atherosclerotic lesion size (47). More emphasis regarding atherogenic mechanisms has been placed on extracellular oxidant processes that have led to the formation of protein adducts. Specific subtypes of lipoxygenases have been consistently linked to the atherogenic process (48). Previous studies have demonstrated that incubation of AngII with mouse peritoneal macrophages and J774 cells upregulated 12/15 lipoxygenase abundance and activity (33). However, we were unable to demonstrate any difference in 12/15 lipoxygenase activity in macrophages from mice with profound renin inhibition. A more general index of oxidant stress has been the serum titers of autoantibodies to oxidatively modified proteins. Again, we were unable to detect any difference in the serum titers of autoantibodies to one of the most commonly described adducts, malondialdehyde. Thus, we were unable to define oxidant stress mechanisms that were associated with the profound reduction in atherosclerosis promoted by renin inhibition.

Deficiency of AT1a receptors has previously been shown to greatly decrease atherosclerotic lesion size in hyperlipidemic mice (6, 7). Although no direct comparisons have been performed, the relative extent of reductions in lesion size in the present study appeared larger during renin inhibition than that previously observed in mice with AT1a receptor deficiency or pharmacological blockade. Direct comparisons of the efficacy of renin inhibition, AT1 receptor blockade, and ACE inhibition on reduction in atherosclerotic lesion size are complicated by the definition of comparable inhibition for these 3 different mechanisms. However, the profound reductions in angiotensin peptides in this study do obviate the interpretation that the reductions in lesion size during AT1a receptor blockade were caused

Table 3
Effects of AT1a receptor deficiency in bone marrow–derived cells in Ldlr^{-/-} mice fed a fat-supplemented diet for 12 weeks

	AT1a receptor ^{+/+}	AT1a receptor ^{-/-}
n	25	24
Body wt (g)	28.0 ± 0.9	29.2 ± 0.7
wbc count (10 ³ /μl)	11.4 ± 0.5	10.5 ± 0.8
Plasma cholesterol (mg/dl)	621 ± 43	671 ± 33
Plasma renin (ng/ml/30 min)	19.8 ± 5.1	27.4 ± 4.8

Values are mean ± SEM.

by enhanced stimulation of AT2 receptors (39, 49, 50). Furthermore, these data demonstrate the benefit of inhibiting the synthesis of all angiotensin peptides on the development of atherosclerosis.

Renin deficiency in bone marrow–derived cells decreases atherosclerosis via the inhibition of monocyte adhesion to endothelial cells in the vascular wall. The major source of systemic concentrations of renin is the renal juxtaglomerular cells. While renin has been detected in several extrarenal tissues, it is unclear whether the levels of renin produced in extrarenal tissues is sufficient to have biological effects (20). In a previous study, we demonstrated that renin protein was detected by immunostaining in mouse atherosclerotic lesions (6). However, the renin detected in lesions may result from delivery of renin from other sources with retention by a receptor-based system (40, 51). In the current study, we demonstrated that cultured macrophages express a complete RAS, including renin mRNA. Furthermore, the creation of chimeric mice with renin deficiency in infiltrating macrophages clearly demonstrated that reductions in atherosclerosis could be achieved through deficiency of macrophage-derived renin. Importantly, reductions in atherosclerosis were seen in chimeric mice repopulated with renin-deficient bone marrow that exhibited no changes in plasma renin concentrations, demonstrating a pivotal role for macrophage-derived renin in atherosclerosis.

AngII has the potential to interact with many cell types to augment the atherogenic process. These include endothelial cells, smooth muscle cells, and macrophages. One potential mechanism by which renin within macrophages could be exerting its effect is the autocrine/paracrine effects of generated angiotensin peptides on macrophages. Our present results demonstrate that the absence of AT1a receptors in bone marrow–derived cells had no effect on lesion size in Ldlr^{-/-} mice. This result is consistent with previous studies in our laboratory demonstrating that AT1a receptor deficiency in bone marrow–derived cells had minimal effects on AngII-induced atherosclerosis (11). These results indicate that macrophages are not the target cell type for AT1a receptors as a mediator of atherosclerosis.

The initiation and progression of atherosclerotic lesions is associated with monocyte adhesion to an intact endothelial surface (52, 53). Consistent with previous studies (54–57), we found that AngII augmented monocyte adhesion to endothelial cells. This was inhibited by incubation of endothelial cells with the AT1 receptor antagonist losartan. Monocyte adhesion to endothelial cells stimulated by macrophages from renin^{+/+} mice was attenuated by macrophages from renin^{-/-} mice. These findings are consistent with macrophage-derived angiotensin peptides contributing to atherosclerosis via the effects on endothelial AT1 receptors. Our studies failed to demonstrate an association of augmented monocyte adhesion with increased endothelial VCAM-1 expression. While there has been a previous report of AngII

increasing VCAM-1 expression in endothelial cells (58), in agreement with the present study, others have also failed to demonstrate increased VCAM-1 (55–57, 59). Thus, the in vitro data are consistent with AngII facilitating monocyte adhesion, although the regulator of this effect has not been identified. Because endothelial cells possess AT1a receptors and the function of this cell type regulates lesion development, future in vivo studies are needed to explore this axis using AT1a receptor–floxed mice.

In conclusion, renin inhibition profoundly decreased formation of atherosclerosis. Importantly, reductions in atherosclerosis were evident in Ldlr^{-/-} mice with deficiency of renin in bone marrow–derived cells, invoking a prominent role for macrophage-derived renin in atherosclerosis.

Methods

Mice and diet

Ldlr^{-/-} (B6.129S7-Ldlr^{em1Her}, stock no. 002207), AT1a receptor^{-/-} (B6.129P2-Agr1a^{em1Unc}, stock no. 002682), and age-matched C57BL/6 male mice (stock no. 000664) were obtained from The Jackson Laboratory. Male renin^{-/-} mice on a C57BL/6 background (N9) were generated as previously described (60). LDLR and AT1a receptor genotypes were determined by PCR as described previously (6). Renin genotyping used the

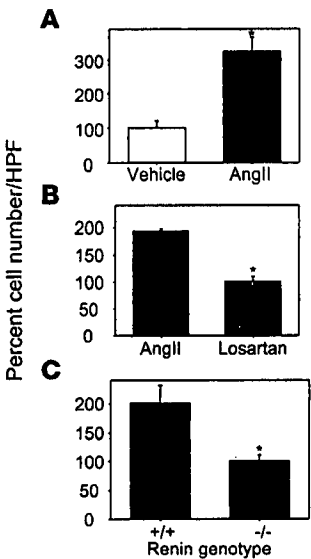


Figure 7
Renin deficiency in mouse macrophages markedly reduced monocyte adhesion to endothelial cells. (A) AngII resulted in increased THP-1 monocyte adhesion to endothelial cells. The adhesion assay was performed by cocubation of 10⁻⁷ M AngII with HUVECs for 18 hours. Values (mean ± SEM) represent cell number of each well relative to vehicle per HPF (n = 4 per group). *P = 0.009 versus vehicle. (B) The AT1 receptor antagonist losartan inhibited AngII-induced THP-1 monocyte adhesion to endothelial cells. The adhesion assay was performed by cocubation of 10⁻⁷ M AngII and 10⁻⁶ M losartan. Losartan was added 2 hours prior to the addition of AngII to cultured HUVECs. Values (mean ± SEM) represent cell number of each well relative to losartan per HPF (n = 3 per group). *P < 0.001 versus AngII. (C) Renin deficiency resulted in decreased THP-1 monocyte adhesion to HUVECs. Cultured peritoneal macrophages from renin^{+/+} or renin^{-/-} mice were cocubated with HUVECs for 24 hours, and an adhesion assay was then performed. Values (mean ± SEM) represent cell number of each well relative to renin^{+/+} per HPF (n = 4 per group). *P = 0.03 versus renin^{+/+}.

following primers: 5'-ACTCTTGTTGCTCTGGAGTCCTT-3', 5'-AGTAGTAGAAGGGGGAGTTGTGG-3', 5'-GCTACCCGTGATATTGCTGAA-3', and 5'-CTTCCCAAAGCTGGCTTATTC-3'. The resultant wild-type and deficient allele bands were 265 and 386 bp, respectively. All mice were backcrossed 10 times into a C57BL/6 background. Littermates were selected for the studies reported in this manuscript.

All mice were maintained in a barrier facility and fed normal mouse laboratory diet. To induce hypercholesterolemia, mice were changed to a diet supplemented with saturated fat (milk fat 21% wt/wt) and cholesterol (0.15% wt/wt, diet no. TD88137; Harlan Teklad). All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Aliskiren administration

Model 2004 Alzet mini-osmotic pumps (Durect Corp.) were implanted into male *Ldlr*^{-/-} or C57BL/6 mice at the age of 6–8 weeks. Pumps were filled with either PBS (*n* = 15) or solutions of aliskiren dissolved in PBS (2.5 mg/kg/d, *n* = 14; 25 mg/kg/d, *n* = 16; 50 mg/kg/d, *n* = 6) and replaced at 28-day intervals to continuously deliver solutions for a total of 12 weeks in *Ldlr*^{-/-} mice (8, 9). Aliskiren (50 mg/kg/d) was infused for 4 weeks in C57BL/6 mice (*n* = 8).

Irradiation and bone marrow repopulation

This procedure was performed as described previously (11). Bone marrow was harvested from *renin*^{+/+} or *renin*^{-/-} and AT1a receptor^{+/+} or AT1a receptor^{-/-} male mice (6–8 weeks old) and injected (1×10^7 cells/mouse) into age-matched male *Ldlr*^{-/-} recipient mice that had been irradiated. Four weeks after bone marrow repopulation, recipient mice were changed to a diet supplemented with fat for 12 weeks.

Systolic blood pressure measurements

Systolic blood pressure was measured using the Visitech tail cuff system (BP-2000 Visitech Systems) as described previously (38, 61). Systolic blood pressure measurements were performed 1 week before osmotic pump implantation and 2, 4, 8, and 12 weeks after implantation or 4, 8, and 12 weeks after bone marrow transplantation.

Measurement of plasma components

Lipid. Plasma cholesterol concentrations and lipoprotein cholesterol distributions were determined as described previously (9).

RAS. Concentrations of angiotensin peptides were determined by resolving plasma by HPLC and performing a radioimmunoassay on the fractions as described previously (62). Plasma renin and aldosterone concentrations were measured as described previously (6). For determination of the IC₅₀ for aliskiren, plasma (8 μ l) from 3-month fat-fed *Ldlr*^{-/-} mice (*n* = 3) was incubated with an excess of rat angiotensinogen in the absence and presence of aliskiren (0.01–100 nM) for 30 minutes at 37°C. AngI was measured by radioimmunoassay (Diagnostic Systems Laboratories).

Aliskiren concentration. Plasma concentrations of aliskiren were measured by liquid chromatography/mass spectrometry as described previously (63).

Oxidation autoantibody titers. Serum autoantibodies to MDA-LDL were measured by ELISA as described previously (64).

Atherosclerosis quantification

Atherosclerosis was quantified both on the aortic intima of the arch and in the root as described previously (65, 66).

Cholesterol content in macrophages

Resident mouse peritoneal macrophages were obtained from male *renin*^{+/+} or *renin*^{-/-} mice (6–8 weeks old) as described previously (67). Cells were incubated with DMEM supplemented with 10% vol/vol FBS and antibiot-

ics (penicillin G, 100 IU/ml, and streptomycin, 100 μ g/ml) overnight. Cells were washed with serum-free DMEM to remove nonadherent cells, and monolayers were incubated in serum-free DMEM in the presence of acetylated LDL (20 μ g/ml; Intracel) for 24 hours. Macrophages were extracted with chloroform/methanol at a 2:1 ratio with 5 α -cholestane added as an internal standard. Lipid extracts were dissolved in methylene chloride. Unesterified cholesterol was quantified by GC-MS using a CP-3800 gas chromatograph and a Saturn 2200 mass spectrometry (Varian) equipped with a DB-5 MS column (J & W Scientific Inc.). Saponification was performed to determine total cholesterol. Cholesterol measurements were normalized to cellular protein.

mRNA abundance of RAS components

RNA was harvested from mouse tissues and peritoneal macrophages using the SV Total RNA Isolation System (Promega). RT-PCR or real-time PCR was performed as described previously (6, 36). mRNA abundances were calculated with normalization to either 18S rRNA or β -actin. Nontemplate and non-RT reactions were used as negative controls.

Immunostaining

Macrophages were detected using rat anti-mouse CD68 (FA-11, catalog no. MCA 1957; Serotec) or rabbit anti-mouse macrophage serum (catalog no. AIA31240; Accurate Chemical Company) as described previously (6). Smooth muscle cells and endothelial cells were detected using rabbit anti- α -SMA (catalog no. ab5694; Abcam) and biotin-conjugated rat anti-mouse CD31 monoclonal antibody (PECAM-1, catalog no. 553371; BD Biosciences – Pharmingen), respectively. Immunostaining was performed on frozen sections, with appropriate negative controls, as described previously (64, 68).

Adhesion assay

HUVECs were isolated from human umbilical cords obtained from the University of Kentucky Labor and Delivery unit and cultured in M199 media supplemented with 20% FBS as described previously (69). Passages 1–5 were grown to confluence in cell culture inserts coated with human fibronectin (catalog no. 354492; BD). Plates were washed with PBS, and monolayers were incubated in M199 media with FBS (1% vol/vol) overnight. Cells were incubated for 24 hours with AngII (10^{-7} M), losartan (10^{-6} M), or peritoneal macrophages from *renin*^{+/+} or *renin*^{-/-} mice placed in the lower chamber. Adhesion assays were performed as described by Huber et al. (70). Briefly, THP-1 cells were stimulated by TNF- α (10 ng/ml) and then labeled with calcein AM (catalog no. C-3099; Invitrogen). Labeled cells (5×10^4 /well) were added to the upper chamber of each well. After incubating for 30 minutes at 37°C, unbound cells were washed off, and bound THP-1 cells were fixed with glutaraldehyde (1% wt/vol). Bound monocytes were counted by an inverted phase-contrast microscope. A minimum of 4 high-power fields (HPFs) of each well were counted by at least 2 observers that were blinded to the experimental design. The percent adhesion was calculated as the mean cell number of each well relative to the mean cell number of vehicle.

Western blotting analyses

Western blotting analyses of angiotensinogen in plasma and cultured macrophage medium, and 12/15 lipoxigenase in macrophages were performed as described previously (6, 71). Western blotting of VCAM-1 in cultured HUVECs was performed using mouse anti-human CD106 (catalog no. 555645; BD Biosciences – Pharmingen).

Statistics

To compare 2 groups on a continuous response variable, we used a 2-sample *t* test. To compare more than 2 groups on a continuous response variable, we used 1-way ANOVA; however, if the assumptions for the ANOVA

were not satisfied, we either performed the ANOVA on log-transformed values or used the Kruskal-Wallis procedure. For repeated-measures data, we fitted an appropriate linear mixed model. Relationships between systolic blood pressure and atherosclerotic lesion size were analyzed using Pearson correlations and linear regression models. A *P* value less than 0.05 was considered statistically significant. Data are presented as mean $\pm$ SEM. SAS version 9.1 (SAS Institute) was used to carry out data analyses.

Acknowledgments

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surements of plasma aliskiren concentrations, John May (University of Kentucky) for performing GC-MS cholesterol assays, and Thomas E. Curry (University of Kentucky) for providing human umbilical cords for HUVECs.

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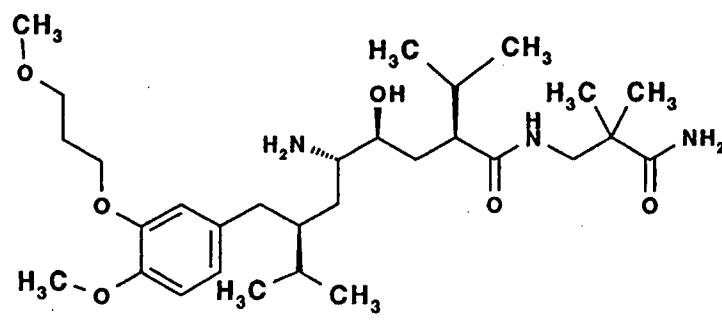
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REIVINDICACIONES

1. Una composición farmacéutica que comprende el inhibidor de renina de la fórmula (I):



o una sal farmacéuticamente aceptable del mismo, y al menos un agente terapéutico seleccionado a partir del grupo que consiste de:

(i) un antagonista del receptor AT_1 el cual es valsartan o una sal farmacéuticamente aceptable del mismo,

(ii) un inhibidor de HMG-Co-A reductasa seleccionado de fluvastatina, pitavastatina ó atorvastatina o una sal farmacéuticamente aceptable de las mismas,

(iii) un inhibidor de la enzima convertidora de angiotensina (ACE) el cual es benazepril o enalapril, o una sal farmacéuticamente aceptable de los mismos,

(iv) un bloqueador del canal de calcio el cual es amlodipina o verapamil ó una sal farmacéuticamente aceptable de los mismos;

(v) un inhibidor de aldosterona-sintasa el cual es fadrozol o una sal farmacéuticamente aceptable del mismo como el enantiómero (+) del clorhidrato de fadrozol,

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y

(vi) un diurético seleccionado de clorothiazida, hidroclorotiazida, methylchlorotiazida y clorotadilon o una sal farmacéuticamente aceptable de los mismos;

y un portador.

2. Una composición de acuerdo con la reivindicación 1, en donde el inhibidor de renina está presente en la forma del hemi-fumarato.

3. Una composición de acuerdo con cualquiera de las reivindicaciones 1 a 2, caracterizada porque comprende el compuesto de la fórmula (I) y valsartan o una sal farmacéuticamente aceptable de los mismos.

4. Una composición de acuerdo con la reivindicación 3, caracterizada porque el compuesto de la fórmula (I) está en la forma de la sal de hemi-fumarato y junto con valsartan.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800176089-2

RECIBO OFICIAL DE CAJA : 09 - 8,521
FECHA : FEBRERO 3 DE 2009

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 03-039889- -00012-0000

Fecha: 2009-03-06 15:12:44 Dep. 2020 NUECREACION
Tra. 11 PATENTEIYII Eje: 379 FASENACIONALII
Act. 444 RESPUESTAREQUE Folios: 51

***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PGO	Vr. PAGO
CLARKE MODET Y CO. COLOMB	CONSIGNACION	BANCO DE BOGOTA	062754387	108004750	02/02/2009	31,570,000.00

***** CONCEPTO *****

CANT.	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICA	
		12 MARCAS Y LEMAS COMERCIALES	111,000.00
		TOTAL :	\$ 111,000.00

SON: CIENTO ONCE MIL PESOS

RESPONSABLE : _____

RECIBO DE CAJA APLICADO AL EXPEDIENTE No. _____

P-1754
49

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCION DE NUEVAS CREACIONES

Radicación No. 03039889
Clasificación Internacional (IPC): A61K31/165
Concepto Técnico No. 413

Patente de Invención ☒ Patente de Modelo de Utilidad ☐
Vía Nacional ☐
Vía PCT (fase nacional) ☒ Cap. I ☐ Cap. II ☒
No. Sol. Internacional PCT/EP01/13241
Fecha de Presentación Internal. 15/11/2001

Como resultado del examen definitivo, realizado con fundamento en el artículo 48 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica:

1. Documentos estudiados

- 1.1. Descripción: Folios: 25 a 80
- 1.2. Reivindicaciones: 1 a 10 Folios 81 a 90
1 a 5 Folios 130 a 132
1 a 4 Folios 202 a 203
- 1.3 Rta. del Solicitante: Folios: 121 a 128 y 154 a 161
- 1.4 Prioridad: Fecha: 17/11/2000, país: Reino unido y Número: 0028151.9

2 Objeto de la invención

- Título modificado de la solicitud: Combinaciones sinérgicas que comprenden aliskiren (inhibidor de renina) y un agente terapéutico tal como valsartan, una estatina, benazepril, enalapril, amlodipino, fadrozol o hidroclorotiazida, para enfermedades cardiovasculares.

El problema planteado en esta solicitud es la necesidad de combinaciones sinérgicas útiles para tratar problemas cardiovasculares.

Para solucionar este problema técnico la solicitud en estudio proporciona combinaciones con efecto terapéutico benéfico y sinérgico sobre hipertensión e insuficiencia cardíaca congestiva.

Clasificación Internacional de Patentes (IPC.): A61K31/165

3 Determinación del Estado de la Técnica

La fecha que se ha tenido en cuenta para determinar el Estado de la Técnica es:

Fecha de presentación de la solicitud prioritaria 17/11/2000

- Consultadas las bases de datos con que cuenta la oficina y las otras fuentes utilizadas para determinar el estado de la técnica no se encontró documento alguno relacionado con el objeto de la solicitud.

4. Análisis de patentabilidad (según requisito) (artículo 14 D486)

Novedad y Nivel inventivo (artículos 16 y 18 D486)

La presente solicitud de patente reivindica:

Reivindicaciones 1-4: Composición farmacéutica que comprende al inhibidor de renina de la fórmula I (aliskiren) y cuando menos un agente terapéutico tal como: valsartan, una estatina, benazepril, enalapril, amlodipino, fadrozol o hidroclorotiazida

- **D₁ WO 91/17771 enseña** composiciones y métodos para mejorar el efecto terapéutico de la baja presión arterial y la insuficiencia cardíaca congestiva en mamíferos. Más específicamente este invento enseña composiciones sinérgicas comprendidas por cantidades de al menos dos agentes terapéuticos seleccionados de un grupo que consiste en un inhibidor de renina, un inhibidor de la enzima convertidora de angiotensina I y un antagonista de angiotensina II. Se incluyen además también métodos para mejorar los efectos sinérgicos. Ver folio 110 abstract, folio 111 reivindicación 1.
- **D₂ WO 92/10097 enseña** composiciones farmacéuticas que comprenden un vehículo farmacéuticamente aceptable con un antagonista del receptor de angiotensina II combinado con otros agentes como diuréticos, bloqueadores del canal del calcio, bloqueadores β adrenoreceptores, inhibidores de renina o un inhibidor de la enzima convertidora de angiotensina y un método para tratar la hipertensión. (Ver folio 112).

La presente solicitud difiere con D₁ y D₂ en que las combinaciones reivindicadas no son sugeridas por dichos documentos, por lo tanto es nueva de acuerdo a lo estipulado en el art. 16 de la Decisión 486 de la Comisión de la Comunidad Andina.

El problema técnico planteado en esta solicitud el cual es la necesidad de combinaciones sinérgicas útiles para tratar problemas cardiovasculares no se había resuelto previamente mediante los compuestos reivindicados. Por esta razón se considera que dichas combinaciones no son evidentes para una persona medianamente versada en la materia y además presentan efectos ventajosos tales como mejor control de la hipertensión que con la monoterapia, menores eventos laterales adversos, disminución de edema periférico, reducción significativa de la proteinuria, hipertrofia cardíaca, supresión de la aldosterona en sangre, evita el desarrollo de hipercalcemia y reducción significativa de arterosclerosis.

- Por lo tanto, también **tiene nivel inventivo** de acuerdo a lo estipulado en el art. 18 de la Decisión 486 de la Comisión de la Comunidad Andina.

Aplicación industrial (artículo 19 D 486)

- De acuerdo con el artículo 19 de la Decisión 486 de la Comisión de la Comunidad Andina se considera que la invención es susceptible de aplicación industrial porque puede ser producida o utilizada en la industria.

Título sugerido para la invención: Combinaciones sinérgicas que comprenden aliskiren (inhibidor de renina) y un agente terapéutico tal como valsartan, una estatina, benazepril, enalapril, amlodipino, fadrozol o hidroclorotiazida, para enfermedades cardiovasculares

5. Conclusión:

En consecuencia y por todo lo expuesto anteriormente, se recomienda **Conceder** las reivindicaciones: 1 a 4 (folios: 202 a 203).

Bogotá, D.C.,

CONCEPTO TECNICO No. 413	EXAMINADOR TECNICO Código No. 202087
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