

Castillo Grau & Asociados

ABOGADOS

Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. S. D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 09-054148- -00014-0000

Fecha: 2013-09-13 16:36:01 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 412 REPOSPRESRECU Folios: 52

Re: Expediente No. 09 054148

Solicitud de patente

**MÉTODOS Y COMPOSICIONES ANTI-PARASÍTICAS
QUE UTILIZAN ÍNDOLES ASOCIADOS CON EL
DIINDOLILMETANO**

Clase: A61K 31/405

RECURSO DE REPOSICIÓN

Actuando en mi calidad de apoderado de la sociedad BIORESPONSE L.L.C, por medio del presente escrito me permito manifestarle que, obrando dentro del término legal, interpongo **RECURSO DE REPOSICIÓN** ante el Superintendente de Industria y Comercio, contra la Resolución No. 43912 del 30 de julio de 2013, proferida por ese Despacho dentro del expediente de la referencia y por medio de la cual se negó la Patente de Invención denominada "MÉTODOS Y COMPOSICIONES ANTI-PARASÍTICAS QUE UTILIZAN ÍNDOLES ASOCIADOS CON EL DIINDOLILMETANO"

OBJETO DE ESTE RECURSO

Que se revoque la Resolución No. 43912 del 30 de julio de 2013 y, en consecuencia, se conceda la Patente de Invención titulada "MÉTODOS Y COMPOSICIONES ANTI-PARASÍTICAS QUE UTILIZAN ÍNDOLES ASOCIADOS CON EL DIINDOLILMETANO" a favor de BIORESPONSE L.L.C.

FUNDAMENTOS DEL RECURSO

1. El 27 de mayo de 2009 se presentó solicitud de entrada en fase nacional de la Patente de Invención titulada "MÉTODOS Y COMPOSICIONES ANTI-PARASÍTICAS QUE UTILIZAN ÍNDOLES ASOCIADOS CON EL DIINDOLILMETANO" a nombre de la sociedad BIORESPONSE L.L.C. Solicitud a la que se le asignó el número de expediente 09 054148.



El extracto de la solicitud fue publicado en la Gaceta de la Propiedad Industrial No. 619 de 19 de agosto de 2010.

3. La sociedad Lafrancol S.A. presentó oposición a la solicitud en referencia argumentando que esta carecía de novedad y nivel inventivo. Argumentos que el Examinador técnico consideró irrelevantes para el análisis de patentabilidad de la solicitud.
4. Mediante oficio No. 3368 de 2013 se requirió al solicitante de la patente en referencia en aplicación del artículo 45 de la Decisión 486 de la Comunidad Andina de Naciones.
5. Dentro del término legal, mi poderdante dio respuesta al requerimiento formulado y presentó nuevas reivindicaciones.
6. Mediante Resolución No. 43912 del 30 de julio de 2013 el Superintendente de Industria y Comercio negó la Patente de Invención en referencia por considerar que las características técnicas esenciales de la solicitud se encontraban reveladas en los siguientes documentos: D1 "COMPOSICIONES ACTIVADORAS INMUNES DE 3.3 - DIINDOLILMETANO" y D2" COMPUESTOS Y COMPOSICIONES QUE UTILIZAN COMPUESTOS INMUNOMODULADORES PARA EL MANEJO Y TRATAMIENTO DE ENFERMEDADES PARASÍTICAS"
7. A criterio de mi cliente, los argumentos expuestos por ese Despacho son erróneos pues las anterioridades D1 y D2 no afectan el nivel inventivo de la mencionada solicitud; para demostrarlo, a continuación rebatiremos cada uno de los argumentos esgrimidos en el acto administrativo objeto de recurso:

- a) *La diferencia que existe entre las composiciones reivindicadas y aquellas reveladas en D2 radica en que dicho documento no se especifica los agentes tipo DIM o sus derivados como agente inmunomodulador.*

Ni la anterioridad D1 ni D2 sugieren que el DIM o cualquier índole relacionado con DIM puede ser efectivo para tratar enfermedades por protozoarios, ni mucho menos sugiere que DIM o cualquier índole relacionado con DIM puede ser efectivo para tratar a un sujeto infectado con un parásito que causa malaria o una patología parecida a la malaria o efectivo para inhibir el crecimiento de dicho parásito en el sujeto.

- b) *Sin embargo, al revisar el capítulo descriptivo y los argumentos aportados por la solicitante, esa diferencia en los agentes tipo DIM o sus derivados no parece conceder un efecto técnico inesperado o una ventaja a las composiciones*



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reclamadas en esta solicitud, ya que no parecen presentar propiedades superiores en comparación con las composiciones reveladas en el estado de la técnica cercano. (Subraya fuera del texto)

En este punto es importante anotar, que al observar la descripción no se muestra una ventaja o un avance técnico asociado a las combinación de uno o mas índoles asociados con DIM y uno o más agentes antiprotozoarios que apoyen el nivel inventivo de la solicitud, por lo cual la solicitud carece de sustento para reconocer nivel inventivo. (Subraya fuera del texto)

Por lo tanto, el problema técnico objetivo que se pretende solucionar es la provisión de composiciones para el tratamiento de enfermedades parasíticas que contengan un inmunomodulador alternativo y un agente antiprotozoario.

Dicho problema ya fue solucionado por D1 que enseña la actividad del compuesto DIM y sus derivados en la mejora de la respuesta inmune (resumen).

Considera mi poderdante que esos argumentos no son ciertos toda vez que se ha demostrado que el DIM es efectivo para inhibir directamente el crecimiento de los parásitos causantes de la malaria, este hallazgo se evidencia en el informe titulado "Ensayo *in vitro* de Diindolilmetano (DIM) y DIM de BioResponse en el ensayo de actividad antimalárica con Hipoxantina marcada con tritio¹", en el cual se describen el diseño experimental y los resultados de un ensayo *in vitro* que mide la inhibición del crecimiento del *Plasmodium falciparum* (en lo sucesivo, *P. falciparum*), un parásito protozoario que causa malaria en seres humanos, por dos preparaciones diferentes de DIM (ver anexo 1)².

Para mayor claridad, a continuación se describe el ensayo de inhibición del crecimiento *in vitro*, es decir, el ensayo de ³H-Hipoxantina, que se usó en el informe, sus fundamentos y su diseño experimental. En el informe se encuentran los resultados del ensayo de Hipoxantina tritiada (³H) (³H-Hipoxantina), el cual se utilizó para valorar la capacidad de dos diferentes preparaciones de DIM de inhibir el crecimiento de dos cepas de parásitos causantes de la malaria.

El ensayo de ³H-Hipoxantina es una prueba bien establecida para medir la actividad de los medicamentos antimaláricos potenciales (ver, p.ej., Andrews et al., 2000, Int, J, Parasitol. 30(6):761-768, en la p. 763, "Andrews", que se anexa a la presente como Anexo 2), es relevante para predecir la sensibilidad de varios parásitos

¹ In vitro Assay of Diindolylmethane (DIM) and BioResponse-DIM in the ³H Hypoxanthine Assay for Anti-malarial Activity

² El ensayo de inhibición del crecimiento *in vitro* del *P. falciparum* y el informe se llevaron a cabo por solicitud del Inventor de la patente en referencia, Dr. Michael A. Zeligs, por Eskitis Institute for Cell and Molecular Therapies, Griffith University.

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patógenos que causan malaria o una patología parecida a la malaria (es decir, parásitos del genus *Plasmodium*) o la inhibición del crecimiento por posibles medicamentos antiparasitarios (ver Andrews en la p. 763, que muestra el uso del ensayo de ^3H -Hipoxantina para medir la inhibición del crecimiento de especies de *Plasmodium falciparum* del genus *Plasmodium* genus por diversos medicamentos; ver también Brasseur el: a], 1998, Antimicrob. Agents Chemother. 42(4):818-820, en pp. 818-814, "Brasseur", anexo 3, la cual muestra el uso del ensayo de ^3H -Hipoxantina para medir la inhibición del crecimiento de la especie *Babesia* del genus *Plasmodium* por varios medicamentos).

Los resultados del ensayo de Hipoxantina *in vitro* son predictivos de la eficacia clínica del medicamento en examen *in vivo* (ver, p.ej., Brasseur et al., 1992, Am. J. Trop. Med. Hyg. 46(1):8- 14, "Brasseur2", anexo 4), en particular, Brasseur et al., en un estudio realizado en el continente africano en un escenario de surgimiento de resistencia al medicamento antimalárico mefloquina, demostró que 37 de 38 pacientes que respondieron al tratamiento *in vivo* con mefloquina estaban infectados con parásitos que eran sensibles a bajas concentraciones del medicamento en el ensayo de ^3H -Hipoxantina *in vitro* (mostraban baja EC_{50} , es decir, la mitad de la máxima concentración efectiva, que es equivalente a la " IC_{50} " a la cual se hace referencia más adelante), y la resistencia al medicamento *in vivo* se asoció con resultados elevados de la EC_{50} en el ensayo de ^3H -Hipoxantina *in vitro* (ver Brasseur2 en la p. 10), así las cosas, los resultados del ensayo de ^3H -Hipoxantina *in vitro* son predictivos de la eficacia *in vivo* de medicamentos antimaláricos potenciales.

Este ensayo también examina la actividad de compuestos de prueba contra la fase hemática del ciclo vital de la malaria, la etapa durante la cual un parásito causante de la malaria, como el *P. falciparum*, infecta los glóbulos rojos. Utiliza la ^3H -Hipoxantina como precursor radiomarcado de los ácidos nucleicos que se retiene solamente en los parásitos en fase de crecimiento. En este ensayo, los glóbulos rojos (también conocidos y mencionados aquí y en el Informe como "eritrocitos") son parasitados en cultivo de tejido por formas asexuadas en fase de multiplicación de un parásito de la malaria como el *P. falciparum*, entonces, los glóbulos rojos parasitados se cultivan en presencia de uno o más compuestos de prueba, un control negativo y un control positivo, a continuación, los glóbulos rojos parasitados se exponen a un ensayo de ^3H -Hipoxantina durante un tiempo determinado, entonces las muestras de glóbulos rojos que contienen parásitos son extraídas del cultivo y cada condición examinada se aísla en un papel de filtro individual, después de un enjuague completo para eliminar la marca de ^3H no contenida como parte del ADN parasitario, los papeles de filtro se examinan en un contador de centelleo que



mide la cantidad de marcador ^3H en cada muestra. Dado que los glóbulos rojos son células que no se dividen y que no contienen núcleos ni su propio ADN y dado que solamente los parásitos en fase de crecimiento acumulan y retienen ^3H en su ADN, la actividad de un compuesto de prueba de inhibición del crecimiento de un Plasmodium se correlaciona con la presencia reducida del marcador ^3H en comparación con las condiciones del cultivo de control que no contiene compuesto de prueba, la inhibición de dicha acumulación del marcador ^3H indica la inhibición directa del crecimiento del parásito por el compuesto de prueba. La concentración del compuesto de prueba requerida para inhibir la incorporación de la ^3H -Hipoxantina en un 50% se informa como el 50% de la concentración inhibitoria (IC_{50}).

El diseño experimental del ensayo de ^3H -Hipoxantina se describe brevemente en la sección "Descripción del ensayo" del informe haciendo referencia a publicaciones científicas que describen el diseño experimental del ensayo en mayor detalle (ver anexo 1, página 1, "Descripción del ensayo", citando las publicaciones científicas enumeradas en la página 2, "Referencias").

El informe sostiene, entre otras cosas, que: "las líneas de *P. falciparum* se cultivaron *in vitro* en eritrocitos humanos O^+ en...medios..."; "los ensayos se llevaron a cabo usando...eritrocitos parasitados...durante 72 horas, usando el método de incorporación de ^3H -Hipoxantina como se describió antes"; y se determinó la IC_{50} de cada compuesto" (ver anexo 1, página 1, "Descripción del ensayo", citando, entre otros, a Andrews) y documenta los resultados de las pruebas de actividad antimalárica de DIM producido por Interpharma Praha (denominado "DIM" en la sección de "Resultados" del Informe), DIM producido por BioResponse, L.L.C. (denominado "BR-DIM" en la sección de "Resultados" del Informe.), matriz de microencapsulación usada en BR-DIM (denominado "Vehículo" en la sección de "Resultados" del Informe, que es un control negativo), y sal de difosfato de cloroquina (denominada "CQ" en la sección de "Resultados" del Informe, que es un control positivo) (ver anexo 1, página 2, "Resultados", y página 1, "Descripción de Materiales Suministrados" y "Descripción del ensayo") y presenta los resultados de tres ensayos independientes para cada una de las dos cepas de *P. falciparum* examinadas, es decir, la cepa resistente a la cloroquina y la cepa sensible a la cloroquina (ver anexo 1, página 1, "Descripción del ensayo").

Finalmente, describimos los resultados del ensayo de inhibición del crecimiento *in vitro* empleado en el informe, que muestran que dos preparaciones de DIM inhiben directamente el crecimiento de los parásitos que causan la malaria.

La sección de "Resultados" del Informe indica que ambas preparaciones de DIM examinadas (es decir, DIM y BR-DIM), y el control positivo, la sal de fosfato de cloroquina, que es un medicamento antimalárico estándar (ver Andrews, en la p. 764, col. 2, 1º párrafo completo, y p. 763, col. 1., párrafo 2), pero no el control negativo (es decir, el "Vehículo"), reducen la acumulación de ³H-Hipoxantina, y por ende inhiben el crecimiento de la cepa de *P. falciparum* resistente a la cloroquina y la cepa sensible a la cloroquina de *P. falciparum* (ver anexo 1, página 2, "Resultados"), en particular, el Informe demuestra que una preparación de DIM ("DIM.") tiene una IC₅₀ de 5,34 (± 0,74) microgramos/mL (µg/mL) en la cepa Dd2 (resistente a la cloroquina) de *P. falciparum* e IC₅₀ de 8,7 (± 1,06) µg/mL en la cepa 3D7 (sensible a la cloroquina) de *P. falciparum*, además, demuestra que otra preparación de DIM ("BR-DIM"), que contiene 30% de Diindolimetano en peso) tiene una IC₅₀ de 20,87 (± 2,28) µg/mL la cepa Dd2 (resistente a la cloroquina) de *P. falciparum* y una IC₅₀ de 17,46 (± 6,05) µg/mL en la cepa 3D7 (sensible a la cloroquina) de *P. falciparum*, dado que la preparación BR-DIM contiene 30% de diindolimetano en peso, esto corresponde a una IC₅₀ de 3,26 µg/mL en la cepa Dd2 y 5,24 µg/mL en la cepa 3D7, respectivamente, para el diindolimetano en BR-DIM.

Estos datos indican que el DIM reduce efectivamente la acumulación de ³H-Hipoxantina y por tanto es efectivo para inhibir directamente el crecimiento de dos cepas de parásitos causantes de la malaria, también indican que las concentraciones de cada una de las dos preparaciones de DIM son efectivas para inhibir la acumulación de ³H-Hipoxantina, y por ende el crecimiento de dos cepas de parásitos causantes de la malaria en 50%.

- c) *La solicitante argumenta en su oficio bajo el radicado No. 09-054148-00010-0000, que la presente solicitud sí presenta nivel inventivo ya que la persona del oficio normalmente versada en la materia al observar las enseñanzas de los documentos D1 y D2 no habría de manera obvia llegado a la presente invención, porque los documentos presentan enseñanzas opuestas a lo aquí revelado y por lo tanto no conducirían a la presente invención.*

Frente a estos argumentos esta Superintendencia se permite aclarar a la solicitante, que al realizar una revisión del capítulo descriptivo, en donde se encuentran ejemplos de fabricación de DIM procesado para Biodisponibilidad oral intensificada, y de la fabricación de diferentes formas farmacéuticas dependiendo de la vía de administración (ver Pág. 47-53), no existe evidencia que permita aportar al nivel inventivo porque la formación contenida no permite que una persona del oficio normalmente versada en la materia pueda reconocer el aporte que se está haciendo la composición que comprende la combinación a la tecnología, ya que no se

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demuestra en la solicitud la verdadera ventaja de la invención de la solicitud con respecto a lo divulgado en el arte previo. Por el contrario, al analizar detalladamente la invención se encuentra que esta se encuentra indiscutiblemente dirigida al uso de una combinación en diferentes patologías y condiciones para el tratamiento y prevención de las infecciones parasitarias, preferiblemente infecciones parasitarias protozoarias, por lo tanto, no se puede reconocer nivel inventivo. Adicionalmente, se reitera que una composición farmacéutica, como tal, se debe definir por los componentes partículas que la integran, así como las cantidades específicas de cada uno de los mismos, que le permitan a la persona del oficio normalmente versada en la materia distinguir las características técnicas esenciales que la hacen novedosa e inventiva frente al arte previo. El hecho de que en la invención se “proponga” una combinación alternativa no es suficiente para demostrar nivel inventivo, como se demostró anteriormente, ya que no se puede identificar cual es la ventaja de estas características frente a las composiciones de combinaciones ya conocidas en el estado del arte, la solicitante no puede atribuir el nivel inventivo a una invención que simplemente realiza modificaciones de rutina en el campo químico-farmacéutico y que no permiten identificar cual es el aporte que ofrecen las composiciones de la invención frente a las del arte previo, cuando se utilizan como agentes en infecciones parasitarias. (Subraya fuera del texto)

Como ya se mencionó, con base en los datos presentados en el Informe, se concluye que DIM muestra actividad inhibitoria del crecimiento contra los parásitos de la malaria, en particular, estos datos indican que el DIM es efectivo para inhibir el crecimiento, la multiplicación y el número de dos cepas de parásitos *P. falciparum* que causan malaria en los seres humanos, es importante anotar que el DIM mostró una actividad todavía más pronunciada contra la cepa más común Dd2 resistente a la cloroquina de parásitos de la malaria que contra la cepa 3D7 de parásitos de la malaria sensible a la cloroquina; estos resultados también muestran que el DIM tiene una actividad inhibitoria directa del crecimiento contra los parásitos de la malaria, independiente de cualquier influencia del sistema inmunológico del huésped.

Como se anotó, los resultados del ensayo de ³H-Hipoxantina obtenidos con un medicamento de prueba son predictivos de la respuesta clínica *in vivo* de los sujetos infectados con parásitos de la malaria al medicamento de prueba (ver Brasseur p.ej., en la p. 10), así las cosas, un índole relacionado con DIM (como el DIM) sería efectivo para inhibir el crecimiento de los parásitos que causan la malaria o una patología similar a la malaria y para tratar a un sujeto infectado con un parásito causante de la malaria o una patología similar a la malaria, descubrimientos que, a criterio de mi cliente, una persona técnica en la materia hubiera considerado inesperados.



8. Por todo lo anterior, y teniendo en cuenta que la solicitud en referencia cumple con los requisitos legales, solicito la concesión de la patente titulada "MÉTODOS Y COMPOSICIONES ANTI-PARASÍTICAS QUE UTILIZAN ÍNDOLES ASOCIADOS CON EL DIINDOLILMETANO" a favor de BIORESPONSE, L.L.C.

9. Adjunto nuevo capítulo reivindicatorio, con modificaciones que resumen las explicaciones dadas en este escrito.

FUNDAMENTOS DE DERECHO

- 1.- Código Contencioso Administrativo
- 2.- Decisión 486 de la Comisión de la Comunidad Andina de Naciones.
- 3.- Las demás normas concordantes.

PRUEBAS

Adjuntamos la declaración presentada ante la Oficina de Marcas y Patentes de Estados Unidos por el Doctor Michael A. Zeligs, inventor de la patente en referencia, con su correspondiente traducción. En dicha declaración se rebaten los argumentos esgrimidos por la Oficina Estadounidense dentro de la patente de invención MÉTODOS Y COMPOSICIONES ANTI-PARASÍTICAS QUE UTILIZAN ÍNDOLES ASOCIADOS CON EL DIINDOLILMETANO, solicitud No. 12/447.307, la cual también se objetó por falta de nivel inventivo con base en las anterioridades DI y D2 y cuyo contenido es aplicable a la presente solicitud.

ANEXOS

- Anexo 1: Informe titulado "Ensayo *in vitro* de diindolilmetano (DM) y DIM de BioResponse en el ensayo de ³H-Hipoxantina de actividad antimalárica" (in vitro Assay of Diindolylmethane (DIM) and BioResponse-DIM in the ³H Hypoxanthine Assay for Anti-malarial Activity), fechado el 8 de febrero de 2012, firmado por el Dr. Andrews, Eskitis Institute for Cell and Molecular Therapies, Griffith University.
- Anexo 2: Andrews et al., 2000, Int. J. Parasitol. 30(6):761-768
- Anexo 3: Brasseur et al., 1998, Antimicrob. Agents Chemother. 42(4):818-820
- Anexo 4: Brasseur et al., 1992, Am. J. Trop. Med. Hyg. 46(1):8-14
- Comprobante de pago por la modificación/corrección a solicitud en trámite relacionada con el capítulo reivindicatorio. Recibo no. 91, 872
- Comprobante de pago correspondiente a las 22 reivindicaciones adicionales del capítulo reivindicatorio que se presenta con este escrito. Recibo no. 91, 868

Castillo Grau & Asociados

ABOGADOS

NOTIFICACIONES

Recibiré notificaciones en la Secretaría de su Despacho o en mi oficina de abogado ubicada en la Carrera 13 No. 37-43 Piso 12 en Bogotá, D.C.

De Usted atentamente,



LUIS FELIPE CASTILLO GIBSON

C.C. No. 79.397.879 de Bogotá

T.P. No. 68.995 del C.S.J.



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 Industria y Comercio SUPERINTENDENCIA	RECIBO DE CAJA	No. 13 - 0091872
		Bogotá D.C., Septiembre 12 de 2013 - 14:11:36

RECIBIDO DE : CASTILLO GRAU & ASOCIADOS LTDA.					NI 800.098.704
*** Soporte del Pago ***					
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*** Conceptos Pagados ***					
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			=====	=====	

SON: **CIENTO VEINTICINCO MIL PESOS MONEDA CORRIENTE**

Responsable: _____

Recibo de Caja-Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

No. 09-054148- -00014-0000
Fecha: 2013-09-13 16:36:01 Dep. 2020 DIR.NUEVASCR
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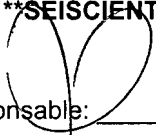
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*** Conceptos Pagados ***				
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				\$660.000.00

SON: **SEISCIENTOS SESENTA MIL PESOS MONEDA CORRIENTE**

Responsable: 

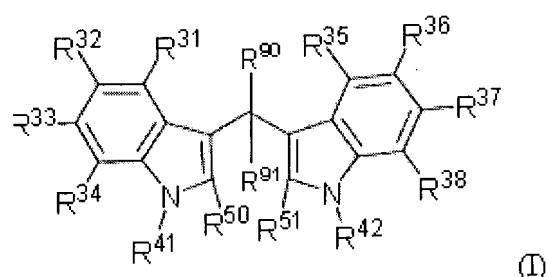
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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

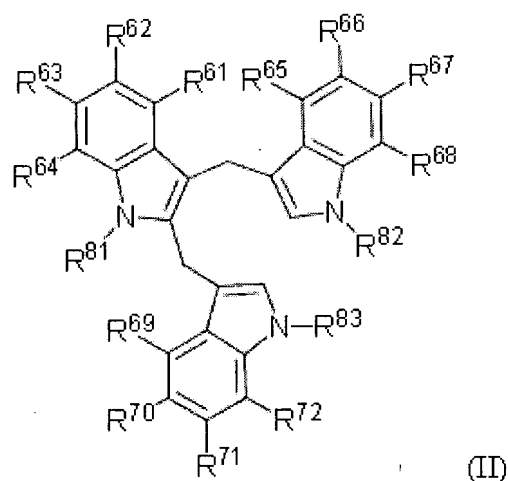
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Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 412 REOSPRESCRECU Folios: 52

LO QUE SE REIVINDICA ES:

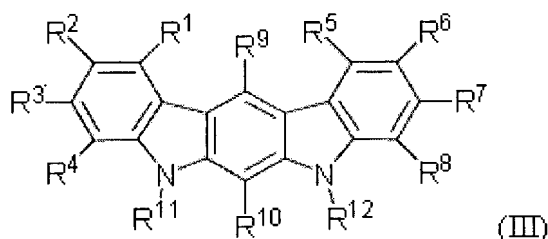
1. Una composición que comprende una cantidad terapéuticamente efectiva de la combinación de uno o más índoles relacionados con DIM y uno o más agentes antiprotzoarios; que se caracteriza porque los uno o más índoles relacionados con DIM se encuentran en una cantidad efectiva para inhibir el crecimiento de un parásito que causa malaria, que se caracteriza porque el mencionado parásito es *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale* o *Plasmodium vivax*; que se caracteriza porque los uno o más índoles relacionados con DIM se seleccionan de entre el grupo compuesto por un compuesto de fórmula I:



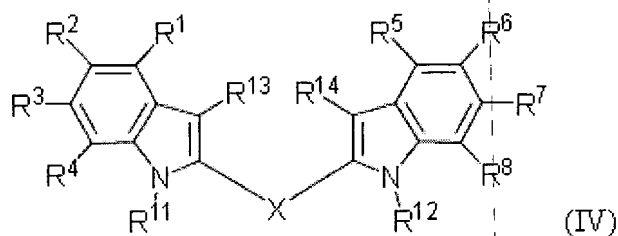
Que se caracteriza porque R32 y R36 son sustituyentes seleccionados independientemente de entre el grupo compuesto por grupos hidrógeno, hidroxilo y metoxi y etoxycarbonilo, R33 y R37 son sustituyentes seleccionados independientemente de entre el grupo compuesto por hidrógeno, hidroxilo y metoxi, R31, R34, R35, R38, R41 y R42 son hidrógeno, R50, R51 son bien sea hidrógeno o metilo y R90, R91 son hidrógeno; un compuesto de fórmula II:



Que se caracteriza porque R62, R63, R66, R67, R70 y R71 son sustituyentes seleccionados independientemente de entre el grupo compuesto por hidrógeno, hidroxilo y metoxi y R61, R64, R65, R68, R69, R72, R81, R82 y R83 son hidrógeno; un compuesto de fórmula (III):



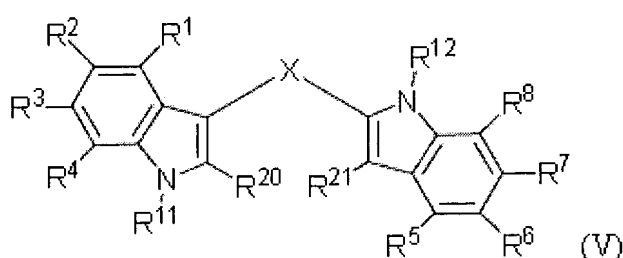
Que se caracteriza porque R1, R2, R3, R4, R5, R6, R7, R8, R9 y R10 son sustituyentes seleccionados independientemente de entre el grupo compuesto por hidrógeno, C1-C24 alquilo, C2-C24 alquenilo, C2-C24 alquinilo, C5-C20 arilo, C6-C24 alcarilo, C6-C24 aralquilo, halo, hidroxilo, sulfhidrilo, C1-C24 alcoxi, C2-C24 alqueniloxi, C2-C24 alquiniloxi, C5-C20 ariloxi, acilo, aciloxi, C2-C24 alcoxicarbonilo, C6-C20 ariloxicarbonilo, halocarbonilo, C2-C24 alquilcarbonato, C6-C20 arilcarbonato, carboxi, carboxilato, carbamoilo, carbamoilo mono-(C1-C24 alquilo) sustituido, carbamoilo di-(C1-C24 alquilo) sustituido, arilcarbamoilo mono sustituido, tiocarbamoilo, carbamido, ciano, isociano, cianato, isocianato, isotiocianato, azido, formilo, tioformilo, amino, mono- y di-(C1-C24 alquilo) sustituido amino, mono- y di-(C5-C20 aril) sustituido amino, C2-C24 alquilamido, C6-C20 arilamido, imino, alquilimino, arilimino, nitro, nitroso, sulfo, sulfonato, C1-C24 alquilosulfanilo, arilsulfanilo, C1-C24 alquilosulfinilo, C5-C20 arilsulfinilo, C1-C24 alquilosulfonilo, C5-C20 arilsulfonilo, fosfono, fosfonato, fosfinato, fosfo, fosfino y combinaciones de los mismos y que además se caracteriza porque dos cualesquiera sustituyentes adyacentes (orto) pueden estar enlazados formando una estructura cíclica seleccionada de entre anillos de cinco miembros, anillos de seis miembros y anillos fusionados de cinco miembros y/o de seis miembros, que se caracteriza porque la estructura cíclica es aromática, alicíclica, heteroaromática, o heteroalíclica y tiene cero a 4 nohidrógeno sustituyentes y cero a 3 heteroátomos y R11 y R12 se seleccionan independientemente de entre el grupo compuesto por hidrógeno, C1-C24 alquilo, C2-C24 alcoxicarbonilo, amino sustituido C1-C24 alquilo, (C1-C24 alquilamino) sustituido C1-C24 alquilo y di-(C1-C24 alquilo)amino sustituido C1-C24 alquilo, con la condición de que por lo menos uno de R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11 y R12 es diferente de hidrógeno y cuando R1, R2, R3, R4, R5, R6, R7 y R8 se seleccionan de entre hidrógeno, halo, alquilo y alcoxi, entonces R11 y R12 son diferentes de hidrógeno y alquilo; un compuesto de fórmula (IV):



Que se caracteriza porque R1, R2, R3, R4, R5, R6, R7 y R8 son sustituyentes seleccionados independientemente de entre el grupo compuesto por hidrógeno, C1-C24 alquilo, C2-C24 alquenilo, C2-C24 alquinilo, C5-C20 arilo, C6-C24 alcarilo, C6-C24 aralquilo, halo, hidroxilo, sulfhidrilo, C1-C24 alcoxi, C2-C24 alqueniloxi, C2-C24 alquiniloxi, C5-C20 ariloxi, acilo, aciloxi, C2-C24 alcoxicarbonilo, C6-C20 ariloxicarbonilo, halocarbonilo, C2-C24 alquilcarbonato, C6-C20 arilcarbonato, carboxi, carboxilato, carbamoilo, carbamoilo mono-(C1-C24 alquilo) sustituido, carbamoilo di-(C1-C24 alquilo) sustituido, arilcarbamoilo mono sustituido, tiocarbamoilo, carbamido, ciano, isociano, cianato, isocianato, isotiocianato, azido, formilo, tioformilo, amino, amino mono- y di-(C1-C24 alquilo) sustituido, amino mono- y di-(C5-C20 aril) sustituido, C2-C24 alquilamido, C5-C20 arilamido, imino, alquilimino, arilimino, nitro, nitroso, sulfo, sulfonato, C1-C24

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alquilosulfanilo, arilsulfanilo, C1-C24 alquilosulfinilo, C5-C20 arilsulfinilo, C1-C24 alquilosulfonilo, C5-C20 arilsulfonilo, fosfono, fosfonato, fosfinato, fosfo, fosfino y combinaciones de los mismos y que además se caracteriza porque dos cualesquiera sustituyentes adyacentes (orto) pueden estar enlazados formando una estructura cíclica seleccionada de entre anillos de cinco miembros, anillos de seis miembros y anillos fusionados de cinco miembros y/o de seis miembros, que se caracteriza porque la estructura cíclica es aromática, alicíclica, heteroaromática, o heteroalíclica y tiene de cero a 4 sustituyentes diferentes de hidrógeno y de cero a 3 heteroátomos, con la condición de que uno pero no los dos de R2 y R6 es amino, amino monosustituido, o amino disustituido; R11 y R12 se seleccionan independientemente de entre el grupo compuesto por hidrógeno, C1-C24 alquilo, C2-C24 alcoxicarbonilo, C1-C24 alquilo amino sustituido, (C1-C24 alquilamino) C1-C24 alquilo sustituido y C1-C24 alquilo di-(C1-C24 alquilo)amino sustituido, R13 y R14 se definen igual que para R1, R2, R3, R4, R5, R6, R7 y R8, con la condición de que por lo menos uno de R13 y R14 es diferente de hidrógeno y X es O, S, arileno, heteroarileno, CR15R16 o NR17 que se caracteriza porque R15 y R16 son hidrógeno, C1-C6 alquilo, o juntos forman =CR18R19, en donde R18 y R19 son hidrógeno o C1-C6 alquilo y R17 es igual a lo definido para R11 y R12; y un compuesto de fórmula (V):



Que se caracteriza porque R1, R2, R3, R4, R5, R6, R7, R8, R11, R12 y X se definen igual que se definieron los compuestos de formula (III), y R20 y R21 se definen igual que para R1, R2, R3, R4, R5, R6, R7 y R8; y que se caracteriza porque el agente antiprotozoario se selecciona de entre el grupo compuesto por: atovaquona, amodiaquina, anfotericina, astemizol, butoconazol, clindamicina, eflornitina, fumagilina, yodoquinol, clioquinol, Etanidazol, Benznidazol, fluoroquinolonas, enoxacina, ciprofloxacina, doxiciclina, melarsoprol, metronidazol, miltefosina, nifurtimox, nitazoxanida, paromomicina, pentamindina, estibogluconato de sodio, gluconato de antimonio, suramina, sal sódica de suramina, tinidazol, pirimetamina, proguanilo, espiramicina, sulfadoxina, artemisinina, an derivado de la artemisinina, dihidroartemisinina, arteméter, artesunato, quinina, una quinolina, halofantrina, mefloquina, lumefantrina, amodiaquina, pironaridina, piperaquina, cloroquina, hidroxiclороquinaa, naftoquina, primaquina, tafenoquina, amodiaquina, a 4-aminoquinolina, un curcuminoide, Genisteína, dehidrosilibina, silibina A, silibina B, isosilibina A, isosilibina B, 8-(1;1)-DMA-kaempferida, luteolia, baicaleína, ácido dihidrobetulínico, quercetina, ácido eriodictiólico, ácido lursólico, ácido oleanólico, un triterpeno, atovaquona, cloroquina, yodoquinol, clioquinol, Ácido jasmónico [ácido 3-oxo-2-(2-pentenil)ciclopentanoacético], jasmonato de metilo [ácido metilo 3-oxo-2-(2-pentenil)ciclopentanoacético], *cis*-jasmona [3-metil-2-(2-pentenil)-2-ciclopenten-1-ona],

yoduro de 3,3'-dihexiloxacarbocianina, estibogluconato de sodio, curcumina, Pirroloquinazolindiamina, Novobiocina, quercetina, ciclosporina, ácido dihidrobetulínico, una campotecina, bortezomib, etopósido, salvicina, una antraciclina, un extracto de *Yucca schidigera*, un té o extracto hecho de *Artemisia annua*, tés o extractos hechos de *Curcuma domestica*, un extracto de ajo, un extracto de raíz de *Uvaria chamae* o *Hippocratea Africana*, un extracto de raíz de *Homalium letestui*, un extracto de *Vernonia brasiliensis*, un extracto de *Sapindus mukorossi* y un extracto de *Acanthospermum australe*.

2. La composición de la reivindicación 1, que se caracteriza porque el mencionado parásito es *Plasmodium falciparum*.
3. La composición de la reivindicación 1 o 2, que se caracteriza porque el sujeto es un ser humano.
4. La composición de cualquiera de las reivindicaciones 2-6, que se caracteriza porque el sujeto es un cordero, ternero, cerdo, conejo o pollo.
5. La composición de cualquiera de las reivindicaciones 1-4, que se caracteriza porque los uno o más indoles relacionados con DIM se seleccionan de entre el grupo compuesto por diindolilmetano, DIM hidroxilados, DIM metoxilados, 2-(Indol-3-ilmetil)-3,3'-diindolilmetano (LTR), LTR hidroxilados, LTR metoxilados, 5,5'-dimetilDIM (5-Me-DIM), 2,2'-dimetilDIM (2-Me-DIM), 5,5'-dicloroDIM (5-Cl-DIM), imidazolil-3,3'-diindolilmetano, imidazolil-3,3'-diindolilmetanos nitro sustituidos, 2,10-dicarbetoxi-6-metoxi-5,7-dihidro-indolo-[2,3-b]carbazol, 6-etoxicarboniloxi-5,7-dihidro-indolo-[2,3-b]carbazol y 2,10-dicarbetoxi-6-etoxicarboniloxi-5,7-dihidro-indolo-[2,3-b]carbazol y 2,6-dicarbetoxi-3,3'-dimetil-13,14-diindolilmetano.
6. La composición de cualquiera de las reivindicaciones 1-5, que se caracteriza porque el agente antiprotozoario se selecciona de entre el grupo compuesto por atovaquona, amodiaquina, anfotericina, astemizol, butoconazol, clindamicina, eflornitina, fumagilina, yodoquinol, clioquinol, Etanidazol, Benznidazol, fluoroquinolonas, enoxacina, ciprofloxacina, doxiciclina, melarsoprol, metronidazol, miltefosina, nifurtimox, nitazoxanida, paromomicina, pentamindina, estibogluconato de sodio, gluconato de antimonio, suramina, sal sódica de suramina, tinidazol, pirimetamina, proguanilo, espiramicina y sulfadoxina.
7. La composición de cualquiera de las reivindicaciones 1-5, que se caracteriza porque el agente antiprotozoario se selecciona de entre el grupo compuesto por artemisinina, dihidroartemisinina, arteméter, artesunato, quinina, una quinolina, halofantrina, mefloquina, lumefantrina, amodiaquina, pironaridina, piperquina, cloroquina, hidroxicloroquina, naftoquina, primaquina, tafenoquina, amodiaquina, a 4-aminoquinolina, un curcuminoide, Genisteína, dehidrosilibina, silibina A, silibina B,

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isosilibina A, isosilibina B, 8-(1;1)-DMA-kaempferida, luteolia, baicaleína, ácido dihidrobetulínico, quercetina, ácido eriodictiólico, ácido lursólico, ácido oleanólico y un triterpeno.

8. La composición de cualquiera de las reivindicaciones 1-5, que se caracteriza porque el agente antiprotazoario se selecciona de entre el grupo compuesto por atovaquona, cloroquina, yodoquinol, clioquinol, ácido jasmónico [ácido 3-oxo-2-(2-pentenil)ciclopentanoacético], jasmonato de metilo [ácido metilo 3-oxo-2-(2-pentenil)ciclopentanoacético], *cis*-jasmona [3-metil-2-(2-pentenil)-2-ciclopenten-1-ona], yoduro de 3,3'-dihexiloxacarbocianina, estibogluconato de sodio, curcumina, Pirroloquinazolidinamina, Novobiocina, quercetina, ciclosporina, ácido dihidrobetulínico, una campotecina, bortezomib, etopósido, salvicina y una antraciclina.
9. La composición de cualquiera de las reivindicaciones 1-5, que se caracteriza porque el agente antiprotazoario se selecciona de entre el grupo compuesto por un extracto de *Yucca schidigera*, un té o extracto hecho de *Artemisia annua*, tés o extractos hechos de *Curcuma domestica*, un extracto de ajo, un extracto de raíz de *Uvaria chamae* o *Hippocratea Africana*, un extracto de raíz de *Homalium letestui*, un extracto de *Vernonia brasiliensis*, un extracto de *Sapindus mukorossi* y un extracto de *Acanthospermum australe*.
10. La composición de cualquiera de las reivindicaciones 1 -9, que se caracteriza porque el índole relacionado con DIM es DIM.
11. La composición de la reivindicación 10, que se caracteriza porque el DIM es DIM procesado.
12. La composición de cualquiera de las reivindicaciones 1-9, que se caracteriza porque el índole relacionado con DIM está microencapsulado con fosfatidilcolina (PC), formando complejos con PC, o convertido en micropartículas y nanopartículas de disolución rápida.
13. La composición de la reivindicación 10 o 11, que se caracteriza porque el DIM está microencapsulado con fosfatidilcolina (PC), formando complejos con PC, o convertido en micropartículas y nanopartículas de disolución rápida.
14. La composición de cualquiera de las reivindicaciones 1-13, que se caracteriza porque la composición está formulada para administración oral, administración intravenosa, administración vaginal, administración rectal o administración tópica.
15. La composición de cualquiera de las reivindicaciones 1-14, que se caracteriza porque la composición está formulada como un suplemento nutricional, alimento enriquecido, mezcla de bebida, o mezcla de té.

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16. La composición de cualquiera de las reivindicaciones 1-15, que además comprende Vitamina-E succinato de polietilenglicol (TPGS), piperina, limonina, D-Limoneno o polietilenglicol.
17. La composición de cualquiera de las reivindicaciones 1-16, que se caracteriza porque la composición está formulada para administración oral.
18. La composición de cualquiera de las reivindicaciones 1-14 y 16, que se caracteriza porque el DIM y el agente antiprotozoario están emulsionados en una crema para administración tópica a la mucosa vaginal o rectal.
19. La composición de cualquiera de las reivindicaciones 1-5 y 10-18, que se caracteriza porque el agente antiprotozoario se selecciona de entre el grupo compuesto por artemisinina, dihidroartemisinina, arteméter, artesunato, quinina, curcumina, cloroquina y mefloquina.
20. La composición de cualquiera de las reivindicaciones 1-19, que se caracteriza porque la cantidad de los uno o más índoles relacionados con DIM se encuentra en el intervalo de 50 a 600 mg por dosis.
21. La composición de la reivindicación 20, que se caracteriza porque los uno o más índoles relacionados con DIM está formulada para administración dos veces al día.
22. La composición de la reivindicación 20 o 21, que se caracteriza porque los uno o más agentes antiprotozoarios se encuentran en la cantidad que varía de 25 a 1000 mg.
23. La composición de cualquiera de las reivindicaciones 20-22, que se caracteriza porque los uno o más índoles relacionados con DIM y los uno o más agentes antiprotozoarios están formulados para administración oral.
24. La composición de la reivindicación 23, la cual además comprende curcumina en la cantidad que varía de 25 a 1000 mg.
25. La composición de la reivindicación 24, la cual además comprende piperina.
26. La composición de la reivindicación 25, la cual está formulada como una tableta o cápsula.
27. La composición de la reivindicación 19, la cual está formulada como una suspensión o emulsión para administración intravenosa.
28. La composición de la reivindicación 27, que se caracteriza porque la cantidad de los uno o más índoles relacionados con DIM se encuentra en el intervalo de 2 a 15 mg/kg.

29. La composición de la reivindicación 14, que se caracteriza porque la composición está formulada para administración tópica vaginal.
30. La composición de la reivindicación 29, que se caracteriza porque los uno o más agentes antiprotozoarios comprenden butoconazol y/o clindamicina.
31. La composición de la reivindicación 29, que se caracteriza porque los uno o más agentes antiprotozoarios comprenden artemisinina o un derivado de la artemisinina.
32. La composición de la reivindicación 30, que se caracteriza porque los uno o más agentes antiprotozoarios además comprenden artemisinina o un derivado de la artemisinina.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Michael A. Zeligs

Confirmation No.: 2877

Application No.: 12/447,307

Art Unit: 1627

Filed: April 27, 2009

Examiner: Wang, Shengjun

For: ANTI-PARASITIC METHODS AND
COMPOSITIONS UTILIZING
DIINDOLYLMETHANE-RELATED
INDOLES

Attorney Docket No: 9439-024-999

DECLARATION OF MICHAEL A. ZELIGS UNDER 37 C.F.R. 1.132Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Michael A. Zeligs, do declare and state that:

1. I am a citizen of the United States residing at 568 Rembrandt Road, Boulder, Colorado 80302.

2. I am the founder and Chief Executive Officer of BioResponse, L.L.C., and BioResponse, L.L.C. is the owner of the entire right, title and interest in, to and under the invention described and claimed in the above-identified application.

3. I earned a Master's degree in Physiology, Graduate School of Biology, University of California, Santa Barbara, California, in 1972, and an M.D. from the College of Medicine, University of California, Irvine in 1977. I have completed specialty medical training in Anesthesiology, Pediatrics, and Molecular Immunology. As a post-graduate fellow in Immunology at the University of California, Irvine, from 1988-1991, I studied inflammation and human immune responses, treated immunocompromized patients, conducted a clinical trial in immune potentiation, assessed immune response through testing in the clinic, and conducted laboratory studies of cytokine driven lymphocyte proliferation. My clinical training has also included training in Tropical Medicine with assignments in three protozoal parasite endemic regions, Hawaii (1978-1980), Peru (1977), and Panama (1974). As part of this training in

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Tropical Medicine, I was directly responsible for diagnosing and treating protozoal parasitic diseases, including Malaria and Leishmaniasis. My academic and technical experience and honors, and a list of my publications, are set forth in my *Curriculum Vitae*, attached hereto as Exhibit 1.

4. I am the sole inventor of the invention described and claimed in the above-identified patent application, Application No. 12/447,307 (the "'307 application"). I understand that the claims of the '307 application are being amended so as to be directed to: (i) methods of treating a subject infected with a parasite that causes malaria or a malaria-like condition comprising administering to the subject an amount of one or more DIM-related indoles (e.g., Diindolylmethane ("DIM")) effective to inhibit growth of such parasite in the subject and one or more anti-protozoal agents (see independent claim 1 and claims depending therefrom); (ii) methods of treating a subject infected with a parasite that causes malaria or a malaria-like condition comprising administering to the subject an amount of one or more DIM-related indoles (e.g., DIM) effective to inhibit growth of such parasite in the subject (see independent claim 28 and claims depending therefrom); and (iii) compositions comprising an amount of one or more DIM-related indoles (e.g., DIM) effective to inhibit growth of a parasite that causes malaria or a malaria-like condition in a subject infected with such parasite and one or more anti-protozoal agents (see independent claim 49 and claims depending therefrom).

5. I have read the Office Action dated August 9, 2011 and the references cited therein, including International Patent Publication No. WO 2006/053160 by Hensel ("Hensel"), U.S. Patent Publication No. US 2006/0100264 by Bjeldanes et al. ("Bjeldanes"), and U.S. Patent No. 6,534,085 by Zeligs ("Zeligs") cited in the Office Action as a basis for the rejection under 35 U.S.C. § 103(a). I understand that the claims of the '307 application are subject to a rejection wherein the Office Action contends that Hensel in view of Bjeldanes and Zeligs would make obvious the methods of treating protozoal disease claimed in the '307 application prior to presently made claim amendments.

6. In my opinion, nothing in the disclosures of Hensel, Bjeldanes and Zeligs suggests that DIM or any DIM-related indole can be effective in treating protozoal disease, much less suggests that DIM or any DIM-related indole can be effective in treating a subject infected with a parasite that causes malaria or a malaria-like condition or effective in inhibiting growth of such parasite in the subject.

7. In my opinion, a person of ordinary skill would have found it unexpected that DIM can be effective in inhibiting growth of malaria-causing parasites. Contrary to the expectations of a person of ordinary skill in the art, DIM was shown to be effective in directly inhibiting growth of malaria-causing parasites. This finding is evidenced in the report entitled "In vitro Assay of Diindolylmethane (DIM) and BioResponse-DIM in the ^3H Hypoxanthine Assay for Anti-malarial Activity" ("Report"), describing the experimental design and results of an *in vitro* assay measuring growth inhibition of *Plasmodium falciparum* (i.e., *P. falciparum*), a protozoan parasite that causes malaria in humans, by two different DIM preparations (see Exhibit 2, attached hereto). The *in vitro* growth inhibition assay of *P. falciparum* was conducted, and the Report was prepared, per my request and on my behalf, by Eskitis Institute for Cell and Molecular Therapies, Griffith University. Paragraphs 8 and 9, below, describe the *in vitro* growth inhibition assay, i.e., the ^3H -Hypoxanthine assay, used in the Report, its rationale and experimental design. Paragraph 10, below, describes the results of the *in vitro* growth inhibition assay used in the Report, which show that two DIM preparations directly inhibit the growth of parasites that cause malaria.

8. The Report describes the results of the Tritiated (^3H) Hypoxanthine (" ^3H -Hypoxanthine") assay, which was utilized to assess the ability of two different DIM preparations to inhibit growth of two strains of malaria-causing parasites. The ^3H -Hypoxanthine assay is a well-established assay for measuring the activity of potential anti-malarial drugs (see, e.g., Andrews et al., 2000, *Int. J. Parasitol.* 30(6):761-768, at p. 763, "Andrews," attached hereto as Exhibit 3). This assay is relevant for predicting the sensitivity of various pathogenic parasites that cause malaria or a malaria-like condition (i.e., parasites of the *Plasmodium* genus) to growth inhibition by potential anti-parasite drugs (see Andrews at p. 763, which shows use of the ^3H -Hypoxanthine assay to measure growth inhibition of *P. falciparum* species of the *Plasmodium* genus by various drugs; see also Brasseur et al., 1998, *Antimicrob. Agents Chemother.* 42(4):818-820, at pp. 818-819, "Brasseur," attached hereto as Exhibit 4, which shows use of the ^3H -Hypoxanthine assay to measure growth inhibition of *Babesia* species of the *Plasmodium* genus by various drugs). The results of the *in vitro* ^3H -Hypoxanthine assay are predictive of the clinical efficacy of the tested drug *in vivo* (see, e.g., Brasseur et al., 1992, *Am. J. Trop. Med. Hyg.* 46(1):8-14, "Brasseur2," attached hereto as Exhibit 5). In particular, Brasseur et al., in a study done in the African continent in a setting of emerging resistance to the anti-malarial drug mefloquine, demonstrated that 37 out of 38 patients responsive to the *in vivo* treatment with

mefloquine were infected with parasites that were sensitive to low concentrations of the drug in the *in vitro* ^3H -hypoxanthine assay (displaying low "EC₅₀," i.e., half maximal effective concentration, which is equivalent to "IC₅₀" referred to hereinbelow), and resistance to the drug *in vivo* was associated with high EC₅₀ results in the *in vitro* ^3H -hypoxanthine assay (see Brasseur2 at p. 10). Thus, the results of the *in vitro* ^3H -Hypoxanthine assay are predictive of the *in vivo* clinical efficacy of potential anti-malarial drugs.

9. The ^3H -Hypoxanthine assay tests the activity of test compounds against the blood stage life cycle of malaria—the stage during which a malaria-causing parasite, such as *P. falciparum*, infects red blood cells. It utilizes ^3H -Hypoxanthine as a radiolabeled nucleic acid precursor which is retained only in growing parasites. In this assay, red blood cells (also known as, and referred herein and in the Report as, "erythrocytes") are parasitized in tissue culture by multiplying asexual forms of a malaria parasite such as *P. falciparum*. Then, the parasitized red blood cells are cultured in the presence of one or more test compound(s), a negative control, and a positive control. Subsequently, the parasitized red blood cells are exposed to ^3H -Hypoxanthine for a period of time. The samples of red blood cells containing parasites are then removed from culture, and each condition tested is isolated on an individual filter paper. After complete rinsing to remove ^3H label not contained as part of parasitic DNA, the filter papers are counted on a scintillation counter measuring the quantity of ^3H label in each sample. Since red blood cells are non-dividing cells which do not contain nuclei or their own DNA, and since only growing parasites accumulate and retain ^3H in their DNA, the activity of a test compound to inhibit growth of a *Plasmodium* parasite correlates with a reduced presence of ^3H label compared to the control culture conditions containing no test compound. Inhibition of such ^3H label accumulation indicates direct inhibition of parasite growth by the test compound. The concentration of the test compound required to inhibit ^3H -hypoxanthine incorporation by 50% is reported as the Inhibitory Concentration 50% ("IC₅₀"). The experimental design of the ^3H -Hypoxanthine assay is briefly described in the "Description of assay" section of the Report with reference to scientific publications describing the experimental design of the assay in more detail (see Exhibit 2, page 1, "Description of assay," citing to scientific publications listed at page 2, "References"). The Report states, *inter alia*, that: "*P. falciparum* lines...were cultured in vitro in O⁺ human erythrocytes in...media...."; "[a]ssays were carried out using...parasitized erythrocytes...over 72 hrs using the ^3H -Hypoxanthine incorporation method as previously described"; and IC₅₀ of each compound "was determined" (see Exhibit 2, page 1, "Description of

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assay," citing, *inter alia*, to Andrews). The Report documents the results of testing the anti-malarial activity of DIM produced by Interpharma Praha (termed "DIM" in the "Results" section of the Report), DIM produced by BioResponse, L.L.C. (termed "BR-DIM" in the "Results" section of the Report), microencapsulation matrix used in BR-DIM (termed "Vehicle" in the "Results" section of the Report, which is a negative control), and chloroquine diphosphate salt (termed "CQ" in the "Results" section of the Report, which is a positive control) (see Exhibit 2, page 2, "Results," and page 1, "Description of Supplied Materials" and "Description of assay"). The Report presents the results of three independent assays for each of the two *P. falciparum* strains tested, i.e., chloroquine resistant strain and chloroquine sensitive strain (see Exhibit 2, page 1, "Description of assay"). The results of the assay are described in paragraph 10 below.

10. The "Results" section of the Report indicates that both DIM preparations tested (i.e., DIM and BR-DIM), and the positive control—chloroquine phosphate salt, which is a standard anti-malarial drug (see Andrews, at p. 764, col. 2, 1st full para., and p. 763, col. 1, para. 2), but not the negative control (i.e., "Vehicle"), reduce ³H-hypoxanthine accumulation in, and thus inhibit the growth of, chloroquine resistant strain of *P. falciparum* and chloroquine sensitive strain of *P. falciparum* (see Exhibit 2, page 2, "Results"). In particular, the Report demonstrates that one preparation of DIM ("DIM") has IC₅₀ of 5.34 (± 0.74) micrograms/ml (μg/ml) in Dd2 (chloroquine resistant) *P. falciparum* strain and IC₅₀ of 8.7 (± 1.06) μg/ml in 3D7 (chloroquine sensitive) *P. falciparum* strain. Further, the Report demonstrates that another preparation of DIM ("BR-DIM," containing 30% of Diindolylmethane by weight) has IC₅₀ of 10.87 (± 2.28) μg/ml in Dd2 (chloroquine resistant) *P. falciparum* strain and IC₅₀ of 17.46 (± 6.05) μg/ml in 3D7 (chloroquine sensitive) *P. falciparum* strain. Since BR-DIM preparation contains 30% Diindolylmethane by weight, this corresponds to an IC₅₀ of 3.26 μg/ml in Dd2 strain and 5.24 μg/ml in 3D7 strain, respectively, for Diindolylmethane in BR-DIM. These data indicate that DIM is effective to reduce ³H-hypoxanthine accumulation in, and thus, is effective to directly inhibit the growth of, two strains of malaria-causing parasites. These data also indicate the concentrations of each of the two DIM preparations effective to inhibit ³H-hypoxanthine accumulation in, and thus the growth of, two strains of malaria-causing parasites by 50%.

11. Based on the data presented in the Report, I conclude that DIM shows growth-inhibitory activity against malarial parasites. In particular, these data indicate that DIM is effective in inhibiting the growth, multiplication and number of two strains of human malaria-causing parasite *P. falciparum*. Notably, DIM showed even greater activity against the more

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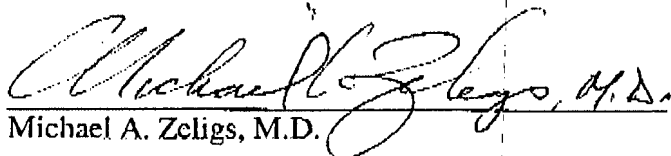
common chloroquine resistant Dd2 strain of malarial parasites than against the chloroquine sensitive 3D7 strain of malarial parasites. I also conclude that these results show that DIM has a direct growth-inhibitory activity against malarial parasites, independent from any influence from the host immune system. As discussed in paragraph 8, the results of the ³H-Hypoxanthine assay obtained for a test drug are predictive of *in vivo* clinical response of subjects infected with a malarial parasite to the test drug (see Brasseur et al., e.g., p. 10). Thus, I conclude that a DIM-related indole (such as DIM) would be effective in inhibiting growth of parasites that cause malaria or a malaria-like condition. I also conclude that a DIM-related indole (such as DIM) would be effective in treating a subject infected with a parasite that causes malaria or a malaria-like condition.

12. In my opinion, a person of ordinary skill in the art would have found it unexpected that DIM can be effective in inhibiting growth of a malaria-causing parasite. I also believe that a person of ordinary skill in the art would have found it unexpected that DIM has a direct growth-inhibitory activity against two strains of human malarial parasites, independent from any influence from the host immune system.

13. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date:

February 9, 2012


Michael A. Zeligs, M.D.

- Exhibit 1: Curriculum Vitae of Michael A. Zeligs, M.D.
Exhibit 2: Report entitled "In vitro Assay of Diindolylmethane (DIM) and BioResponse-DIM in the ³H Hypoxanthine Assay for Anti-malarial Activity," dated February 8, 2012, signed by Dr. Andrews, Eskitis Institute for Cell and Molecular Therapies, Griffith University
Exhibit 3: Andrews et al., 2000, Int. J. Parasitol. 30(6):761-768
Exhibit 4: Brasseur et al., 1998, Antimicrob. Agents Chemother. 42(4):818-820
Exhibit 5: Brasseur et al., 1992, Am. J. Trop. Med. Hyg. 46(1):8-14

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OFICINA DE MARCAS Y PATENTES DE ESTADOS UNIDOS

Solicitud de: Michael A. Zeligs
No. de Solicitud: 12/447.307
Radicada: 27 de abril de 2009

Confirmación No.: 2877
Unidad de estado de la Técnica: 1627
Examinador: Wang, Shengjun

Para: MÉTODOS Y COMPOSICIONES ANTIPARASITARIAS QUE UTILIZAN INDOLES
RELACIONADOS CON DIINDOLILMETANO

Legajo de Apoderado No: 9439-024-999

DECLARACIÓN DE MICHAEL A. ZELIGS CONFORME A 37 C.F.R. 1.132

Comisionado de Patentes
P.O. Box 1.450
Alexandria, VA 223 13-3 450

Yo, Michael A. Zeligs, me permito declarar lo siguiente:

1. Soy ciudadano estadounidense domiciliado en 568 Rembrandt Road, Boulder, Colorado 80302.
2. Soy cofundador y Director Ejecutivo de BioResponse, L.L.C., y BioResponse, L.L.C. es el propietario de todos los derechos, titularidad e interés en la invención descrita y reivindicada en la solicitud arriba identificada.
3. Obtuve una Maestría en Fisiología en la Escuela de Posgrados de Biología de la Universidad de California en Santa Bárbara, California, en 1972, y obtuve mi diploma de Médico del College of Medicine, Universidad de California, Wine en 1977. Hice estudios de especialización en Anestesiología, Pediatría e Inmunología Molecular. En condición de becario de posgrado en Inmunología en la Universidad de California, Irvine, de 1985-1991, hice estudios sobre inflamación y respuesta inmunológica humana, traté pacientes inmunocomprometidos, realicé estudios clínicos en potenciación de la inmunidad, valoré la respuesta inmunitaria mediante pruebas clínicas, y llevé a cabo estudios de laboratorio sobre la proliferación de los linfocitos impulsada por citocinas. Mi formación clínica también incluye entrenamiento en Medicina Tropical con trabajo de campo en tres regiones de parasitismo endémico por protozoarios, Hawái 1978-1980, Perú, 1977, y Panamá, 1974. Como parte de este entrenamiento en Medicina Tropical fui directamente responsable del diagnóstico y tratamiento de enfermedades parasitarias por protozoarios, incluidas malaria y Leishmaniasis. Mi experiencia y honores académicos y técnicos, y una lista de mis publicaciones, aparecen en mi Curriculum Vitae, que se anexa a la presente como Evidencia 1.
4. Soy el inventor único de la invención descrita y reivindicada en la solicitud de patente arriba identificada, Solicitud No. 12/447.307 (la "solicitud '307"). Entiendo que las reivindicaciones de la solicitud '307 están siendo modificadas para que se refieran a: (i) métodos para tratar a un sujeto infectado con un parásito que causa malaria o una patología parecida a la malaria que comprende administrarle al sujeto una cantidad de uno o más indoles relacionados con el DIM (p.ej., Diindolilmetano ("DIM")) efectiva para inhibir el crecimiento de dicho parásito en el sujeto y uno o más agentes antiprotozoarios (ver la reivindicación independiente 1 las y reivindicaciones que dependen de la misma); (ii) métodos para tratar a un sujeto infectado con un parásito que causa malaria o una patología parecida a la malaria que comprende administrarle al sujeto una cantidad de uno o más indoles relacionados con el DIM (p.ej., DIM) efectiva para inhibir el crecimiento de dicho parásito en el sujeto (ver la reivindicación independiente 28 y las reivindicaciones que dependen de la misma); y (iii) composiciones que comprenden una cantidad de uno o más indoles relacionados con el DIM (p.ej. DIM) efectivas para inhibir el crecimiento de un parásito que causa malaria o una patología parecida a la malaria en un sujeto infectado con dicho parásito y uno o más agentes antiprotozoarios (ver la reivindicación independiente 49 y las reivindicaciones que dependen de la misma).

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5. He leído el Oficio fechado el 9 de agosto de 2011 y las referencias citadas en él, incluidas la Publicación Internacional de Patente No. WO 2006/053160 por Hensel ("Hensel."), la Publicación Internacional de Patente No. US 2006/0100264 por Bjeldanes et al., ("Bjeldanes") y la patente estadounidense No. 6.534.085 de Zeligs ("Zeligs") citada en el Oficio como base para el rechazo según lo previsto en 35 U.S.C. # 103(a). Entiendo que las reivindicaciones de la solicitud '307 son objeto de un rechazo en el cual el Oficio sostiene que Hensel, a la luz de Bjeldanes y Zeligs, convertiría en obvios los métodos de tratar enfermedades por protozoarios a que hace referencia la solicitud '307 antes de las modificaciones de las reivindicaciones que aquí se hacen.
 6. En mi opinión, nada en las revelaciones de Hensel, Bjeldanes y Zeligs hace pensar que el DIM o cualquier indol relacionado con DIM puede ser efectivo para tratar enfermedades por protozoarios, ni mucho menos sugiere que DIM o cualquier Indol relacionado con DIM puede ser efectivo para tratar a un sujeto infectado con un parásito que causa malaria o una patología parecida a la malaria o efectivo para inhibir el crecimiento de dicho parásito en el sujeto.
 7. En mi opinión, la persona medianamente versada en la materia técnica correspondiente habría encontrado inesperado que el DIM pueda ser efectivo para inhibir el crecimiento de los parásitos causantes de la malaria. Contrario a las expectativas de la persona medianamente versada en la materia técnica correspondiente, se ha demostrado que el DIM es efectivo para inhibir directamente el crecimiento de los parásitos causantes de la malaria. Este hallazgo se evidencia en el informe titulado "Ensayo *in vitro* de Diindolilmetano (DIM) y DIM de BioResponse en el ensayo de actividad antimalárica con Hipoxantina marcada con tritio" ("Informe"), en el cual se describen el diseño experimental y los resultados de un ensayo *in vitro* que mide la inhibición del crecimiento del *Plasmodium falciparum* (en lo sucesivo, *P. falciparum*), un parásito protozooario que causa malaria en seres humanos, por dos preparaciones diferentes de DIM (ver Evidencia 2, anexa a la presente). El ensayo de inhibición del crecimiento *in vitro* del *P. falciparum* se llevó a cabo y el Informe fue preparado por solicitud mía y a mi nombre, por Eskitis Institute for Cell and Molecular Therapies, Griffith University. En los Parágrafos 8 y 9 a continuación se describe el ensayo de inhibición del crecimiento *in vitro*, es decir, el ensayo de ³H-Hipoxantina, que se usó en el informe, sus fundamentos y su diseño experimental. En el Parágrafo 10, más adelante se describen los resultados del ensayo de inhibición del crecimiento *in vitro* empleado en el informe, que muestran que dos preparaciones de DIM inhiben directamente el crecimiento de los parásitos que causan la malaria.
 8. El informe describe los resultados del ensayo de Hipoxantina tritiada (³H) (³H-Hipoxantina), el cual se utilizó para valorar la capacidad de dos diferentes preparaciones de DIM de inhibir el crecimiento de dos cepas de parásitos causantes de la malaria. El ensayo de ³H-Hipoxantina es una prueba bien establecida para medir la actividad de los medicamentos antimaláricos potenciales (ver, p.ej., Andrews et al., 2000, Int. J. Parasitol. 30(6):761-768, en la p. 763, "Andrews", que se anexa a la presente como Evidencia 3). Este ensayo es relevante para predecir la sensibilidad de varios parásitos patógenos que causan malaria o una patología parecida a la malaria (es decir, parásitos del genus *Plasmodium*) o la inhibición del crecimiento por posibles medicamentos antiparasitarios (ver Andrews en la p. 763, que muestra el uso del ensayo de ³H-Hipoxantina para medir la inhibición del crecimiento de especies de *Plasmodium falciparum* del genus *Plasmodium* genus por diversos medicamentos; ver también Brasseur et al., 1998, Antimicrob. Agents Chemother. 42(4):818-820, en pp. 818-814, "Brasseur", anexa a la presente como Evidencia 4, la cual muestra el uso del ensayo de ³H-Hipoxantina para medir la inhibición del crecimiento de la especie *Babesia* del genus *Plasmodium* por varios medicamentos). Los resultados del ensayo de Hipoxantina *in vitro* son predictivos de la eficacia clínica del medicamento en examen *in vivo* (ver, p.ej., Brasseur et al., 1992, Am. J. Trop. Med. Hyg. 46(1):8- 14, "Brasseur2", que se anexa a la presente como Evidencia 5). En particular, Brasseur et al., en un estudio realizado en el continente africano en un escenario de surgimiento de resistencia al medicamento antimalárico mefloquina, demostró que 37 de 38 pacientes que respondieron al tratamiento *in vivo* con mefloquina estaban infectados con parásitos que eran sensibles a bajas concentraciones del medicamento en el ensayo de ³H-Hipoxantina *in vitro* (mostraban baja EC₅₀, es decir, la mitad de la máxima concentración efectiva, que es equivalente a la "IC₅₀" a la cual se hace referencia más adelante), y la resistencia al medicamento *in vivo* se asoció con resultados elevados de la EC₅₀ en el ensayo de ³H-Hipoxantina *in vitro* (ver Brasseur2

en la p. 10). Así las cosas, los resultados del ensayo de ^3H -Hipoxantina *in vitro* son predictivos de la eficacia *in vivo* de medicamentos antimaláricos potenciales.

9. El ensayo de ^3H -Hipoxantina examina la actividad de compuestos de prueba contra la fase hemática del ciclo vital de la malaria, la etapa durante la cual un parásito causante de la malaria, como el *P. falciparum*, infecta los glóbulos rojos. Utiliza la ^3H -Hipoxantina como precursor radiomarcado de los ácidos nucleicos que se retiene solamente en los parásitos en fase de crecimiento. En este ensayo, los glóbulos rojos (también conocidos, y mencionados aquí y en el Informe como "eritrocitos") son parasitados en cultivo de tejido por formas asexuadas en fase de multiplicación de un parásito de la malaria como el *P. falciparum*. Entonces, los glóbulos rojos parasitados se cultivan en presencia de uno o más compuestos de prueba, un control negativo, y un control positivo. A continuación, los glóbulos rojos parasitados se exponen a un ensayo de ^3H -Hipoxantina durante un tiempo determinado. Entonces las muestras de glóbulos rojos que contienen parásitos son extraídas del cultivo, y cada condición examinada se aísla en un papel de filtro individual. Después de un enjuague completo para eliminar la marca de ^3H no contenida como parte del ADN parasitario, los papeles de filtro se examinan en un contador de centelleo que mide la cantidad de marcador ^3H en cada muestra. Dado que los glóbulos rojos son células que no se dividen y que no contienen núcleos ni su propio ADN, y dado que solamente los parásitos en fase de crecimiento acumulan y retienen ^3H en su ADN, la actividad de un compuesto de prueba de inhibición del crecimiento de un Plasmodium se correlaciona con la presencia reducida del marcador ^3H en comparación con las condiciones del cultivo de control que no contiene compuesto de prueba. La inhibición de dicha acumulación del marcador ^3H indica la inhibición directa del crecimiento del parásito por el compuesto de prueba. La concentración del compuesto de prueba requerida para inhibir la incorporación de la ^3H -Hipoxantina en un 50% se informa como el 50% de la concentración inhibitoria (IC_{50}). El diseño experimental del ensayo de ^3H -Hipoxantina se describe brevemente en la sección "Descripción del ensayo" del informe haciendo referencia a publicaciones científicas que describen el diseño experimental del ensayo en mayor detalle (ver Evidencia 2, página 1, "Descripción del ensayo", citando las publicaciones científicas enumeradas en la página 2, "Referencias"). El informe sostiene, entre otras cosas, que: "las líneas de *P. falciparum* se cultivaron *in vitro* en eritrocitos humanos O^+ en...medios..."; "los ensayos se llevaron a cabo usando...eritrocitos parasitados...durante 72 horas, usando el método de incorporación de ^3H -Hipoxantina como se describió antes"; y se determinó la IC_{50} de cada compuesto" (ver Evidencia 2, página 1, "Descripción del ensayo", citando, entre otros, a Andrews). El Informe documenta los resultados de las pruebas de actividad antimalárica de DIM producido por Interpharma Praha (denominado "DIM" en la sección de "Resultados" del Informe), DIM producido por BioResponse, L.L.C. (denominado "BR-DIM" en la sección de "Resultados" del Informe), matriz de microencapsulación usada en BR-DIM (denominado "Vehículo" en la sección de "Resultados" del Informe, que es un control negativo), y sal de difosfato de cloroquina (denominada "CQ" en la sección de "Resultados" del Informe, que es un control positivo) (ver Evidencia 2, página 2, "Resultados", y página 1, "Descripción de Materiales Suministrados" y "Descripción del ensayo"). El Informe presenta los resultados de tres ensayos independientes para cada una de las dos cepas de *P. falciparum* examinadas, es decir, la cepa resistente a la cloroquina y la cepa sensible a la cloroquina (ver Evidencia 2, página 1, "Descripción del ensayo"). Los resultados del ensayo se describen en el párrafo 10 a continuación.
10. La sección de "Resultados" del Informe indica que ambas preparaciones de DIM examinadas (es decir, DIM y BR-DIM), y el control positivo, la sal de fosfato de cloroquina, que es un medicamento antimalárico estándar (ver Andrews, en la p. 764, col. 2, 1º párrafo completo, y p. 763, col. 1., párrafo 2), pero no el control negativo (es decir, el "Vehículo"), reducen la acumulación de ^3H -Hipoxantina, y por ende inhiben el crecimiento de la cepa de *P. falciparum* resistente a la cloroquina y la cepa sensible a la cloroquina de *P. falciparum* (ver Evidencia 2, página 2, "Resultados"). En particular, el Informe demuestra que una preparación de DIM ("DIM.") tiene una IC_{50} de 5,34 ($\pm 0,74$) microgramos/mL ($\mu\text{g/mL}$) en la cepa Dd2 (resistente a la cloroquina) de *P. falciparum* e IC_{50} de 8,7 ($\pm 1,06$) $\mu\text{g/mL}$ en la cepa 3D7 (sensible a la cloroquina) de *P. falciparum*. Además, el Informe demuestra que otra preparación de DIM ("BR-DIM"), que contiene 30% de Diindolilmetano en peso) tiene una IC_{50} de 20,87 ($\pm 2,28$) $\mu\text{g/mL}$ la cepa Dd2 (resistente a la cloroquina) de *P. falciparum* y una IC_{50} de 17,46 ($\pm 6,05$) $\mu\text{g/mL}$ en la cepa 3D7 (sensible a la cloroquina) de *P. falciparum*. Dado que la preparación BR-DIM contiene 30% de diindolilmetano en peso, esto corresponde a

una IC₅₀ de 3,26 µg/mL en la cepa Dd2 y 5,24 µg/mL en la cepa 3D7, respectivamente, para el diindolilmetano en BR-DIM. Estos datos indican que el DIM reduce efectivamente la acumulación de ³H-Hipoxantina y por tanto es efectivo para inhibir directamente el crecimiento de dos cepas de parásitos causantes de la malaria. Estos datos también indican que las concentraciones de cada una de las dos preparaciones de DIM son efectivas para inhibir la acumulación de ³H-Hipoxantina, y por ende el crecimiento de dos cepas de parásitos causantes de la malaria en 50%.

11. Con base en los datos presentados en el Informe, concluyo que DIM muestra actividad inhibitoria del crecimiento contra los parásitos de la malaria. En particular, estos datos indican que el DIM es efectivo para inhibir el crecimiento, la multiplicación y el número de dos cepas de parásitos *P. falciparum* que causan malaria en los seres humanos. Es importante anotar que el DIM mostró una actividad todavía más pronunciada contra la cepa más común Dd2 resistente a la cloroquina de parásitos de la malaria que contra la cepa 3D7 de parásitos de la malaria sensible a la cloroquina. También concluyo que estos resultados muestran que el DIM tiene una actividad inhibitoria directa del crecimiento contra los parásitos de la malaria, independiente de cualquier influencia del sistema inmunológico del huésped. Como se dice en el parágrafo 8, los resultados del ensayo de ³H-Hipoxantina obtenidos con un medicamento de prueba son predictivos de la respuesta clínica *in vivo* de los sujetos infectados con parásitos de la malaria al medicamento de prueba (ver Brasseur p.ej., en la p. 10). Así las cosas, concluyo que un indol relacionado con DIM (como el DIM) sería efectivo para inhibir el crecimiento de los parásitos que causan la malaria o una patología similar a la malaria. También concluyo que el indol relacionado con DIM (como DIM) sería efectivo para tratar a un sujeto infectado con un parásito causante de la malaria o una patología similar a la malaria.
12. En mi opinión, la persona medianamente versada en la materia técnica correspondiente habría encontrado inesperado que el DIM pudiera ser efectivo para inhibir el crecimiento de un parásito causante de la malaria. También creo que la persona medianamente versada en la materia técnica correspondiente habría encontrado inesperado que el DIM tuviera una actividad inhibitoria directa del crecimiento contra dos cepas de parásitos de la malaria humana, con independencia de cualquier influencia del sistema inmunológico del huésped.
13. Manifiesto que todas las declaraciones hechas en este documento con base en mi propio conocimiento son ciertas y que creo que las declaraciones hechas según mi leal saber y entender son ciertas y, además, que estas declaraciones se hacen con pleno conocimiento de que las manifestaciones intencionalmente falsas y similares acarrear penas pecuniarias o de cárcel o ambas, según lo previsto en la Sección 1001 del Título 18 del Código de los Estados Unidos y que dichas declaraciones intencionalmente falsas pueden poner en peligro la validez de la solicitud o cualquier patente expedida en respuesta a ella.

Fecha: 9 de febrero de 2012
Michael A. Zeligs, MD

Evidencia 1: Curriculum Vitae de Michael A. Zeligs, MD

Evidencia 2: Informe titulado "Ensayo *in vitro* de diindolilmetano (DM) y DIM de BioResponse en el ensayo de ³H-Hipoxantina de actividad antimalárica", fechado el 8 de febrero de 2012, firmado por el Dr. Andrews, Eskitis Institute for Cell and Molecular Therapies, Griffith University.

Evidencia 3: Andrews et al., 2000, Int. J. Parasitol. 30(6):761-708

Evidencia 4: Brasseur et al., 1998, Antimicrob. Agents Chemother. 42(4):818-820

Evidencia 5: Brasseur et al., 1992, Am. J. Trop. Med. Hyg. 46(1):8-14

ANEXO 1

Report to:	Michael A. Zeligs, M.D. CEO, BioResponse, LLC P.O. Box 288, Boulder, CO USA 80306 Phone: 303-447-3841 Fax: 303-938-8003 Email: zeligsmd@bioresponse.com Report
Report by:	Dr Kathy Andrews Eskitis Institute for Cell and Molecular Therapies, Griffith University
Name of Project	In vitro Assay of Diindolymethane (DIM) and BioResponse-DIM in the ³ H Hypoxanthine Assay for Anti-malarial Activity
Description of Supplied Materials	1. 10g Diindolymethane (Dietary supplement); Interpharma Praha, a. s.; Certificate of analysis 1268/09; Lot # 00303009 (DIM) 2. 10g BioResponse Diindolymethane powder; Bioresponse® Nutrients; Lot # 027909 (BR-DIM) 3. 10g BR-DIM Microencapsulation Matrix Vehicle Control; Bioresponse® Nutrients; Lot # 011806 (Vehicle)
Dissolution of supplied powders	All powders were prepared as 20mg/ml stocks in 80% DMSO/20% Phosphate buffered saline (PBS), according to information supplied by the client. 80% DMSO was selected as in a lower concentration of DMSO (50%) DIM and BR-DIM powders did not dissolve even after extensive vortexing. All powders and solutions were stored at 4°C protected from light, as requested. Solutions were not stored and used beyond 7 days.
Description of assay	<i>P. falciparum</i> in vitro growth inhibition assay. <i>P. falciparum</i> lines Dd2 (chloroquine resistant) [1] and 3D7 (chloroquine sensitive) [2] were cultured <i>in vitro</i> in O⁺ human erythrocytes in RPMI 1640 media (Gibco BRL) supplemented with 10% heat inactivated human serum, as previously described with minor modifications [3]. Assays were carried out using synchronous ring-stage parasitized erythrocytes (0.25% parasitaemia/2.5% haematocrit) over 72 hrs using the ³H-hypoxanthine incorporation method, essentially as previously described [4]. Chloroquine diphosphate salt (CQ; Sigma-Aldrich) was assessed in all assays as a positive antimalarial control. The final concentrations of each compound required to inhibit ³H-hypoxanthine incorporation by 50% (IC₅₀), in comparison to DMSO vehicle controls (0.6% DMSO), was determined by linear interpolation of inhibition curves as described previously [5]. Three independent assays were carried for each <i>P. falciparum</i> line, each in triplicate wells.

Results		<i>P. falciparum</i> IC ₅₀ (µg/ml)		<i>P. falciparum</i> IC ₅₀ (µM)	
		3D7	Dd2	3D7	Dd2
	DIM	8.70 (±1.06)	5.34 (± 0.74)	35.35	21. 68
	BR-DIM	17.46 (±6.05)	10.87 (±2.28)	ND	ND
	Vehicle	92.96 (±26.65)	89.11 (±10.12)	ND	ND
	CQ	0.01 (±0.00)	0.08 (±0.02)	0.02	0.16
	ND=not determined				
NOTE: Raw data (corrected counts per minute (CCPM)) can be found in the attached Excel file.					
Notes/Discussion	1. The poor solubility of DIM and BR-DIM dictated the highest concentration able to be tested in the in vitro assay (taking into account DMSO toxicity). The highest concentration tested was 150µg/ml. 2. DIM and BR-DIM visibly came out of solution in the first three wells of each assay (150, 75, and 37.5 µg/ml), even after extensive mixing via aspiration. No precipitate was observed for the Vehicle control wells. 3. No lysis of erythrocytes was observed in any assay well at the start or end of experiments (visual inspection only). 4. The Vehicle control did not achieve 100% inhibition in any assay at the highest concentration tested (150µg/ml).				
References	1. Wellems, T., Oduola, A., Fenton, B., Desjardins, R., Panton, L., and do Rosario, V. (1988) <i>Rev Brasil Genet</i> 11, 813-825 2. Walliker, D., Quakyi, I. A., Wellems, T. E., McCutchan, T. F., Szarfman, A., London, W. T., Corcoran, L. M., Burkot, T. R., and Carter, R. (1987) <i>Science</i> 236(4809), 1661-1666 3. Trager, W., and Jensen, J. B. (1976) <i>Science</i> 193(4254), 673-675. 4. Andrews, K. T., Walduck, A., Kelso, M. J., Fairlie, D. P., Saul, A., and Parsons, P. G. (2000) <i>Int J Parasitol</i> 30(6), 761-768 5. Huber, W., and Koella, J. C. (1993) <i>Acta Trop</i> 55(4), 257-261				
Signature/Date	 Dr Kathy Andrews 8 th February 2012				

ANEXO 2

Anti-malarial effect of histone deacetylation inhibitors and mammalian tumour cytodifferentiating agents

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Received 20 November 1999; received in revised form 13 March 2000; accepted 13 March 2000

Abstract

The histones of *Plasmodium falciparum* represent a potential new target for anti-malarial compounds. A naturally occurring compound, apicidin, has recently been shown to inhibit the in vitro growth of *P. falciparum*. Apicidin was shown to hyperacetylate histones, suggesting that its mode of action is through histone deacetylase inhibition. We have tested the ability of known histone deacetylase inhibitors, mammalian tumour suppressor compounds, and cytodifferentiating agents to inhibit the in vitro growth of a drug sensitive and resistant strain of *P. falciparum*. Seven of the tested compounds had μM IC₅₀ values, and trichostatin A, a histone deacetylation inhibitor and cytodifferentiating agent, was active at low nM concentrations. One compound, suberic acid bisdimethylamide, which selectively arrests tumour cells as opposed to normal mammalian cells, had an in vivo cytostatic effect against the acute murine malaria *Plasmodium berghei*, and one round of treatment with the compound failed to select for resistant mutations. These results suggest a promising role for histone deacetylase inhibitors and cytodifferentiating agents as antimalarial drug candidates. © 2000 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Anti-malarial; Histone deacetylation inhibitor; *Plasmodium berghei*; *Plasmodium falciparum*; SBHA

1. Introduction

Malaria parasites are responsible for 1–2 million deaths annually, with the most severe forms attributed to infection with *Plasmodium falciparum* [Thirteenth Programme Report of the UNDP/World Bank/WHO Special programme for research and training in tropical diseases (1995–1996)]. The incidence of drug resistance is continuing to increase, resulting in multi-drug

resistant strains of malaria. The search for new anti-malarial therapies to combat this serious world-wide health problem is therefore a high priority.

The histones of *P. falciparum* have recently been proposed as targets for drug treatment of blood stage parasites [1,2]. They are present in abundance, are antigenic, and may have a role to play in the pathology of malaria [2]. Histones are nuclear core proteins that play an important role in transcriptional regulation via the continuous acetylation/deacetylation of the ϵ -amino group of specific lysine residues. The sequences of the H2A, H2B, and H3 histone genes of *P. falciparum* have been identified from cDNA clones and have a high degree of homology with histones from other organisms [3–5]. In addition, nuclease mapping of chromatin has shown that nucleosomal structures are present in *P. falciparum* [6,7], and more

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recently a *P. falciparum* histone deacetylase homologue was cloned and sequenced [8].

Apicidin, a cyclic tetrapeptide isolated from *Fusarium* spp. has been shown to have an irreversible effect on the in vitro development of Apicomplexan parasites, including *Plasmodium* species [1]. It has been suggested that apicidin acts on parasites by inhibiting histone deacetylase, thus interfering with the continuous acetylation/deacetylation process and preventing cell proliferation. While apicidin was shown to be effective against Apicomplexan parasites, this compound also has an antiproliferative effect on mammalian cells [1]. Therefore, potential inhibitors of histone deacetylase with selective toxicity for the malaria parasite are required if compounds such as apicidin are to be useful anti-malarial agents.

Other inhibitors of histone deacetylase include sodium *n*-butyrate, trichostatin A (TSA) and hexamethylene bisacetamide (HBMA) [9,10]. These compounds also have differentiating activity in some mammalian tumour cell lines [11–13]. Recently, more potent analogues of HBMA have been identified such as azelaic bishydroxamic acid (ABHA) and the analogues of suberic acid bisdimethylamide (see Fig. 1 for structures). These analogues have up to 100 times more differentiating activity in murine erythroleukemic cell lines, human promyelocytic cells (HL-60), and human colon carcinoma cells than HBMA [14]. ABHA and related compounds have also been shown to be highly selective for tumour cell lines in vitro [15] and in vivo [16], and to act as histone deacetylase inhibitors [16,17]. The high potency of ABHA combined with the increased selectivity for transformed human cells and stability compared with naturally occurring compounds, such as TSA [16], suggests that this compound, and its analogues, may be useful not only against melanomas, but as anti-parasitic agents.

We have investigated the effect of ABHA and sev-

eral of its analogues on the in vitro growth of a drug sensitive and drug resistant strain of *P. falciparum*, and the effect of suberohydroxamic acid (SBHA) on the in vivo growth of the murine malaria *Plasmodium berghei*. Since in some organisms, methylation of cytidine modulates the effects of histone acetylation, we have also examined the effects of the methylation inhibitor 5-azacytidine on the in vitro growth of a drug sensitive and resistant strain of *P. falciparum*.

2. Materials and methods

2.1. Materials and strains

In vitro studies were carried out using *P. falciparum* strains 3D7 [18] and Dd2 [19]. Polymerase chain reaction size polymorphism analysis of the MSP1, MSP2 and GLURP genes [20] confirmed the identities of the two parasite strains.

The murine malaria *P. berghei* QIMR strain [21] was used for in vivo experiments in female BALB/c mice (Animal Resources Centre, Western Australia). Human O⁺ erythrocytes and human serum (HS) was a gift from the Brisbane Red Cross Blood Bank.

2.2. Parasite culture

Asexual *P. falciparum* parasites were cultured essentially as described by Trager and Jensen [22] and maintained in a synchronous state by sorbitol treatment [23] and Percoll[®] (Pharmacia Biotech) gradient purification [24]. *P. berghei* QIMR was maintained by routine passage through female BALB/c mice.

2.3. Compounds

In vitro: Pyrimethamine (Sigma) was prepared as a

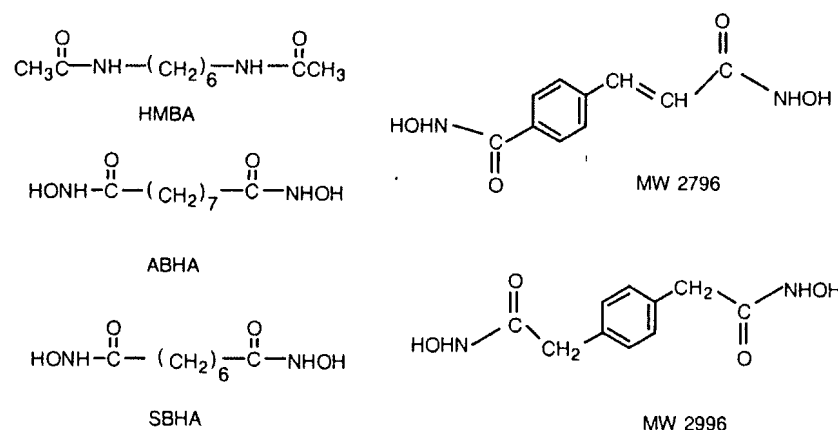


Fig. 1. Chemical structures of suberic acid bisdimethylamide analogues.

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10 mM stock in 0.5% lactic acid, chloroquine (diphosphate salt) (Sigma) as a 10 mM stock in PBS, and mefloquine (Hoffmann La Roche) as a 10 mM stock in 70% ethanol. Stock solutions were prepared in dimethyl sulfoxide (DMSO) at 50 mg/ml for the following compounds: SBHA, salicylhydroxamic acid (Aldrich), MW2796, MK4 (9-anilidoazelaic-1-hydroxamic acid), MK0028 (4-chlorobenzoyl-L-phenylalanylglycine hydroxamic acid), and MW2996 (synthesised at the University of Queensland Centre for Drug Design and Development). Trichostatin A (TSA) (Sigma) was stored at 1 mg/ml in methanol. Desferrioxamine mesylate (DFX) (CIBA-Geigy) and HMBA (Sigma) were prepared as 50 mM stocks in DMSO and PBS, respectively. ABHA was diluted to 5 mg/ml in PBS. Azelaic acid was prepared as a 25 mg/ml stock in PBS. TSA, pyrimethamine, chloroquine, and mefloquine were stored at -70°C . All other compounds were stored at 4°C . 5-azacytidine (Sigma) was prepared fresh as a 10 mM stock solution in RPMI 1640 (without serum) immediately prior to use.

In vivo: Chloroquine (diphosphate salt) was diluted to 1 mg/ml in PBS. A 20 mg/ml stock mixture of SBHA was prepared in PBS. The mixture was heated to 55°C and vortexed extensively to dissolve the SBHA. The stock solution was stored at 4°C and reheated, vortexed, then cooled to room temperature prior to use *in vivo*.

2.4. Determination of *in vitro* growth inhibition

Test compounds were diluted in six replicate wells of a flat-bottomed 96-well micro titre plate (Costar[®], Corning Incorporated) in hypoxanthine-free RPMI 1640 medium containing 10% human serum. Synchronised *P. falciparum* ring stage parasites were added to the wells at 0.5% parasitaemia and 3% haematocrit. Replicate control wells contained parasites without test compound or uninfected erythrocytes to measure maximal growth rates and zero growth rates, respectively. Plates were incubated at 37°C in a gas mixture of 5% O_2 and 5% CO_2 for 48 h until the parasites had gone through one invasion cycle, followed by the addition of 1 μCi of [$^3\text{H}(\text{G})$]-hypoxanthine monohydrochloride (NEN[™] Life Science Products Inc.) to five of the six replicate wells. The sixth well received an equal amount of complete RPMI 1640 medium containing 10% HS. The plates were incubated for a further 24 h, then harvested onto $102 \times 258 \text{ mm}^2$ glass fibre filter mats (Wallac) using a 1295-004 Betaplate[™] 96-well Harvester (Wallac). Incorporation of [$^3\text{H}(\text{G})$]-hypoxanthine was detected using a 1205 Betaplate[™] Liquid Scintillation Counter (Wallac). The effect of the test compounds on parasitaemia was also determined microscopically by examination of stained thin films

prepared from the control wells. IC50 values were calculated using a non-linear regression analysis, fitting the natural log of the drug concentration to the counts per minute (cpm) using a four-parameter logistic regression (SigmaPlot v4.00).

2.5. *Plasmodium berghei* anti-malarial studies

To determine the effect of SBHA on the murine malaria *P. berghei* QIMR strain, four groups of six female BALB/c mice (average weight 19.8 g) were injected i.p. with 10^6 parasite-infected erythrocytes from an infected mouse. This inoculum was determined from preliminary experiments and resulted in a reproducible infection in mice. Drug treatment was initiated either 2 h or 48 h p.i. SBHA was diluted to 20 mg/ml in PBS and the mice received 4 mg i.p. injections twice a day for three consecutive days. Control groups received an equivalent volume of PBS or 0.2 mg of chloroquine (dissolved at 1 mg/ml in PBS) 2 h p.i., twice a day, for three consecutive days. The parasitaemia was monitored from day 4 p.i. by daily microscopic examination of stained thin blood films. SBHA treated mice that showed a recrudescence of parasitaemia were re-treated with a single 8 mg daily dose of the drug. Mice were euthanased on day 19 p.i. or earlier if the parasitaemia could not be controlled by treatment.

Further *in vivo* studies were carried out as described above, with the following treatments: One group of mice was treated with a single 8 mg dose of SBHA, or vehicle alone, 2 h p.i. A second group of mice was

Table 1

In vitro IC50 values for *P. falciparum* strains 3D7 and Dd2. IC50 values calculated using a non-linear regression analysis of five replicates for each sample concentration. Standard errors (SEs) omitted for clarity

Compound	3D7	Dd2
TSA	11. nM	7.9 nM
SBHA	1.3 μM	0.8 μM
MW2796	0.9 μM	1.1 μM
DFX	11 μM	8 μM
MK4	ND	5.8 μM
ABHA	4.9 μM	3.2 μM
Salicylhydroxamic acid	41 μM	37 μM
MK0028	46 μM	47 μM
HMBA	1.4 mM	0.8 mM
MW2996	> 446 mM	> 446 mM
Azelaic Acid	> 531 mM	> 531 mM
5-azacytidine (rings)	0.91 mM	0.94 mM
5-azacytidine (schizonts)	1.3 mM	1.4 mM
Pyrimethamine	0.019 μM	87 μM
Chloroquine	0.022 μM	0.77 μM
Mefloquine	119 nM	80 nM

treated with 16 mg of SBHA per day in two 8 mg doses (or the vehicle alone for the control group) twice a day for three consecutive days p.i. Mice in these

groups were euthanased after the parasitaemia had reached 15%.

3. Results and discussion

This study showed that synthetic, stable, histone deacetylase inhibitors were active against drug sensitive and drug resistant strains of *P. falciparum* in vitro, and cured mouse malaria in vivo. Most of the histone deacetylase inhibitors and mammalian tumour suppressor agents tested on 3D7 and the multi-drug resistant Dd2 in vitro gave significant growth inhibition on both lines, and where inhibition was observed, each compound gave similar 50% inhibitory concentration (IC₅₀) values for both lines (Table 1). The standard anti-malarial drugs pyrimethamine and chloroquine gave the expected IC₅₀s, and although Dd2 has been reported as resistant to mefloquine, the IC₅₀ values we obtained were similar to the IC₅₀ for the reportedly sensitive 3D7 strain. However, both are within the range of IC₅₀ values previously reported for the radio-metric assay method [25].

Of the differentiating agents tested, TSA gave the most potent in vitro growth inhibition of *P. falciparum* at nanomolar concentrations (Table 1). TSA has been previously shown to cause hyperacetylation of histones from *P. falciparum*-infected erythrocytes [1]. In this study, several analogues of the less potent differentiating agent HMBA had inhibitory effects at micromolar concentrations against both the drug sensitive 3D7 and multi-drug resistant Dd2 strains in vitro (Table 1). One of these analogues, ABHA, a known inhibitor of histone deacetylase [16], is greater than 100 times more potent than HMBA [14], and is selective in its ability to arrest tumour cells as opposed to normal cell lines [15]. SBHA is an analogue of ABHA and was four times more potent than ABHA on *P. falciparum* in vitro (Table 1). This compound also selectively arrests tumour cells in comparison to normal cell lines (PG Parsons—unpublished). This selectivity, combined with its in vitro activity against *P. falciparum* and availability, prompted us to explore the in vivo effect of the compound on the lethal murine malaria *P. berghei*.

Mice infected with *P. berghei* QIMR strain and treated with 8 mg of SBHA per day for three days showed a 3–4 day delay in the onset of parasitaemia compared with control animals (Fig. 2A) regardless of whether they were treated 2 h or 48 h p.i. (Fig. 2B and C). Two individuals in each group did not develop parasitaemias until around day 14 p.i. (Fig. 2B and C). Mice that developed parasitaemias of up to 25% were able to be treated and the parasitaemia controlled with 8 mg of SBHA per day (Fig. 2B and C, indicated with a bold line). One individual in the group treated with SBHA 48 h p.i. recrudesced under continual 8 mg per

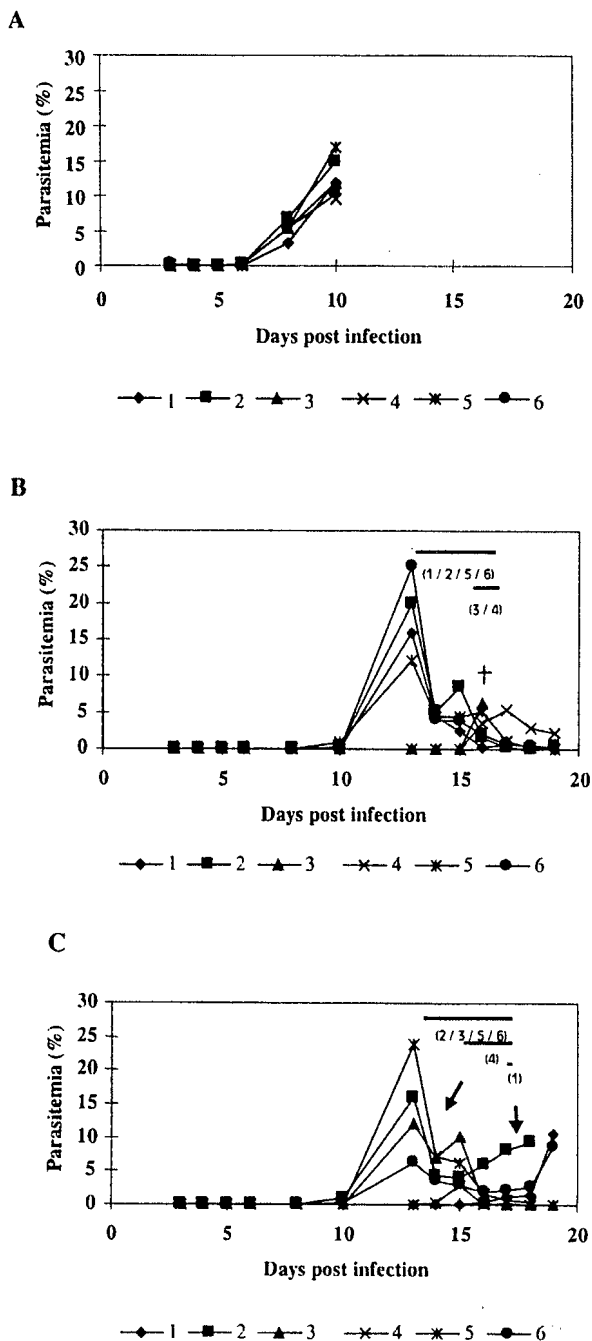


Fig. 2. The effect of SBHA on *P. berghei* QIMR strain in vivo. Female BALB/c mice were infected with 10^6 *P. berghei* QIMR strain parasites and treated 2 h (B) or 48 h (C) p.i. with two daily 4 mg doses of SBHA for three consecutive days. Mice in a control group (A) were administered PBS alone. Bold line represents daily 8 mg doses of SBHA for individual mice (indicated in brackets). Cross indicates mouse found dead on day 17. Blood was collected from two mice (C, mouse 2 and 5, indicated with arrows) on day 18 for further studies.

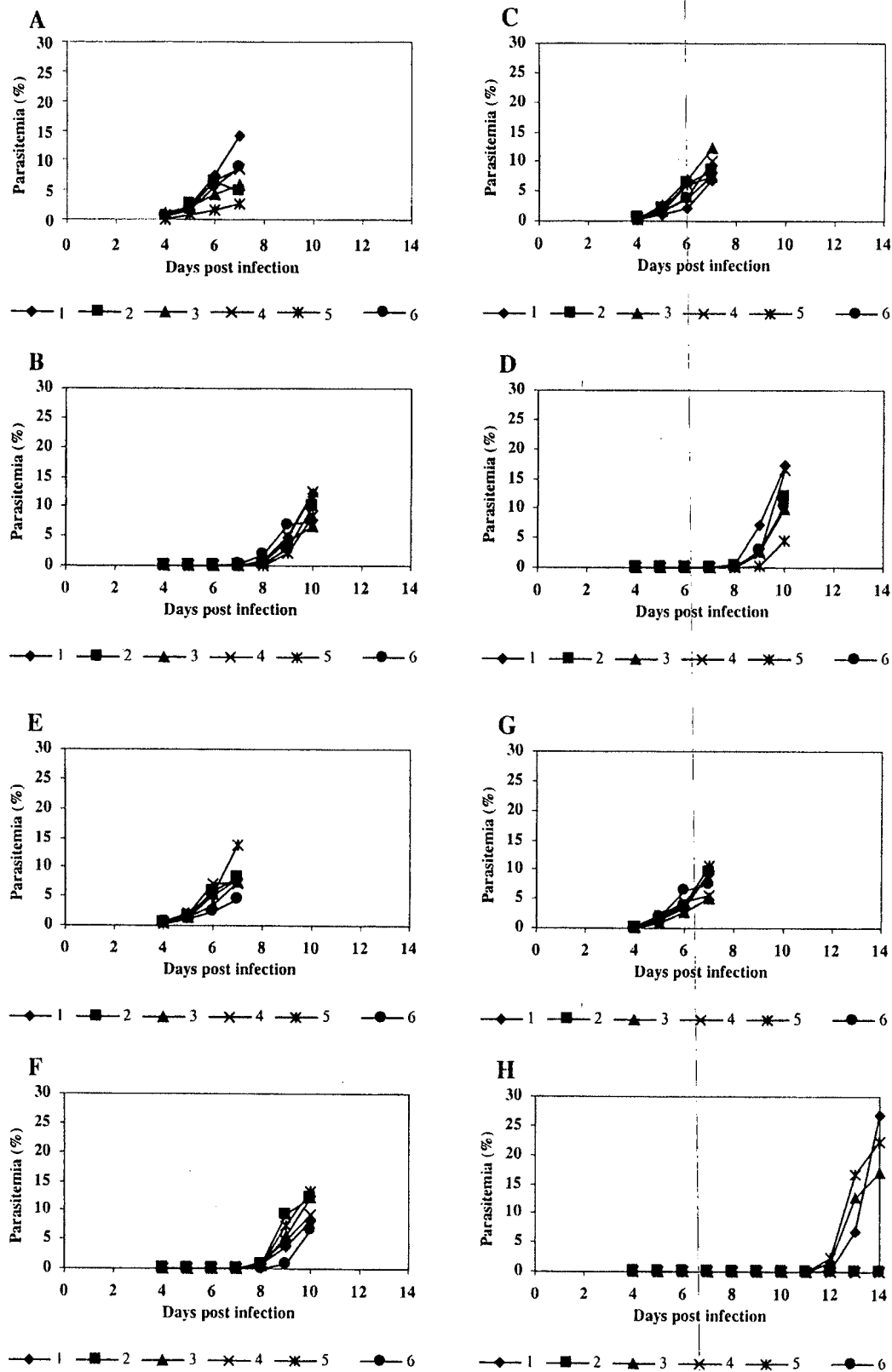


Fig. 3. In vivo effect of SBHA on *P. berghei* QIMR strain parasites that showed no recrudesence or recrudesence under SBHA treatment. Female BALB/c mice were infected with 10^6 parasites passed from mice that showed no recrudesence (A and B) or recrudesence (C and D) of parasitaemia under SBHA treatment (see Fig. 2). Additional groups were inoculated with 10^6 *P. berghei* QIMR strain parasites (E–H). Mice were treated with single 8 mg doses of SBHA 2 h p.i. for 3 days (B, D, and F), or with two 8 mg doses per day for 2 h p.i. for 3 days (H). Control groups received equivalent volumes of PBS alone (A, C, E, and G). Parasitaemia was monitored from day 4 p.i.

day SBHA treatments (Fig. 2C, mouse 2). Blood from this mouse, and from another mouse in which the parasitaemia was controlled by SBHA treatment, was collected on day 18 (Fig. 2C, mouse 2 and 5 indicated with arrows) and stored at -70°C in Fenwall[®] Glycerolyte[™] 57 solution (Baxter). Blood from these two mice and from the original *P. berghei* QIMR strain was thawed and injected into female BALB/c mice. Blood was collected from the parasitaemic mice 4 days p.i., and used to prepare an inoculum for a second experiment to test whether resistant parasites had been generated (Fig. 3). Mice infected with these parasites were treated with 8 mg SBHA per day, for 3 days p.i. or control groups with PSB alone (Fig. 3A–F). While all groups showed the previously observed 3–4 day delay in the onset of parasitaemia compared with control animals (Fig. 3B, D and F compared with A, C and E, respectively), there was no noticeable difference between the SBHA treated groups. This result suggests that the parasites that recrudesced under SBHA treatment were not highly resistant to SBHA. An additional group of mice treated with 16 mg of SBHA showed a 7–8 day delay in the onset of parasitaemia compared with control animals (Fig. 3H and G, respectively), and three of the six mice failed to develop parasites (Fig. 3H). Blood from these three mice was collected on day 18 p.i. and used to inoculate uninfected mice and again no parasites were observed up to 4 weeks later. Taken together, these results suggest that continual administration of SBHA has a cytostatic effect on *P. berghei* infected mice, but is unable to kill all parasites. At the higher 16 mg per day dose of SBHA, the mice showed physical effect of the drug treatment (rough coats). This effect persisted until around 3–4 days after SBHA treatment, suggesting that this period of time is required for the drug to be cleared from the mouse. This would also explain the observed delay in the onset of parasitaemias. Even at these high concentrations of SBHA, only parasitaemias in 50% of the mice were controlled, suggesting that more potent analogues of SBHA are required for further studies. In the recently published paper on the fungus-derived histone deacetylase inhibitor, apicidin [1], the in vivo effect of the compound on *P. berghei* infected mice was also determined. Mice were infected with the parasite and then treated with apicidin 2 h p.i. The parasitaemia was monitored on day 6 p.i. While this compound appeared to be effective at low mg/kg concentrations, based on the delays in the onset of parasitaemia observed here, the true effect of apicidin may not be apparent at the time point selected by the authors.

Three individuals in the group treated with 16 mg of SBHA per day failed to develop parasites (Fig. 3H), suggesting that SBHA may be able to kill parasites when administered at high enough doses. This result

was confirmed by an in vitro experiment. *Plasmodium falciparum* 3D7 parasite cultures were treated with 10%, 50% and 100% inhibitory concentrations of SBHA and after 48 h growth, the cultures were washed to remove the drug, then viability assessed by ability to grow over the next 48 h. SBHA killed parasites at approximately the expected frequencies, suggesting that the drug was not acting by slowing growth, but was lethal to the parasites.

As SBHA is a mammalian tumour suppressing agent and several analogues have been shown to cause tumour differentiation, we tested the ability of SBHA to differentiate asexual blood stage parasites into gametocytes in vitro. 3D7 parasite cultures were treated with 10%, 50% and 100% inhibitory concentrations of SBHA for 48 h, followed by media changes every 48 h for 10 days. No gametocytes were detected in SBHA treated cultures or in untreated parasite cultures. Likewise, no gametocytes were detected on stained thin films from mice treated with SBHA in vivo.

The effect of the methyltransferase inhibitor 5-azacytidine on the in vitro growth of *P. falciparum* was also determined. This compound has been shown to cause hypomethylation of DNA in mammalian systems, bacteria, plants and fungi. Hypomethylation of DNA has been shown to relieve repression of expression in different organisms. A link between histone acetylation and methylation in the control of cellular regulation has been suggested by several observations. DNA fractions, derived from HeLa cells, representing nonmethylated CpG-rich islands had hyperacetylated H3 and H4 [26]. Conversely, on the highly methylated inactive X chromosome of female mammals, the histones H3 and H4 were hypoacetylated [27–30]. The methylation inhibitor 5-azacytidine and the histone deacetylase inhibitor sodium butyrate have also been shown to relieve repression in episomal sequences introduced into animal cells [31] and more recently in genes from the fungus *Neurospora* [32]. TSA was also able to selectively demethylate genes from *Neurospora*, indicating a possible link between protein acetylation and DNA methylation in this system [32]. The link between histone deacetylase activity and methylation has also been strengthened by the finding that the mammalian methyl-CpG-binding protein, MeCP2, associates with the transcriptional repressor mSin3A and histone deacetylases, and that TSA is able to relieve transcriptional repression in vivo [33]. Sodium butyrate, TSA, and the cytosine methylation inhibitor 5-aza-2'-deoxycytidine have also been found to de-repress silent rRNA genes from members of the plant genus *Brassica* [34].

Early studies on *P. falciparum* have demonstrated the in vitro effect of a number of direct or indirect methylation inhibitors, including S-isobutyl adenosine

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[35], 3-deazaadenosine [36], 5'-deoxy-5'-(isobutylthio)-3-deazaadenosine [36], sinefungin [36], Neplanocin A [37], SIBA [39,38] and deaza-SIBA [38]. We have found that the cytidine analogue 5-azacytidine was unable to inhibit parasite growth in vitro below 0.5 mM and that there was very little difference in IC₅₀ values when the compound was added to ring or schizont stage parasites (Table 1). The possible additive or synergistic effects of 5-azacytidine and a histone deacetylase inhibitor were not explored in this study and should not be excluded. To date, the methylation status of *P. falciparum* remains speculative. This lack of definitive methylation may account for the relatively high amounts of 5-azacytidine required for inhibition of parasite growth in vitro. While individual genes may be methylated and their expression potentially controlled by their methylation status, as in other organisms, these genes may not be essential for parasite growth. In addition, *P. falciparum* may possess a unique and/or non-homologous methylation enzyme that is unaffected by 5-azacytidine. Furthermore, a number of different DNA base modifications have been reported in different organisms (reviewed in [39]). For example, the eukaryote *Trypanosoma brucei*, which like *P. falciparum* possesses a family of highly variant antigens, has been shown to have an unusual DNA base modification called DNA base J (β -D-glucosyl-hydroxymethyluracil) [39–41].

SBHA and its analogues represent a potential class of new anti-malarial compounds. Unlike apicidin, which affects mammalian HeLa cells in vitro (1), SBHA is selective for tumour cells as opposed to normal cells. We have shown the in vitro effect of SBHA and several analogues at micromolar concentrations, and the in vivo effect of SBHA on an acute murine malaria, *P. berghei*. The compounds tested here provide a useful tool for the study of histone acetylation in transcriptional control, and more potent analogues exploiting the existing selectivity may be useful anti-malarial agents.

Acknowledgements

This work was supported by a grant from the Australian National Health and Medical Research Council. Compounds MW2796 and MW2996 were synthesised by Mike West at the University of Queensland Centre for Drug Design and Development. Size polymorphism PCR was carried out by Dr Qin Cheng. We are grateful to Dr Helen Leonard, Dr Qin Cheng, Dr Dave Fitzpatrick and Sarah McNicol for their helpful discussion and to Ms Qinghui Lin for her excellent technical assistance.

References

- [1] Darkin-Rattray SJ, Gurnett AM, Myers RW, et al. Apicidin: A novel antiprotozoal agent that inhibits parasite histone deacetylase. *Proc Natl Acad Sci USA* 1996;93:13143–7.
- [2] Longhurst HJ, Holder AA. The histones of *Plasmodium falciparum*: identification, purification and a possible role in the pathology of malaria. *Parasitology* 1997;114:413–9.
- [3] Creedon KA, Kaslow DC, Rathod PK, Wellem TE. Identification of a *Plasmodium falciparum* histone 2A gene. *Mol Biochem Parasitol* 1992;54:113–6.
- [4] Bennett BJ, Thompson J, Coppel RL. Identification of *Plasmodium falciparum* histone 2B and histone 3 genes. *Mol Biochem Parasitol* 1995;70:231–3.
- [5] Longhurst HJ, Holder AA. The sequence of *Plasmodium falciparum* histone H3. *Mol Biochem Parasitol* 1995;69:111–3.
- [6] Lanzer M, Wetheimer SP, de Bruin D, Ravetch JV. Chromatin structure determines the sites of chromosome breakages in *Plasmodium falciparum*. *Nucleic Acids Res* 1994;22:3099–103.
- [7] Cary C, Lamont D, Dalton JP, Docrig C. *Plasmodium falciparum* chromatin: nucleosomal organisation and histone-like proteins. *Parasitol Res* 1994;80:255–8.
- [8] Joshi MB, Lin DT, Chiang PH, Goldman ND, et al. Molecular cloning and nuclear localization of a histone deacetylase homologue in *Plasmodium falciparum*. *Mol Biochem Parasitol* 1999;99:11–9.
- [9] Riggs MG, Whittaker RG, Neumann JR, Ingram VM. *n*-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. *Nature* 1977;268:462–4.
- [10] Yoshida M, Kijima M, Akita M, Beppu T. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J Biol Chem* 1990;265:17174–9.
- [11] Leder A, Leder P. Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. *Cell* 1975;5:319–22.
- [12] Kruh J. Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol Cell Biochem* 1982;42:65–82.
- [13] Yoshida M, Nomura S, Beppu T. Effects of trichostatin on differentiation of murine erythroleukemia cells. *Cancer Res* 1987;47:3688–91.
- [14] Breslow R, Jursic B, Yan ZF, et al. Potent cytodifferentiating agents related to hexamethylenecisacetamide. *Proc Natl Acad Sci USA* 1991;88:5542–6.
- [15] Parsons PG, Hansen C, Fairlie DP, et al. Tumor selectivity and transcriptional activation by azelaic bishydroxamic acid in human melanocytic cells. *Biochem Pharmacol* 1997;53:1719–24.
- [16] Qiu L, Kelso MJ, Hansen C, West ML, Fairlie DP, Parsons PG. Antitumour activity *in vitro* and *in vivo* of selective differentiating agents containing hydroxamate. *Br J Cancer*, in press.
- [17] Richon VM, Emiliani S, Verdin E, et al. A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases. *Proc Natl Acad Sci USA* 1998;95:3003–7.
- [18] Walliker D, Quakyi IA, Wellem TE, et al. Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science* 1987;236:1661–6.
- [19] Wellem TE, Oduola AMJ, Fenton B, Desjardins R, Panton LJ, do Rosario VE. Chromosome size and variation occurs in cloned *Plasmodium falciparum* on *in vitro* cultivation. *Rev Brasil Genet* 1988;11:813–25.
- [20] Ranford-Cartwright LC, Taylor J, Umasunthar T, et al. Molecular analysis of recrudescence parasites in a *Plasmodium falciparum* drug efficacy trial in Gabon. *Trans R Soc Trop Med Hyg* 1997;91:719–24.
- [21] Saul A, Prescott N, Smith F, Cheng Q, Walliker D. Evidence of cross-contamination among laboratory lines of *Plasmodium berghei*. *Mol Biochem Parasitol* 1997;84:143–7.

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- [22] Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976;193:673–5.
- [23] Lambros A, Vanderberg P. Synchronization of *Plasmodium falciparum* stages in culture 1979;65:418–20.
- [24] Lambros A, Vanderberg P. Synchronization of *Plasmodium falciparum* stages in culture. 1979; 65:418–20.
- [25] Peel SA, Merritt SC, Hendy J, Baric RS. Derivation of highly mefloquine-resistant lines from *Plasmodium falciparum* in vitro. *Am J Trop Med Hyg* 1993;48:385–97.
- [26] Tazi J, Bird A. Alternative chromatin structure of CpG islands. *Cell* 1990;60:909–20.
- [27] Jeppesen P, Turner BM. The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell* 1993;74:281–9.
- [28] O'Neill LP, Turner BM. Histone H4 acetylation distinguishes coding regions of the human genome from heterochromatin in a differentiation-dependent but transcription-independent manner. *EMBO J* 1995;14:3946–57.
- [29] Keohane AM, O'Neill LP, Belyaev ND, Lavender JS, Turner BM. X-Inactivation and histone H4 acetylation in embryonic stem cells. *Dev Biol* 1996;180:618–30.
- [30] Boggs BA, Connors B, Sobel RE, Chinault AC, Allis CD. Reduced levels of histone H3 acetylation on the inactive X chromosome in human females. *Chromosoma* 1996;105:303–9.
- [31] Hsieh C-L. Dependence of transcriptional repression on CpG methylation density. *Mol Cell Biol* 1994;14:5487–94.
- [32] Selker EU. Trichostatin A causes selective loss of DNA methylation in *Neurospora*. *Proc Natl Acad Sci USA* 1998;95:9430–5.
- [33] Nan X, Ng HH, Johnson CA, et al. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 1998;393:386–9.
- [34] Chen ZJ, Pikaard CS. Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. *Genes Dev* 1997;11:2124–36.
- [35] Trager W, Robert-Gero M, Lederer E. Antimalarial activity of S-isobutyl adenosine against *Plasmodium falciparum* in culture. *FEBS Lett* 1978;85:264–6.
- [36] Trager W, Tershakovec M, Chiang PK, Cantoni GL. *Plasmodium falciparum*: Antimalarial activity in culture of sinefungin and other methylation inhibitors. *Exp Parasitol* 1980;50:83–9.
- [37] Whaun JM, Miura GA, Brown ND, Gordon RK, Chiang PK. Antimalarial activity of Neoplanocin A with perturbations in the metabolism of purines, polyamines and S-adenosylmethionine. *J Pharmacol Exp Ther* 1986;236:277–83.
- [38] Whaun JM, Brown ND, Chiang PK. Effects of two methyladenosine analogues, SIBA and deaza-SIBA, on *P. falciparum*-infected red cells. *Prog Clin Biol Res* 1984;155:143–57.
- [39] Gommers-Ampt JH, Borst P. Hypermodified bases in DNA. *FASEB J* 1995;9:1034–42.
- [40] Gommers-Ampt J, Lutgerink J, Borst P. A novel DNA nucleotide in *Trypanosoma brucei* only present in the mammalian phase of the life-cycle. *Nucl Acids Res* 1991;19:745–51.
- [41] Gommers-Ampt JH, van Leeuwen AL, de Beer AL, et al. β -D-glucosyl-hydroxymethyluracil: A novel modified base present in the DNA of the parasitic protozoan *T. brucei*. *Cell* 1993;75:1129–36.

ANEXO 3

In Vitro Evaluation of Drug Susceptibilities of *Babesia divergens* Isolates

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Received 18 August 1997/Returned for modification 31 October 1997/Accepted 27 January 1998

The susceptibilities of three bovine and two human *Babesia divergens* isolates to antimicrobial agents were evaluated in vitro by a tritiated hypoxanthine incorporation assay. The MICs at which 50% of isolates are inhibited (MIC₅₀s) for mefloquine (chlorhydrate), chloroquine (sulfate), quinine (chlorhydrate), clindamycin (phosphate), pentamidine (isethionate), phenamidine (isethionate) plus oxomemazine (chlorhydrate), lincocmycin (chlorhydrate monohydrate), and imidocarb (dipropionate) were determined. Except for imidocarb, the MIC₅₀s observed for the different isolates were close. Imidocarb and the combination of phenamidine plus oxomemazine exhibited the highest in vitro activity, while antimalarial agents such as mefloquine, chloroquine, and quinine were inactive. Other drugs had intermediate activities. The data support further in vitro evaluation of antimicrobial agents active against *B. divergens* for the improvement of therapeutic strategies.

Babesiosis is due to an intraerythrocytic protozoan parasite transmitted by tick bites to a wide variety of wild and domestic animals, including cattle, horses, dogs, and rodents. In Europe, *Babesia divergens* is considered the most pathogenic agent of bovine babesiosis and is responsible for important economic losses in beef- and milk-producing breeds. Current therapy includes the use of imidocarb dipropionate, but it remains debatable whether differences in outcomes among individual animals are due to variations in the sensitivities of the isolates to drugs or to host factors in animals which are not highly inbred (11).

In humans, 19 of the 24 cases of babesiosis reported in Europe were due to *B. divergens*, and all occurred in splenectomized patients. The initial therapeutic trials with antimalarial drugs for the treatment of this condition, characterized by severe symptoms and high mortality rates, have had limited success. More recently, complete parasite clearance and recovery were obtained with the combination of clindamycin and quinine in association with exchange transfusion (6).

Limited information is available on the antimicrobial susceptibility of *Babesia* spp. in vitro. The first reports of the use of tritiated nucleic acid precursors for the screening of drugs (7) showed a positive correlation between in vitro and in vivo activities in animals (10). More recently, in vitro assays have been proposed for determination of the susceptibilities of *Babesia bovis* and *Babesia bigemina* to drugs (4, 12). The aim of this work was to compare the antimicrobial susceptibilities of *B. divergens* isolates of bovine and human origin by a tritiated hypoxanthine incorporation assay.

MATERIALS AND METHODS

***B. divergens* isolates.** Five *B. divergens* isolates were tested. Three isolates were of bovine origin and were obtained from different geographical areas in Europe:

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Muenchen (Munich, Germany), Weybridge 8843 (Weybridge, United Kingdom), and Taylor (Ireland). Two isolates were obtained in France from patients admitted to Rouen University (isolate Rouen) and Le Mans (isolate Le Mans) hospitals. Isolates were maintained in human erythrocyte (RBC) cultures and infected 10 to 20% of the RBCs. The isolates were then suspended in RPMI 1640 medium (Gibco, Cergy, France) supplemented with 40% (vol/vol) fetal calf serum and 15% (vol/vol) dimethyl sulfoxide and frozen in liquid nitrogen.

***B. divergens* cultures.** After thawing and washing, 0.2 ml of packed infected RBCs was injected intraperitoneally into 2-month-old female gerbils (*Meriones unguiculatus*) (Centre D'élevage R. Janvier, Le Genest-Saint-Isle, France) 2 days after an intraperitoneal injection of 2 mg of dexamethasone (Solutdecadron; Merck-Sharp-Dohme, Paris, France). When the level of parasitemia reached 50% (i.e., 2 to 3 days later), parasitized RBCs were collected by cardiac puncture. Human RBCs were collected from healthy type O- and Rh-positive volunteer blood donors, placed in preservative-free heparin (Heparine Leo, Saint-Quentin-Les-Yvelines, France), and washed by serial centrifugations in RPMI 1640 medium. The buffy coat and the upper part of the RBC pellets were discarded to avoid leukocyte contamination, and 0.2 ml of packed human RBCs was poured into 50-ml plastic flasks (Nunc, Roskilde, Denmark) containing 5 ml of RPMI 1640 medium supplemented with HEPES (25 mM; pH 7.4; Sigma, St. Louis, Mo.), gentamicin (12 µg/ml), and 10% (vol/vol) decomplexed pooled human serum from type O- and Rh-positive donors. A volume of 0.01 ml of parasitized packed gerbil RBCs was added. The cultures were maintained at 37°C in a 5% CO₂ atmosphere and were supplemented with human RBCs twice a week to adjust the level of parasitemia to between 1 and 2%.

In vitro assays for drug susceptibility. Drug susceptibility assays were performed in 96-well culture microplates (Falcon, Meylan, France) in the presence of a final RBC concentration of 1.8% (vol/vol) and serial dilutions of drugs. Each duplicate well received 200 µl of an RBC suspension and 50 µl of each drug dilution in RPMI 1640 medium. For each experiment, controls consisted of four wells containing infected RBCs without drug (i.e., wells to which 50 µl of RPMI 1640 alone was added) and two wells containing 200 µl of noninfected RBCs.

[³H]hypoxanthine (specific activity, 4 Ci/mmol; Amersham, Les Ulis, France) was added (0.4 µCi/well). The plates were incubated at 37°C for 48 h in a 5% CO₂ atmosphere, the cells were harvested on glass fiber filters (Whatman GF/C) with a cell harvester, and the incorporated radioactivity (in counts per minute) was counted in a liquid scintillation spectrometer.

Five milliliters of RBCs from each well were stained with Giemsa stain, and 5,000 RBCs were microscopically examined to determine morphologically the percentage of infected RBCs.

Antimicrobial agents. From a stock solution in 70% ethanol, nine dilutions of the following antimicrobial agents were prepared in RPMI 1640 medium: mefloquine chlorhydrate (0.3 to 0.017 µg/ml; Hoffmann-La Roche-Basle, Basel, Switzerland), chloroquine sulfate (6 to 0.023 µg/ml; Rhône Poulenc, Paris, France), quinine chlorhydrate (1.2 to 0.047 µg/ml; Assistance Publique, Paris, France), clindamycin phosphate (250 to 0.98 µg/ml; Upjohn, Val de Reuil, France), pentamidine isethionate (10 to 0.07 µg/ml; Rhône Mérieux, Toulouse, France),

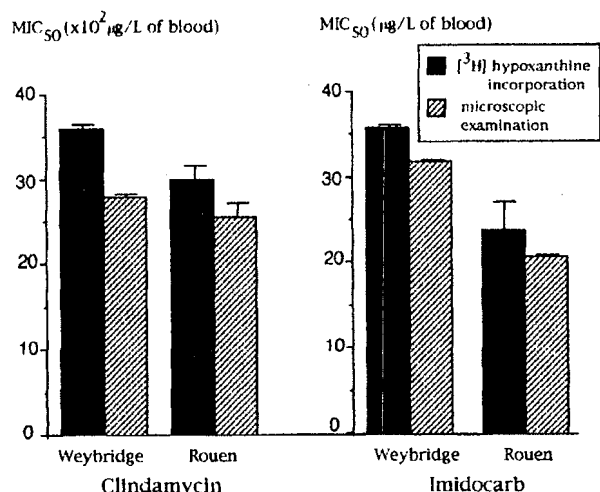


FIG. 1. Comparison of MIC₅₀s of clindamycin and imidocarb for the Weybridge 8843 bovine isolate and the Rouen human isolate obtained in the [³H]hypoxanthine incorporation assay and by microscopic examination. Results are expressed as mean values for 15 independent experiments (one bar indicates 1 SD).

phenamidine isethionate and oxomemazine chlorhydrate (2 to 0.008 µg/ml; Oxopirvedine; Rhône Mérieux, Lyon, France), lincomycin chlorhydrate monohydrate (4,000 to 15.6 µg/ml; Lincocine; Upjohn, Val de Reuil, France), and imidocarb dipropionate (25 to 0.098 µg/ml; Carbesia; Pitman-Moore, Meaux, France). A volume of 50 µl of each dilution was added to the wells. Control wells were filled with 50 µl of medium without drug.

Expression of results and statistical analysis. The amount of parasite-dependent tritiated hypoxanthine incorporated was obtained by subtracting the amount of background incorporation observed in noninfected cultures. For each drug, a concentration-versus-[³H]hypoxanthine incorporation curve was established, and the inhibitory activity was expressed as the minimum concentration (wt/vol) of drug which either inhibited tritiated hypoxanthine incorporation or decreased the proportion of morphologically infected RBCs by 50% of the value in control cultures without drug (the concentration at which 50% of isolates are inhibited [MIC₅₀]). MIC₅₀s were expressed in micrograms per liter of blood with a 50% hematocrit.

Variance analysis was performed by the Fisher and Yates chi-square test or the Student *t* test, thus assuming normal, like distributions of values. The significance of *r* values was used to study linear correlation.

RESULTS

In preliminary experiments, tritiated hypoxanthine incorporation was measured in cultures maintained in the absence of antimicrobial agents (15 microplates, 24 wells/microplate). For the noninfected wells, the mean ± 1 standard deviation (SD) level of incorporation was 191 ± 52 cpm/well, and for infected wells without drug, the mean ± 1 SD level of incorporation was 11,707 ± 2,575 cpm/well. No significant difference was found between isolates (*P* > 0.1; data not shown). One SD accounted for less than 5 and 17% of the mean for each microplate and for the pooled data for the 15 microplates, respectively. The variations for the cultures maintained in the presence of the different antimicrobial agents are summarized in Fig. 1 to 3. In the case of clindamycin, it was verified with the Le Mans isolate that a good correlation was found between MIC₅₀s obtained with 18 independent microplates (*r* = 0.7; *P* < 0.01).

A detailed comparison of the MIC₅₀s obtained by the tritiated hypoxanthine incorporation assay and microscopic counting was performed for the Weybridge 8843 and Rouen isolates in the presence of clindamycin or imidocarb. As indicated in Fig. 1, no significant difference in the MIC₅₀s was observed in 15 independent experiments (*P* > 0.1). This was confirmed for

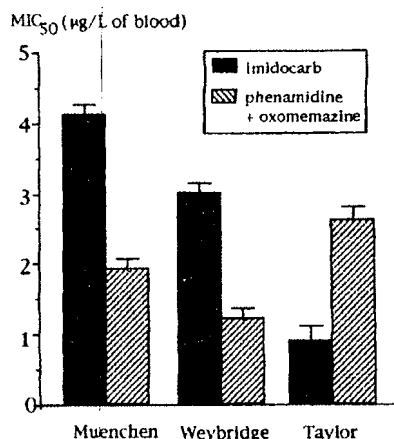


FIG. 2. MIC₅₀s of phenamidine plus oxomemazine and of imidocarb for bovine *B. divergens* isolates. Results are expressed as mean values for eight independent experiments (one bar indicates 1 SD).

all isolates in the presence of the other antimicrobial agents (*P* > 0.1; data not shown).

From these data, the MIC₅₀s of clindamycin and imidocarb varied from 2,200 to 3,400 µg/liter and from 27 to 34 µg/liter, respectively. Imidocarb, a drug restricted to the treatment of babesiosis in animals, was efficient against both human and bovine *B. divergens* isolates (Fig. 1 and 2). The MIC₅₀s of imidocarb and clindamycin for bovine and human isolates were not significantly different; however, MIC₉₀s determined by using probit/log concentration regression curves suggested that they had higher levels of activity against human isolates than against bovine isolates (14,100 and 28,800 µg/liter, respectively). The combination of phenamidine plus oxomemazine was more effective than imidocarb against the bovine isolates Muenchen and Weybridge 8843 but not against the bovine isolate Taylor (Fig. 3). None of the *B. divergens* isolates tested was found to be sensitive to antimalarial agents (Table 1). Pentamidine isethionate inhibited *B. divergens* in vitro, and lincomycin was active and had a high MIC₅₀ (Table 1).

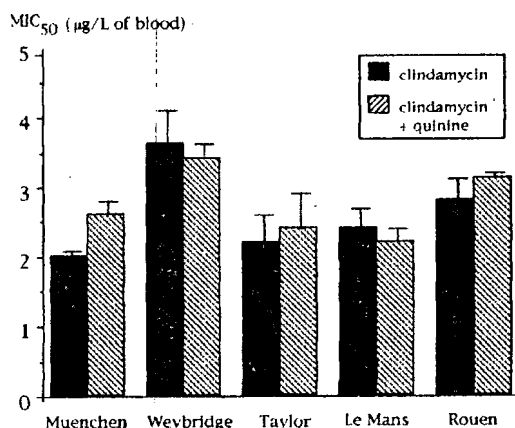


FIG. 3. MIC₅₀s of clindamycin and clindamycin plus quinine for bovine and human isolates of *B. divergens* in 18 independent experiments. Results are expressed as mean values for 18 independent experiments (one bar indicates 1 SD).

TABLE 1. In vitro activities of mefloquine, chloroquine, quinine, pentamidine, and lincomycin against bovine and human *B. divergens* isolates

Isolate source and isolate	MIC (μg/liter of blood) ^a				
	Meflo- quine	Chloro- quine	Quinine	Pent- amidine	Lincomycin
Bovine isolates					
Muenchen	>24	>48	>96	5.1 ± 1	70,000 ± 2,100
Weybridge 8843	ND	ND	>96	ND	ND
Taylor	>24	>48	>96	2.5 ± 0.4	32,000 ± 3,800
Human isolates					
Le Mans	>24	>48	>96	2.2 ± 0.0	37,000 ± 4,700
Rouen	ND	ND	>96	ND	38,000 ± 2,500

^a Results are for two to six triplicate experiments. ND, not determined.

DISCUSSION

In this report a 48-h in vitro assay was used to evaluate the susceptibilities of human and bovine *B. divergens* isolates to antimicrobial agents. Continuous cultures of *B. divergens* were maintained as described previously (7), and the level of incorporation of tritiated hypoxanthine was measured in the presence of different concentrations of drugs. This assay was easier to perform than microscopic counting and was found to be reliable and sensitive. The MIC₅₀s did not differ significantly from the values obtained by microscopic examination. Uptake of tritiated purines such as hypoxanthine was previously proposed for the screening of drug activities against *Babesia* spp. with short-term cultures of blood from experimentally infected animals (8, 9). In the present culture assay, which uses human instead of bovine RBCs, higher levels of tritiated hypoxanthine incorporation were obtained.

Bovine babesiosis is generally treated with imidocarb dipropionate. For bovine isolates, the MIC₅₀ of this compound was significantly lower than the maximum (C_{max}) concentration in serum of 6.6 mg/liter observed in cattle receiving a dose of 17 mg/100 kg of body weight. This is consistent with the high level of activity of imidocarb against bovine babesiosis. Similar MIC₅₀s, i.e., 0.87 μg/ml of medium, have been reported for *B. bovis* (15). In addition, the comparable results obtained for human and bovine isolates suggest that imidocarb is possibly a candidate agent for the treatment of human *B. divergens* babesiosis.

The combination of phenamidine isethionate plus oxememazine chlorhydrate, which is currently restricted for use in the treatment of canine babesiosis, was found to be more active than imidocarb in vitro.

The treatment of human *B. divergens* babesiosis with antimalarial drugs has had limited success. Of the seven patients treated with chloroquine for several weeks, four died and three recovered (1, 5, 10, 14). A treatment based on blood exchange and the administration of clindamycin plus quinine has been successfully used in six patients. Our results confirm the susceptibility of *B. divergens* to clindamycin in vitro. The in vivo efficacy of quinine is controversial (3), since some of the patients treated with quinine alone died and others recovered after several weeks. The very high MIC₅₀s of quinine found in this study suggest that this compound is not useful for treatment. The MIC₅₀s of antimalarial drugs found in this study were higher than the cutoff limits of sensitivity for *Plasmodium falciparum* (8.6, 2.7, and 1 μg/liter of blood for quinine, chloroquine, and mefloquine, respectively). Interestingly, it has been shown in a recent study that atovaquone, which has anti-

malarial activity, was more active than imidocarb for the treatment of bovine *B. divergens* babesiosis (13).

All isolates were susceptible to pentamidine isethionate in vitro. For the Rouen isolate, the MIC₅₀ (2.2 ± 0.08 μg/liter of blood) was higher than the C_{max} (0.2 to 0.5 μg/liter of blood) resulting from an injection of 4 mg/kg, which is the maximum dose that can be used in humans due to the renal toxicity of this compound (16). This may explain the treatment failures reported for patients treated with pentamidine alone (17) or with pentamidine and chloroquine (2). No data on the pharmacokinetics of pentamidine isethionate in cattle are available, and it cannot be stated whether pentamidine isethionate can be proposed for use in the treatment of bovine babesiosis. The MIC₅₀ of lincomycin found in this study is higher than the C_{max} values obtained in humans (2 to 7 μg/liter of blood), which suggests that lincomycin probably has limited use for the treatment of this disease.

Data suggest that tritiated hypoxanthine incorporation by *B. divergens* in human RBC cultures provides an accurate and convenient means of evaluating the antibabesial activities of drugs, further drug screening, detecting resistant isolates, and comparing in vitro activities with in vivo activities.

REFERENCES

1. Bayle, J., H. Dumon, J. J. Verdot, and R. Muratore. 1979. Human piroplasmiasis. One case. Presse Med. 8:3674.
2. Beck, P., A. Gorenflot, J. P. Estebe, A. Marchand, and A. Roquilly. 1987. Insuffisance respiratoire aigüe d'un cas de babésiose humaine. Bull. Soc. Fr. Parasitol. 5:16.
3. Brasseur, P., S. Lecoubiet, N. Kapel, L. Favenec, and J. J. Ballet. 1996. Quinine in the treatment of *Babesia divergens* infections in humans. Eur. J. Clin. Microbiol. Infect. Dis. 15:840-841.
4. Brockelman, C. R., and P. Tan-Ariya. 1991. Development of an in vitro microtest to assess drug susceptibility of *Babesia bovis* and *Babesia bigemina*. J. Parasitol. 77:994-997.
5. Enrican, J. H., H. Williams, I. A. Cook, W. M. Lancaster, J. C. Clark, and L. P. Joyner. 1979. Babesiosis in man: report of a case from Scotland with observations on the infecting strain. J. Infect. 1:227-234.
6. Gorenflot, A., P. Brasseur, G. Bonmarchand, D. Laneelle, and D. Simonin. 1988. Two cases of severe human babesiosis treated with success. Presse Med. 19:335.
7. Gorenflot, A., P. Brasseur, E. Precigout, M. L'Hostis, A. Marchand, and J. Schrevel. 1991. Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil, man. Parasitol. Res. 73:3-12.
8. Irvin, A. D., and E. R. Young. 1977. Possible in vitro test for screening drugs for activity against *Babesia* and other blood protozoa. Nature (London) 169:407-409.
9. Irvin, A. D., and E. R. Young. 1979. Further studies on the uptake of tritiated nucleic acid precursors by *Babesia* spp. of cattle and mice. Int. J. Parasitol. 9:109-112.
10. Jadin, J. B., and P. Girault. 1981. Babesiosis and rickettsiosis, p. 132-135. In Society of Protozoologists (ed.), Parasitological topics special publication no. 1. Society of Protozoologists, Lawrence, Kans.
11. Mallick, K. P., S. K. Dwivedi, and N. K. Srivastana. 1987. A report on the occurrence of hemoprotozoan infections in rural livestock. Indiana J. Parasitol. 11:25-26.
12. Nott, S. E., W. J. O'Sullivan, A. M. Gero, and A. S. Bagnara. 1990. Routine screening for potential babesicides using cultures of *Babesia bovis*. Int. J. Parasitol. 20:797-802.
13. Pudney, M., and J. S. Gray. 1997. Therapeutic efficacy of atovaquone against the bovine intraerythrocytic parasite, *Babesia divergens*. J. Parasitol. 83:307-310.
14. Rabinovich, S. A., Z. K. Voronina, and N. I. Stepanova. 1978. Première découverte de babésiose humaine en URSS: courte analyse des cas décrits dans la littérature. Mosk. Med. Parasitol. 47:97-107.
15. Rodriguez, R. I., and A. J. Trees. 1996. In vitro responsiveness of *Babesia bovis* to imidocarb dipropionate and the selection of a drug-adapted line. Vet. Parasitol. 62:35-41.
16. Roebush, T. K., R. H. Rubin, E. R. Wolpw, P. B. Cassaday, and M. G. Schultz. 1979. Neurologic complications following treatment of human *Babesia microti* infection with dimidazene aceturate. American J. Med. Hyg. 28:184-189.
17. Telford, S. R., A. Gorenflot, P. Brasseur, and A. Spielman. 1992. Babesial infections in humans and wildlife, p. 17. In J. P. Kreier (ed.), Parasitic protozoa. Academic Press, Inc., New York, N.Y.

ANEXO 4

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MULTI-DRUG RESISTANT FALCIPARUM MALARIA IN CAMEROON IN 1987-1988 II. MEFLOROQUINE RESISTANCE CONFIRMED IN VIVO AND IN VITRO AND ITS CORRELATION WITH QUININE RESISTANCE

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Abstract. To further document the phenomenon of *Plasmodium falciparum* resistance to mefloquine formerly described in Cameroon, complementary in vivo and in vitro studies were conducted. Two hundred six *P. falciparum* isolates were studied in vitro using an isotopic microassay with mefloquine solutions prepared daily. Using the cutoff limit of 30 nmol, 26 (20%) of 133 isolates from the northern part of the country were defined as being resistant to mefloquine. In contrast, only one of 73 isolates collected in the southern part of the country was resistant. In vivo 7-day assays were performed in the northern area in 57 asymptomatic *P. falciparum* carriers (age range 1-10 years) who were given a single 25 mg/kg dose of mefloquine (Lariam). Among 46 cases in which followup was possible, *P. falciparum* asexual parasites were cleared within five days in 38 cases, by days 6 and 7 in two cases, and remained detectable up to day 7 in six cases. Thus, these latter patients have a RII-RIII level of resistance by in vivo criteria. No resistance was found in 40 additional patients studied similarly in the southern region. These observations were made before any mefloquine drug pressure was exerted in the country, but results of cross-resistance and drug consumption studies support the hypothesis that in the northern region, where a close correlation ($r = 0.67$) was found between the response to quinine and mefloquine, the more frequent use of quinine may have induced a primary quinine resistance and a secondary mefloquine resistance (without chloroquine resistance). Chloroquine drug pressure in the southern region, which is 2.5 times higher than in the northern region, possibly led to a primary chloroquine resistance and a secondary quinine resistance (without mefloquine resistance).

Mefloquine (Lariam), a quinoline derivative that has structural similarities with quinine, was first introduced for clinical use in 1985. In the African continent, consumption started in 1988, but is believed to have remained low due to the high cost price of this new drug.

In 1986, we studied the in vitro response to mefloquine of 44 *Plasmodium falciparum* isolates originating from four areas of Cameroon.¹ This survey showed a high degree of sensitivity to the drug in malaria parasites from the southwestern region of the country, and also revealed the unanticipated presence of numerous isolates apparently resistant to mefloquine in other areas, particularly in the north.

In the present study, conducted at the end of 1987 and in 1988, we investigated the status of the response of malaria parasites to mefloquine both by in vitro and in vivo methods. The study of cross-resistance between three major anti-

malarial drugs and a study of drug consumption habits strongly suggest that the emergence of resistance to mefloquine in the absence of specific drug pressure may be related to the use, or more likely the misuse, of quinine.

MATERIALS AND METHODS

The areas and the patients studied are the same as those described previously by Brasseur and others.² In vitro studies were performed in October 1987, and complementary in vivo/in vitro studies were performed in October 1988.

The in vitro 48-hr isotopic microtest using ³H-hypoxanthine incorporation is similar to that used with quinine and chloroquine² and was carried out simultaneously. In view of the previous doubts about the stability of mefloquine solutions,³ the solutions we used in our plates were freshly prepared every day from mefloquine hy-

drochloride powder (Hoffmann-LaRoche, Basel, Switzerland) that had been aliquoted in Paris and taken to the field.

In vivo 7-day assays were conducted in 55 patients (25 boys and 30 girls) with an age range of 1–10 years (mean 4.4 years). Their weights ranged from 5 to 30 kg. Most of the subjects were asymptomatic parasite carriers, except for five cases in whom a mild fever ($< 38.5^{\circ}\text{C}$) was observed. They were given a single 25 mg/kg dose of mefloquine, using 250-mg tablets. All remained under medical supervision for three hours after receiving the drug and were re-examined at 24-hr intervals. Parasitemia was followed using daily blood smears for eight days. Day 0 and day 3 blood samples, stored at -20°C , were used to determine mefloquine blood concentration by high-performance liquid chromatography. Of these 55 patients, 44 had a parasitemia high enough to warrant simultaneous performance of an in vitro isotopic microtest.

In the various areas under study, we attempted to estimate both the consumption of antimalarial drugs and the dose regimens that were most frequently used. This was done first by collecting data on all drugs imported into the country by governmental and private suppliers (Laborex and Socapharm, Douala, Cameroon) and by recording the quantities delivered to each city in the country. In the villages studied, further investigations were aimed at detecting other sources of drugs, particularly those imported from neighboring countries. Second, in each of the health centers in the areas studied, including hospitals, dispensaries, and small drug stores, questionnaires were used to record the quantities and regimen of each drug used. These data were used to double-check the information available from the suppliers. Third, with the assistance of an interpreter, we investigated self medication in the families of each patient studied.

RESULTS

A total of 234 in vitro mefloquine assays were performed, of which 206 (88%) were successful. The results (Figures 1 and 2) confirm and extend the data collected in 1985–86¹. Almost all of the isolates collected from the southern region were found to be mefloquine-sensitive. Only one of the 73 isolates from Limbe and Kribi (located in the southwestern Cameroon) was resistant to mefloquine, showing a rather high concentration

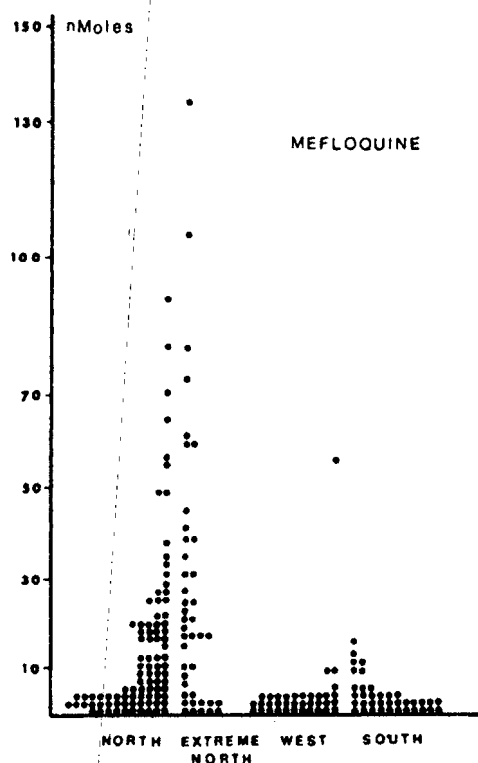


FIGURE 1. Sensitivity to mefloquine in 206 isolates studied in vitro. Concentrations effective at 50% (EC_{50}) are expressed in nanomoles/liter.

effective at 50% (EC_{50}) of 56 nmol, but the values for 68 of the 72 isolates were below 10 nmol.

In contrast, a large proportion of the 133 isolates from the north region responded poorly to mefloquine. Twenty-six isolates had an EC_{50} above the cutoff limit of 30 nmol, and 19 others could be considered as borderline with EC_{50} values in the range of 20–30 nmol. Prevalence of resistance was almost three times higher around Maroua (located in extreme northern Cameroon) than around Garoua (14/41 [34%] versus 11/83 [13%]). In the city of Maroua, 10 (53%) of 19 isolates were mefloquine-resistant by in vitro criteria. The highest EC_{50} value recorded, 134 nmol, was twice as high as the most resistant case found in 1986,¹ and more than four times above the cutoff limit. The mean EC_{50} and EC_{90} values were 3 and 49 nmol, respectively, in the southern region and 16 and 752 nmol, respectively, in the northern region.

During the in vivo assays, mefloquine was not well tolerated; vomiting occurred in six children

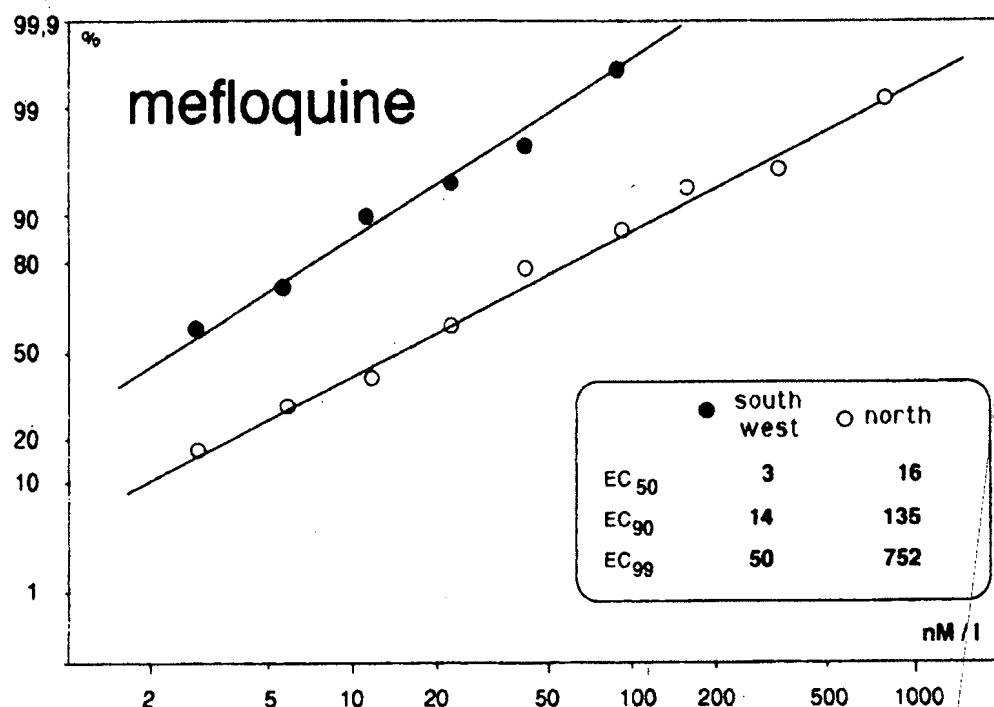


FIGURE 2. Probit regression lines of mefloquine resistance. EC = effective concentration.

(11%) who were subsequently excluded from the study. Dizziness was recorded in 26% of the patients, diarrhea in 7%, severe abdominal pains in 6%, and bradycardia in 4.4%.

Parasitemia was cleared within seven days (mean 2.8 days) in 40 of the 46 patients receiving follow up. Asexual parasites were detectable up to day 6 in one of these 40 patients, and in another patient up to day 5. In the six remaining cases (ages 2–7), parasites remained detectable up to day 7. Clearance of the parasites was obtained with Fansidar. Parasites counts indicated one case with an RIII level of resistance, and five others with RII levels of resistance. Blood levels of mefloquine in day 3 samples were > 500 nmol. Based on these results, six of these 46 infected individuals could be considered as having parasites with RII-RIII levels of mefloquine resistance. Five of these six patients originated from the extreme north district of Cameroon, around Maroua, and one was from Garoua. Forty-two of the 46 isolates could be simultaneously studied in vitro. Two of the six in vivo-resistant cases had parasites with high EC₅₀ values in vitro (79 and 46 nmol), one had a borderline EC₅₀ value

(25 nmol), one had a moderately high EC₅₀ value (18 nmol), and two were not tested in vitro. In the two cases classified as in vivo sensitive despite the fact that parasitemia was cleared only by day 5 or day 6, the EC₅₀ values in vitro were 22 and 24 nmol, respectively. Among the 38 other patients who were considered as harboring sensitive parasites on the basis of in vivo studies, one had parasites with a borderline EC₅₀ value (30 nmol), and all of the remaining patients had EC₅₀ values below 30 nmol (mean EC₅₀ ± SD = 6.5 ± 4.3 nmol).

Since most of the isolates tested for their in vitro response to mefloquine had also been studied in vitro with chloroquine and quinine,² the cross-resistance, or simultaneous resistance, to two or three of these antimalarials could be evaluated in each area. The results, summarized in Figure 3, are clear-cut; all the isolates resistant to mefloquine from the northern region are chloroquine-sensitive (Figure 3 N₁), but the chloroquine-resistant isolates found mainly in the southern region appear to be mefloquine-sensitive without exception (Figure 3 S₁). Thus, there is no indication in the two areas studied of cross-

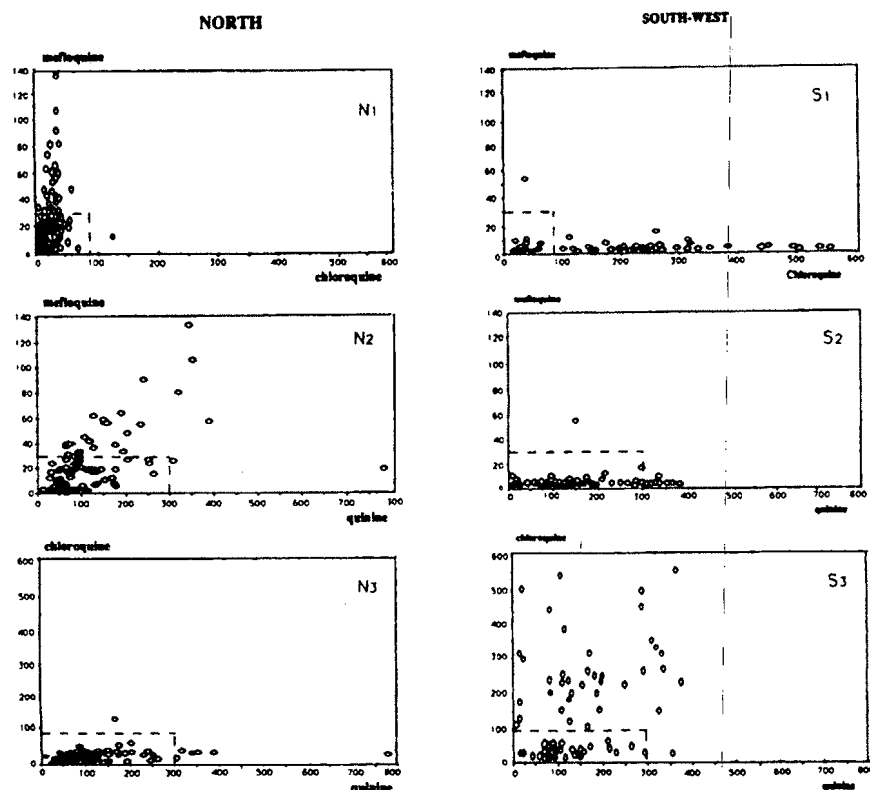


FIGURE 3. Comparison of the responses to chloroquine, quinine, and mefloquine in isolates studied in the dry northern area (N) and the wet southwestern area (S) of Cameroon.

resistance between these two major drugs; on the contrary, there is a strong negative correlation in the response to them in the southern region ($r = -0.08$, $P < 0.01$). In contrast, a high degree of cross-resistance between quinine and mefloquine is observed among the isolates from the northern region ($r = 0.67$, $P < 0.01$) (Figure 3 N₂), but not from the southern region (Figure 3 S₂). In the latter area, where chloroquine-resistant cases are highly prevalent, some degree of cross-resistance occurs between quinine and chloroquine ($r = 0.3$, $P < 0.02$) (Figure 3 S₃). Figure 3 also clearly shows the difference between northern Cameroon, where quinine-resistance coexists with mefloquine-resistance, and the southwestern region, where quinine and chloroquine resistance coexist.

Of interest are the relative degrees of mefloquine and quinine resistance in isolates from the northern region. There is a correlation of the response to the two drugs, which can be seen in

Figure 3 N₂. However, true mefloquine resistance is seen in isolates, most of which show only a decrease in sensitivity to quinine (i.e., EC_{50} values between 100 and 300 nmol, which is below the cut-off limit for quinine that corresponded to EC_{50} values above 30 nmol, which are greater than the cut-off limit for mefloquine). The practical importance of this observation is that RII–RIII levels of resistance to mefloquine exist *in vivo*, while for now, only RI levels were found for quinine.

Analysis of data from the drug consumption study led to the conclusion that the total drug pressure is greater in the southern region than in the north, although quinine is more widely used in the north than in the south. Some of the results are summarized in Table 1, which shows the total amounts, from various origins, of 4-amino-quinolines and quinine sold in the two areas in 1985. Quinine accounted for 5.6% of all antimalarials consumed in the southwestern region, and for

TABLE 1
Estimations of total amounts of antimalarial drugs sold in the 2 areas studied in 1985*

	Quinine	4-amino-quinolines	Sulfadoxine pyrimethamine	Total
Southwest	590 (5.6)	9,396 (88.7)	607 (5.7)	10,593
North	354 (15)	1,966 (83)	46 (2)	2,366

* Values are the number (%) of drugs sold in each category, including tablets, injectable solutions, suppositories, oral solutions, and all salts and brands available (expressed in kg/year). 4-amino quinolines include Nivaquine, Flaviquine, Camoquine, and Resochin.

15% of those consumed in the northern region. Since 1987, its consumption has increased to 13.8% in the southern region, probably because of the emergence of chloroquine resistance in this region in early 1985. However, the 1985 figures are more relevant to this study because they reflect the earlier drug pressure that may have contributed to the selection of the resistant parasites, which were detected by our study performed in 1987.

In addition, it appears that in those areas, quinine regimens are most inappropriate, particularly in the northern region; a single intramuscular injection of 100 mg of quinine base is usually given, followed the next day by the oral administration of 200–300 mg of chloroquine. In such a regimen, the ratio of quinine:chloroquine is about 1:2–1:3. Taking into account that the quinine:chloroquine ratio of drugs sold in the northern region is approximately 1:5, this would suggest that nearly half of the treatments given in the northern region contained quinine, as opposed to one of six in the southern region. When the amounts of chloroquine sold are compared with the number of inhabitants in the two areas, it appears that the consumption of chloroquine per person is 2.5 times higher in the south region (1.8 g/person/year) than in the northern region (0.7 g/person/year).

DISCUSSION

Both in vivo and in vitro results clearly confirm our earlier impression that mefloquine-resistant parasites are highly prevalent in Cameroon¹. The foci of resistance appear to be geographically restricted, with three of four cases coming from Maroua in the extreme northern region. In our in vitro assay, we chose the relatively high cutoff limit of resistance of 30 nmol, while the limit defined by other investigators ranged from 20 to 29 nmol.^{4–8} However, 20% of the 133 isolates studied were above this limit of 30 nmol (10% were above 50 nmol), a very high

prevalence considering the fact that this level of resistance was detected in 1987, long before any mefloquine drug pressure was exerted in the area. In vivo methods have shown the prevalence of resistance to be 13%, which is a very high value considering that the 7-day in vivo assay does not, in most cases, enable the detection of resistance at the RI level, but only of the higher RII and RIII responses.

Mefloquine resistance has already been reported in African isolates, and was previously suspected in several in vitro studies. Among nine African strains adapted to culture and in which drug sensitivity may have changed upon cultivation, a reduced sensitivity was reported in two strains originating from Nigeria and Sierra Leone.⁸ One isolate from Chad was considered resistant,⁹ and two of seven isolates from Guinea we studied were also resistant.¹⁰ Recently, resistance was also reported in several Nigerian isolates tested in vitro,¹¹ and in six Tanzanian isolates.¹² Among 291 isolates studied in Paris, resistance was found in some originating from Mali and Senegal, but this suspicion was not confirmed in 50 cases studied in vivo.¹³ In 1986, resistance in southern Cameroon was not detected by the in vitro method, but it was found in isolates from the northern region.¹ One case, confirmed by both in vivo and in vitro methods, was reported in a traveler from Tanzania.⁶ Several acute malarial attacks were observed in Europeans living in northern Cameroon who ingested 250 mg of mefloquine/week; however, blood levels of the drug were not measured (V. Lavoue, unpublished data).

Since mefloquine solutions may change upon storage or drying, we performed our assays with dilutions prepared from pre-dosed material. Regardless of a possible in vitro bias, the present in vivo assays confirm that our suspicions based on in vitro studies were correct. Compared with other areas of the world, such as Thailand, where mefloquine resistance has been reported,^{4,5} it is

impressive that such high resistance levels have already been reached in Cameroon.

Cross-resistance studies yielded particularly clear-cut results. In two groups of isolates, one characterized by a high prevalence of chloroquine resistance, and the other by a high prevalence in mefloquine-resistant parasites, no single case of simultaneous resistance to the two drugs was observed. This observation has important public health implications, although it may be limited only to Cameroon, and may not persist for any specific period.

Quinine resistance occurred in the two groups of isolates, but was strongly correlated in the one with mefloquine resistance and in the other with chloroquine resistance. These data, taken together with the results of our earlier study,¹ and with the observation that quinine consumption is greater in the northern region of the country, lead us to formulate the following hypothesis. Specifically, two types of quinine-resistant parasites may exist in Cameroon, which probably differ in the way resistance was induced. The southern region type corresponds to isolates in which chloroquine drug pressure has probably been a factor in the selection of chloroquine resistance, and in some instances, in a secondary cross-resistance to quinine. This idea is supported by the correlation that our group and others found between chloroquine and quinine resistance,^{1, 14-17} and by the fact that in early 1985, quinine resistance was not prevalent in Cameroon,¹⁴ but appeared by the end of 1985 at exactly the same time and at the same speed as chloroquine resistance (Figure 3).^{1, 2}

The northern region type of quinine resistance is represented by a geographically distinct group with exposure to a relatively higher quinine drug pressure and a lower chloroquine pressure, and which may have generated either a primary quinine resistance or more frequently, a decrease in sensitivity to quinine. This phenomenon may have led to a secondary mefloquine resistance, since it occurred in the absence of mefloquine pressure. This view is supported by the structural similarities between the two drugs, by the strong correlation we found between the responses to them, and by the very frequent use of quinine at low doses in the same area. This situation is reminiscent of that observed along the Thai-Cambodian border in 1982, in which mefloquine resistance was found in areas where a high prevalence of quinine resistance existed, long before

mefloquine was introduced into Thailand.⁵ Therefore, selection, rather than importation, of mefloquine-resistant parasites would seem to be possible in the African context.

Whatever the true reasons for mefloquine resistance in this area, an essential finding that needs to be stressed is the difference in levels of resistance to quinine and mefloquine. This is shown in the results in Figure 3 N, and by the results of *in vivo* sensitivity assays. There is a parallelism in the responses to the two drugs, but RII and RIII levels of resistance to mefloquine have been found to occur in isolates that are not resistant to quinine, but which have only a moderately decreased sensitivity to the natural compound. Even when quinine resistance exists, its level is moderate and would probably correspond only to an RI level *in vivo*. No well-documented case of high level resistance to this drug has been reported. From a clinical point of view, this means that all isolates would respond to quinine treatment with an initial clearance of parasites and symptoms even if RI resistance that is associated with a late recrudescence may occur occasionally. In contrast, for several of the isolates we studied, treatment with mefloquine was unable to ensure the same initial clearance.

In view of the possibility of true failures, of its poor tolerance, and its high price, mefloquine does not appear to be recommendable as an initial therapeutic choice or as a prophylaxis. Despite its short half-life, quinine, because of the absence of RII-RIII resistance, would appear to be the antimalarial drug with the greatest clinical efficacy.

Acknowledgments: The authors are grateful to Hoffmann-LaRoche for supplying the mefloquine used in this study, and to D. Fiddock for reviewing the manuscript.

Financial support: This study was supported by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases grant no. 880149 and in part by grant no. 870319.

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REFERENCES

1. Brasseur P, Kouamouo J, Brandicourt O, Moyou-Somo R, Druilhe P, 1988. Patterns of in vitro resistance to chloroquine, quinine, and mefloquine of *Plasmodium falciparum* in Cameroon, 1985-1986. *Am J Trop Med Hyg* 39: 166-172.
2. Brasseur P, Kouamouo J, Moyou-Somo R, Druilhe P, 1992. Multi-drug resistant falciparum malaria in Cameroon in 1987-1988. I. Stable figures of prevalence of chloroquine and quinine resistant isolates in the original foci. *Am J Trop Med Hyg* 45: 1-7.
3. Druilhe P, Danis M, Mazier D, Felix H, Gentilini M, 1982. Biological activity of mefloquine and its metabolites on *Plasmodium falciparum* continuous culture. *Mol Biochem Parasitol (suppl)*: 725.
4. Childs GE, Pang L, Wimonwatrawatee T, Pooyindee N, Nanakorn A, Limchitsee S, Webster HK, 1987. In vitro mefloquine resistance of *Plasmodium falciparum* isolated from the Burmese border region of Thailand. *Southeast Asian J Trop Med Public Health* 18: 438-442.
5. Boudreau EF, Webster HK, Pavanand K, Thosingha L, 1982. Type II mefloquine resistance in Thailand. *Lancet* ii: 1355.
6. Bygbjerg IC, Shapira A, Flach SH, Gomme G, Jepsen S, 1983. Mefloquine resistance of falciparum malaria from Tanzania enhanced by treatment. *Lancet* i: 774-775.
7. Hengy C, Garrigue G, Abissegue B, Ghogomu NA, Gazin P, Gelas H, Kouka-Bemba D, Lebras J, Jambou R, 1989. Surveillance de la chimiosensibilité de *Plasmodium falciparum* a Yaounde et ses environs (Cameroun). *Bull Soc Pathol Exot* 82: 217-223.
8. Oduola AMJ, Milhous WK, Salako LA, Walker O, Desjardins RE, 1987. Reduced in vitro susceptibility to mefloquine in west African isolates of *Plasmodium falciparum*. *Lancet* ii: 1304-1305.
9. Sarrouy J, Bernard J, Doury JC, Gemenez-Espinos M, Moulinas JM, Bureau P, 1988. Premier cas de paludisme a *Plasmodium falciparum* résistant a la chloroquine et de sensibilité diminuée a la mefloquine au retour du Tchad. *Bull Epidemiol Hebdomadaire* 47: 187.
10. Brasseur P, Pathe-Diallo M, Druilhe P, 1987. Low sensitivity to chloroquine and quinine of *Plasmodium falciparum* isolates from Guinea in March 1986. *Am J Trop Med Hyg* 37: 452-454.
11. Salako LA, Fadeke Aderounmu A, 1987. In vitro chloroquine and mefloquine-resistant *Plasmodium falciparum* in Nigeria. *Lancet* i: 572-573.
12. Kilimali VAEB, Mkufya AR, Kilawa WL, 1989. Low resistance of *Plasmodium falciparum* to mefloquine in Tanga region, Tanzania. *Trans R Soc Trop Med Hyg* 83: 162-164.
13. Simon F, Lebras J, Gaudebout C, Girard PM, 1988. Reduced sensitivity of *Plasmodium falciparum* to mefloquine in west Africa. *Lancet* i: 467-468.
14. Brasseur P, Druilhe P, Kouamouo J, Brandicourt O, Danis M, Moyou SR, 1986. High level of sensitivity to chloroquine of 72 *Plasmodium falciparum* isolates from southern Cameroon in January 1985. *Am J Trop Med Hyg* 35: 711-716.
15. Druilhe P, Brandicourt O, Chongsuphajasiddhi T, Berthe J, 1988. Activity of a combination of three cinchona bark alkaloids against *Plasmodium falciparum* in vitro. *Antimicrob Agents Chemother* 32: 250-254.
16. Chongsuphajasiddhi T, Sabchareon A, Attanath P, 1981. In-vivo and in vitro sensitivity of falciparum malaria to quinine in Thai children. *Ann Trop Paediatr* 1: 21-26.
17. Webster HK, Boudreau EF, Pavanand K, Yongvanitch K, Pang LW, 1985. Antimalarial drug susceptibility testing of *Plasmodium falciparum* in Thailand using a microdilution radioisotope method. *Am J Trop Med Hyg* 34: 228-235.