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EFECTO BIOLÓGICO DE NUEVOS COMPUESTOS DE SÍNTESIS Y NATURALES FRENTE A TRIPANOSOMATIDOS

TESIS DOCTORAL

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I-INTRODUCCIÓN

Los tripanosomas son los agentes etiológicos de numerosas enfermedades, que afectan tanto a la salud humana como a la animal y vegetal. Hoy día las tripanosomiasis están consideradas como una de las siete plagas del siglo XXI.

Entre dichas enfermedades se encuentran la tripanosomiasis americana o enfermedad de Chagas (Oliveira, 2000), y la Leishmaniasis (Dedet, 2002).

Una de las parasitosis más importantes en el hombre, tanto por su prevalecía como la gravedad de su cuadro clínico es la enfermedad de Chagas, cuyo agente etiológico es el protozoo flagelado *Trypanosoma cruzi*, descubierto por Carlos Chagas en 1909. La enfermedad es adquirida por tripomastigotas invasivas las cuales son transmitidas por los vectores insectos, transfusiones de sangre, transplacentariamente o a través de transplantes de órganos (Brener y Gazzinelli 1997). Se extiende por Centro y Sudamérica, concretamente entre las latitudes 42ºLN y 46ºLS, que corresponde al área de distribución de los vectores triatominos de hábitos antropofílicos.

Se estima que existen entre 16 y 20 millones de personas infectadas en Sudamérica, así como 120 millones de individuos expuestos, con más de 80 mil defunciones anuales (http://www.who.int/tdr/diseases/chagas/default.htm. Acceso el 15 de Enero del 2009; Teixeira ARL y col 2006). Al margen de estas cifras, no hay que olvidar la repercusión social que acompaña a las enfermedades de carácter crónico. Las alteraciones cardíacas y/o digestivas que sufren de por vida los individuos afectados interfieren considerablemente con su capacidad laboral; además, el coste médico derivado y el absentismo laboral consecuente suponen pérdidas de miles de millones de Euros en muchos países centro y sudamericanos, ya de por sí deficitarios.

Las leishmaniasis son otro grupo de importantes enfermedades de regiones tropicales y subtropicales, producidas por parásitos protozoarios pertenecientes al género *Leishmania*, y transmitida por insectos dípteros de los géneros *phlebotomus*, en el Viejo Mundo y, *Lutzomyia* en el Nuevo Mundo (Laison, 1987 y Berman, 1980).

Esta considerada como una de las siete enfermedades prioritarias que afectan al Continente Latinoamericano, para la Organización Mundial de la Salud (OMS), son afecciones cosmopolitas o endémicas que originan importantes problemas en la salud

pública, debido a la amplia diversidad en las formas clínicas (producción de lesiones cutáneas, mucosas o viscerales) (Murray y col.,2000; Osorio y col 2008), y especialmente a aquellas personas que tienen la forma mucocutánea, a las que provocan mutilaciones que son difíciles de tratar, por lo que producen un impacto psicológico en las personas afectadas (Brazil, 1976).

La leishmaniasis representa un serio obstáculo para el desarrollo socioeconómico de 88 países de los que 72 están en vías de desarrollo, como Perú y México. Por lo cual, la OMS ha reconocido a esta enfermedad como un problema de salud pública global. Se estima que unos 350 millones de personas pueden estar en riesgo de contraer la enfermedad y que unos 14 millones están infectados en todo el mundo. Según la OMS anualmente aparecen de 1.5 a 2 millones de casos nuevos aunque oficialmente solo se reconocen 600.000, se considera que 500.000 sufrirán leishmaniasis visceral y casi un millón y medio sufrirán leishmaniasis cutánea (OPS/OMS, 2003; Osorio y col 2008).

A pesar de lo alarmante de la situación, las posibilidades de control son escasas. Como es general entre las enfermedades parasitarias, las perspectivas de disponer de una vacuna son muy remotas, estando actualmente la quimioterapia lejos de paliar las consecuencias de la carencia de una inmunoprofilaxis eficaz. Se puede afirmar que hasta el momento no existe un fármaco del todo efectivo e inocuo contra el mal de Chagas, dado que los suministrados hasta el momento no son por completo eficaces en todas las fases de la enfermedad, producen graves efectos segundarios o ambas cosas. El agente más prescrito en el tratamiento de esa enfermedad es un derivado de la Nitrofurfurilidina (3-metil-4-5´-nitro-furfurilidenamino-tetrahidrol[1,4] -tiazino-1,1-dióxido) conocido, también, como **Nifurtimox**. El **Benznidazol** (N-bencil-2-nitro-1-imidazolacetamida) ha prescrito con cierto éxito para el tratamiento de la enfermedad de Chagas. (http://www.who.int/tdr/diseases/default.htm.,2008; Croft y col 2005). Los medicamentos usados para el tratamiento de la leishmaniasis, no son efectivos en todos los casos y la mayoría presenta efectos tóxicos segundarios. Los antimoniales pentavalentes, como el antimoniato de meglumina y el estibogluconato de antimonio y sodio son los fármacos de elección para todas las formas clínicas. (Croft, 1999; Becerril, 2008; Osorio y col. 2008.).

Además de producir índices muy variables de curación (no son eficaces contra todo los estados de la enfermedad ni contra todas las cepas patógenas), los antichagasicos y los antileishmaniasis, requieren ser administrados durante periodos prolongados, con la consiguiente aparición de efectos colaterales adversos, que obligan en muchos casos a interrumpir el tratamiento.

De otra parte, la mayoría de los afectados pertenecen a áreas endémicas rurales, donde el tratamiento sintomático, cirugía para pacientes con megaformaciones intestinales son procedimientos en la mayoría de los casos impracticables, en virtud de su elevado coste. Todo ello hace que siga siendo necesaria la búsqueda de nuevos fármacos activos frente al parásito.

El agente tripanocida ideal debería ser:

- selectivo y potente, tanto contra los amastigotes intracelulares como contra los tripomastigotes extracelulares;
- de acción tripanocida rápida y completa;
- efectivo para impedir la evolución de las formas agudas e indeterminadas de la infección;
- inocuo para la persona tratada y no debe producir efectos deletéreos;
- no debe inducir resistencia del parásito al medicamento.

Y su farmacodinamia debe alcanzar niveles afectivos tripanocidas de concentración de la droga en el plasma sanguíneo, en fluidos biológicos y en tejidos. Su modo de acción debe ser estable y, de preferencia, efectivo por vía de administración oral.

II-OBJETIVOS

El equipo de investigación en el que se ha desarrollado el presente trabajo se encuentra dedicado a estudios de tipo farmacológico. El cribado de nuevas moléculas de síntesis o de extractos vegetales, ha sido la base de nuestro trabajo.

Los objetivos prioritarios de la investigación planteada se centran en:

1. Selección de nuevos compuestos de síntesis y naturales activos que ofrezcan una alternativa a los fármacos y quimioprofilácticos existentes frente a *T. cruzi, L. peruviana, y L. braziliensis*
2. Realizar pruebas de citotoxicidad sobre células vero y macrófagos con los productos más activos.
3. Comparar la acción de estos productos con las drogas comerciales "Glucantime", "benznidazol" y "pentostam".
4. Estudiar la posible inhibición de la capacidad de invasión celular así como la de multiplicación, la diferenciación de las formas flageladas a formas amastigotas, la diferenciación de estas últimas a formas flageladas del parásito.
5. Estudiar su posible mecanismo de acción, a través del efecto de estos productos sobre la síntesis de macromoléculas de los parásitos; determinar su acción a nivel de las principales vías metabólicas y determinar las alteraciones ultraestructurales.

III-REVISIÓN BIBLIOGRÁFICA

III-1 Género *Tripanosoma cruzi*

III-1.1 Generalidades

La enfermedad de Chagas está causada por *T.cruzi*, un parásito protozoario flagelado que es transmitido en un 80% vectorialmente (un insecto hematófago de la familia Triatominae) y en un 20% por transfusión de sangre contaminada o por vía congénita. Las otras vías de transmisión son excepcionales (accidentes, transplante de órganos, otros vectores, etc) y no representan una importancia significativa en términos de salud pública (Figueroa y col., 1998; Gutiérrez y col., 2000). Los humanos y un gran número de especies animales, tanto domésticos como salvajes, constituyen los reservorios naturales de *T. cruzi*.

En humanos, la enfermedad presenta dos fases, una aguda que aparece poco tiempo después de la infección y, la otra crónica tras un periodo silencioso o asintomático que puede durar varios años. Las lesiones de la fase crónica afectan irreversiblemente órganos internos como el corazón, el esófago, el colon y el sistema nervioso periférico. Recientes estudios han puesto de manifiesto que un 27% de los individuos infectados desarrollan síntomas cardiacos que los pueden conducir a una muerte súbita, un 6% trastornos digestivos, principalmente megavísceras y un 3% trastornos del sistema nervioso periférico (Charmina y col., 2007).

III-1.2 Morfología y ciclo biológico

III-1.2.1 Morfología

Los flagelados Tripanosomátidos tienen la posibilidad de pasar a lo largo de su ciclo vital por varias formas (fenómeno conocido como Polimorfismo), distinguibles entre si por la zona del nacimiento del flagelo y la posición relativa entre el núcleo y el kinetoplasto. Estas formas reciben los nombres de amastigotas, epimastigotas y tripomastigotas.

En el hospedador mamífero *T. cruzi* aparece en las formas **tripomastigotas** y **amastigotas**, mientras que, en el vector se desarrolla como **epimastigotas** y **tripomastigotas metacíclicas.**

El estudio detallado de su ultraestructura **(**Hoare, 1972**)** revela asimismo la existencia de un núcleo bien visible y una única mitocondria, alargada, en cuyo extremo posterior aparece un nuevo orgánulo con valor taxonómico llamado kinetoplasto, que contiene alrededor del 30 % del material nuclear de la célula. Otras estructuras, como los glicosomas, con una función metabólica semejante a la que desempeñan en otros eucariotas los peroxisomas, son sólo visibles al microscopio electrónico.

*__Tripomastigota.__ Se encuentra exclusivamente en la sangre (en el plasma) y nunca se divide; por esta razón el número de formas sanguíneas es siempre reducido. Desde el punto de vista morfológico y fisiológico es similar a la forma tripomastigota de otras especies. Son pequeñas (15-20 µm) y termina posteriormente en punta; el kinetoplasto es subterminal y voluminoso. En las extensiones teñidas adquieren forma de C o de hoz. Presenta pleomorfismo con formas delgadas, rechonchas e intermedias.

*__Amastigota.__ Se encuentra en el citoplasma de determinadas células (en una vacuola parasitófora), principalmente macrófagos, células musculares, etc., donde se multiplica continuamente por fisión binaria. Es esferoidal u oval de pequeño diámetro (2-5 µm) y con un flagelo externo muy corto.

*__Epimastigota.__ Es la forma replicativa, no infectiva para el ser humano o mamífero, flagelados anchos, muy móviles, con el kinetoplasto entre el núcleo y el flagelo libre, con 20 a 25 µm de longitud. Este estadio morfológico se multiplica de manera profusa en el intestino de los triatominos para dar lugar a los tripomastigotas metacíclicos.

***Tripomastigota metacíclicas.** Es una forma no replicativa pero infectiva para el ser humano u otros mamíferos. Tiene forma alargada y mide unas 20 a 25 µm de longitud. Se distingue por presentar un núcleo vesiculoso y hacia la parte posterior de éste se halla el kinetoplasto de forma casi siempre esférica. El flagelo, con su membrana ondulante, se observa a lo largo del cuerpo del parásito y surge libremente en el extremo posterior.

III-1.2.2 Ciclo biológico

El parásito presenta un ciclo de vida heteroxeno, que requiere la presencia de dos hospedadores: vector (un hospedador invertebrado en el que se desarrollan varias fases de su ciclo biológico) y el hospedador definitivo. El vector se trata de hemípteros hematófagos de las especies *Triatoma infestans*, *Tri. dimidiata*, *Rhodnius prolixus* y *Panstrongylus megistus*, entre otros (Vargas, 2005**).** El parásito penetra en el hospedador definitivo (el hombre u otro mamífero doméstico o selvático) a través de las heces contaminadas con las formas tripomastigotas metacíclicas**,** comportamiento característico de los parásitos pertenecientes a la sección *Stercoraria*. En el nuevo hospedador, las formas metacíclicas pueden invadir inmediatamente las células en la puerta de entrada o ser diseminadas por la circulación linfática hasta otras localizaciones, transformándose en amastigotas. Las amastigotas se multiplican intracelularmente, y sufren una nueva transformación hasta tripomastigotas, que ocasionan la lisis de la célula, permitiendo su salida al torrente sanguíneo. Estas tripomastigotas pueden infectar otras células colindantes, pero carecen de capacidad multiplicativa, ya que la única forma replicativa en el vertebrado es la forma amastigota intracelular. Los triatominos nacen libres de infección, infectándose al alimentarse de hospedadores infectados. Las tripomastigotas migran al intestino medio del insecto, donde se transforman en epimastigotas. Allí se multiplican por fisión binaria un gran número de veces, dando lugar nuevamente a tripomastigotas metacíclicas, que migran al intestino posterior, desde donde son excretadas con las heces en el momento de la picadura, comenzando de nuevo el ciclo **(**Stevens y col., 1999; 2001**)**.

FASES EN EL TRIATOMINO **FASES EN EL HOMBRE**

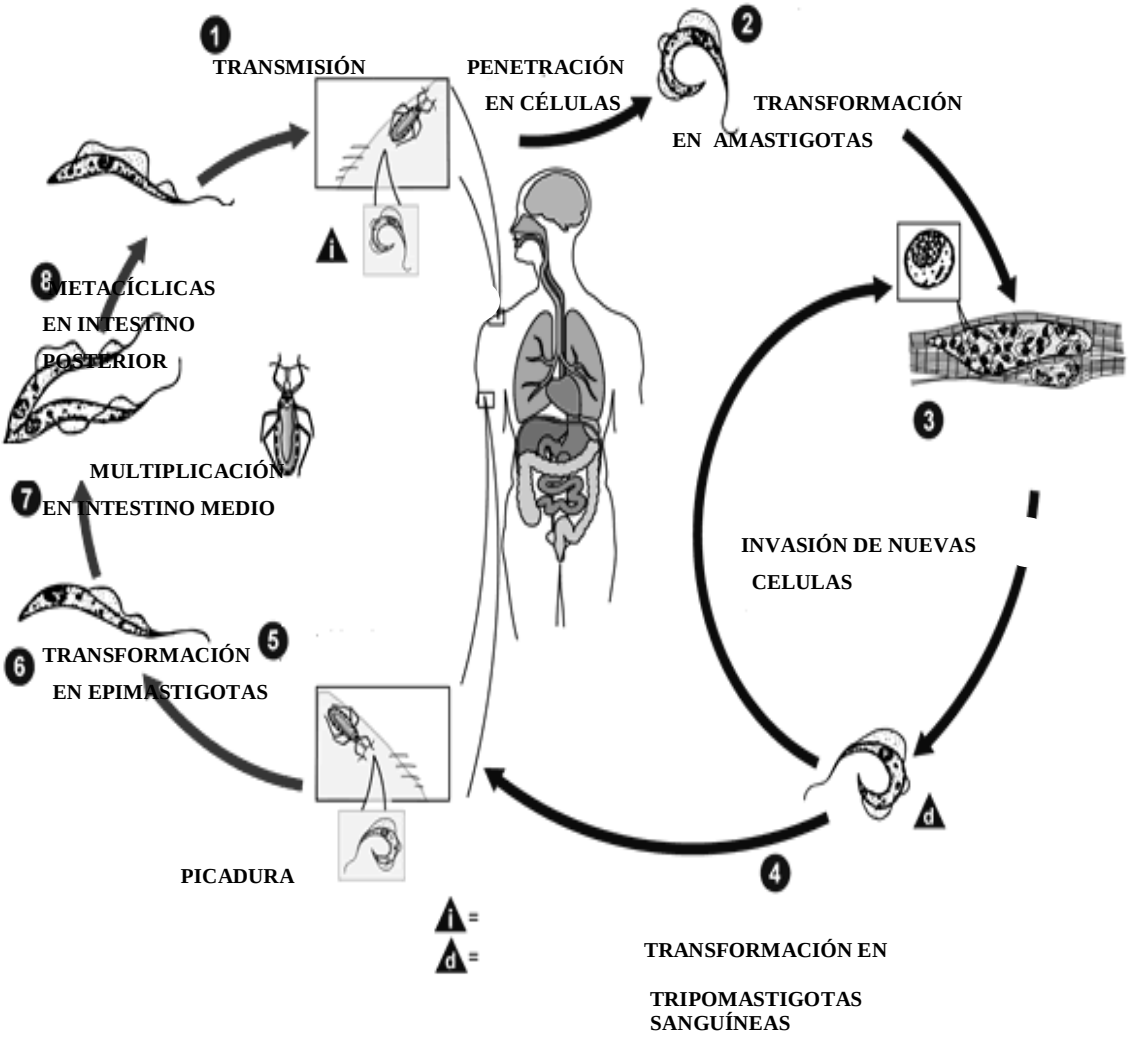

Fig.1: Ciclo biológico de *T. cruzi*. Fuente: **(**CDC, 2005**).**

I: estadio infectivo; d: estadio diagnóstico.

III-1.3 Manifestaciones clínicas y Diagnóstico

III-1.3.1 Manifestaciones clínicas

En la evolución de la enfermedad de Chagas se distinguen tres fases diferentes:

1. **Fase aguda**. Durante la cual se pueden presentar los siguientes síntomas: fiebre, malestar, linfadetis, hepatomegalia y esplenomegalia. Si el sitio primario de la infección es el ojo, se presenta edema unilateral de los párpados y conjuntivitis (Lopez-Antuñano, 1997; Reina-San-Martin y col., 2000; Becerril, 2008).

2. **Fase subclínica (indeterminada)** con baja parasitemia y sin sintomatología clínica. Se encuentran focos de miocarditis y disminución de neuronas del plexo parasimpático (Lopez-Antuñano, 1997; Reina-San-Martin y col., 2000; Becerril, 2008).

3. **Fase crónica**. Los síntomas crónicos en adultos sobre todo, son el resultado de arritmias y dilatación del corazón y colon. También se ha descrito el crecimiento anormal del hígado, intestino, esófago, útero y vejiga. (Lopez-Antuñano, 1997; Reina-San-Martin y col., 2000; Becerril, 2008).

III-1.3.2 Diagnóstico

En las etapas tempranas de desarrollo de la enfermedad suelen utilizarse métodos de diagnóstico directo, fundamentalmente la observación al microscopio óptico de un frotis de sangre periférica, el xenodiagnóstico (que requiere la disponibilidad de triatominos libres de infección cultivados en laboratorio) y el hemocultivo. El diagnóstico en la fase crónica es algo más complejo y para ello se suele recurrir a técnicas inmunológicas (que detectan la presencia de anticuerpos frente al parásito) como la IFI (Inmunofluorescencia Inderecta) o la ELISA (Enzyme linked inmunosorbent assay), o bioquímicas (que detectan ciertas moléculas del parásito como indicadores de la infección), además de otros métodos de uso más complejo y menos extendido, como el test de fijación de complemento, la hemaglutinación indirecta o la PCR. Los falsos positivos debidos a la existencia de reacciones cruzadas con otros protozoos, fundamentalmente con *Trypanosoma rangeli* y con especies pertenecientes a los géneros *Leishmania* spp. y

Plasmodium spp., representan las principales desventajas de estos métodos, especialmente cuando el paciente procede de una región geográfica en la que existe concomitancia de infecciones. Otros inconvenientes son su fiabilidad, sobre todo en casos en los que la parasitación es baja o la infección muy reciente, su coste y su lentitud **(**Punukollu y col., 2007**).**

III-1.4 Tratamiento

La enfermedad de Chagas sigue siendo el mayor problema de salud en varios de los países de Latinoamérica; en efecto, los pacientes con la enfermedad crónica severa empeoran progresivamente y con frecuencia mueren debido a un fallo cardiaco. Como hemos mencionado anteriormente, hasta hoy no existe ningún tratamiento completamente efectivo para esta enfermedad (Urbina, 1999; Charmina y col., 2007) por lo que permanece prácticamente incurable. A esto hay que unirle el poco interés de la industria farmacéutica para desarrollar nuevos fármacos antichagásicos. (Coura y De Castro, 2002). Casi todos los fármacos disponibles en la actualidad para el tratamiento de la enfermedad de Chagas, son efectivos durante la fase aguda pero casi no tienen efecto durante la fase crónica. Además, la existencia de cepas de *T. cruzi* resistentes a estos fármacos explica el bajo índice de curación en los pacientes chagásicos. Por lo tanto, la búsqueda de nuevos fármacos contra la enfermedad de Chagas está plenamente justificada.

Las drogas actualmente usadas para el tratamiento etiológico de la enfermedad de Chagas son el nitrofurano nifurtimox (Lampit®, Bayer) y el nitroimidazol benznidazol (Rochagan®, Radanil®, Roche), cuya actividad anti-*T. cruzi* fue descubierta empíricamente hace más de tres décadas.

El Nifurtimox (1972-1992) es un derivado de la Nitrofurfurilidina (3-metil-4-5´-nitro-furfurilidenamino-tetrahidrol[1,4]-tiazino-1,1 dióxido), es eficaz en los casos agudos y crónicos tempranos, Su mecanismo de acción se basa en la inhibición del desarrollo intracelular del parásito actuando sobre la vía de la reducción del grupo nitro de la molécula a radicales nitroaniónicos, que a su vez reaccionan con el oxigeno molecular para generar metabolitos reducidos del mismo, altamente tóxicos (radicales

superoxido, peroxido e hidroxilo). Estudios por Docampo y colaboradores han demostrado que el *T. cruzi* es deficiente en algunos de los mecanismos de detoxificación de metabolitos de oxigeno, particularmente del peroxido de hidrogeno, y es por ende mas susceptible al stress oxidativo que las células de vertebrados (Docampo y col., 1990). Este medicamento puede ocasionar graves efectos segundarios, entre los que se encuentran alteraciones del sistema nervioso central y del digestivo. Estos efectos secundarios se pueden presentar hasta en el 70% de los casos, cuando el tratamiento se administra por períodos prolongados.

El benznidazol (N-bencil-2-nitro-1-imidazolacetamida) disponible desde 1975, se ha prescrito con cierto éxito para el tratamiento de la enfermedad de Chagas (Lopez-Antuñano, 1997 y Marco, 2008), su mecanismo de acción parece actuar por una vía diferente, resultante de la reacción de sus derivados nitroreducidos con macromoléculas como ADN, ARN, proteínas y lípidos insaturados (Docampo, 1990; Maya y col., 2007).

Estos resultados indican que la actividad antiparasítica de estos dos compuestos esta indisolublemente asociada a su toxicidad hacia el hospedero vertebrado. Ambas drogas son activas en la fase aguda (hasta un 80% de éxitos terapéuticos, definidos como cura parasitológica radical indicada por negativización de todas las pruebas parasitológicas y serológicas) (Cançado, 1999), así como en la fase crónica temprana de la enfermedad (típicamente niños y adolescentes, hasta 60% de curas) (de Andrade y col., 1996; de Andrade y col., 1998; Sosa Estani y Segura, 1999; Sosa Estani y col., 1998; Silveira y col., 2000; Solari y col., 2001).

Mas aun, la eficacia antiparasitaria de los compuestos varia según la región geográfica, probablemente como resultado de la diferente susceptibilidad intrínseca a las drogas de las cepas del *T. cruzi* que circulan en diferentes zonas endémicas. (Andrade y col., 1992; Cançado, 1999; Brunton y col., 2006). Asimismo, estos compuestos presentan frecuentemente efectos colaterales deletéreos, que incluyen anorexia, vómitos, polineuropatía periférica y dermopatía alérgica, que pueden conllevar a la interrupción del tratamiento (Cançado, 1999). Sin embargo, la mayor limitación de los tratamientos actualmente disponibles en su baja eficacia ($\geq$ 80% de fracasos terapéuticos) en la fase crónica establecida de la enfermedad, que es la presentación clínica mas frecuente en Latino América (Cançado, 1999). Las razones de la marcada diferencia en la eficacia de

estas drogas en las dos fases de la enfermedad no están claras aun, pero posiblemente resulten de la inadecuada farmacocinética de estos compuestos frente a la ubicación de los parásitos en tejidos profundos en la fase crónica de la infección (Urbina, 1999a; Urbina, 2002; Maya y col., 2006).

Algunos estudios han demostrado que el tratamiento con benznidazol en pacientes crónicos, aunque incapaz de inducir cura parasitológica en la mayoría de los pacientes, conllevó a una marcada reducción en la ocurrencia de cambios electrocardiográficos y a una menor frecuencia de deterioro del cuadro clínico de los pacientes (Bahia-Oliveira y col., 2000; Viotti y col., 1994).

Sin embargo, aunque existe un amplio consenso en el sentido de que a todos los pacientes seropositivos deben recibir tratamiento específico para eliminar o reducir su carga parasitaria, muchos médicos mantienen serias reservas con relación al uso de nifurtimox o benznidazol en pacientes crónicos, debido a la desfavorable relación riesgo /beneficio de esas drogas (Urbina y col., 2006)

Durante las décadas pasadas se han probado centenares de productos experimentales con diferentes estructuras químicas para el tratamiento de las infecciones producidas por *T. cruzi*, pero sólo algunos de ellos han completado la fase preclínica con éxito relativo pero serios efectos adversos como: polineuritis y anemia hemolítica, relacionados con deficiencia de glucosa-6-fosfato deshidrogenasa y depresión de la médula ósea, evitaron su uso.

Entre otros compuestos que han mostrado actividad anti-chagasica y a modo de ejemplo mencionaremos:

Actinomicina D

Formas metacíclicas viables de la cepa Y de *T. cruzi* tratadas con Actinomicina D mostraron las siguientes modificaciones: a) inhibición de la replicación en medio de cultivo, en sangre y en tejidos de ratón normal; b) incapacidad para penetrar en células Vero e imposibilidad de replicarse dentro de macrófagos normales; c) retención de su inmunogenicidad y de la habilidad de proteger al ratón contra una infección virulenta, y

d) tampoco indujeron lesiones histológicas como las que se describen en la enfermedad de Chagas experimental.(Da-Cruz y col., 1991). La Actiomicina D es una sustancia que inhibe la RNA polimerasa, con el consiguiente bloqueo de la síntesis de DNA y de proteínas. Provocando hipofunción, disfunción y muerte. Se ha evitado el uso de esa droga porque provoca una citotoxicidad generalizada (a nivel de la medula ósea, tubo digestivo, anejos cutáneos, aparato digestivo, aparato reproductor, neoplasias secundarias, etc.)(www.apuntesdeanatomia.org/ap/lesio.htm. acceso el 20 de Octubre del 2009).

Azoles y derivados

Los azoles constituyen un conjunto de fármacos caracterizados por poseer un heterociclo nitrogenado (imidazol; triazol, etc) en su estructura. Son fungistáticos, frecuentemente utilizados para el tratamiento de candidiasis y otras micosis. En 1964 Abitbol y col., obtuvieron resultados positivos con la Anfotericina B en pacientes chagásicos de los cuales 4 negativizaron el xenodiagnóstico. Sin embargo, el tratamiento de la enfermedad de Chagas con Anfotericina B no se repitió, posiblemente por la toxicidad de este micostático. En 1981 Docampo y col.**,** comprobaron que el Miconazole y el Econazole inhiben el crecimiento del *T. cruzi* in vitro y modifican su contenido en esteroles, en particular del ergosterol, un esterol vegetal cuya presencia en el *T. cruzi* es notable. La mayor parte de estos estudios se realizaron in vitro e in vivo, utilizando el modelo murino de la enfermedad de Chagas. La acción antiparasitaria se manifestó en todos los ensayos (McCabe y col., 1984; McCabe y col., 1987; Beach y col., 1986; Urbina y col., 1988; Lazardi y col., 1990 y Maldonado y col., 1993).

En resumen, si bien algunos azoles, son parcialmente eficaces como antichagásicos, el éxito de la quimioterapia de la enfermedad de Chagas con los azoles está supeditado a los resultados que se obtengan con los nuevos bis-triazoles. (Stoppani, 1999). Los efectos secundarios de los azoles, en general, que han sido descritos en humanos son hepatotoxicos, disfunción endocrina, reducción de los niveles de cortisol e insuficiencia adrenal (Singh y Sivakumar, 2004).

Agentes quelantes

Algunos agentes quelantes y sus derivados son tan efectivos o superiores al Benznidazol, compuesto usado para suprimir la reproducción de epimastigotas de *T. cruzi* (Rodríguez y col., 1995). Los compuestos más efectivos incluyeron: N,N,N',N'-tetracis(2-piridilmetil) etilenediamina; sodio dietilamina -N-carboditioato; piperidina-N-carboditioato y varios de sus análogos; un número de otros carboditioatos con dos grupos no polares en el nitrógeno, y disulfito de tetraetiltiuram, una prodroga de sodio dietilamina-N-carboditioato y ampliamente usada en el tratamiento del alcoholismo. La introducción de grupos agregados polares iónicos o no iónicos en la molécula quelatora generalmente resulta en la pérdida de la actividad tripanocida. Los agentes quelantes comercialmente disponibles que no han exhibido actividad son la D-penicilamina, el ácido meso -2,3-dimercaptosuccínico y el tetrahidroclorhidrato de trietilentetramina. Los datos de dosis-respuesta *in vitro* indican que algunos de estos compuestos inhiben los epimastigotas de *T. cruzi* a concentraciones tan bajas como 0.625 mg/ml. Este hecho podría tener gran importancia al impedirse la formación de tripomastigotas a expensas de los epimastigotas. Se propone que el mecanismo de acción de estos compuestos tiene base en su habilidad de interferir con el metabolismo de los metales esenciales en sitios intracelulares del epimastigote (hierro, cobre o zinc). El desarrollo de quelantes específicamente diseñados para romper selectivamente el metabolismo de metales esenciales de *T. cruzi* debe producir una generación nueva de drogas para el tratamiento de la enfermedad de Chagas. (Docampo y Vercesi, 1989).

Allopurinol y pirazol pirimidinas

El *T. cruzi* es un organismo incapaz de sintetizar purinas (adenina; guanina), pero es capaz de sintetizar compuestos pirimidinicos (timina; citosina y ciracilo). La importación de bases púricas por el *T. cruzi* depende de un transporte específico en el que participan las quinasas. Por lo tanto, para sintetizar los nucleótidos que constituyen el ADN y el ARN, el *T. cruzi* debe utilizar purinas pre-formadas por el organismo huésped (Hammond y col., 1984), de manera que, la síntesis de nucleosidos y nucleótidos por el parásito es un buen blanco terapéutico (Christopherson y Lyons, 1990).

El *T. cruzi* utiliza la adenina, y la hipoxantina para sintetizar acido adenilico (AMP) mientras que la guanina y la xantina son utilizadas para sintetizar acido guanilico (GMP). La conversión de la xantina y la hipoxantina en las bases aminadas depende de enzimas específicas que utilizan al adenil succinato como dador del grupo anión (Spector y col., 1981**)**. Esas enzimas podrían también constituir blancos terapéuticos.

Por otra parte, la síntesis de los nucleótidos púricos es catalizada por la hipoxantina-fosforibosil transferasa y enzimas similares que, pueden utilizar también sustratos no-fisiológicos, por ejemplo, el Allopurinol (4-hidroxi-pirazol-(3,4d)-pirimidina) y otras pirazol pirimidinas. Como consecuencia de esas reacciones se forman nucleosidos y nucleotidos no-fisiológicos, que son tóxicos para el parásito pues inhiben la síntesis de los nucleótidos fisiológicos y por consiguiente, la del ADN y el ARN. Estas inhibiciones tienen consecuencias farmacológicas, que se expresan como inhibición de la multiplicación in vitro, la reducción de la parasitemia y la prolongación de la sobrevivencia de los ratones infectados según el modelo murino. La administración oral de los nucleótidos del Allopurinol o de los análogos de las pirimidinas también disminuye la parasitemia de ratones infectados pero no produce cura parasitológica. La acción tripanocida varía según la estructura de la base nitrogenada. La síntesis de nucleosidos o nucleótidos artificiales con diferentes pirimidinas, entre ellos 3'-azido-3'-deoxitimidina, (conocido como Zidovudina) produce nucleótidos con acción terapéutica antichagásica. El ensayo de 59 análogos de la purina como inhibidores de la hipoxantina-ribosil transferasa permitió sintetizar 8 moléculas con gran afinidad por la transferasa **(**Eakin y col., 1997**)**, estructuras caracterizadas por los grupos sustituyentes en las posiciones 6 y 8 de la purina. El Allopurinol y sus derivados han sido ensayados para el tratamiento de las diferentes formas clínicas de la enfermedad de Chagas **(**Nakajima-Shimada y col., 1996). Para obtener efectos notables se requieren dosis relativamente altas de Allopurinol lo que puede producir intolerancia. El tratamiento reduce la parasitemia en forma que puede ser total y también negativiza el xenodiagnósticos **(**Gallerano y col., 1991**)**. Sin embargo, esos efectos suelen ser transitorios y al cabo de cierto tiempo, la sintomatología reaparece.

Derivados nitroimidazoles

Petray, Morilla, Corral y Romero en 2004 evaluaron la actividad tripanocida in vitro de un 2-nitroimidazol; el etanidazol (EZL, SR-2508) sobre tripomastigotes de la cepa RA en células Vero y macrófagos murinos J774 (infectados en una proporción de 10:1) y la compararon con la actividad de Benznidazol.

El tratamiento con EZL (concentraciones de 3 a 243 µM) provocó una reducción dosis-dependiente en el número de tripomastigotes RA y fue más activo contra amastigotes (la forma intracelular). No se reportaron cambios en la viabilidad de células Vero y de macrófagos J774 .Etanidazol fue menos potente para eliminar al parásito que el fármaco utilizado como referencia; Benznidazol, pero considerando los efectos secundarios, de estudios anteriores se ha comprobado que etanidazol es menos neurotóxico in vivo, en cambio está bien documentado la diversidad de efectos secundarios que produce la administración de Benznidazol.

Dialquilaminas

Aran y cols. en 2005 sintetizaron una serie de nuevos 3-alcoxi y 3-hidroxi-[ω-(dialquilamino) alquil]-5-nitroindazoles y determinaron sus actividades antichagásicas in vitro contra la forma de amastigote de cadena Brener. Algunos compuestos tuvieron un porcentaje de inhibición del crecimiento de amastigotes entre 80 y 100 a 25µM de concentración y esta actividad citotóxica fue específica, ya que a esta misma concentración la citotoxicidad contra macrófagos fue del 0 al 10%.

Derivados de Indazol

Gerpe y cols., en 2006 sintetizaron una serie de derivados N-oxido de indazol y determinaron sus actividades antichagásicas in vitro contra epimastigotes y tripomastigotes de las cepas Brener y Tulahuen a diferentes concentraciones. De los resultados obtenidos resalta el compuesto 3-Ciano-2-(4-iodofenil)-2H-indazol Nóxido, el cual exhibió alta actividad antichagásica sobre las dos cepas y los dos estadíos de tripanosoma utilizados y el hecho de que la presencia del grupo N-oxido es importante

para la actividad antichagásica. Esta investigación reporta por primera vez la actividad antichagásica de 5- nitroindazoles e indazoles N-óxido.

Estos grupos de fármacos con utilidad variada ofrecen otra alternativa de estudio para la quimioterapia antichagásica, en los que resulta necesario enfocarse también en los mecanismos de acción para su actividad tripanocida. Basándose en los resultados obtenidos, en algunos casos se pueden continuar los estudios en modelos animales, o basarse en la estructura de los fármacos para el diseño de compuestos con mayor efectividad antiparasitaria y menor citotoxicidad para el organismo huésped.

Actividad tripanocida de productos naturales

La naturaleza despliega ante nosotros un poderoso arsenal químico, el cual permanece mayormente inadvertido, pero que se manifiesta en las interacciones entre diferentes especies o entre individuos de una misma especie. Los organismos (plantas, insectos) necesitan defenderse de microorganismos y de otros organismos predadores o competidores, y en muchos casos recurren a compuestos químicos específicamente diseñados para tales fines: los “metabolitos secundarios” o “productos naturales”, como más comúnmente se los conoce. Desde la antigüedad, el hombre ha venido utilizando los productos naturales principalmente de plantas terrestres, con fines terapéuticos (medicina tradicional) o de defensa (venenos y toxinas). Este conocimiento derivó en el estudio sistemático de los productos naturales de plantas. A pesar de los grandes avances en el área de la química combinatoria, la mayor parte de las drogas medicinales actualmente en uso siguen siendo productos naturales o análogos basados en ellos. Esto es debido a la enorme diversidad estructural de los productos naturales y a que son naturalmente biosintetizados en forma esteroespecífica, lo cual les confiere propiedades estructurales únicas. Esta diversidad estructural de sustancias es uno de los requerimientos principales para lo que se conoce como “drug discovery”. La importancia de los productos naturales como compuestos “cabeza de serie” ha sido cabalmente comprendida por los laboratorios medicinales, lo cual ha promovido un renovado interés por la preservación de los recursos naturales especialmente en países en vías de desarrollo donde estas enfermedades son endémicas. (Garcia-Barriga, 1986, Rutter, 1990 y Kayser, 2003).

Entre las plantas con actividad tripanocida podemos desatacar las familias *Fabaceae*, *Passifloraceae* y *Ancistrocladeae*. La mayoría de los compuestos activos aislados de estas familias son alcaloides quinoleínicos. Es importante destacar *Pasiflora incarnata* (*Passifloraceae*) con un IC50 = 0.25 µg/ml contra las formas epimastigotes del parásito y *Ancistrocladus tanzaniensi* con un IC50 entre 1.5 a 1.8 µg/ml.

En algunas publicaciones se ha mostrado la diversidad de productos naturales con actividad antiprotozoaria, los cuales incluyen moléculas activas contra los agentes etiológicos de la malaria, leishmaniosis, la enfermedad de Chagas y tripanosomiasis africana. Los alcaloides, según las nuevas estrategias experimentales, se agrupan de acuerdo a su estructura en 4 categorías: 1) quinolinas, isoquinolinas y derivados, 2) análogos indólicos y derivados, 3) alcaloides esteroidales y 4) otros alcaloides. (Osorio y col., 2006).

III-1.5 POSIBLES BLANCOS TERAPEUTICOS

En los últimos años, la tendencia respecto al desarrollo de nuevos fárrnacos está cambiando de un "screening" aleatorio hacia un diseño más racional o dirigido hacia enzimas o moléculas "dianas". Las investigaciones realizadas en las dos últimas décadas en bioquímica y biología molecular en tripanosomátidos, han revelado algunos "blancos" terapéuticos potenciales, sustancias que son esenciales para los parásitos y están ausentes en el huésped vertebrado (Vargas y col., 1999). Entre otros: el metabolismo de glucosa, homeostasis del calcio, Superoxido dismutasa, tripanotiona reductasa, el metabolismo de esteroles y el de aminoácidos, cistein proteinasa, la captura de Purinas y dihidrofolato reductasa etc...

Inhibición del metabolismo de glucosa

La glucólisis comienza ha ser considerada como un potencial "blanco" terapéutico, para el diseño de nuevos fármacos antitripanocidas (Vargas, 1999). La degradación de glucosa a piruvato es el único suplemento de ATP de los tripanosomátidos que viven en la sangre de mamíferos. Por lo que, la inhibición de la glucolísis conlleva la muerte rápida de estos parásitos (Mesa-Valle y col., 1996). Además, la gran separación evolutiva entre estos tripanosomas y humanos, y la inusual organización de la ruta

glucolítica (compartimentación en glicosomas) en los parásitos (Castilla y col.,1994), han dotado a las enzimas glicosomales de estos parásitos, de propiedades distintivas que pueden ser explotadas, sobre la base de su estructura o mecanismo catalítico, para el diseño de inhibidores selectivos (Castilla y col., 1996 y Fernández-Ramos y col., 2000) A pesar de la poca información sobre el metabolismo de carbohidratos en *T. cruzi*, se ha demostrado la presencia de enzimas glicolíticas, entre las cuales se encuentra la gliceraldehido 3-fosfato deshidrogenasa (GAPDH) y también se han establecido las diferencias estructurales de la contraparte en mamíferos. En diversos estudios se han mostrado resultados satisfactorios de compuestos que inhiben GAPDH de *T. cruzi* de manera selectiva in vitro, pero faltan estudios para evaluar los efectos de esta inhibición específica sobre el parásito y si se comprueba que dicha inhibición conlleva a la muerte del mismo, continuar con estudios de toxicidad en células huésped e in vivo (De Marchi y cols., 2004; Moyersoen y cols., 2004).

La homoeostasis del calcio

Entre otros "blancos" terapéuticos potenciales se encuentra la regulación de la homeostasis del calcio en los parásitos: el mecanismo de transporte del calcio en las mitocondrias; el papel del calcio en la regulación del metabolismo en las mitocondrias, la regulación de la concentración del calcio citosólico; el papel del calcio en la invasión por parásitos intracelulares de las células huésped y en el mecanismo de acción de las drogas tripanocidas, así como el estudio de las bombas de calcio presentes en la membrana plasmática y en los organelos intracelulares (Benaim y Col 1991; Vercesi y Docampo, 1992).

La Superoxido dismutasa

Se sabe que la presencia de un parásito en su hospedador desencadena una serie de mecanismos dirigidos a su eliminación, entre los que están, la producción de radicales libres capaces de alterar la integridad de las membranas, proteínas y ácidos nucleicos del parásito. A su vez, estos organismos han desarrollado mecanismos detoxificantes de alta eficacia a fin de adaptarse a su hospedador (Luque y col., 2000). La enzima Superoxido dismutasa (SOD) es considerada como la primera línea de defensa de los parásitos frente a las especies activas del oxígeno, por su capacidad de degradar al ión superóxido. Así,

los organismos que carecen de ella muestran una menor proporción de crecimiento, un promedio de vida menor, hipersensibilidad frente a los compuestos producidos en el ciclo oxido-reductor e incremento de la mutagénesis espontánea y del índice de mortalidad (Salas y col., 2001).

Inhibidores del metabolismo de la tripanotiona

Los tripanosomátides poseen un sistema redox centrado en el tripanotiona (N1,N8-bis(glutationil)-espermidina); un tiol de bajo peso molecular que consiste en dos moléculas de glutatión unidos por espermidina. En estos organismos el tripanotión es mantenido como dihidrotripanotión por la actividad de la enzima tripanotión reductasa (TR). Debido a la función esencial del tripanotión en *T. cruzi* y a las diferencias bioquímicas existentes con su contraparte mamífera (glutatión) y con las enzimas involucradas en su metabolismo, este se considera como uno de los blancos farmacológicos para el diseño de fármacos anti-T. cruzi. Tripanotión reductasa (TR) es la enzima más estudiada y conocida del metabolismo tripanotión, ha sido identificada y aislada en todos los miembros tripanosomátides y se considera como una enzima esencial de estos organismos. La determinación de la estructura del centro activo de TR ha permitido la búsqueda y diseño de inhibidores de esta enzima como una alternativa de nuevos tratamientos antichagásicos.

Un primer grupo de inhibidores reportados fue llamado “sustratos subversivos” o inhibidores turncoat como nitrofuranos y naftoquinonas (Augustyns y cols, 2001; Rodrigues y de Castro, 2002; Schmidt y Krauth-Siegel, 2002). Estos compuestos que convierten una enzima anti-oxidativa en una prooxidativa; son reducidos por TR a su respectivo radical y este reacciona con oxígeno para producir radicales aniónicos superóxido. Concomitantemente, se inhibe la reducción de tripanotión disulfuro (Augustyns y col, 2001; Schmidt y Krauth-Siegel, 2002). La capacidad de TR de catalizar la reducción de compuestos que de manera espontánea se reoxidan produciendo superóxido, constituye una subversión del papel fisiológico normal de la enzima (Henderson y col., 1988).

Los alcaloides alcaloides bisbenzilisoquinolinas (BBIQ) mostraron actividad in vivo en la fase aguda de la enfermedad, e incluso con algunos se obtuvo la cura

parasitológica. De este mismo grupo de compuestos, ceferantina y dafnolina tuvieron una actividad anti-T. cruzi igual e incluso mayor que la de Benz pero con menos efectos colaterales. Se pueden considerar estos resultados como alentadores, y pueden servir de apoyo para dilucidar completamente el mecanismo de acción sobre el parásito.

Compuestos como los sustratos subversivos, neuroepilépticos tricíclicos y lignanos pueden servir como base para la síntesis y desarrollo de compuestos a partir de sus estructuras, además también resulta importante investigar el mecanismo de acción de dichos compuestos.

A partir de los resultados mostrados de butionin sulfoximina (BSO) y las combinaciones con los fármacos actuales, resulta un candidato probable para estudios in vitro e in vivo de combinaciones con fármacos que también inhiban el metabolismo del tripanotion a diferente nivel o incluso con fármacos que afecten otro blanco del parásito.

El metabolismo de esteroles

El metabolismo de esteroles de *T. cruzi* también ofrece diferencias con la contraparte mamífera y esto es importante debido a la necesidad de ergosterol para la viabilidad y proliferación celular en todos los estadíos del ciclo de vida del parásito (Barret y col., 2003; Rodrigues y de Castro, 2002; Urbina, 2002; Urbina y Docampo, 2003). Los inhibidores de la biosíntesis de ergosterol (EBIs) revisados actúan en diferentes niveles de la biosíntesis de ergosterol; en la C-14 desmetilación dependiente de citocromo P-450 de lanosterol (azoles: imidazol; derivados triazoles: ketoconazol, fluconazol, itraconazol, albaconazol, posaconazol, TAK-187, D0870; derivados de FTI-2220) y sobre la escualeno sintetasa o escualeno epoxidasa (alilaminas: naftinina y terbinafina; derivados quinuclidinas: 3-(Bifenil-4-il)-3-hidroxiquinuclidina, E5700 y ER-119884; derivados relacionados a fenoxicarb: WC-9), los cuales han mostrado actividad anti-T. cruzi potente in vitro e in vivo, además de sinergismo en la combinación de terbinafina y derivados triazoles, y también a nivel de la oxidoescualeno ciclasa (inhibidores OSC bajo investigación como antifúngicos y controladores de colesterol), con resultados in vitro prometedores; a nivel de la 24-SMT (azasteroles) con resultados de actividad anti-T. cruzi in vitro e in vivo; a nivel de la PFT (farnesil- O-NH-PA ester, farnesil-NH-PMM, rCrVFM, Tipifarnib; Bisfosfonatos:

pamidronato, alendronato, risedronato, 1-hidroxialquil-1, 1-bisfosfonatos, 1-amino-1,1-bisfosfonatos y derivados de ariloxietil tiocianato) con resultados in vitro y en el caso de risedronato también resultados in vivo satisfactorios. A pesar de que algunos de los compuestos mencionados mostraron menor efectividad que los fármacos empleados como referencia, tienen a su favor que los efectos secundarios también son menores, además que ya existen fármacos en el mercado o en desarrollo como antimicóticos, antifúngicos o anticancerígenos, con lo que se disminuyen costos y tiempos de investigación y producción.

Inhibidores de cisteína proteasas (cruzipaina, cruzaina)

El *T. cruzi* se caracteriza por poseer una cisteína-proteasa, la cruzipaina (GP59/51) que constituye una fracción importante de las proteínas celulares**.** La enzima se asemeja a la papaina y tiene 35-45% de homología con una catepsina I. La cruzipaína se encuentra en todas las formas del parásito, tiene función metabólica esencial y es necesaria para la transformación de amastigotas en tripomastigotas. Además intervendría en la penetración de los tripomastigotas en las células del huésped. La estructura de la cruzipaina y de su gene la califican como blanco para posibles quimioterápicos especialmente, por la reactividad del tiol de la cisteína, susceptible a inhibidores específicos (diazometano y fluorometil acetona) (Meirelles y col., 1992; Harth y col 1993; Mc Kerrow y col., 1995). Cuando la estructura peptídica del inhibidor satisface la especificidad molecular del centro activo de la cruzipaina, se forma un compuesto covalente cruzipaína-inhibidor, cataliticamente inactivo (Khabnadieh y col 2005). Estudios con varios péptidos sintéticos han demostrado que los inhibidores más potentes son aquellos que poseen grupos hidrofóbicos voluminosos en la posición P1 (primer residuo vecino al diazometano) y un residuo aromático próximo. Los parásitos afectados por estos inhibidores muestran la membrana plasmática rota, mitocondrias hinchadas y cromatina desorganizada. Se han observado efectos citotóxicos de los péptidos inhibidores de la cruzipaina en amastigotas cultivados y en el modelo murino (Engel y col., 1998; Engel y col., 1998). En resumen, la obtención de péptidos inhibidores de la cisteína-proteasa pueden constituir un avance significativo para la quimioterapia de la enfermedad de Chagas por la capacidad de esos péptidos (o seudopéptidos) para curar el Chagas crónico y por su inocuidad para el organismo huésped. Esos péptidos podrían

constituir el fármaco de elección para el tratamiento de todas las formas de la enfermedad.

Entre algunas de las limitaciones de este blanco se pueden mencionar la resistencia de algunas cepas de *T. cruzi*, pero a pesar de esto, en el 2002, la corporación farmacéutica Celera Genomic (http://www.celera.com/celera/pr _1056752136) anunció que el Instituto Un Mundo Saludable (IOWH) y el Institutos Nacional de Salud de EUA (NIH) habían iniciado el desarrollo del compuesto CRA-3316, inhibidor de cruzipaín específico, como un nuevo tratamiento potencial para la enfermedad de Chagas. Este fármaco se reporta como el único en desarrollo en la fase I/II. Este compuesto, también conocido como APC-3316 o K-777, es de los compuestos investigados por Engel y col. en diversos de sus artículos; el N-metil-piperazina-urea-F-hF-vinilsulfona-fenil (Mu-F-hF-VSΦ) (Engely col; 2000).

III-2. Género *Leishmania*

III-2.1. Generalidades

Los protozoos parásitos del género *Leishmania* son los agentes patógenos responsables de las Leishmaniasis. Estas parasitosis constituyen un grupo heterogéneo de enfermedades transmitidas por insectos de la subfamilia *Phlebotominae* (flebotominos) que afectan a humanos y a otros mamíferos, con afectación visceral y tegumentaria.

Se conocen unas 30 especies de *Leishmania* de las que unas 20 pueden causar enfermedad en humanos. Las Leishmaniasis humanas se presentan bajo distintas formas clinicas:

a) Leishmaniasis cutánea en la que los parásitos quedan aislados en los macrófagos de la zona de la picadura, debido a la instauración de una respuesta inmune de tipo celular.
b) Leishmaniasis mucocutánea, por diseminación de los parásitos hacia las mucosas.

c) Leishmaniasis visceral, en la que los parásitos se diseminan a órganos internos por vía linfática o sanguínea, ya que se produce un fallo de la respuesta de tipo celular.

Un gran número de animales, tanto domésticos como silvestres, actúan como hospedadores principales y reservorios de las especies de *Leishmania* que afectan a humanos (Ashford, 2000). Entre ellas destaca el reservorio canino cuya sintomatología es frecuentemente a la vez cutánea y visceral (Dereure, 1999).

Las Leishmaniasis se distribuyen ampliamente en todos los continentes excepto en la Antártida (mapas de distribución disponibles en el sitio web de la OMS: http://www.who.int/leishmaniasis/leishmaniasis_maps/en/index.html). Por ello la OMS considera estas afecciones cosmopolitas o endémicas y como una de las siete enfermedades prioritarias (OMS.1990) (OMS, 1996).

La enfermedad se caracterizaba por malestar general, accesos febriles, anemia grave, atrofia muscular y esplenomegalia. El tratamiento de la leishmaniasis es complicado y la enfermedad presenta una morbilidad sustancial por lo que a menudo se requieren terapias expeditivas. En la leishmaniasis se vienen utilizando derivados pentavalentes del antimonio que tienen un papel fundamental en la terapia mundial desde hace más de 70 años.

III-2.2. Morfología y ciclo biológico

III-2.2.1. Morfología

La morfologia de *Leishmania* está determinada por un ciclo de vida complejo ya que los parásitos están expuestos a diferentes ambientes (extra e intracelulares). Estos parásitos son digenéticos o heteroxenos, muestran dos estadíos básicos en su ciclo: uno extracelular con la morfología promastigota, que se desarrolla en el hospedador invertebrado (Phlebotomino) y, el estadio intracelular con la forma amastigota que aparece en el hospedador vertebrado (Trager, 1953).

La forma promastigota constituye la forma extracelular que se desarrolla en el aparato digestivo del flebotomo (vector de la enfermedad) y constituye la forma infectiva para el hospedador vertebrado. Tiene forma alargada, de 15 a 20 µm de longitud y presenta un flagelo libre en su parte anterior que puede llegar a medir el doble de la longitud del cuerpo del parásito. Al microscopio electrónico muestra un núcleo central, ribosomas, retículo endoplasmático, aparato de Golgi, una única mitocondria y el kinetoplasto que aparece como un cuerpo electrodenso en la zona anterior (Lofgren, 1950; Crowther y col., 1954).

La forma amastigota se observa en los tejidos parasitados de los hospedadores vertebrados, generalmente en el interior de las células del sistema mononuclear fagocitario (SMF). En el interior de estas células se pueden encontrar las amastigotas englobadas en un vacuola (vacuola parasitófora) que se forma tras la fusión del lisosoma y el fagosoma una vez que el parásito ha sido internalizado (Chang y Dwyer, 1978). Las amastigotas son formas ovoides con un tamaño comprendido entre 2 y 5µm de diámetro (Crowther y col., 1954). Estas formas presentan un núcleo central y un kinetoplasto alargado, pudiéndose apreciar la existencia de bolsillo flagelar y un flagelo vestigial (Chang y Dwyer, 1978; Magill, 1999).

Durante el desarrollo del parásito en el flebotomo vector, *Leishmania* adopta varias morfologías intermedias entre la forma flagelada y la aflagelada. Las formas descritas y enumeradas por orden de aparición a lo largo de esta transformación son las promastigotas procíclicas (aparecen entre las 24 y 48 tras la ingesta), nectomonas (48 - 72 horas), leptomonas (4 - 7 días), haptomonas (5 - 7 días) y metacíclicas (7 -14 días), estas últimas son las responsables de la transmisión de la infección al vertebrado (Chang y col., 1990; Kamhawi, 2006). Además de diferencias morfológicas entre promastigotas y amastigotas, existen variaciones antigénicas y metabólicas; entre ellas la expresión de proteínas de membrana (Sadick y Raff, 1985), metabolismo de la glucosa, choque respiratorio y receptores de membrana como el de manosa/fructosa (Channon y col., 1984).

III-2.2.2.Ciclo biológico

Cuando el flebotomo ingiere sangre con macrófagos infectados con amastigotas de un individuo parasitado comienza el desarrollo del parásito en el interior de su aparato digestivo. Durante un periodo inicial de 6 a 9 días (dependiendo de la especie) *Leishmania* se desarrolla diferenciándose en los distintos estadíos descritos anteriormente hasta convertirse en promastigotas metacíclicas, en un proceso conocido como metaciclogénesis (Kamhawi, 2006). La sangre de la que se alimenta el vector permanece contenida inicialmente en la región abdominal del aparato digestivo gracias a la membrana peritrófica, al tercer día de desarrollo, los parásitos migran hacia la zona anterior del intestino medio. Una vez en el intestino, las promastigotas avanzan hacia la zona bucal pasando por los distintos estadios del desarrollo. (Cunningham, 2002; Kamhawi, 2006).

La transmisión de la infección se produce cuando el flebotomo infectado se alimenta sobre un nuevo hospedador vertebrado y le inocula promastigotas metacíclicas durante la ingesta.

Una vez en el interior del hospedador vertebrado las promastigotas metacíclicas son fagocitadas por los macrófagos e incluidas en una vacuola parasitófora en la que las promastigotas se transforman en amastigotas. Éstos se multiplican por fisión binaria en el interior del macrófago hasta que es lisado, liberándose al exterior de la célula e infectando nuevas células (Handman, 1999; Cunningham, 2002; Handman y Bullen, 2002).

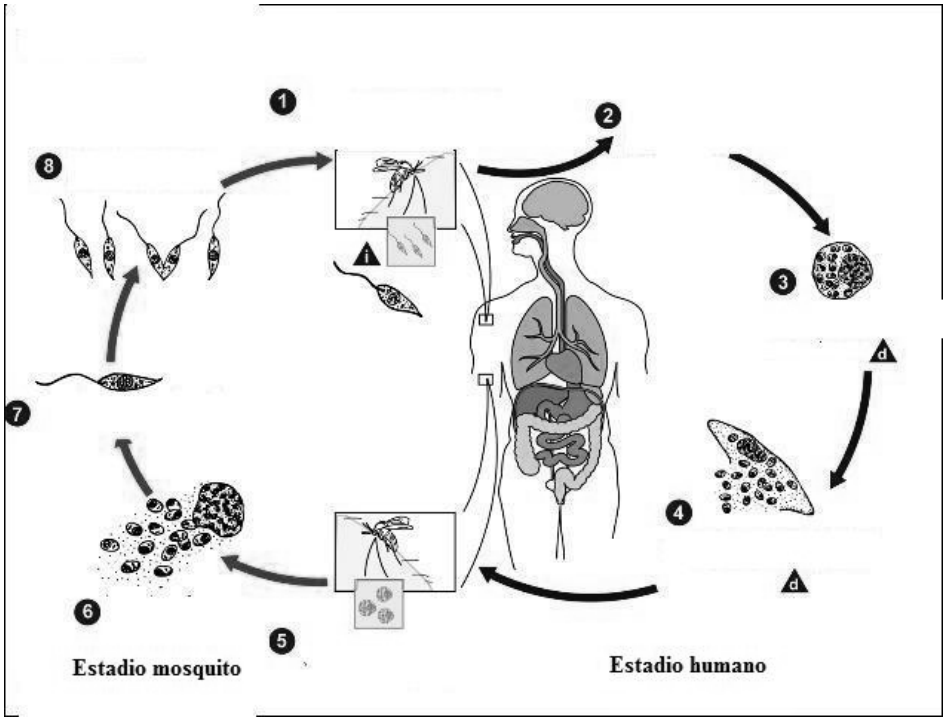

Fig.2: CICLO BIOLÓGICO DE LEISHMANIA.

(Fuente: Centers for Disease Control and Prevencion Nacional Center for Infeccious Diseases. Division of Parasitic Diseases.2005)

Estadio humano:

1- El mosquito toma la sangre (inyecta el promastigote en la piel);
2- La promastigota es fagocitado por el macrófago;
3- La promastigota se transforma a amastigota en el interior del macrófago;
4- La amastigote se multiplica en las células de diversos tejidos (incluyendo los macrófagos).

Estadio mosquito:

5- El mosquito toma la sangre (ingiere macrófagos infectados con amastigotas);
6- Ingestión de la células infectadas;
7- La amastigota se transforma a estadio promastigota en el intestino del mosquito;
8- Se divide en el intestino y migra hacia la proboscis.

I: estadio infectivo; d: estadio diagnóstico.

III-2.3 Manifestaciones clínicas y diagnóstico

III-2.3.1 Manifestaciones clínicas

Dependiendo de la especie de *Leishmania* se puede producir una infección cutánea, mucocutánea o visceral.

La leishmaniosis cutánea, llamada también botón de Oriente, es la forma más frecuente del padecimiento. Puede presentarse en dos formas clínicas con pronóstico y características inmunológicas opuestas: la leishmaniosis cutánea localizada (LCL) y la leishmaniosis cutánea difusa (LCD). La LCL se distingue por la presencia de úlceras únicas o múltiples, redondeadas, de bordes indurados, fondo limpio e indoloro que aparecen 15 a 20 días después de la picadura del vector infectado. Algunas veces los pacientes con LCL curan de manera espontánea en un lapso de seis meses a dos años, excepto cuando la lesión ocurre en la oreja, donde es crónica y mutilante (García-Almagro, 2005; Marco, 2008).

En el polo opuesto se encuentra la LCD, que se caracteriza por falta de respuesta inmune celular hacia antígenos de *Leishmania*, lo que permite la diseminación del parásito por el líquido tisular, la linfa o la vía sanguínea con desarrollo de lesiones nodulares en toda la piel, salvo en el cuero cabelludo.

En el continente europeo, sobre todo en el este de África, ambas formas, LCL y LCD, las produce *L. aethiopica.* En el continente americano hay tres especies del complejo *L. mexicana* que pueden causar ambas formas clínicas: *Leishmania (L.) mexicana, L. (L.) amazonensis* y *L. (L.) pifanoi.* (García-Almagro, 2005; Marco, 2008).

La leishmaniosis mucocutánea (LMC) o espundia, cursa con invasión y destrucción de la mucosa nasofaríngea y puede ser muy desfigurante. Las especies causante de esta forma clínica pertenecen al complejo *L. braziliensis*: *L. braziliensis, L. guyanensis, L. panamensis* y *L. peruviana.* Esta forma clínica se desarrolla después de que desaparezcan las lesiones cutáneas y en ocasiones puede presentarse hasta 20 años después. Las lesiones se caracterizan por tener escasos parásitos y los daños son

secundarios a la reacción inflamatoria que ocurre en las mucosas nasal, bucal y faríngea, y llevan a la degeneración del tabique nasal. Los tratamientos son muy prolongados y los pacientes casi siempre sufren el rechazo de su comunidad debido a las destrucciones mutilantes que les confiere un aspecto de leprosos.

La leishmaniosis visceral (LV), también llamada kala azar en la India o enfermedad negra en virtud de la hiperpigmentación observada en pacientes de esta región, es la forma más severa, consecuencia del fallo de la respuesta inmune de tipo celular del hospedador. La expresión clínica se caracteriza por un síndrome febril, debilidad, sudor nocturno, pérdida de peso, pancitopenia, leucopenia, anemia, hemorragias, hepatoesplenomegalia y afectación renal (Osman y col, 2000, Alvar, 2001, Collin y col., 2004, Murray y col., 2005 y Marco, 2008).

La LV es letal en el 100% de los casos si no recibe tratamiento, y aun con éste la mortalidad puede llegar al 15 %. En la región del Mediterráneo, la LV ha surgido como un problema de salud pública, sobre todo en individuos inmunosuprimidos como los pacientes con VIH. En esta región los perros forman parte importante del reservorio natural. Después da la recuperación de la LV, es posible observar la leishmaniasis cutánea posterior a kala azar, que cursa con nódulos cutáneos que contienen abundantes macrófagos infectados, los cuales pueden curarse con terapia muy prolongada.

Las manifestaciones clínicas resultan de una combinación de factores del parásito y factores genéticos del hospedador (Osman y col., 2000, Collin y col., 2004 y Marco, 2008).

III-2.3.2 Diagnóstico

El diagnóstico clínico de la leishmaniasis es complicado debido al amplio espectro de manifestaciones clínicas, a menudo inespecífico, que puede mostrar la enfermedad. El diagnóstico determinado por la presencia de lesiones debe ser confirmado con las siguientes pruebas que demuestren la presencia del parásito de manera directa o indirecta. (Reithinger y Dujardin, 2006).

- *Prueba de leishmanina o la intradermorreacción de Montenegro*. Es una prueba de hipersensibilidad celular tardía a antígenos de *Leishmania*.
- *Observación microscópica del parásito,* basada sobre la observación al microscopio de las formas amastigotas en preparaciones teñidas de productos patológicos.
- *Cultivo in vitro* de la muestra en medios NNN o RMPI-1640 suplementado con suero fetal bovino, método directo que permite el aislamiento del parásito y facilita su detección. Los cultivos se mantienen a 24-26 ºC, se examinan al microscopio en busca de promastigotes una o dos veces por semana.
- *Xenodiagnóstico*. Consiste en la inoculación del aspirado de la lesión en animales susceptibles (hámster dorado, ratón BALB/c) y permite recuperar e identificar el parásito. El desarrollo de la lesión puede tardar varios meses.
- *Pruebas serológicas*. Hacen posible la detección de anticuerpos específicos contra la *Leishmania*:

  • La prueba ELISA es un método cuantitativo que permite determinar el nivel y el isotipo de anticuerpos presentes. Los pacientes con LCD y LV cursan con hipergammaglobulinemia con títulos elevados de IgG e IgM. (Guillén L. y Col., 2002)

  • La inmunofluorescencia indirecta (IFI) es un método semicuantitativo que establece la presencia de anticuerpos anti-*Leishmania*. (Guillén L. y Col., 2002).

  • La inmunoelectrotransferencia (Western blot) identifica de modo adicional antígenos específicos.

  • La inmunohístoquímica con anticuerpos anti-*Leishmania* permite identificar el parásito dentro de las células.
- *Pruebas moleculares*. Se basan en la detección del ADN del parásito en tejidos mediante la reacción en cadena de la polimerasa (PCR) con el uso de oligonucleótidos específicos del género y especie de *Leishmania*. Esta técnica es útil cuando hay escasos parásitos en las lesiones y además determina un diagnóstico específico de la especie. (Iniesta et al., 2002).

El diagnóstico de LV se confirma mediante biopsia esplénica o punción de médula ósea. Ambos procedimientos son traumáticos y requieren un centro hospitalario.

El diagnóstico diferencial de la leishmaniosis cutánea debe descartar padecimiento como lepra, esporotricosis, paracoccidioidomicosis, tuberculosis y cáncer (carcinoma basocelular). En el caso de la leishmaniosis visceral, las enfermedades que deben descartar son: histoplasmisis, enfermedad de Chagas, paludismo, brucelosis, mononucleosis infecciosa, tuberculosis y fiebre tifoidea atípica. (Olivier y Col., 2005; Sacks y Col., 2002; Woelbing y Col., 2006).

III-2.4 Tratamiento

El control farmacológico de la leishmaniasis ha sido un aspecto de interés desde el inicio de la descripción de la enfermedad. En general los resultados no han sido muy favorables a pesar de que durante las últimas décadas se han estudiado una gran cantidad de posibles fármacos contra la leishmaniasis. Sin embargo, aún no existe ningún compuesto que sea capaz de eliminar completamente los parásitos, que no muestre toxicidad ni ofrezca resistencias.

Los fármacos recomendados para el tratamiento de esta enfermedad son:

Antimoniales

En 1912 se trató en Brasil el primer caso de leishmaniasis con una forma de antimonial trivalente (SbIII). A partir de 1920 se dispuso de los antimoniales pentavalentes (SbV) y desde 1945 del estibogluconato sódico (Ramos y Segovia, 1997).

Los antimoniales pentavalentes constituyen el tratamiento clásico para la leishmaniasis visceral siendo administrado por vía parenteral. Existen dos formulaciones en el mercado que contienen la forma pentavalente del compuesto: antimoniato de meglumina, comercializado con el nombre de Glucantime® (Aventis, Francia), que contiene 85 mg de SbV/ml y el Estibogluconato de sodio, conocido como Pentostam® (GlaxoSmithKline, Reino Unido) con 100mg de SbV/ml (Croft y Yardley, 2002).

El mecanismo de acción de los compuestos antimoniales se basa en su capacidad para inhibir selectivamente enzimas necesarios para la glicolisis y la oxidación de los

ácidos grasos del parásito (Baneth y Shaw, 2002). A pesar de su posible toxicidad, los fármacos antimoniales logran la cura clínica en un 90% de los casos aunque la remisión de los síntomas y recidivas ocurre con frecuencia (Gramiccia y col., 1992; Ikeda-García y col., 2007). Además, en los últimos años se ha incrementado el número de resistencias por lo que se ha limitado su uso tanto en los casos caninos como humanos de la enfermedad y ha aumentado el estudio de los mecanismos que conducen a la resistencia (Carrió y Portús, 2002; Croft y col., 2006).

Alopurinol

El Alopurinol es un análogo de purina (hipoxantina) que fue utilizado por primera vez contra la leishmaniasis en los años 80. Su mecanismo de acción se basa en la inhibición de enzimas de la ruta de las purinas como la xantina oxidasa. Este compuesto es metabolizado por *Leishmania* produciendo un análogo de inosina inactivo, frenando así el metabolismo de las purinas (Nelson y col., 1979).

La mayor ventaja que ofrece el Alopurinol es que no suele generar efectos secundarios, por lo que se recomienda su uso en casos con nefritis crónica debida a la leishmaniasis (Plevraki y col., 2006). Sin embargo, se ha demostrado que este compuesto por sí solo no es capaz de eliminar por completo los parásitos del organismo (Cavaliero y col., 1999; Koutinas y col., 2001) y no previene la infección de los individuos sanos (Saridomichelakis y col., 2005).

Anfotericina B (anB)

La anB es un antibiótico que pertenece al grupo de los polienomacrólidos que se obtiene de la fermentación del hongo *Streptomyces nodosus*. Su capacidad leishmanicida ya fue descubierta en los años 60 aunque es usado principalmente como fungicida, especialmente para el tratamiento de micosis sistémicas. Su acción se basa en su capacidad para unirse al ergosterol formando poros en la membrana celular de *Leishmania*, resultando en la pérdida de iones y produciendo la muerte celular (Ramos y col., 1996; Walker y col., 1998; Croft y col., 2006). La anfotericina B se comercializa con el nombre de Fungizona® (Bristol-Myers-Squibb, Estados Unidos), La anB tiene una gran capacidad leishmanicida; sin embargo su toxicidad es muy elevada, lo que ha

restringido su uso. Los efectos tóxicos se producen debido a la similitud existente entre el ergosterol y el colesterol de las células de mamíferos, lo que hace que se produzcan uniones inespecíficas generando daños en el hospedador. La anB suele usarse como fármaco de segunda elección aunque está aumentando su utilización debido al creciente número de resistencias a los compuestos antimoniales (Baneth y Shaw, 2002; Croft y Yardley, 2002; Croft y col., 2006).

En los últimos años se han desarrollado otro tipo de formulaciones de anB que han conseguido disminuir su toxicidad y reducir su precio. Un ejemplo son las nanoesferas de poli (épsilon-caprolactona) que han sido empleadas in vitro contra amastigotes de *L. donovani* (Espuelas y col., 2002). Por otra parte, los conjugados de anfotericina B con arabinogalactano (Golenser y col., 1999) y los complejos bilaminares de fosfolípidos y apoliproteína (Nelson y col., 2006) han resultado poco tóxicos y con alta capacidad leishmanicida contra *L. major* en infecciones murinas. (Loiseau y col., 2002).

Por otra parte, se han ensayado nuevas formulaciones de anB basadas en la modificación del estado de agregación. Por ejemplo, se han realizado estudios centrados en la utilización de anB sometida previamente a calor. Con este tratamiento se consigue la superagregación del fármaco, lo que conduce a un cambio en la distribución del mismo (Kwong y col., 2001) y una reducción de la toxicidad (Petit y col., 1999; Bau y col., 2003).

Finalmente, se ha llevado a cabo un estudio basado en la modificación de los grupos aldehído de la anB. Este compuesto resultó ser menos tóxico sobre las células macrofágicas RAW264.7 conservando la capacidad leishmanicida de la anB contra *L. major* (Sokolsky-Papkov y col., 2006).

Aminosidina o Paromomicina

La Aminosidina es un antibiótico aminoglucósido producido por *Streptomyces rimosus*, usado para el tratamiento de enfermedades bacterianas y con demostrada capacidad antiparasitaria (Baneth y Shaw, 2002). Las formulaciones para administración oral son recomendadas contra las amebiosis intestinales. Las formas inyectables se usan contra la leishmaniasis visceral mientras que las formulaciones

tópicas han sido utilizadas contra formas cutáneas de la enfermedad (Croft y Coombs, 2003). Sin embargo Vexenat y col. no consideraron la aminosidina como un fármaco recomendable para sustituir a los antimoniales (Vexenat y col., 1998).

Pentamidina

La pentamidina es el nombre común del isotionato de Pentamidina, una diamidina aromática usada para el tratamiento de la neumonía causada por *Pneumocystis carinii*.

Posteriormente, se demostró que este fármaco también posee actividad anti-leishmanicida (Croft y Brazil, 1982). La formulación de isotionato de Pentamidina, administrada por vía parenteral, ha sido comercializada con el nombre de Pentacarinat®. La Pentamidina también ha sido comercializada en forma de dimetasulfonato de Pentamidina con el nombre de Lomidine®. (Rhalemet y col., 1999; Méndez y Alunda, 2000; Baneth y Shaw, 2002; Guerin y col., 2002).

La Pentamidina interactúa con el ADN, modificando su conformación (Nguewa y col., 2005); además inhibe varias rutas metabólicas del metabolismo de las poliaminas (Reguera y col., 1994; Calonge y col., 1996; Johnson y col., 1998). La Pentamidina causa irritación muscular en la zona de inyección. Además, se pueden observar otros efectos secundarios como hipotensión, taquicardia o vómitos (Baneth y Shaw, 2002). Se han ensayado formulaciones de Pentamidina basadas en la encapsulación del principio activo en nanoesferas de ácido Poliláctico o de Polimetacrilato. Las nanoesferas de ácido Poliláctico mostraron una eficacia tres veces superior a la observada para las formas libres en infecciones de ratones BALB/c con *L. infantum*. No observandose toxicidad como en el tratamiento con la forma libre de la Pentamidina (Durand y col., 1997).

Alquilfosfocolinas

La Hexadecilfosfocolina o Miltefosina es un análogo de alquil lisofosfolípido. Este compuesto fue desarrollado como anticancerígeno por su capacidad de inducir la apoptosis celular selectiva de las células tumorales. Una formulación tópica fue comercializada con el nombre de Miltex® para el tratamiento de las lesiones cutáneas

causadas por los tumores de mama o linfomas. Posteriormente, la capacidad leishmanicida de este compuesto provocó que se comercializara en India una formulación oral con el nombre de Impavido® (Zentaris, Alemania) para su uso contra la leishmaniasis visceral humana.(Duijsings y col., 2004; Sindermann y Engel, 2006).

Varios derivados de alquil lisofosfolípido como la miltefosina, edelfosina o ilmofosina han mostrado propiedades líticas sobre formas promastigote y amastigote de varias especies de *Leishmania* (Escobar y col., 2002; Azzouz y col., 2005).

Azol y derivados

Los derivados azoles fueron desarrollados originalmente como compuestos antifúngicos debido a su capacidad de inhibición de la ruta de biosíntesis de los esteroles. *Leishmania* comparte este lípido de membrana con los hongos, por lo que también es sensible a estos fármacos (Croft y Coombs, 2003), aunque se ha demostrado que son menos eficaces que los antimoniales pentavalentes, al menos en las formas caninas (Baneth y Shaw, 2002). Los compuestos azoles consiguen lisar el parásito al alterar la composición de su membrana lo que aumenta su permeabilidad. No existen notables diferencias entre especies del parásito en cuanto a la sensibilidad a estos compuestos (Beach y col., 1988).

Los efectos secundarios que han sido descritos en humanos son hepatotoxicidad, disfunción endocrina, reducción de los niveles de cortisol e insuficiencia adrenal (Singh y Sivakumar, 2004).

Varios derivados azoles (metronidazol, ketoconazol, fluconazol e itraconazol) han sido valorados en ratones BALB/c infectados con *L. infantum*, resultando menos eficaces que los antimoniales ya que no se observaron reducciones significativas de la carga parasitaria (Gangneux y col., 1999). Estudios de toxicidad realizados en humanos han demostrado que el itraconazol es mejor tolerado que el ketoconazol aunque su eficacia no fue mejor que la conferida por el placebo (Singh y Sivakumar, 2004).

Los derivados de azoles han mostrado mayor eficacia al ser utilizados en combinación con otros compuestos anti-Leishmania. El metronidazol en combinación

con espiramicina fue empleado para tratar 27 perros infectados naturalmente con *L. infantum.* Los resultados obtenidos mostraron una reducción en el título de anticuerpos así como en la carga parasitaria, aunque no se logró la eliminación completa de la infección (Pennisi y col., 2005).

Sitamaquina

La sitamaquina es una 8-aminoquinoleína conocida anteriormente como WR6026 (Glaxo Smith Kline), compuesto que fue sintetizado por primera vez en 1944 (Guerin y col., 2002). Este compuesto es un análogo de la primaquina, compuesto usado contra la malaria desde los años 50, de administración oral y con amplio espectro de actividad antiprotozoaria (Yeates, 2002; Edison, 2008).

Su mecanismo de acción está basado en su capacidad para provocar un rápido colapso del potencial de membrana de la mitocondria de *Leishmania* (Taylor y col., 1991). Datos obtenidos en humanos han mostrado que la Sitamaquina es, en general, bien tolerada aunque pueden producirse vómitos, dolor abdominal, cefaleas o trastornos renales (Ramos y Segovia, 1997; Pérez-Victoria y col., 2006).

Imiquimod

La imidazoquinolona o imiquimod es un compuesto antivírico utilizado originalmente contra los papilomavirus que causan verrugas genitales. Este principio activo es el ingrediente principal de la crema comercializada como Aldara (3M Pharmaceuticals) usada contra queratosis, carcinoma de células basales o verrugas genitales (Croft y Coombs, 2003; Davis y Kedzierski, 2005).

El mecanismo de acción del imiquimod se basa en su capacidad para inducir la producción de citoquinas (IFNγ, TNFα, IL1, IL6 o IL8) que activan la producción de óxido nítrico en los macrófagos, favoreciendo la destrucción de los amastigotes intracelulares (Testerman y col., 1995; Buates y Matlashewski, 1999).

Compuestos experimentales

La investigación en el campo de la quimioterapia continúa en la actualidad puesto que no se ha desarrollado un compuesto que produzca la curación total de la enfermedad. Hasta el momento se han ensayado gran cantidad de compuestos con actividad leishmanicida in vitro con resultados prometedores, siendo algunos de ellos seleccionados para ser evaluados in vivo.

La capacidad leishmanicida de compuestos aislados de plantas se ha estudiado en gran detalle. Formas monoméricas y diméricas de las naftoquinonas, compuestos fenólicos de origen botánico, han sido probadas in vitro sobre formas promastigote de distintas especies de *Leishmania* mostrando actividad anti-Leishmania (Kayser y col., 2000).

También se ha estudiado la capacidad leishmanicida de ciertos alcaloides extraídos de plantas tradicionalmente usadas como plantas medicinales. Estos compuestos se han ensayado in vitro sobre formas promastigote y amastigote de *L. infantum* demostrando su capacidad leishmanicida (Di Giorgio y col., 2004). La capacidad anti-Leishmania de 43 compuestos alcaloides fue probada, reduciendo la lista a dos compuestos activos, se ensayaron sobre formas promastigote y amastigote de *L. infantum* obteniendo prometedores resultados (González et al., 2005). También se realizó un estudio in Vitro que probaba la capacidad leishmanicida de un exudado de *Aloe vera* sobre *L. donovani* (Dutta y col., 2007).

Estudios recientes se han centrado en el análisis de la capacidad leishmanicida de las Cistatinas, inhibidores naturales de las Cisteín proteasas, enzimas muy abundantes en *Leishmania*. Se ha demostrado la capacidad leishmanicida de Cistatinas de pollo sobre *L. donovani* tanto en infecciones in vitro sobre macrófagos peritoneales como in vivo sobre ratones BALB/c. Se han relacionado las características leishmanicidas de las Cistatinas con su poder para inducir la producción de NO (Das y col., 2001; Mukherjee y col., 2007). Resultados similares fueron obtenidos en *L. major* tanto in vitro como in vivo (Selzer y col., 1999).

IV-METODOLOGÍA Y RESULTSDOS

1,4-Bis(alkylamino)benzo[g]phthalazines able to form dinuclear complexes of Cu(II) which as free ligands behave as SOD inhibitors and show efficient in vitro activity against Trypanosoma cruzi

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1,4-Bis(alkylamino)benzo[*g*]phthalazines able to form dinuclear complexes of Cu(II) which as free ligands behave as SOD inhibitors and show efficient in vitro activity against *Trypanosoma cruzi*

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Abstract—The synthesis of a new series of 1,4-bis(alkylamino)benzo[*g*]phthalazines **1**–**3** is reported, and their ability to form dinuclear complexes with Cu(II) assayed. The geometry of the complexes is dependent on the nature of the electron-donor sites at the sidechains. Compounds **1** and **2**, that contain sp^3 or sp^2 nitrogens at the end of the alkylamino groups, originate monopodal dinuclear complexes which seem to include endogenous OH bridges, and the sidechains seem to actively participate in complexation. However, the substitution of nitrogen by oxygen in **3** leads to a tripodal dinuclear complex in which the sidechains are not involved. The in vitro antiparasitic activity on *Trypanosoma cruzi* epimastigotes and amastigotes and the SOD activity inhibition have been evaluated for compounds **1**–**3**, and, as expected, **1** and **2** show in all cases relevant results, whereas **3** is always the less active among the three substrates tested.

1. Introduction

Parasitic diseases represent an increasing threat to human health. Leishmaniasis and Chagas disease are major causes of mortality in tropical areas, and account for more than one million deaths a year.[1,2] Chagas disease, produced by protozoan parasite of the order *Trypanosoma cruzi*,[3] is endemic in 21 countries, and it has been calculated that, only in Central and South America, about 18 millions of people are infected and 100 millions are on risk.[4] Currently available chemotherapy is unsatisfactory because of its limited efficacy in the prevalent chronic stage of the disease ant toxic side effects.[3] However, the last years have witnessed an impressive increase in our understanding of the biochemistry and molecular biology of parasites, so that a major challenge is present for developing new antiparasitic drugs on the basis of that knowledge.

It has been shown that, in general, parasitic protozoan survival is closely related to the ability of enzymes for evading toxic free radical damage originated by their host.[5] One of these mechanisms involves superoxide dismutases (SOD). It seems that these enzymes act as scavengers that protect trypanosomatides like *Leishmania tropica* and *T. cruzi* from the damning effects of the superoxide anions or hydroxyl radicals produced in O_2 reduction.[6,7] It has been suggested that an increase in superoxide due to the inhibition of SOD would affect both the growth and survival of parasited cells.

Three distinct types of SOD have been isolated from various species of organisms, and each of them contains

Keywords: 1,4-Bis(alkylamino)benzo[*g*]phthalazines; *Trypanosoma cruzi* SOD inhibitors; Dinuclear copper complexes; OH endogenous bridges; Antiparasitic activity.
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different transition metals as the prosthetic group: Cu/Zn, Fe and Mn. The metal ligands are in all cases aminoacid residues, varying in composition according to the type of species or even subspecies considered. Maybe the most widely present in different organisms is the homodimeric CuZnSOD, usually containing one copper and one zinc ion bridged by imidazolate and bound to imidazole units from histidines.[8] However, it has been shown that only the copper ion is essential for achieving catalysis, since the species E_2Zn_2SOD is completely inactive, whereas Cu_2E_2SOD presents an activity only slightly lower than that one of the native enzyme.[9]

Our hypothesis is that molecules with a high ability for the complexation of transition metal ions could compete with SOD for them and would inhibit the protective action of the enzyme, favouring the cell death and assuming an antiparasitic activity.[10] In consequence, and taking into account the ability shown by 1,4-disubstituted phthalazines for the complexation of metallic cations like Cu(II), Co(II), or Zn(II),[11] we have designed a new series of benzo[*g*]phthalazine derivatives with two flexible sidechains containing nitrogen and oxygen atoms as shown in Figure 1. The pyridazine nitrogens should behave as active sites for interacting with the metallic cations, and the presence of the four electronegative centres in the polyaminic sidechains linked by two or three carbon atoms should allow the simultaneous complexation with two ions.

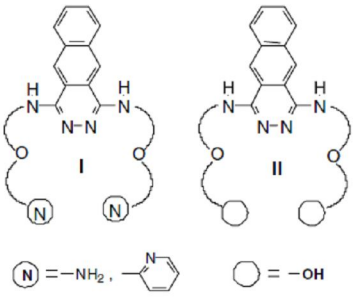


Figure 1.

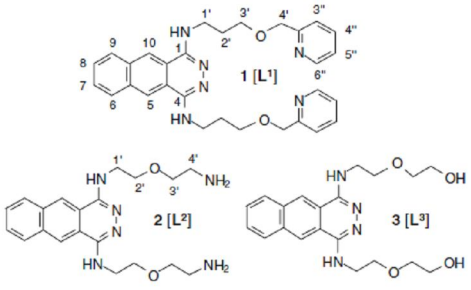
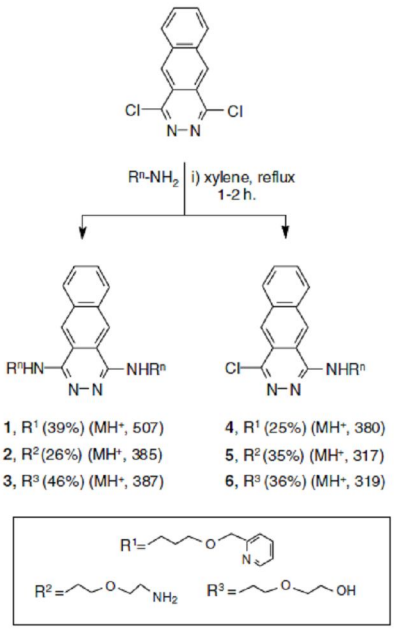


Figure 2.

Moreover, we have systematically modified the nature of the complexation sites located at the end of the aliphatic chains (Fig. 2). So, podands **1** and **2**, containing, respectively, sp^2 (pyridine) and sp^3 (NH_2) nitrogens, were prepared in a first step. In order to evaluate the role of those terminal groups in complexation, we have designed further a third podand, functionalized at the end with OH groups (**3**). In this work, we describe the synthesis of compounds **1**–**3**, their ability for complexing Cu(II) as a model for transition metal ions, and also their parasitic activity against *T. cruzi* and the inhibition of superoxide dismutase.

2. Results and discussion

The preparation of compounds **1**–**3** was performed according to the methodology shown in Scheme 1. Nucleophilic substitution of the starting 1,4-dichlorobenzo-[*g*]phthalazine with 3-(2-pyridilmethoxy)propylamine, 2-(2-aminoethoxy)ethylamine, or 2-(2-aminoethoxy)ethanol under 1–2 h reflux of xylene afforded the respective mixtures of the 1,4-bisalkylaminobenzo[*g*]phthalazines **1**–**3** and their monoalkylamino substituted analogues **4**–**6**. Conditions selected corresponded to a modification of the classic method of Körmendy[12] devised by our group.[13] Among a wide range of solvents assayed, xylene showed to be determinant in obtaining better comparative yields of compounds **1**–**3**. The mixtures of mono- and di-substituted products were carefully separated in every case by flash column chromatography and isolated as yellow solids or oils in yields ranging from 25% to 45%.



Scheme 1.

The amine required for the preparation of **1** is not commercially available, and was prepared by the reaction of 2-hydroxymethylpyridine with sodium hydride and further treatment of the alkoxide with *N*-(3-bromopropyl)phthalimide. Hydrolysis of the imide with hydrazine in methanol afforded the desired 3-(2-pyridilmethoxy)propylamine in 50% whole yield. It should also be noted that compound **1** was initially obtained in the hydrochloride form, and the free compound was liberated by passing through a basic aluminium oxide column chromatography using chloroform as the eluent.

All the new compounds synthesized were unequivocally identified by their analytical and IR, FAB MS, ^{1}H NMR and ^{13}C NMR spectroscopic data, as shown in Section 4. Scheme 1 displays the molecular ions obtained from the mass spectra of **1–6**, which agree in all cases with the proposed structures.

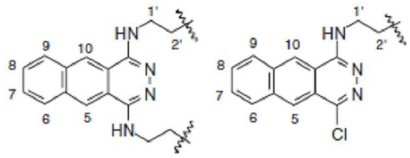
The mono- and diaminoalkyl substitution products are easily differentiated in both ^{1}H and ^{13}C NMR spectra on the basis of signals corresponding to rings A and B of the polyheterocyclic moiety (Table 1). Protons H_5 and H_{10}, which appear as an unique singlet in **1–3**, lose their equivalence in their monosubstituted analogues, their chemical shifts differing in about 0.2 ppm in the ^{1}H NMR spectra and 9 ppm in the ^{13}C analogues. Furthermore, the amino substituted carbons are clearly more deshielded than those ones linked to chlorine atoms in the ^{13}C NMR spectra.

The vigorous stirring of a methanol solution of ligands **1–3** in the presence of copper(II) perchlorate hexahydrate at room temperature (molar ratio 1:2) led to the formation of green precipitates that were isolated as stable solids in the cases of ligands **1** and **2** (**7** and **8**, respectively) and a highly hygroscopic compound from **3** (**9**), and were identified as the desired complexes. Analytical data for the complex **7** (mp 200–205 °C, 74% yield) gave a formula $C_{31}H_{38}Cl_2Cu_2N_6O_{13}$, that tentatively fitted to $\{[Cu_2\ L^1]\cdot(OH)_2\cdot(ClO_4)_2\cdot MeOH\}$. For complex **8** (mp >300 °C, 39% yield) the formula $C_{20}H_{33}Cl_3Cu_2N_6O_{17}$ was obtained, that corresponded to $\{[Cu_2\ L^2]\cdot(OH)\cdot(ClO_4)_3\cdot(H_2O)_2\}$, and complex **9** (10% yield) afforded $C_{62}H_{86}Cl_4Cu_2N_{12}O_{30}$ assigned to $\{[Cu_2(L^3)_3]\cdot(ClO_4)_4\cdot(MeOH)_2\}$.

The formulae proposed from elemental analysis were confirmed by the FAB mass spectral data. Results obtained show that the complexes of ligands **1** and **2**, which have chain ends containing sp^2 or sp^3 nitrogen atoms, include two copper ions for one ligand unit in both cases, as desired. The fragmentation sequences confirm the presence of perchlorate ions, water molecules and/or hydroxyl groups and indicate the order in which all of them are lost.

The mass spectrum of the complex **7** presents a molecular ion peak MH^+ $m/z = 901$ corresponding to one ligand, two copper ions, two hydroxyl groups, two perchlorate units and one molecule of methanol. The

Table 1. Comparison of the most significant ^{1}H and ^{13}C NMR (δ, CD_3OD, ppm) data for the mono- (**4–6**) and bis-alkylamino substituted (**1–3**) derivatives



Compound	1	4	2	5	3	6
H_5 (s)	8.55	8.36	8.59	8.48	8.59	8.28
H_{10} (s)	8.55	8.47	8.59	8.64	8.59	8.46
H_6 (m)	7.98	7.96[a]	8.05	8.05	8.05	7.91
H_9 (m)	7.98	7.87[a]	8.05	8.05	8.05	7.91
H_7 (m)	7.65	7.59	7.62	7.66	7.61	7.58
H_8 (m)	7.65	7.59	7.62	7.66	7.61	7.58
$H_{1'}$ (s)	3.66	3.67	3.56	3.58	3.63	3.65
$H_{2'}$ (m)	2.12	2.10	3.81[b]	3.81	3.72[b]	3.73[b]
C_1	150.9	155.6	151.3	155.4	151.3	155.9
C_4	150.9	146.4	151.3	146.2	151.3	146.8
C_{4a}	119.9	123.8	120.0	123.3	120.0	123.9
C_{10a}	119.9	119.4	120.0	118.8	120.0	119.4
C_5	123.4	126.8	123.8	126.1	123.8	126.7
C_{10}	123.4	124.5	123.8	123.8	123.8	124.5
$C_{1'}$	40.6	40.9	43.1	42.7	43.2	43.4
$C_{2'}$	30.1	30.0	70.2	70.0	70.9	70.5

[a] Interchangeable signals.
[b] Triplet.

fragmentation pattern shows initial loss of methanol, followed by two successive losses of perchlorate, the two hydroxyl groups and, finally, the copper ions to give the protonated ligand peak. In a similar way, **8** has a MH^+ $m/z = 865$ fitting to one ligand, two copper units, three perchlorates, one hydroxyl group and two water molecules and exhibits two successive losses of perchlorate, followed by the hydroxyl and water units, the third perchlorate and the two copper ions. All these data not only confirm the proposed molecular formulae but also suggest that the water molecules and/or hydroxyl groups are linked to the copper ions and not located in the outer coordination sphere.

Although we could not dispose of the adequate crystals for performing X-ray diffraction analyses, the geometries of the copper complexes **7** and **8** have been studied by means of a suite of complementary techniques such as IR, electronic and EPR spectroscopies, thermal analysis by pyrolitic decomposition and magnetic susceptibility measures.

The electronic spectra of both compounds (Table 2) exhibit broad bands at, respectively, 648 and 663 nm that are indicative of Cu(II) d–d transitions and suggest the presence of CuN_2O_3 chromophores with a square-pyramidal coordination geometry.[14] This assert is supported by the EPR spectra of both compounds, that show very neat nuclear spin coupling. Hyperfine coupling constant values in the parallel region $A_{\parallel}$ are estimated as 140 (**7**) and 171 G (**8**), quite similar to the calculated values for D_4 symmetry in each metal atom. Calculated values for $g_{\parallel} = 2.28$ and $g_{\perp} = 2.05$ in **7** or $g_{\parallel} = 2.25$ and $g_{\perp} = 2.02$ in **8** are compatible with elongated octahedral or square-based pyramidal geometries with Cu(II) atoms showing interaction in the fundamental $d_{x^2-y^2}$ state with a weak but significant coupling ($G < 4.0$).[15]

On the other hand, the IR spectra of **7** and **8** show that the perchlorate ions are not coordinated to the metal ions. It is known that when ClO_4^- groups are located in a metal complex as ions with Td point symmetry, they have four normal modes of vibration, whereas coordination leads to C_{3v} or C_{2v} point symmetries and rise to nine frequencies of vibration.[16] As can be seen in Table 3, both **7** and **8** exhibit four bands corresponding to $\nu(ClO_4)$.

Furthermore, the ν(NH) bands of the sidechain terminal nitrogens of **8** are shifted to lower frequencies in the complex with respect to the free ligand, indicating that they are coordinated.[17] The participation of the pyridazine nitrogens is deduced from the shift of the ν(CN) band to higher frequencies. This last behaviour is also found in the case of **7**, and involvement of the pyridine nitrogen is observed on the basis of a similar shift and confirmed from the out- and in-plane pyridine ring deformation bands.

Table 2. Electronic and EPR spectra, and magnetic moment of complexes **7** and **8**

	Electronic spectra λ_{max} (nm)	EPR spectra g values	μ_{eff} (rt) (BM)
7	648	$g_{\parallel} = 2.28$ $g_{\perp} = 2.05$	0.61
8	663	$g_{\parallel} = 2.25$ $g_{\perp} = 2.02$	0.81

Table 3. Selected IR spectral data for complexes **7** and **8** and their free ligands (cm^{-1})

	$Cu(ClO_4)_2$	**1**	**7**	**2**	**8**
ν(OH, H_2O)	—	—	3407	—	3526, 3403
ν(NH)	—	—	—	3323	3226
ν(CN)	—	1551	1571	1551	1578
$\nu(ClO_4)$	1136	—	1144	—	1141
	—	—	1121	—	1120
	1105	—	1109	—	1108
	1087	—	1086	—	1088
ν(CuO)	—	—		—	392
ν(CuN)	—	—		—	324

The high frequency region shows bands assignable to coordinated and bridged OH and coordinated water molecules in **8**, and broad bands over 3400 cm^{-1} found in **7** can be attributed to any of both types of oxygen coordination.

Finally, Cu–O and Cu–N bond vibrations can be seen clearly in **8**,[18] whereas they cannot be observed in the IR spectrum of **7** due to overlapping with bands corresponding to the ligand skeleton.

Concerning the thermal analysis, it should be noted that the pyrolitic decomposition of **8** begins only at 190 °C, confirming that the water molecules and the hydroxyl ion must be coordinated. The loss of the perchlorate ions occurs at 250 °C and is followed by the elimination of CO_2, H_2O, CO, hydrocyanic, and isocyanic acids to give a residue of CuO. However, **7** begins to lose water between 40 and 200 °C, and this can be attributed to a condensation of the OH ligands forming an oxo bridge and losing water. Methanol is also liberated at low temperatures (40–70 °C). Further behaviour is similar to that commented for **8**.

Last, magnetic susceptibility essays performed in the 5–300 K range showed antiferromagnetic exchange between the copper atoms, supporting the existence of endogenous OH bridges.[19] This is more clearly seen in the case of **8**, with a $-2J$ value of 165.25 cm^{-1} that allows to calculate a Cu(OH)Cu angle of 100° and a Cu–Cu distance of 3.2 Å fitting for seven-membered nitrogen chelates. The magnetic moment at rt is of 0.81 BM. This value is lower in complex **7**, in which 0.61 BM was obtained.

On the basis of all the evidence compiled in the previous paragraphs, we think that the structures of both **7** and **8** could be tentatively assigned as shown in Figure 3. In the two dinuclear complexes the copper atoms appear as pentacoordinated and should adopt a square pyramidal geometry.[15] In complex **7**, each metallic ion should interact with two sp^2 nitrogens from the pyridine and

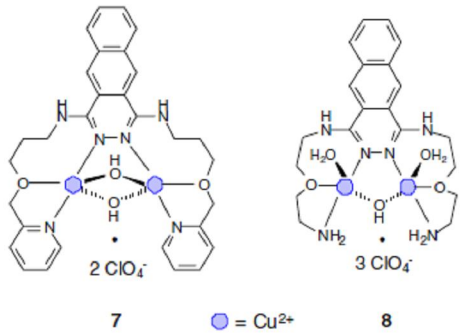


Figure 3.

pyridazine rings and one oxygen atom belonging to the sidechain, and should exhibit two endogenous bridges through hydroxyl groups. That kind of double bridging has been found in a variety of dinuclear copper complexes of nitrogenated heterocycles.[20]

Complex **8** seems to interact in the same way with the ligand, if we change the pyridine sp^2 nitrogen by the sp^3 of the primary amino group. An endogenous OH bridge should also connect the two copper atoms, and the second hydroxo bridge should be replaced by two water molecules, as has been described for benzimidazole and oxazole derivatives,[21] and proposed for the active site of oxyhaemocyanin.[22]

Concerning to the copper complex **9**, formed from ligand **3**, in which the terminal nitrogens have been replaced by OH groups, elemental analysis indicates the presence of three ligand units and two copper ions, in striking contrast to the dinuclear complexes found for ligands **1** and **2** (see Fig. 4). That kind of arrangement has been previously reported for Cu(II) complexes with pyridazine units.[23] The FAB mass spectrum is in accordance with that, because it shows a MH^+ at a very high *m/z* 1748, that corresponds to three ligands, two coppers, four perchlorates and two methanol molecules. The fragmentation pattern shows the successive loss of one ligand unit, one perchlorate anion, the two methanol molecules and another perchlorate, followed by the two remaining perchlorates, the two copper ions and, finally, the second ligand to give one protonated ligand as the base peak. Therefore, it seems that the replacement of the terminal nitrogen complexation sites

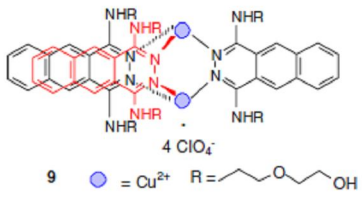


Figure 4.

by oxygen substantially modifies the nature of the complex formed. Unfortunately, **9** was not obtained as a solid, but as a jelly substance. The complex solidifies on standing in a vacuum desiccator but in contact with air becomes jelly again, probably due to fast coordination with water molecules. This fact prevented further study of its geometry by other techniques.

Due to the interest of confirming the crucial structural differences originated in complexation when the nitrogen sites at the end of the sidechains are replaced by oxygen, we decided to prepare the complex of ligand **3** with Zn(II), as both metals exhibit usually a similar behaviour in their complexation geometries. The addition of a methanol solution of anhydrous zinc chloride over a methanol solution of **3** originated the fast formation of a yellow precipitate of the complex **10** which was isolated as a stable solid with mp = 185–189 °C. Elemental analysis gave a molecular formula $C_{80}H_{106}O_{17}N_{16}Zn_2Cl_4$ tentatively fitting to $\{[Zn_2(L^3)_4]\text{-}Cl_4\cdot H_2O\}$.

The FAB mass spectrum of **10** exhibits a molecular peak with *m/z* = 1821, that is in accordance with the proposed formula after loss of the water molecule, and shows the initial and successive losses of four chloride ions, followed by one ligand unit, the two zinc atoms and, finally, two more ligands. The IR spectrum supports the coordination of the metallic ions with the pyridazine units, because the free ligand *ν*(CN) bands at 1508 and 1553 cm^{-1} are respectively shifted at 1554 and 1579 cm^{-1} in complex **10**.[17] However, the *ν*(CO) bands are not significantly modified, suggesting that the oxygen atoms are not participating. On the other hand, the wide and strong ligand *ν*(OH) band at 3315 cm^{-1} moves to 3357 cm^{-1} in **10**, with a shoulder ca. 3300 cm^{-1}, indicating strong hydrogen bonding of the lateral chains[24] instead of participation in complexation. Finally, a weak new band found in the far IR spectrum of the complex at 362 cm^{-1} should be assigned to the *ν*(ZnN) mode.[25]

The replacement of Cu(II) by Zn(II) allowed the inclusion of ^1H and ^{13}C NMR spectral data in structural considerations. When comparing the ^{13}C spectra of the podand **3** and its complex **10**, measured in DMSO-d_6, it is shown that the signals corresponding to the side-chain carbons neighbouring the OH group ($C_{3'}$ and $C_{4'}$) are practically unaffected by complexation ($\Delta\delta = 0.07$ and 0.09 ppm, respectively), whereas those ones of the two remaining methylenes and the aromatic $C_1$and C_4 exhibit more consistent variations ranging from 0.3 to 1.0 ppm. The same pattern is found in the comparison of the ^1H spectra, confirming that side chain oxygens are not taking part in complexation.

In consequence, it seems that the change in the nature of the donor sites at the sidechains causes a remarkable modification in the geometry of complexation and, as will be commented below, this fact could be responsible for differences in the biological activities of the podands.

Table 4. In vitro activity of 1,4-bis(alkylamino)benzo[g]phthalazines **1–3** on *Trypanosoma cruzi* epimastigotes

Compound	IC_{50} (μg/mL)			Toxicity IC_{50}[a] (μg/mL)
	24 h	48 h	72 h	
Benznidazole	—	—	4.12	3.53
1	56.75	12.85	3.25	6.25
2	n.d.	15.61	12.02	67.32
3	75.43	62.64	30.60	93.52

IC_{50}, concentration required to give 50% inhibition, calculated by linear regression analysis from K_c values at the concentrations employed (1, 10 and 25 μg/mL). n.d., not determined.
Note. Average of three separate determinations.
[a] On Vero cells at 72 h of culture.

3. Biological evaluation

In a first step, the effect of podands **1–3** on the in vitro growth of *T. cruzi* epimastigotes was measured, since we thought that the complexing ability of these compounds could support trypanocidal activity. In fact, very good inhibitory activity against the same parasite has been reported for quinone derivatives containing polyaminic chains related to those present in our compounds.[26] The growth inhibition of the parasite was measured at different times according to established procedures (see Section 4). Results obtained are displayed in Table 4 with benznidazole as the reference drug. The three compounds tested were active in this biological

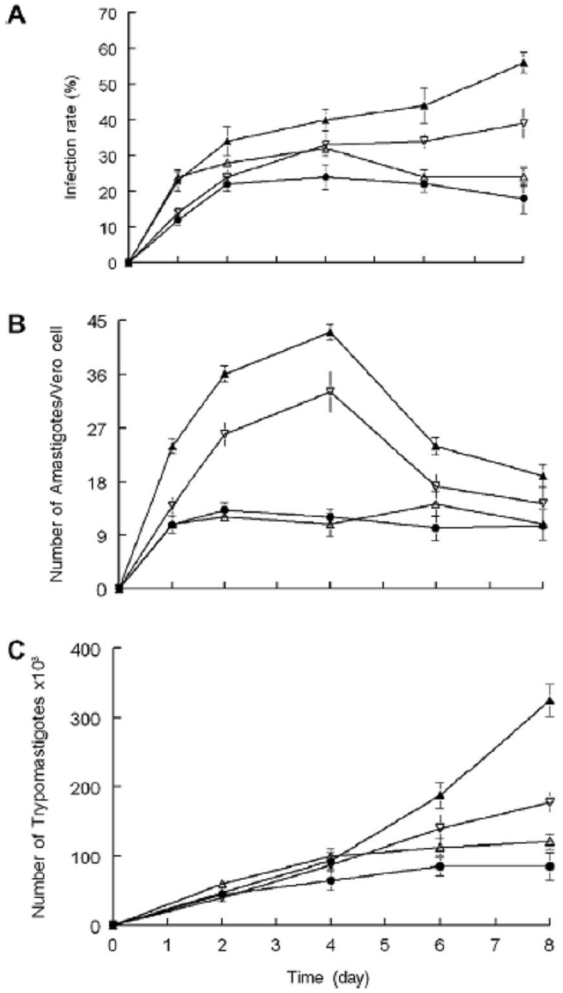


Figure 5. Effect of compounds **1–3** on the infection rate and *T. cruzi* growth. (A) rate of infection. (B) mean number of amastigotes per infected Vero cell. (C) number of trypomastigotes in the culture medium. Symbols: ▲, control; ▽, **1**; △, **2**; ●, **3** (at 5 μg/mL concn). The values are means of four separate experiments and the bars represent the standard deviation.

system. Compound **1** exhibited the highest toxicity against *T. cruzi* epimastigotes with IC_{50} values within the range of the reference drug. Compound **2** was also active, with lower potency than **1**. The antitrypanocidal activity observed for **2** was not associated to host cell toxicity (IC_{50} = 67.32 μg/mL). Compound **3** showed in all cases a remarkably lesser activity than **1** and **2**, and this fact could be related to the different mode of complexation of metal ions discussed above for that compound.

Figure 5 illustrates *T. cruzi* propagation in Vero cells (with and without co-addition of the test compounds). When 1×10^5 Vero cells were incubated for 2 days and then infected with 1×10^6 metacyclic forms, the parasites invaded the cells and underwent morphologic conversion to amastigotes within 1 day after infection. On days 1, 2, 4, 6 and 8, the rate of host-cell infection increased to 23%, 34%, 40%, 44%, and 56%, respectively. When compound **1** was simultaneously added to the infected Vero cells with *T. cruzi* metacyclic forms (5 μg/mL), the infection rate significantly decreased (35%) with respect to the control on day 2, reaching a 68% inhibition on day 8. Compound **2** at 5 μg/mL significantly inhibited the percentage of infected cells with a 57% inhibition on culture day 8 (Fig. 5A).

The average number of amastigotes per infected cell increased to 36 on day 2 and 43 on day 4, decreasing to 24 on day 6 and 19 on day 8, for the control experiment (Fig. 5B). Compounds **1** and **2** were highly inhibitory of *T. cruzi* amastigote replication in Vero cells in vitro (Fig. 5B) when simultaneously added to the cell culture infected with *T. cruzi* metacyclic forms. The addition of 5 μg/mL of **1** and **2** markedly lowered the amastigote number per infected cell to 12 and 10.8 on day 4 and 10.5 and 10.8 on day 8 (44% and 43% reduction in amastigote number with respect to the control for culture day 8, compounds **1** and **2**, respectively). The decrease in the average amastigote numbers on day 5 for the control experiment coincided with the increase in trypomastigote numbers in the medium (Fig. 5C). The number of trypomastigotes in the medium was 1×10^4 on day 2 and 3.25×10^6 on day 8. Compounds **1** and **2** gave, respectively, 74% and 65% reductions in trypomastigote numbers (Fig. 5C).

These results prompted us to evaluate the inhibitory effect of podands **1**–**3** on SOD activity, in order to test their potential as competitors for the metallic ions of the enzyme. We have used epimastigote forms from the Maracay strain of *T. cruzi* that excrete FeSOD when cultured in calf foetal serum.[27] Data obtained for the inhibition activity of the enzyme are displayed in Table 5.

Significant inhibition values of the enzyme activity ($p < 0.025$) are found for **1** and **2**. Podand **2** shows a 100% inhibition at a 25 μg/mL dosis, whereas **1** achieves 64% at 1 μg/mL. As in the previous test, the behaviour of **3** markedly differs from those ones of the other two podands, showing a much lesser activity against the parasitic SOD. All those results might be indicating that one of the action mechanisms of these compounds is blocking the metal ion of the enzyme, giving place to a substantial reduction of its activity.

Table 5. In vitro inhibition of Fe–SOD in *Trypanosoma cruzi* epimastigotes: 23.36 ± 4.21 U/mg

Compound	1 μg/mL	10 μg/mL	25 μg/mL
1	64**	68**	75**
2	48*	73**	100**
3	27	34*	53**

Values are the average of five separate determinations. Significant differences between the activities of the control homogenate and that one incubated with the tested compounds were obtained according to the Newman–Keuls test.

* $p < 0.05$.

** $p < 0.025$.

Table 6. Cu Zn–SOD activity inhibition (%) in human erythrocytes: 23.36 ± 4.21 U/mg

Compound	1 μg/mL	10 μg/mL	25 μg/mL
1	0	7	11
2	0	11	17
3	0	0	0

A high degree of activity against the SOD of the parasite should be nothing if the same pattern was found for human SOD without any discrimination. Therefore, we have also tested the effect of compounds **1**–**3** over CuZn-SOD belonging to human erythrocytes (Table 6).

Inhibition percentages at the higher dosages are very small in all cases for human SOD, and all the three compounds are fully inactive at the dosis of 1 μg/mL. These results enhance the potential antiparasitical interest of the alkylaminobenzo[*g*]phthalazine derivatives studied in this work.

In definitive, we have prepared a new series of compounds with complexing ability towards transition metal ions, that behave as SOD inhibitors and in which the mode of complexation could be related to the enzyme inhibition patterns and antiparasitical activity observed, although more work should be required in order to prove that relation.

4. Experimental

4.1. General

The starting compounds 2-(2-aminoethoxy)ethanol and 2-(2-aminoethoxy)ethylamine were purchased from Aldrich and used without further purification. 1,4-Dichlorobenzo[*g*]phthalazine was obtained from 2,3-naphthalenedicarboxylic acid following a method previously described.[28] All the reactions were monitored using thin layer chromatography (TLC) on precoated aluminium sheets of silica gel and compounds were detected with UV light (245 nm). Flash column chromatography was performed in the indicated solvent supported on silica

gel (particle size 0.040–0.063 mesh). Melting points were determined in Gallenkamp or Kofler apparatus and are uncorrected. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra at 75 MHz at room temperature employing CD_3OD and/or DMSO-d_6 as solvents. Chemical shifts are reported in ppm from TMS (δ scale). All assignments were performed on the basis of ^{1}H–^{13}C heteronuclear multiple quantum coherence experiments (gHSQC and gHMBC). Mass spectra were recorded by electronic impact (EI) at 70 eV, or by the fast atomic bombardment (FAB) technique with a VC Auto Spec spectrometer using a *m*-nitrobenzyl alcohol (NBA) matrix. EPR spectroscopy measurements were made at 300 and 120 K with a Bruker ER-200D spectrometer, equipped with variable temperature device. IR spectra were recorded on a Perkin-Elmer 597 spectrometer (4000–400 cm^{-1} range) and on an IR Bruker IFS 144C spectrometer (700–100 cm^{-1} range). Electronic spectra were recorded on a Perkin-Elmer Lambda 15 spectrometer, equipped with diffuse reflectance device, in the near infrared region, suspending the product in Nujol over filter paper. Magnetic susceptibility in range 4–100 K or 4–300 K was measured on a Squid magnetometer from Quantum Design equipped with helium cryostat. Magnetic susceptibility experimental values were corrected for diamagnetic contributions and TIP (temperature independent paramagnetism) was estimated as 100×10^{-8} emu mol^{-1} per Cu(II) ion.[28]

TGA/DTG analysis profiles (pyrolysis, 300–1000 K, with IR and MS investigation of evolved gases) were recorded under a 100 mL min^{-1} air flow using a Shidmazu TGA-DTG-50H Thermobalance coupled to a Nicolet Magma 550 FT-IR apparatus and a Fisons Thermolab mass spectrometer.

4.2. Synthesis of 3-(2-pyridilmethoxy)propylamine

To a stirred solution of 2-pyridilmethanol (6.0 g, 55 mmol) in anhydrous DME (150 mL) at 60 °C, an 80% suspension of sodium hydride in oil (1.65 g, 55 mmol) was added under argon. After 30 min, a solution of *N*-(3-bromopropyl)phthalimide (14.7 g, 57 mmol) in DME (100 mL) was added dropwise, the resulting mixture heated at 60 °C during 4 h and then stirred overnight at rt. After that, the reaction mass was treated with an aqueous 15% ammonium chloride solution until neutral pH. The aqueous phase was separated and extracted several times with chloroform. Evaporation of the solvent in the organic phase gave dark brown syrup that was flash column chromatographed using ethyl acetate/ethanol (10:1) as the eluent. The fraction of R_f 0.70 afforded 3.04 g (19%) of yellow oil. The so-obtained phthalimide was added over a 0.2 M hydrazine solution in methanol and the mixture was heated for 4 h at 40 °C. The solvent was removed under reduced pressure and the remaining white solid was purified by flash column chromatography ($CHCl_3$/EtOH/28% aqueous NH_4OH; 1:1:0.1) to give 0.82 g (50%) of the desired amine as an oil (R_f = 0.39). Anal. calcd for $C_9H_{14}N_2O$: C, 65.06; H, 8.43; N, 16.85. Found: C, 65.17; H, 8.62; N, 16.58; IR (KBr, cm^{-1}) 3400–3000, 3380, 3060, 2960, 2880, 1710, 1640, 1600, 1580, 1480, 1440, 1360, 1140, 1050, 670; ^{1}H NMR (CD_3OD): δ 8.48 (d, 1H, H-6′), 7.83 (t, 1H, H-4′), 7.50 (d, 1H, H-3′), 7.33 (m, 1H, H-5′), 4.59 (s, 2H, H-4), 4.44 (sa, 2H, NH_2), 3.64 (t, 2H, H-3), 2.83 (t, 2H, H-1), 1.85 (q, 2H, H-2); ^{13}C NMR (CD_3OD): δ 159.80 (C-2′), 149.88 (C-6′), 139.24 (C-4′), 124.48 (C-3′), 123.56 (C-5′), 74.41 (C-4), 70.44 (C-3), 40.19 (C-1), 32.83 (C-2); MS (FAB), *m*/*z* (%): 167 (MH^+, 1), 149 (2), 137 (1), 108 (90), 93 (100), 77 (8).

4.3. Synthesis of the 3-(2-pyridilmethoxy)propylamine derivatives 1 and 4

A solution of 1,4-dichlorobenzo[*g*]phthalazine (194 mg, 0.78 mmol) and 3-(2-pyridilmethoxy)-propylamine (520 mg, 3.13 mmol) in xylene (50 mL) was refluxed for 2 h. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The solid residue was purified by flash column chromatography (toluene/$CHCl_3$/EtOH, 2:1:1). The appropriate fractions (monitored by TLC) were combined to give two alkylamination products.

4.3.1. 1,4-Bis-[3-(2-pyridilmethoxy)propylamino]benzo-[*g*]phthalazine (1). The most retained fraction (R_f = 0.13) afforded an oil which was identified as the hydrochloride of **1**. The chromatographic treatment of that compound over basic aluminium oxide using chloroform as the eluent gave place to 100 mg (39%) of a yellow oil identified as the free polyamine **1**. Anal. calcd for $C_{30}H_{32}O_2N_6{\cdot}H_2O$: C, 68.44; H, 6.46; N, 15.96. Found: C, 68.56; H, 6.31; N, 15.83; IR ($CHCl_3$, cm^{-1}) 3600–3000, 1649, 1551; 1506, 1118, 1048; ^{1}H NMR (CD_3OD): δ 8.55 (s, 2H, H-5, H-10), 8.41 (d, 2H, H-6″), 7.98 (m, 2H, H-6, H-9), 7.71 (t, 2H, H-4″), 7.65 (m, 2H, H-7, H-8), 7.50 (d, 2H, H-3″), 7.23 (m, 2H, H-5″), 4.60 (s, 4H, H-4′), 3.75 (t, 4H, H-3′), 3.66 (m, 4H, H-1′), 2.12 (m, 4H, H-2′); ^{13}C NMR (CD_3OD): δ 159.62 (C-2″), 150.90 (C-1, C-4), 149.47 (C-6″), 138.82 (C-4″), 135.32 (C-5a, C-9a), 129.81 (C-6, C-9), 129.01 (C-7, C-8), 124.06 (C-3″), 123.45 (C-5, C-10), 123.32 (C-5″), 119.90 (C-4a, C-10a), 74.25 (C-4′), 70.66 (C-3′), 40.57 (C-1′), 30.14 (C-2′); MS (FAB), *m*/*z* (%): 509 (MH^+, 1), 399 (4), 277 (5), 263 (21), 210 (3), 91 (100).

4.3.2. 1-[3-(2-Pyridilmethoxy)propylamine]-4-chlorobenzo[*g*]phthalazine (4). The less retained fraction (R_f = 0.55) afforded 73 mg (25% yield) of **4** as a yellow oil. Anal. calcd for $C_{21}H_{19}ON_4Cl$: C, 66.58; H, 5.02; N, 14.79. Found: C, 66.50; H, 5.23; N, 14.99; IR (Cl_3CH, cm^{-1}) 3600–3000, 3340, 2950, 1635, 1580, 1440, 750; ^{1}H NMR (CD_3OD): δ 8.47 (s, 1H, H-10), 8.36 (s, 1H,H-5), 8.35 (d, 1H, H-6″), 7.96 (m, 1H), 7.87 (m,1H), 7.67 (t, 1H, H-4″), 7.59 (m, 2H, H-7,H-8), 7.43 (d, 1H, H-3″), 7.19 (t, 1H, H-5″), 4.55 (s, 2H, H-4′), 3.69 (t, 2H, H-3′), 3.67 (t, 2H, H-1′), 2.10 (m, 2H, H-2′); ^{13}C NMR (CD_3OD): δ 159.76 (C-2″), 155.59 (C-1), 149.80 (C-6″), 146.45 (C-4), 139.12 (C-4″), 136.09 (C-9a), 135.97 (C-5a), 130.31/130.28 (C-6/C-9), 130.14 (C-7, C-8), 126.81 (C-5), 124.46 (C-10), 124.39 (C-3″), 123.78 (C-4a), 123.61 (C-5″),

119.37 (C-10a), 74.54 (C-4′), 70.59 (C-3′), 40.92 (C-1′), 30.04 (C-2′); MS (IE) *m/z* (%): 379, (M^+, 83), 343 (2), 286 (80), 270 (100), 229 (19).

4.4. Synthesis of the 2-(2-aminoethoxy)ethylamine derivatives 2 and 5

A solution of 1,4-dichlorobenzo[*g*]phthalazine (100 mg, 0.40 mmol) and 2-(2-aminoethoxy)ethylamine (860 mg, 8.30 mmol) in xylene (50 mL) was refluxed for 1 h. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The solid residue was purified by flash column chromatography ($CHCl_3$/EtOH/28% aqueous NH_4OH, 1:1:0.2) to give the two alkylamination products.

4.4.1. 1,4-Bis-[2-(2-aminoethoxy)ethylamino]benzo[*g*]-phthalazine (2). The most retained fraction ($R_f = 0.54$) afforded 40 mg (26%) of **2** as a yellow solid with mp 60–63 °C. Anal. calcd for $C_{20}H_{28}O_2N_6 \cdot H_2O$: C, 59.70; H, 7.46; N, 20.79. Found: C, 59.11; H, 6.90; N, 19.92; IR (KBr, cm^{-1}) 3500–3300, 1631, 1551; 1506, 1114, 1032; ^{1}H NMR (CD_3OD): δ 8.59 (s, 2H, H-5, H-10), 8.05 (m, 2H, H-6, H-9), 7.62 (m, 2H, H-7, H-8), 3.81 (t, 4H, H-2′), 3.73 (t, 4H, H-3′), 3.56 (t, 4H, H-1′), 2.80 (t, 4H, H-4′); ^{13}C NMR (CD_3OD): δ 151.27 (C-1, C-4), 135.62 (C-5a, C-9a), 130.10 (C-6, C-9), 129.31 (C-7, C-8), 123.73 (C-5, C-10), 120.09 (C-4a, C-10a), 73.54 (C-3′), 70.82 (C-2′), 43.13 (C-1′), 42.84 (C-4′); MS (FAB), *m/z* (%): 385 (MH^+, 57), 342 (10), 298 (56), 279 (39), 211 (100).

4.4.2. 1-[2-(2-Aminoethoxy)ethylamino]-4-chlorobenzo-[*g*]phthalazine (5). The less retained fraction ($R_f = 0.65$) afforded 44 mg (35%) of **5** as a yellow oil. Anal. calcd for $C_{16}H_{17}ON_4Cl$: C, 57.40; H, 5.68; N, 16.74. Found: C, 57.26; H, 5.43; N, 16.51; IR (KBr, cm^{-1}) 3600–3100, 3050; 2920; 1629, 1560, 1430, 746; ^{1}H NMR (CD_3OD): δ 8.64 (s, 1H H-10), 8.48 (s, 1H, H-5), 8.05 (m, 2H, H-6, H-9), 7.66 (m, 2H, H-7, H-8), 3.81 (m, 4H, H-2′, H-3′), 3.58 (t, 2H, H-1′), 2.84 (t, 2H, H-4′); ^{13}C NMR (CD_3OD): δ 155.43 (C-1), 146.25 (C-4), 135.58 (C-9a), 135.36 (C-5a), 129.81 (C-6, C-9), 129.67 (C-7, C-8), 126.12 (C-5), 123.83 (C-10), 123.27 (C-4a), 118.83 (C-10a), 72.62 (C-3′), 70.00 (C-2′), 42.74 (C-1′), 42.00 (C-4′); MS (FAB), *m/z* (%): 317 (MH^+, 88), 289 (13), 274 (15), 256 (17), 229 (100).

4.5. Synthesis of the 2-(2-aminoethoxy)ethanol derivatives 3 and 6

A solution of 1,4-dichlorobenzo[*g*]phthalazine (284 mg, 1.14 mmol) and 2-(2-aminoethoxy) ethanol (1.75 g, 17.10 mmol) in xylene (50 mL) was refluxed for 1 h. The reaction mixture was cooled to room temperature and the solvent removed under reduced pressure. The residue was purified by flash column chromatography (chloroform/toluene/ethyl acetate/methanol, 1:1:2:3) to give the two alkylamination products.

4.5.1. 1,4-Bis[2-(2-hydroxyethoxy)ethylamino]benzo[*g*]-phthalazine (3). The most retained fraction ($R_f = 0.10$) afforded 115 mg (46%) of a yellow solid with mp 129–132 °C. Anal. calcd for $C_{20}H_{26}N_4O_4 \cdot H_2O$: C, 59.40; N, 13.86; H, 6.43. Found: C, 60.10; N, 14.04; H, 6.74; IR (KBr, cm^{-1}) 3500–3000, 3315, 1629, 1553, 1508, 1123; ^{1}H NMR (CD_3OD): δ 8.59 (s, 2H, H-5, H-10), 8.05 (m, 2H, H-6, H-9), 7.61 (m, 2H, H-7, H-8), 3.83 (t, 4H, H-4′), 3.72 (t, 8H, H-2′, H-3′), 3.63 (t, 4H, H-1′); ^{1}H NMR (DMSO-d_6): δ 8.82 (s, 2H, H-5, H-10), 8.11 (m, 2H, H-6, H-9), 7.70 (m, 2H, H-7, H-8), 3.67 (s, 4H, H-4′), 3.64 (t, 4H, H-3′), 3.50 (m, 4H, H-2′), 3.34 (t, 4H, H-1′); ^{13}C NMR (CD_3OD): δ 151.34 (C-1, C-4), 135.61 (C-5a, C-9a), 130.10 (C-6, C-9), 129.32 (C-7, C-8), 123.79 (C-5, C-10), 120.04 (C-4a, C-10a), 73.78 (C-3′), 70.93 (C-2′), 62.55 (C-4′), 43.18 (C-1′); ^{13}C NMR (DMSO-d_6): δ 148.60 (C-1, C-4), 133.17, 128.56, 127.71, 122.22, 118.00 (C aromat.), 72.16 (C-3′), 68.90 (C-2′), 60.20 (C-4′), 41.12 (C-1′); MS (FAB), *m/z* (%): 387 (MH^+, 14), 386 (M^+, 21), 355 (6), 298 (56), 253 (8), 210 (100).

4.5.2. 1-[2-(2-Hydroxyethoxy)ethylamino]-4-chlorobenzo[*g*]-phthalazine (6). The less retained fraction ($R_f = 0.78$) afforded 133 mg (36%) of a yellow solid with mp 136–138 °C. Anal. calcd for $C_{16}H_{16}N_3O_2Cl \cdot H_2O$: C, 57.23; H, 5.36; N, 12.52; Cl, 10.00. Found: C, 57.41; H, 5.24; N, 12.38; Cl, 10.78; IR (Cl_3CH, cm^{-1}) 3500–3000, 1520, 1450, 1370, 1300; ^{1}H NMR (CD_3OD): δ 8.46 (s, 1H, H-10), 8.28 (s, 1H, H-5), 7.91 (m, 2H, H-6, H-9), 7.58 (m, 2H, H-7, H-8), 3.83 (t, 2H, H-4′), 3.77 (t, 2H, H-3″), 3.73 (t, 2H, H-2′), 3.65 (t, 2H, H-1′); ^{13}C NMR (CD_3OD): δ 155.93 (C-1), 146.81 (C-4), 136.16 (C-9a), 135.99 (C-5a), 130.36 (C-6, C-9), 130.24 (C-7, C-8), 126.74 (C-5), 124.54 (C-10), 123.86 (C-4a), 119.39 (C-10a), 74.00 (C-3′), 70.54 (C-2′), 62.73 (C-4′), 43.37 (C-1′); MS (FAB), *m/z* (%): 319 (MH^+, 5), 272 (10), 256 (12), 229 (100).

4.6. Synthesis of the Cu(II) dinuclear complexes

4.6.1. General method. A solution of 0.30 mmol of copper(II) perchlorate hexahydrate in dry methanol (2 mL) was added dropwise over a solution of the corresponding ligand (0.15 mmol) in 2 mL of the same solvent. A green precipitate slowly appeared during the addition. After 30 min stirring at rt, the solid complex was separated by centrifugation and exhaustively dried by heating under vacuum.

4.6.2. Complex 7 $\{[Cu_2L^1](OH)_2\ (ClO_4)_2 \cdot CH_3OH\}$. Yield 100 mg, 74%. Mp 200–205 °C. Anal. calcd for $C_{31}H_{38}Cl_2\ Cu_2N_6O_{13}$: C, 41.33; H, 4.22; N, 9.33; Cl, 7.88. Found: C, 41.82; H, 4.37; N, 9.79; Cl, 7.92; IR (KBr, cm^{-1}) 3400, 3280, 1551, 1506, 1144, 1121, 1109, 1086, 626; MS (FAB), *m/z* (%): 901 (MH^+, 0.01), 870 (0.5), 771 (0.5), 735 (4.5), 671 (4), 635 (4), 571 (82), 509 (L^1H^+, 100).

4.6.3. Complex 8 $\{[Cu_2L^2](OH)(ClO_4)_3 \cdot 2H_2O\}$. Yield 54 mg, 39%. Mp > 300 °C. Anal. calcd for $C_{20}H_{33}Cl_3\ Cu_2N_6O_{17}$: C, 27.74; H, 3.81; N, 9.71, Cl, 12.31. Found: C, 27.61; H, 3.63; N, 9.40, Cl, 12.08; IR (KBr, cm^{-1}) 3526, 3403, 3315, 3226, 1651, 1627, 1578, 1551, 1120, 1108, 1088, 940, 636, 626, 392, 324; MS (FAB), *m/z* (%): 865 (MH^+, 0.1), 765 (0.2), 665 (1.5), 611 (1.5), 447 (5.5), 385 (L^2H^+, 100).

4.6.4. Complex 9 {[$Cu_2(L^3)_3$] $(ClO_4)_4 \cdot 2CH_3OH$}. A careful concentration of the reaction mixture to half volume, followed by refrigeration during 24 h, and further centrifugation yielded 45 mg (10%) of the brilliant green complex. Anal. calcd for $C_{62}H_{86}Cl_4\ Cu_2N_{12}O_{30}$: C, 42.58; H, 4.92; N, 9.61, Cl, 8.12. Found: C, 42.27; H, 4.98; N, 9.18, Cl, 8.59; IR (KBr, cm^{-1}) 3600–3100, 3320, 1630, 1350, 1110, 900, 760, 630; MS (FAB), *m/z* (%): 1748 (MH^+, 0.1), 1362 (0.1), 1263 (8), 1099 (0.5), 999 (2.5), 898 (2), 837 (0.8), 773 (7), 387 (L^3H^+, 100).

4.7. Synthesis of the Zn(II) dinuclear complex 10 {[$Zn_2L^3_4$]$Cl_4 \cdot H_2O$}

A solution of 0.36 mmol of anhydrous zinc(II) chloride in methanol (2.5 mL) was added dropwise over a solution of the corresponding ligand (0.18 mmol) in methanol (2 mL). After 30 min stirring at rt, the solid complex was separated by filtration and exhaustively dried by heating under vacuum. Yield 80 mg, 21%. Mp 185–189 °C. Anal. calcd for $C_{80}H_{106}O_{17}\ N_{16}Zn_2Cl_4$: C, 52.32; H, 5.82; N, 12.20; Cl, 7.72. Found: C, 51.84; H, 5.81; N, 12.41; Cl, 7.45; IR (KBr, cm^{-1}) 3357, 1628, 1579, 1132, 1102, 1032, 362, 293, 283, 181, 136; ^1H NMR (DMSO-d_6): δ 8.97 (s, 2H, H-5, H-10), 8.22 (m, 2H, H-6, H-9), 7.84 (m, 2H, H-7, H-8), 3.85 (s, 4H, H-4′), 3.76 (t, 4H, H-2′), 3.72 (m, 4H, H-3′), 3.65 (t, 4H, H-1′); ^{13}C NMR (DMSO-d_6): δ 148.28 (C-1, C-4), 133.80, 129.72, 128.82, 125.24, 117.62 (C aromat.), 72.23 (C-3′), 67.84 (C-2′), 60.11 (C-4′), 41.70 (C-1′); MS (FAB), *m/z* (%): 1821 ($MH^+ - H_2O$ (0.1), 1749 (0.1), 1719 (0.2), 1672 (0.1), 1295 (0.2), 1223 (0.3), 1160 (0.1), 773 (7), 387 (L^3H^+, 100).

4.8. Biological Tests

4.8.1. Parasite strain, culture and trypanocidal in vitro studies. The Maracay strain of *T. cruzi* was isolated from a clinical specimen at the Institute of Malariology and Environmental Health in Maracay (Venezuela). Epimastigotes were cultivated in liquid Trypanosoma medium (MTL) supplemented with 10% inactivated foetal calf serum (IFCS, Sebak) at 28 °C. To obtain the parasite suspension for the trypanocidal assay, the epimastigote culture (in exponential growth phase) was concentrated by centrifugation at 1500 rpm for 10 min and the number of flagellates were counted in an haemocytometric chamber and distributed into aliquots of 2×10^6 parasites/mL.

The compounds were dissolved in methanol (Panreac, Barcelona, Spain) at a concentration of 1%, after assayed as non-toxic and without inhibitory effects on the parasite growth. The compounds were dissolved in the culture medium, and the dosages used were the following: 25, 10, and 1 μg/mL. The effect of each compound against epimastigote forms, as well as the concentrations, was evaluated at 24, 48 and 72 h, using a Neubauer haemocytometric chamber.

4.8.2. Cell culture and cytotoxicity tests. Vero cells (Flow) were grown in minimal essential medium (MEM) (Gibco) supplemented with 10% IFCS and adjusted to pH 7.2, in a humidified 95% air–5% CO_2 atmosphere at 37 °C for 2 days. In the test for cytotoxicity, cells were placed in 25 mL cone-based bottles (Steriling) and centrifuged at 1500 rpm for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1×10^5 cells/mL. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 μL per well, and incubated for 2 days at 37 °C in humid atmosphere enriched with 5% CO_2. The medium was removed, and the fresh medium was added together with the product to be studied (at concentrations of 25, 10 and 1 μg/mL). The cultures were incubated for 72 h. The vital stain Trypan blue (0.1% in phosphate buffer) was used to determine cell viability. The number of dead cells was recorded, the percent viability calculated in comparison to that of the control culture, and the IC_{50} obtained by linear regression analysis from the K_c values at the employed concentrations.

4.8.3. Transformation of epimastigotes to metacyclic forms. To induce metacyclogenesis, parasites were cultured at 28 °C in modified Grace's medium (Gibco) for 12 days according to the methods described by Osuna et al.[29] Twelve days after cultivation at 28 °C, metacyclic forms were counted in order to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

4.8.4. Amastigote-Vero cells assay. The experimental procedure was started with Vero cells maintained in MEM in a humidified 95% air–5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 1×10^5 cells/well in 24-well microplates (NUNC) with rounded coverslips on the bottom and cultivated for 2 days.

Vero cells cultured as above were infected in vitro with *T. cruzi* metacyclic forms at a ratio of 10:1. The drugs (5 μg/mL concentrations) were added immediately after infection and were incubated for 3 h at 37 °C in a 5% CO_2. The non-phagocytosed parasites and the drugs were removed by washing and then the infected cultures were grown for 8 days in fresh medium. Fresh culture medium was added every 48 h.

In all cases, the drug activity was determined from the percentage of infected cells and number of amastigotes per Vero cell infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected Vero cells and the mean number of amastigotes per infected cell were determined by analysing more than 100 host cells distributed in randomly chosen microscopic fields. Values are means of four separate determinations. The number of trypomastigotes in the medium was determined as described previously.[30]

4.8.5. SOD enzymatic inhibition by the alkylaminobenzo[g]phthalazines 1, 2 and 3. The parasites cultured as described above were suspended (0.5–0.6 g wt mL^{-1}) in 3 mL of buffer 1 (0.25 M sucrose, 25 mM Tris–HCl, 1 M EDTA, pH 7.8) and disrupted by 3 cycles of sonic disintegration, 30 s each at 60 V. The sonicated

ıomogenate was centrifuged at 1500*g* for 10 min at ł °C, and the pellet was washed three times with ice-cold ŚTE buffer 1, for a total supernatant fraction of 9 mL. This fraction was centrifuged (2500*g* for 10 min at ł °C), the supernatant collected and solid ammonium ulfate added. The protein fraction, which precipitated ɔetween 35% and 85% salt concentration, was centri-ıuged (9000*g* for 20 min at 4 °C), redissolved in 2.5 mL ɔf 20 mM potassium phosphate buffer (pH 7.8) contain-ng 1 mM EDTA (buffer 2) and dialysed in a Sephadex G-25 column (Pharmacia, PD 10), previously balanced vith buffer 2, bringing it to a final volume of 3.5 mL fraction of the homogenate).[31] The protein concentra-ions were determined by the Bradford method.

ron superoxide dismutase (Fe–SOD) activity was deter-nined by NAD(P)H oxidation according to Paoletti and Mocali.[32] One unit was the amount of enzyme required o inhibit the rate of NAD(P)H reduction by 50%. CuZnSOD from human erythrocytes used in these as-ays was obtained from Boehringer (Mannheim), while ıll the coenzymes and substrates came from Sigma Chemical Co. Data obtained were analysed according o the Newman–Keuls test.

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fficient Inhibition of Iron Superoxide Dismutase and of *Trypanosoma cruzi* Growth by Benzo[*g*]phthalazine Derivatives Functionalized with One or Two Imidazole Rings.

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Efficient Inhibition of Iron Superoxide Dismutase and of *Trypanosoma cruzi* Growth by Benzo[*g*]phthalazine Derivatives Functionalized with One or Two Imidazole Rings

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The synthesis and trypanosomatic behavior of a new series of 1,4-bis(alkylamino)benzo[*g*]phthalazines **1**−**4** containing the biologically significant imidazole ring are reported. In vitro antiparasitic activity against *Trypanosoma cruzi* epimastigotes is remarkable, especially for compound **2**, whereas toxicity against Vero cells is very low. Conversion of epimastigotes to metacyclic forms in the presence of the tested compounds causes significant decreases in the amastigote and trypomastigote numbers. Fe-SOD inhibition is noteworthy, whereas effect on human Cu/Zn-SOD is negligible.

Introduction

Despite significant advances in the last years, infections and parasitic diseases account for about 25% of the global burden disease.[1] Among them, American trypanosomiasis, also known as Chagas disease, is a potentially fatal, chronic illness that currently affects about 20 million people throughout Mexico and Central and South America and is responsible for about 40000 deaths and 850000 new infections a year.[2,3] It is caused by the hemoflagellate protozoan parasite, *Trypanosoma cruzi*. Additionally, humans can contract the disease by transfusions using contaminated blood of persons who carry the parasite, but do not have active Chagas. The increasing immigration of people from Latin America to other countries is a risk factor for the propagation of Chagas disease in other areas of the world.[4] Because a vast majority of people infected will not develop clinical symptoms for ten to twenty years, blood donors may unknowingly spread the disease.

At present, the chemotherapy developed against trypanosomiasis is unsatisfactory due to the toxicity and limited efficacy of the drugs currently used. Therefore, the development of more effective drugs, particularly against the chronic form, is an urgent priority. Some nitrofurans and nitroimidazoles, like benznidazole, are of variable efficacy in short-term cases, causing anorexia, vomiting, and allergic dermopathy as the major side effects. The chronic infection usually results in cardiac failure and death of the host, and there are no cures for that phase of the disease.[3,5]

The drugs currently used are thought to act by inducing oxidative stress within the parasite through the formation of toxic oxygen metabolites. Benznidazole is capable of generating superoxide radical and hydrogen peroxide, and nifurtimox generates nitro-radicals.[2] However, *Trypanosoma cruzi* possesses a variety of enzymatic antioxidant defenses. Among them, superoxide dismutase (SOD[a]) plays a relevant role.[3] SODs are a group of metal-containing enzymes that have a vital antioxidant role conferred by their scavenging ability for the superoxide anion. Three major classes of SOD have been described on the basis of their prosthetic groups: Fe, Mn, or Cu/Zn. The Fe-SOD, which is found in prokaryotes and some plants, appears to be the enzyme normally associated with trypanosomatides.[6] The chemical reaction of superoxide anion with the prosthetic group of SOD occurs in two steps. The first one begins with the oxidized form of the enzyme (Fe^{3+}) binding $O_2^{\bullet-}$, and follows with protonation and release of molecular oxygen. In the second one, the reduced form (Fe^{2+}) binds a superoxide anion and a proton to liberate hydrogen peroxide, returning to the original oxidized state.[7]

Therefore, compounds with the ability of inhibiting the protective action of SOD are good targets for antiparasitic activity affecting both the growth and survival of parasitic cells. Due to the predominant role of the prosthetic groups, competitive complexation of the metal ion of SOD could be an efficient way of deactivating the antioxidant effect of the enzyme.

Based on all the above, we have previously described[8] the synthesis of two series of 1,4-bis(alkylamino)benzo[*g*]phthalazines with structures **I** and **II** (Figure 1) and also their ability for the formation of dinuclear complexes with transition metal ions. The antiparasitic activity of these compounds against *Trypanosoma cruzi* and their Fe-SOD/activity inhibition were studied. The results obtained showed that compounds **I**, with sp^3 and sp^2 nitrogens at the end of the flexible side-chains, have higher activities than the analogues **II**, with terminal hydroxyl groups. This could be related to the remarkable modification in the geometry of the complexation observed when the side-chains

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[a] Abbreviations: SOD, superoxide dismutase; TLC, thin-layer chromatography; TMS, tetramethylsilane, MEM, minimal essential medium; MTL, liquid *Trypanosoma* medium; IFCS, inactivated fetal calf serum; EDTA, ethylendiaminetetraacetic acid, STE, Tris NaCl EDTA; NAD(P)H, nicotinamide adenine dinucleotide phosphate.

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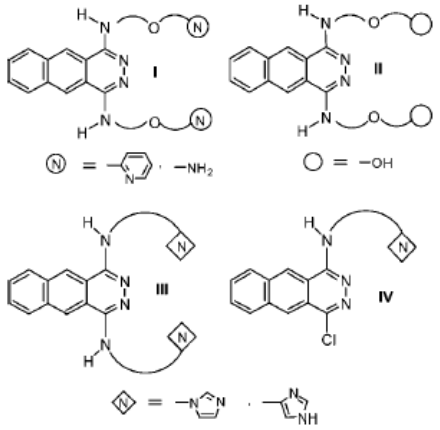


Figure 1. Previous (**I** and **II**) and new models (**III** and **IV**) for the design of benzo[*g*]phthalazine derivatives with potential antiparasitic activity.

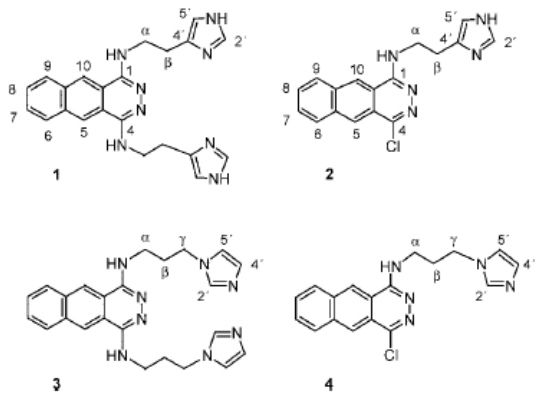


Figure 2. New imidazole containing structures synthesized for testing against SOD and *T. cruzi*.

nitrogens are replaced by oxygens. As a result, dinuclear complexes are not formed.

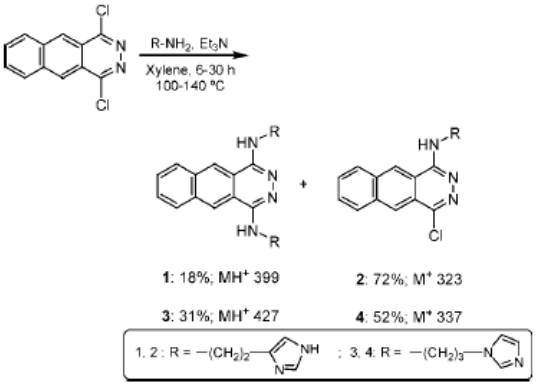
In accordance with those findings and considering both the biological significance of the imidazole ring and the basicity of its nitrogen atoms, we have devised now a new series of benzo[*g*]phthalazine derivatives **III** and **IV** (Figure 1). They have been functionalized at the end of the ethylenic or propylenic side-chains with imidazole rings, linked through carbon or nitrogen. The oxygen atoms present in **I** and **II** have been suppressed in the new design because they are not essential for metal ions complexation.

Results and Discussion

Synthesis. The preparation of compounds **1–4** (Figure 2) was performed from 1,4-dichlorobenzo[*g*]phthalazine as shown in Scheme 1, using similar experimental conditions to those reported by our group for the synthesis of **I** and **II** and other analogues.[8,9]

Thus, nucleophilic substitution at the C-1 and C-4 positions of the starting compound with 2-(imidazol-4-yl)ethylamine under reflux of xylene (140 °C) for 10 h afforded a mixture of the bis- and monoalkylamination products **1** and **2**, in 18 and 72% yield, respectively. In a similar way, but reducing the reflux

Scheme 1. Synthesis of the Mono and Bis(alkylamino)benzo[*g*] phthalazine Derivatives



time to 6 h, the reaction with 3-(imidazol-1-yl)propylamine afforded **3** in 31% yield. Unexpectedly, the corresponding monoalkylamino derivative **4** was obtained in very low yield (6%). To improve these results, the reaction was carried out at 100 °C for 30 h. Under these conditions, compound **4** was obtained in 52% yield. Isolation of the pure compounds from the reaction mixtures was performed by flash chromatography on silica gel with a CCl_3H/MeOH mixture of increasing polarity. The monosubstituted derivatives were eluted faster than their disubstituted analogues. All these compounds were obtained as the free polyamines, with the exception of **1**, isolated as the dihydrochloride, that was filtered through a basic alumina column to give the free amine.

All the compounds synthesized were unequivocally identified on the basis of their EI or FAB mass spectra, and IR, ^{1}H NMR, and ^{13}C NMR spectroscopies. The resonance spectra signals were assigned according to heteronuclear multiple quantum coherence experiments. The mono and bisalkylamination products could be easily differentiated in both ^{1}H and ^{13}C spectra by comparing the H_5/H_{10} and C_1/C_4 signals at rings B and A of the benzo[*g*]phthalazine moiety. Disubstituted compounds exhibited an unique signal for C_1 and C_4, and also for H_5 and H_{10} as a singlet. On the contrary, C_1 and C_4 gave clearly different signals in their monosubstituted analogues, being the carbon linked to the chlorine atom shielded about 9–10 ppm. In a similar way, H_5 and H_{10} appeared as two singlets separated by 0.3 ppm, and the proton in the neighborhood of the chlorine was also shielded with respect to that one coplanar with nitrogen. Neat differences were also observed among the hydrogens at ring C in both types of derivatives because H_6 and H_9 exhibited a symmetrical pattern only in the disubstituted compounds.

The mass spectra of the monosubstituted **2** and **4** showed both the loss of chlorine from the molecular ion, and the presence of fragments containing chlorine. Molecular ions corresponding to the formulas of the proposed structures were found for compounds **1**−**4**.

Biological Evaluation. In a first step, the inhibitory effect of podands **1**−**4** on the in vitro growth of *T. cruzi* epimastigotes was measured at different times following established procedures (see Supporting Information). Results obtained are displayed in Table 1 using benznidazole as the reference drug and including toxicity values against Vero cells. The four compounds tested resulted to be active against epimastigotes. After 72 h of exposure, **1** and **4** show IC_{50} values of 14.2 and 13.7 μM, respectively, close to those found for benznidazole. However,

Table 1. In Vitro Activity of 1,4-Bis(alkylamino)- (**1** and **3**) and 1-(Alkylamino)-4-chlorobenzo-[g]phthalazines (**2** and **4**) on *Trypanosoma cruzi* Epimastigotes

compound	IC_{50} (μM) 24 h	48 h	72 h	toxicity IC_{50}[a] (μM)
benznidazole			15.8	13.6
1	61.1	35.1	14.2	88.7
2	108.5	34.6	<0.3	213.0
3	53.4	34.8	<0.2	69.3
4	51.0	46.9	13.7	145.8

[a] On Vero cells after 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at concentrations employed (2.5, 25, and 125 μM). Note: Average of three separate determinations.

2 and **3** are much more active (IC_{50} <0.3 and 0.2 μM) than **1** and **4**. All the compounds tested are much less toxic against Vero cells than benznidazole. It is worth mentioning that the monosubstitued compound **2** exhibits an inhibitory concentration of 213.0 μM after 72 h of culture. That is 16 times higher than the corresponding value measured for benznidazole (13.6 μM).

Figure 3 illustrates *T. cruzi* propagation in Vero cells (with and without coaddition of the test compounds). When 1×10^5 Vero cells were incubated for 2 days and then infected with 1×10^6 metacyclic forms, the parasites invaded the cells and underwent morphologic conversion to amastigotes within 1 day after infection. During days 1−8, the rate of host-cell infection increased gradually, reaching a 76% of infected cells on day 8. When the four compounds tested were simultaneously added to the infected Vero cells with *T. cruzi* metacyclic forms (10 μM), the infection rate significantly decreased (more than 50%), being especially remarkable the behavior of **2**, **3**, and **4**, with a decrease of the infection of 81, 84, and 74%, respectively, compared with the control substance (Figure 3A).

The average number of amastigotes per infected cell increased on the fourth day of culture, decreasing significantly afterward until day 8 (Figure 3B). This behavior is due to the rupture of the Vero cells with the subsequent release of amastigotes and further transformation into trypomastigotes. The addition of compounds **2** and **3** (10 μM) remarkably diminished the amastigote numbers per infected cell to give reductions of 55 and 44%, respectively, on day 8 with respect to the control culture. The number of trypomastigotes in the medium was 5.3 $\times 10^4$ on day 8, and it was substantially reduced (over 50%) in the presence of **2** and **3** (Figure 3C).

These results prompted us to evaluate the inhibitory effect of **1**−**4** on SOD activity to test their potential as competitors for the metallic ions of the enzyme. We have used epimastigote forms from the Maracay strain of *Trypanosoma cruzi* that excrete Fe-SOD when cultured over calf fetal serum.[10] Data obtained for the inhibition activity of the enzyme are displayed in Table 2.

Significant inhibition values of the enzyme activity are found for all the compounds tested (Table 2). Two of them (**2** and **3**) show a 100% inhibition at 125 μM dose, whereas the monoalkylamino substituted derivative **4** exhibits a 94% inhibition. Compound **4** even gives an 80% inhibition at 2.5 μM concentration, while this value for compounds **1** and **2** fluctuate around 50%. These results could be interpreted considering that the remarkable high activity of compound **2** against epimastigotes of *T. cruzi* obtained in vitro is not only due to the blocking of the metal ion of SOD and that other action mechanisms could be also involved in the trypanosomatic activities observed.

In any case, a good degree of activity against the SOD of the parasite would be of no value if the same pattern was found for human SOD without any discrimination. Therefore, we have also tested the effect of compounds **1**−**4** over CuZn-SOD from human erythrocytes (Table 3).

The results obtained show that inhibition percentages at the higher dosages are very small in all cases for human SOD, and

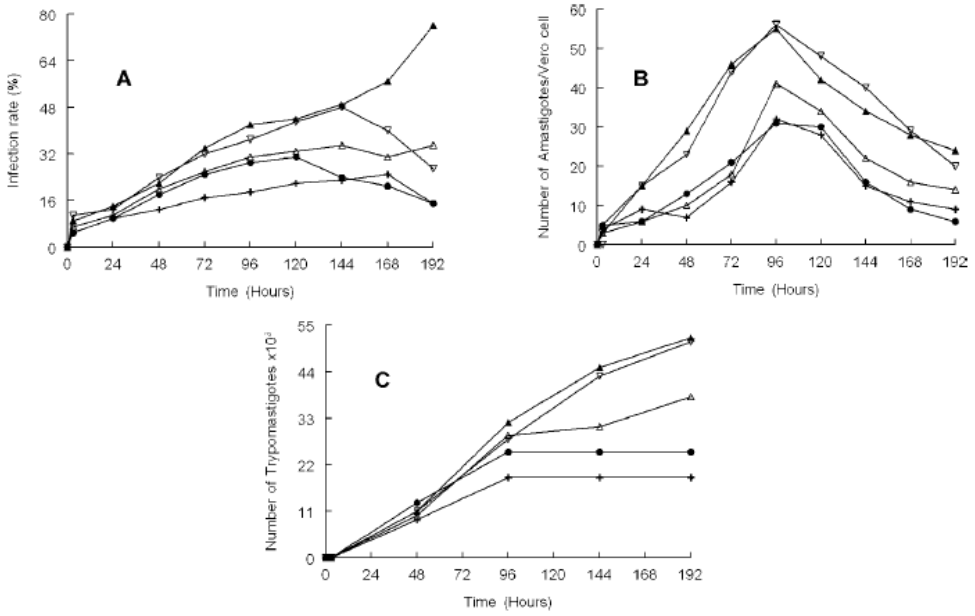


Figure 3. Effect of compounds **1**−**4** on the infection rate and *T. cruzi* growth. (A) Rate of infection. (B) Mean number of amastigotes per infected Vero cell. (C) Number of trypomastigotes in the culture medium. -▲-, control; -Δ-, **1**; -+-, **2**; -●-, **3**; -▽-, **4** (10 μM concentration). The values are means of three separate experiments.

Table 2. In Vitro Inhibition (%) of Fe-SOD in *Trypanosoma cruzi* Epimastigotes: 16.20 ± 1.69 Unit/mg Protein[a]

cmpd	2.5 μM	25 μM	125 μM
1	46	53	86
2	50	91	100
3	32	42	100
4	80	93	94

[a] Values are the average of five separate determinations. Differences between the activities of the control homogenate and the one incubated with the tested compounds were obtained according to the Newman−Keuls test.

Table 3. CuZn-SOD Activity Inhibition (%) in Human Erythrocytes: 23.36 ± 4.21 Unit/mg Protein

cmpd	2.5 μM	25 μM	125 μM
1	0	5	6
2	0	0	11
3	2	12	18
4	7	22	27

all the compounds are nearly inactive at the lower dose employed (2.5 μM) in the test. These results enhance the potential antiparasitical interest of the alkylaminobenzo[*g*]phthalazine derivatives studied in this work.

In conclusion, we have prepared a new series of benzo[*g*]phthalazine derivatives **1**−**4** containing one or two imidazole rings as the key structural feature. These compounds exhibit excellent antiparasitic in vitro properties against *T. cruzi* epimastigotes and remarkably low toxicity against Vero cells. Furthermore, they are nearly inactive against human SOD, but active against Fe-SOD, although inhibition of this latter enzyme may not be the main factor for the activities observed. Although the results obtained here are very promising, further studies are necessary to determine the mechanism involved in the activity pattern observed for compounds **1**−**4**.

Experimental Section

The starting amines: 3-(imidazol-1-yl)propylamine and 2-(imidazol-4-yl)ethylamine (histamine) were purchased from Aldrich and used without further purification. 1,4-Dichlorobenzo[*g*]phthalazine was obtained from 2,3-naphthalenedicarboxylic acid following a method previously described.[11]

Synthesis of 1 and 2. A solution of 1,4-dichlorobenzo[*g*]phthalazine (386 mg, 1.56 mmol), 2-(imidazol-4-yl)ethylamine (692 mg, 6.23 mmol), and 473 mg (4.68 mmol) of triethylamine in xylene (60 mL) was heated at 120–130 °C for 10 h. The reaction mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The solid residue was purified by column chromatography with a polarity increasing chloroform/methanol mixture as eluent to obtain both products.

1,4-Bis[2-(imidazol-4-yl)ethylamino]benzo[*g*]phthalazine (1). The last eluted fraction afforded 239 mg of the dihydrochloride of **1** as a yellow solid, mp 218−220 °C (d). ^{1}H NMR (CD_3OD): δ 8.98 (s, 2H, H-5, H-10), 8.20 (m, 2H, H-6, H-9), 7.83 (m, 2H, H-7, H-8), 7.81 (s, 2H, H-2′), 7.02 (s, 2H, H-5′), 3.79 (t, 4H, Hα), 3.11 (t, 4H, Hβ). MS (FAB) *m*/*z* (%): 471 (M^+, 3), 436 (4), 399 (M^+ + 1 − 2HCl, 25), 398 (19), 317 (7), 304 (14), 223 (13), 210 (8), 179 (13). Anal. ($C_{22}H_{22}N_8 \cdot 2HCl$) C, H, N, Cl.

Flash column chromatography treatment of the hydrochloride of **1** on basic alumina using a polarity increasing mixture of chloroform/methanol as eluent gave 108 mg (18%) of free **1** as a yellow solid (R_f = 0.28, $CHCl_3$/MeOH/NH_4OH, v/v, 7:3:1), mp 123–125 °C. ^{1}H NMR (DMSO-d_6): δ 11.82 (br s, 2H, NH), 8.78 (s, 2H, H-5, H-10), 8.11 (m, 2H, H-6, H-9), 7.69 (m, 2H, H-7, H-8), 7.54 (s, 2H, H-2′), 6.88 (1 s, 2H, H-5′), 3.67 (t, 4H, Hα), 2.94 (t, 4H, Hβ). MS (FAB) *m*/*z* (%): 399 (M^+ + 1, 100), 398 (M^+, 38), 331 (10), 317 (21), 304 (5), 223 (9), 210 (12), 179 (11). Anal. ($C_{22}H_{22}N_8 \cdot 3H_2O$) C, H, N.

1-[2-(Imidazol-4-yl)ethylamino]-4-chlorobenzo[*g*]phthalazine (2). The less-retained fraction (R_f = 0.62) afforded 381 mg (72%) of **2** as a yellow solid, mp 240–245 °C (d). ^{1}H NMR (DMSO-d_6): δ 9.05 (s, 1H, H-10), 8.74 (s, 1H, H-5), 8.35 (m, 1H, H-9), 8.16 (m, 1H, H-6), 7.78 (m, 2H, H-7, H-8), 7.68 (s, 1H, H-2′), 6.92 (s, 1H, H-5′), 3.79 (t, 2H, Hα), 2.98 (t, 2H, Hβ). MS (FAB) *m*/*z* (%) 323 (M^+, 15), 256 (29), 242 (74), 229 (92), 213 (4), 179 (100), 151 (28). Anal. ($C_{17}H_{14}N_5Cl \cdot 4H_2O$) C, H, N.

Synthesis of 3 and 4. A solution of 1,4-dichlorobenzo[*g*]phthalazine (400 mg, 1.61 mmol), 3-(imidazol-1-yl)propylamine (0.77 mL, 6.45 mmol), and 489 mg (4.84 mmol) of triethylamine in xylene (25 mL) was refluxed for 6 h. Work-up of the reaction mixture by the same procedure used for compounds **1** and **2** allowed the isolation of the two substitution products.

1,4-Bis[3-(imidazol-1-yl)propylamino]benzo[*g*]phthalazine (3). The last eluted fraction (R_f = 0.23, Cl_3CH/MeOH/NH_4OH, v/v, 7:3:0.1) afforded 215 mg (31%) of a yellow solid, which was identified as **3**, mp 122–124 °C. ^{1}H NMR (DMSO-d_6): δ 8.81 (s, 2H, H-5, H-10), 8.14 (m, 2H, H-6, H-9), 7.71 (m, 2H, H-7, H-8), 7.70 (s, 2H, H-2′), 7.20 (s, 2H, H-5′), 6.89 (s, 2H, H-4′), 4.11 (t, 4H, Hγ), 3.39 (t, 4H, Hα), 2.13 (q, 4H, Hβ). MS (FAB) *m*/*z* (%) 427 (M^+ + 1, 100), 426 (M^+, 19), 359 (47), 263 (22), 250 (11), 179 (13). Anal. ($C_{24}H_{26}N_8 \cdot 3H_2O$) C, H, N.

1-[3-(Imidazol-1-yl)propylamino]-4-chlorobenzo[*g*]phthalazine (4). The less-retained fraction (R_f = 0.44, Cl_3CH/MeOH, v/v, 4:1) afforded 31 mg (6% yield) of **4** as a yellow solid: mp 217–218 °C. ^{1}H NMR (DMSO-d_6): δ 9.03 (s, 1H, H-10), 8.74 (s, 1H, H-5), 8.36 (m, 1H, H-9), 8.17 (m, 1H, H-6), 7.97 (m, 1H, H-8), 7.80 (m, 1H, H-7), 7.71 (s, 1H, H-2′), 7.25 (s, 1H, H-5′), 6.91 (s, 1H, H-4′), 4.13 (t, 2H, Hγ), 3.55 (t, 2H, Hα), 2.18 (q, 2H, Hβ). MS (EI) *m*/*z* (%) 337 (M^+, 12), 302 (21), 221 (2), 234 (70), 194 (22), 179 (100), 151 (18). Anal. ($C_{18}H_{16}N_5Cl \cdot 2H_2O$) C, H, N, Cl. The yield of compound **4** was substantially improved by increasing the reaction time to 30 h and diminishing the reaction temperature to 100 °C. Under these conditions, **4** was obtained in 52% yield, while compound **3** was not detected.

Biological Tests. The biological evaluation tests have been performed according to procedures previously described in the literature or developed by the authors following standard methods. Experimental conditions used for trypanocidal in vitro studies, cell culture and cytotoxicity tests, transformation of epimastigotes to metacyclic forms,[12] amastigote Vero cells assays,[13] and also Fe-SOD and human SOD enzymatic ihhibition studies[14,15] are explained in detail in the Supporting Information.

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Supporting Information Available: Details on chemical procedures and instruments used for isolation and identification of the newly synthesized compounds, combustion analysis, and also on IR and ^{13}C NMR data. Methodology followed in the biological evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Copper (II) Complexes of [1,2,4]Triazolo [1,5-a]Pyrimidine Derivatives

as

Potential Anti-Parasitic Agents

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Copper (II) Complexes of [1,2,4]Triazolo [1,5-a]Pyrimidine Derivatives as Potential Anti-Parasitic Agents

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Abstract: Anti-proliferative effects are described for newly synthesised copper (II) complexes of two triazolo-pyrimidine derivatives (1,2,4-triazolo-[1,5-a]pyrimidine, **tp**, and 5,7-dimethyl 1,2,4-triazolo-[1,5-a]pyrimidine, **dmtp**) against to *Trypanosoma cruzi* and *Leishmania (Viannia) peruviana*. Of the compounds assayed, those that presented the ligand **tp** and auxiliary ligand 1,10-phenanthroline (**C24b**, **C49)** were most highly active against to *T. cruzi* with IC_{50} within the range of the reference drug benznidazole. These compounds, together with **C35** were the most effective against *L. (V.) peruviana* with an IC_{50} greater than that presented by reference drugs (Pentostam and Glucantim). These compounds were not toxic to the host cell. IC_{25} diminished the infection capacity and severely reduced the multiplication of intracellular forms of *T. cruzi*, and *L. (V.) peruviana*. In the case of *T. Cruzi*, the transformation to trypomastigote was seriously depressed. Copper (II) complexes **C24b**, **C49** and **C35**, acted on the energy metabolism of the parasites at the level of the NAD^+/NADH balance and at the level of the organelle membranes, causing degradation and cell death.

Keywords: *Trypanosoma* cruzi, Leishmania *(Viannia) peruviana*, triazolo-pyrimidine derivatives, anti-parasitic agents, action mechanism.

INTRODUCTION

Parasitic diseases affect hundreds of millions people around the world, mainly in underdeveloped countries. Chagas' disease (*Trypanosoma cruzi*) and leishmaniasis (*Leishmania* spp.) alone are responsible for an infected population of nearly 30 million, with more than 400 million at risk [1]. *Trypanosoma cruzi* is a blooded-flagellated protozoan responsible of the Chagas' disease, a vectorial-transmission sickness which passes through two successive stages, the acute and the chronic phase. This latter form affects more than 16 million people, especially in Latin America, and represents the first cause of cardiac lesions in young, economically productive adults in Latin American countries where the disease is endemic [2]. Leishmaniasis is a vector-borne disease caused by obligate intramacrophage protozoa which is spread by the bite of infected sandflies. This disease has a broad spectrum of clinical syndromes ranging from self-healing cutaneous lesions to lethal visceral consequences, and it is prevalent on four continents and considered endemic in 88 countries [3, 4].

Since parasitic protozoa are eukaryotic, they share many common features with their mammalian host, making the development of effective and selective drugs a hard task. Despite the great effort to discover single targets that afford selectivity, many of the drugs used today have serious side effects. Diseases caused by *Trypanosomatidae* share a similar state regarding drug treatment [5]. Drugs currently used in the treatment of Chagas' disease include two nitroaromatic heterocyles, Nifurtimox (4-(5-nitrofurfurylidenamino)-3-methylthiomorpholine-1,1-dioxide) (Nfx, recently discontinued by Bayer) and Benznidazole (*N*-benzyl-2- (2-nitro-1*H*-imidazol-1-yl) acetamide) (Bnz, Rochagan, Roche), introduced empirically over three decades ago. Both drugs are active in the acute phase of the disease but efficacy is very low in the established chronic phase. Moreover, differences in drug susceptibility among different *T. cruzi* strains lead to varied parasitological cure rates according to the geographical area [6]. The drugs of choice for the treatment of leishmaniasis are sodium stibolgluconate (Pentostam), meglumine antimoniate (Glucantime), pentamidine (1,5-di-(4-amidinophenoxy)pentane) (Ptd) and liposomal amphotericin B, but these sometimes meet with failure [4]. Currently, WHO/TDR is developing a research programme with Miltefosine (hexadecylphosphorylcholine), a very promising leishmanocidal drug, but new therapeutic alternatives should be found in order to increase the pharmaceutical arsenal [7, 8]. The specific chemotherapy currently employed for the treatment of these diseases has serious limitations due to lack of effectiveness, toxic side effects, growth of drug-resistance, and high costs. Thus, it is urgent to develop new chemotherapeutic agents that are more effective, safe, and accessible [9].

For the development of more effective and reliable agents, a large number of compounds bearing nitrogen-containing fused heterocyclic skeletons, such as 4-anilino-

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quinazolines, pyrazolopyrimidines, triazolopyrimidines, pyrrolopyrimidines, pyrazolopyridazines, and imidazopyrazines, have been discovered and many of them exhibit excellent anti-cancer, anti-microbial and anti-protozoal activity [10-14].

Recently, 1,2,4-triazolo[1,5-*a*]pyrimidines have aroused increasing attentions from chemical and biological standpoints since they have proven to be promising due to their potential activities, mainly against parasites [15-18].

In view of the above findings, and as a continuation of our effort [19, 20] to identify new candidates in designing potent, selective, and less toxic antitrypanosomatids, we report here of some new 1,2,4-triazolo [1,5-*a*]pyrimidines and several copper (II) metal complexes of these compounds, which have been developed and examined for antiproliferative *in vitro* activity against *T. cruzi* (epimastigote, amastigote and trypomastigote forms) and *L. (V.) peruviana*. (promastigote and amastigote forms). Unspecific mammal cytotoxicity of the most active compounds was evaluated *in vitro*, and less toxic derivatives have been submitted to a more thorough study of their possible action mechanism.

EXPERIMENTAL

Chemistry

The compounds assayed have been synthesised in the Department of Inorganic Chemistry of the Faculty of Science of the University of Granada (Spain) while the triazolopyrimidine derivatives (**tp**: 1,2,4-triazolo-[1,5-a]pyrimidine and **dmtp**: 5,7-dimethyl-1,2,4-triazolo-[1,5-a]pyrimidine) were purchased from Aldrich (Figs. **1** and **2**).

$[Cu(H_2O)(phen)(tp)_2](ClO_4)_2{\cdot}H_2O$ (**C24b**) and $[Cu(H_2O)(phen)(dmtp)_2](ClO_4)_2$ (**C33c**): Firstly, a green solution was obtained by mixing an aqueous solution of $Cu(ClO_4)_2{\cdot}6H_2O$ (1mmol, 10mL) and another one of the appropriate ligand **tp** (**C24b**) or **dmtp** (**C33c**) (1mmol, 10mL). After adding 1,10-phenanthroline dissolved in ethanol (1mmol, 5mL), a green precipitate of $Cu(phen)_2(ClO_4)_2{\cdot}H_2O$ immediately appeared, which was filtered and then washed with water and ethanol. The filtered solution was allowed to evaporate at room temperature and, after three days, blue crystals of **C24b** and **C33c** suitable for X-ray studies were isolated.

$[Cu(NO_3)(H_2O)(phen)(tp)](NO_3)$ (**C35**): The complex was obtained by mixing three solutions, 10 mL each, one containing 1 mmol of $Cu(NO_3)_2{\cdot}3H_2O$, another 1 mmol of **tp** and the third 1 mmol of 1,10-phenanthroline. The first two solutions were aqueous and the last was ethanolic. After a few days, blue crystals suitable for X-ray study were obtained.

$[Cu(H_2O)_2(en)(tp)_2](ClO_4)_2$ (**C38**) and $[Cu(H_2O)_2(en)(dmtp)_2](ClO_4)_2$ (**C41**): Firstly two aqueous solutions of $Cu(ClO_4)_2{\cdot}6H_2O$ (1mmol, 10mL) and the appropriate ligand (**tp** (**C38**) or **dmtp** (**C41**)) (1mmol, 10mL) were mixed. After adding 0.5 mmol (34 µL) of ethylenediamine, it was left to stand at room temperature, and blue crystals suitable for X-ray studies were isolated.

$[Cu_2(OH)(H_2O)_{2.5}(tp)_5](ClO_4)_3(H_2O)_{1.5}$ (**C49**): Two solutions of $Cu(ClO_4)_2{\cdot}6H_2O$ (1mmol, 10ml) and **tp** (1mmol, 10ml) in 0.01M NaOH were mixed. The mixture was allowed to evaporate at room temperature and, after one week, light blue crystals suitable for x-ray studies resulted. In the all cases, the experimental chemical-analysis data showed excellent agreement with the theoretical ones.

Fig. (**1**) shows the molecular structure of **tp** and the crystal structures for copper(II) complexes containing it. Fig. **2** shows the basic skeleton of **dmtp** and the crystal structures for copper(II) complexes containing it. (For simplicity, we have omitted the non-coordinated water molecules and non-coordinated inorganic anions in the crystal structures of the complexes).

All assayed compounds (**C24b**, **C33c**, **C35**, **C38**, **C41**, **C49**) were copper(II) complexes containing a 1,2,4-triazolo [1,5-a]pyrimidine derivative (**tp** or **dmtp**), a classical N-donor chelate (ethylenediamine, **en** or 1,10-phenanthroline, **phen**) and inorganic anions (NO_3^- or ClO_4^-) as auxiliary ligands. Spectroscopic and structural characterization (X-ray diffraction studies) of these new complexes have been described [21].

All compounds assayed, both for, the assays against the *T. cruzi* epimastigotes, as well as, against the *L. (V.) peruviana* promastigotes, were dissolved in dimethyl sulfoxide (DMSO, Panreac, Barcelona, Spain) at a concentration of 0.1%. Afterwards, this was assayed as non-toxic and without inhibitory effects on parasite growth, as previously demonstrated [19]. The compounds were dissolved in the culture medium, and the dosages used were: 100, 50, 25, 10 and 1 µM. The effect of each compound at these concentrations against those of the two forms of the parasites were evaluated at 24, 48 and 72 h, using a Neubauer haemocytometric chamber. The inhibitory effects were expressed as IC50, i.e. the concentration required to give 50% inhibition, calculated by linear regression from the *K*c values at the concentrations used.

Parasite Strain, Culture

The Maracay strain of *T. cruzi* was isolated at the Institute of Malariology and Environmental Health in Maracay (Venezuela). Epimastigote forms were obtained in biphasic blood-agar NNN medium (Novy-Nicolle-McNeal) supplemented with Minimal Essential Medium (MEM) and 20% inactivated foetal bovine serum and afterwards reseeded in a monophasic culture (MTL), following the method of Luque *et al.* [22].

For anti-leishmania assays, *L. (V.) peruviana* (MHOM/PE/1984/LC26) were used. The promastigote forms were cultured at 28ºC in RPMI 1640 medium (Flor Laboratories, Irvine, UK) in Roux flasks (Corning, USA) of 75 cm^2 in surface area, supplemented with 10% inactivated calf serum following the methodology of Gonzalez *et al.* [23].

Cell Culture and Cytotoxicity Tests

Vero cells (Flow) were grown in MEM (Gibco) supplemented with 10% inactivated foetal calf serum and adjusted to pH 7.2, in a humidified 95% air-5% CO_2 atmosphere at 37°C for 2 days. For the cytotoxicity test, cells were placed in 25-ml colie-based bottles (Sterling), and centrifuged at

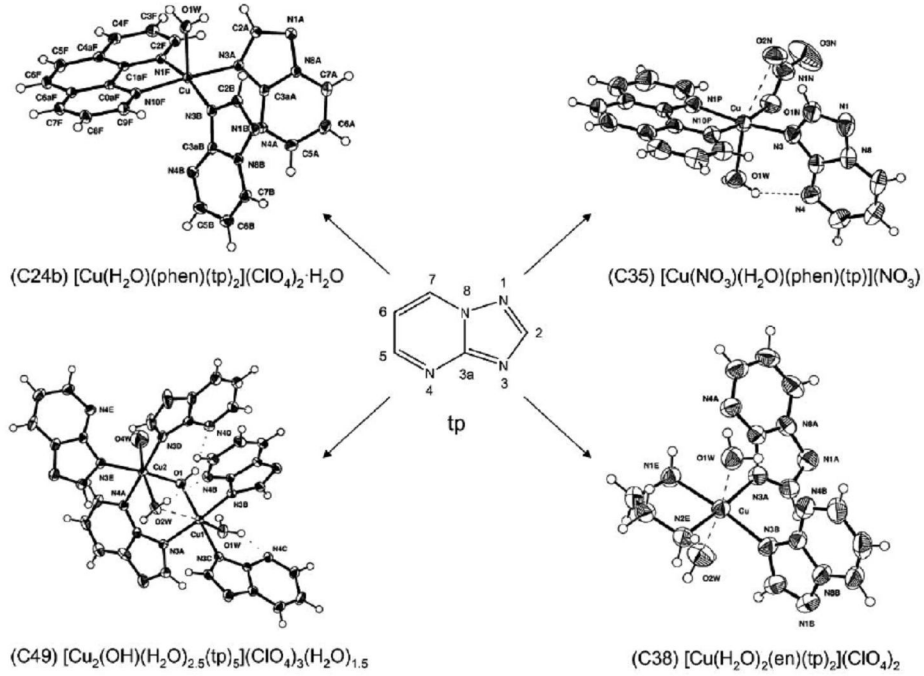

Fig. (1). The molecular structure of **tp** and the crystal structures for copper(II) complexes containing it.

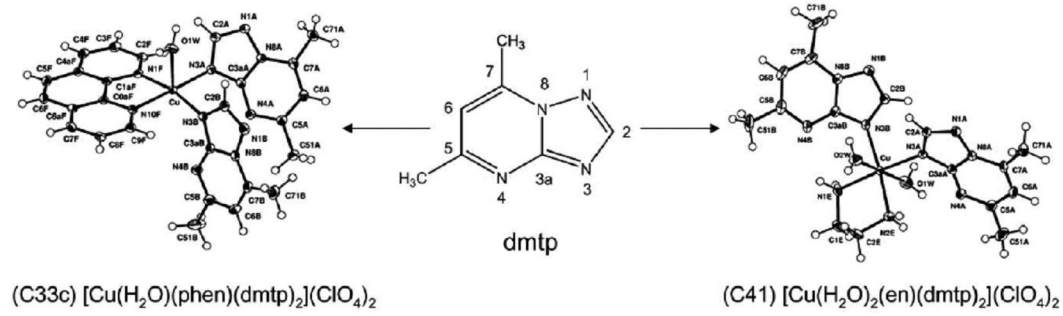

Fig. (2). The molecular structure of **dmtp** and the crystal structures for copper(II) complexes containing it.

100 ***g*** for 5 min. The culture medium was removed, and fresh medium was added to a final concentration of 1 x 10^5 cells/ml. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 µl/well and incubated for 2 days at 37°C in humid atmosphere enriched with 5% CO_2. The medium was removed, and the fresh medium was added together with the product to be studied (at a concentration 100, 50, 25, l0 and 1 µM). The cultures were incubated for 72 h. The vital stain trypan blue (0.1% in phosphate buffer) was used to determine cell viability. The number of dead cells was recorded, and the percent viability was calculated in comparison to that of the control culture, and the IC_{50} calculated by linear regression analysis from the Kc values at the concentrations employed.

Macrophage line J774.2 (ECACC number 91051511) was obtained from a tumour in a female BALB/c rat in 1968. Macrophages were kept in the laboratory by cryopreservation in liquid nitrogen and then by successive subcultures in RPMI medium. For the cytotoxicity test, macrophages were placed in 25mL cone-based bottles (Sterling), and centrifuged at 100 ***g*** for 5 min. The culture medium was removed and Hank's solution was added to a final concentration of 10^5 cells/mL. This cell suspension was distributed in a way similar to that described above for the Vero cells.

Transformation of Epimastigote to Metacyclic Forms

As a means of inducing metacyclogenesis, parasites were cultured at 28°C in modified Grace's medium (Gibco) for 12

days as described previously[31]. Twelve days after cultivation at 28°C, metacyclic forms were counted in order to infect Vero cells. The proportion of metacyclic forms was around 40% at this stage.

Amastigote-Cell Assay

Vero and J774.2 Macrophage cells were cultured in MEM medium in a humidified 95% air-5% CO_2 atmosphere at 37°C. Cells were seeded at a density of 1 x 10^5 cells/ well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultivated for 2 days. Afterwards, the cells were infected *in vitro* with metacyclic forms of *T. cruzi* and promastigotes forms of *L. (V.) peruviana*, at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection, and were incubated for 6 h at 37°C in a 5% CO_2. The non-phagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 8 days in fresh medium. Fresh culture medium was added every 48 h.

The drug activity was determined from the percentage of infected cells and the number of amastigotes per cells infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing more than 100 host cells distributed in randomly chosen microscopic fields. Values are the means of four separate determinations. In the case of *T. cruzi*, the number of trypomastigotes in the medium was determined as described previously [24].

Ultrastructural Alterations

The parasites, at a density of 5 x 10^6 cells/ml, were cultured in their corresponding medium, containing the drugs at the IC_{25} concentration. After 72 h, the cultures were centrifuged at 400 ***g*** for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Pellets were prepared for transmission-electron microscopy following the technique of Luque *et al.* [22].

Metabolite Excretion

Cultures of *T cruzi* epimastigotes and *L. (V.) peruviana* promastigotes (initial concentration 5 x 10^5 cells/ml) received IC_{25} of the triazolo-pyrimidine compounds (except for control cultures). After incubation for 72 h at 28° C, the cells were centrifuged at 400 ***g*** for l0 min. The supernatants were collected to determine excreted metabolites by nuclear magnetic resonance spectroscopy (^{1}H-NMR) as previously described by Fernandez-Becerra *et al.* [25]. The chemical displacements were expressed in parts per million (ppm), using sodium 2,2dimethyl-2-silapentane-5-sulfonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Fernandez-Becerra *et al.* [25].

RESULTS AND DISCUSSION

Previous studies indicate that newly synthesised 1,2,4-triazolo[1,5-a]pyrimidine derivatives are promising chemotherapeutic drugs in the treatment of diseases caused by member of the Trypanosomatidae [19,20]. Currently, we are evaluating the toxic activity of triazolo-pyrimidine compounds and several of its copper(II) complexes against *T. cruzi* and *L. (V.) peruviana*.

The inhibitory effect of eight triazolo-pyrimidine compounds on the *in vitro* growth of *T. cruzi* epimastigote and promastigote forms of *L. (V.) peruviana* was measured at different times following established procedures (see Experimental section). The results are displayed in Table **1** for benznidazole used as the reference drug against *T. cruzi*, and Pentostam and Glucatim against *L. (V.) peruviana*, including toxicity values against Vero and macrophage cells. Against *T. cruzi* after 72 h of exposure, the 8 compounds assayed showed IC_{50} values very close to those of benznidazole (reference drug) and even in some cases presented IC_{50} values significantly lower than those of benznidazole (**C33c** with a IC_{50} of around 11.06 µM, **C49** with a IC_{50} of 10.58 µM, and **C35** and **C24b** with a IC_{50} < 1.00 and 13.00 µM, respectively). The two trizolo pyrimidine compounds which act as ligand of the complexes (**tp** and **dmtp**) presented inhibition values slightly higher than the IC_{50} of the reference drug. The other two complexes (**C38** and **C41**) also presented a significant inhibitory effect on *T. cruzi* growth. Of the 8 compounds assayed, **tp**, **dmtp**, **C24b** and **C49** were much less toxic against Vero cells than was benznidazole. It bears mentioning that these compounds exhibited an inhibitory concentration (toxicity IC_{50} on Vero cells) of 98.98, 93.34, 50.19, and 154.20 µM after 72 h of culture, respectively. This represents IC_{50} values of 4- to 5-fold higher than the corresponding value of benznidazole (14.59 µM). On the contrary, the compounds **C33c**, **C35**, **C38**, and **C41**, were quite toxic against Vero cells.

As with *T. cruzi*, the complexes **C24b** and **C49** were quite effective at inhibiting growth of the promastigote forms of *L. (V.) peruviana* cultured *in vitro* (Table **1**), these being lower than the IC_{50} of the reference drugs. These two complexes also have low toxicity values, the IC_{50} on J774.2 macrophages cells being some seven-fold or more higher than for the two reference drugs. The triazolo pyrimidine derivatives **tp** and **dmtp** also proved effective in their growth inhibition of *L. (V.) peruviana* and showed low toxicity. The complexes **C35** was the most effective on the growth of *L. (V.) peruviana* (IC_{50} < 0.010 µM) and needed some 100-fold more concentration to affect the macrophage cells.

In most studies on activity assays of new compounds against parasites, forms that develop in vectors are used (epimastigotes in the case of *T. cruzi* and promastigotes in *L. (V.) peruviana*), for the ease of working with these forms *in vitro*; however, in this study, we have included the effect of these compounds on the forms that are developed in the host (amastigotes and trypomastigotes), the study of which is of great importance, given that the final aim is to determine the effects in the definitive host. For this type of work and studies on the action mechanism, we selected the products that had the greatest inhibitory effect on the *in vitro* growth of the parasites and that at the same time had less toxic effect on Vero cells and macrophages, using the IC_{25} of each product as the test dosage.

When 1x10^5 Vero cells were incubated for 2 days and then infected with 1x10^6 metacyclic forms, obtained in the

Table 1. ***In Vitro*** **Activity of Triazolo-Pyrimidine Derivatives on** ***Trypanosoma cruzi*** **Epimastigote and Promatigote Forms of L. (V.) peruviana**

	IC_{50} (μM)		Toxicity IC_{50} (μM)	
	T. cruzi	*L. (V.) peruviana*	on Vero cells (μM)[a]	on J774.2 Macrophages cells[a]
Compound				
Benznidazole	15.83	-	14.59	-
Pentostam	-	11.32	-	9.56
Glucatim®	-	15.33	-	25.61
tp	18.93	20.72	98.98	124.54
dmtp	26.81	24.57	93.34	106.56
C24b	13.00	11.06	50.19	77.09
C33c	11.06	7.76	9.54	14.08
C35	< 1.00	< 0.010	<1.00	1.96
C38	17.93	8.41	7.98	12.35
C41	23.33	25.35	29.03	30.75
C49	10.58	12.17	154.20	186.61

[a]On Vero and J774.2 Macrophages cells at 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at the employed concentrations (1,10, 25, 50 and 100 μM) at 72 h culture
Note: Average of four separate determinations.

way described in the experimental section (control experiment; Fig. **3a**), the parasites invaded the cells and underwent morphological conversion to amastigotes within 1 day after infection. On days 4, 6, 8, and 10, the rate of host-cell infection increased to 14, 44, 73, and 110%, respectively. When triazolo-pyrimidine derivatives were added simultaneously to the infection of Vero cells with *T. cruzi* metacyclic forms (IC_{25} concentration), the infection rate significantly decreased with respect to the control, reaching a 63 and 67% for the complexes **C24b** and **C49**, respectively on day 10. However, the two free triazolopyrimidine derivatives were the ones that inhibited the invasion of Vero cells by the metacyclic forms to a lesser extent, although values continued to be significant (44 and 55% **dmtp** and **tp**, respectively). In the control experiments, the average number of amastigotes per infected cell increased to 85.6 amastigotes/cell on day 7, decreasing to 50 on day 10 (Fig. **3b**). The four triazolo-pyrimidine derivatives inhibited *T. cruzi* amastigote replication in Vero cells *in vitro*. Thus, the addition of a concentration equivalent to the IC_{25} of these compounds produces a markedly lowered the amastigote number per infected cell, reaching 80% reduction in amastigote number with respect to control for cultures on day 10.

The decrease in the average amastigote number on day 7 for the control experiment coincided with the increase in trypomastigote numbers in the medium. This behaviour was due to the rupture of the Vero cells with the subsequent release of amastigotes and future transformation into trypomastigotes. The number of trypomastigotes in the medium was 1.8 x 10^6 parasites on day 10. Meanwhile, the four compounds gave 60 to 70% reduction in trypomastigote numbers, respectively (Fig. **3c**).

When 1 x 10^5 J774.2 macrophages cells were incubated for 2 days and then infected with 1 x 10^6 promastigotes forms of *L. (V.) peruviana* (Fig. **4a**), the parasites invaded the cells and underwent morphological conversion to amastigotes from the first 3 hours of infection. There was a continued increase in the number of invaded cells up to the last day of the assay, when values reached 80% infected cells. When the compounds were added simultaneously to the infection of macrophages cells with *L. (V.) peruviana* (IC_{25} concentration), the infection rate significantly fell with respect to the control. Complex **C35** produced the highest percentage of inhibition (92 %) on day 10. The average number of amastigotes per cell, in the control experiment (Fig. **4b**) increased from the first 3 h of infection to day 5 of culture, when the value of 72 amastigotes/cell was reached. Towards day 6, there was a decline apparently corresponding to the rupture of the cells and release of amastigotes into the medium, these again invading new cells. On day 7, a new increase in parasitized cells was observed, followed by a new decline on day 8, etc. When the triazolo-prymidine compounds were added to the medium containing the macrophages and *L. (V.) peruviana*, a behaviour very similar to that of the control experiment was found, although there was a clear inhibition of the number of amastigotes that replicated by macrophage infection. With complex **C35**, 91% inhibition was reached, this being the highest inhibition, for cultures of 10 days.

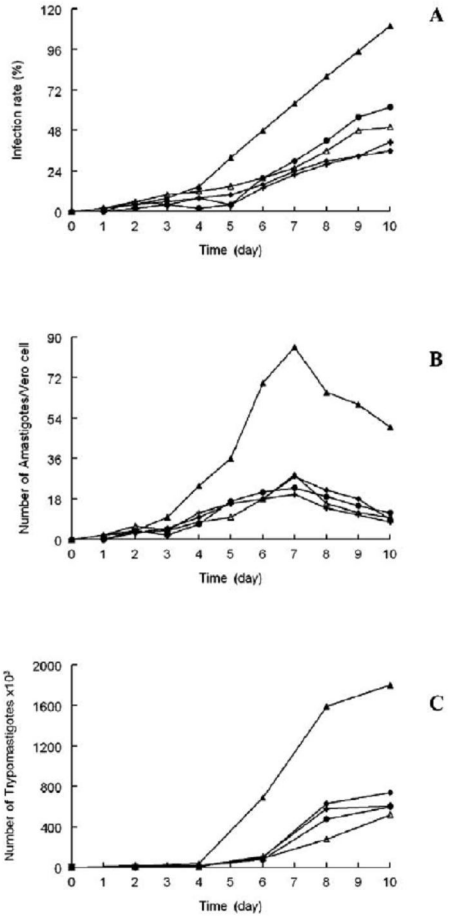

Fig. (3). Effect of 1,2,4-triazolo-[1,5-a]pyridimine derivatives on the infection rate and *T. cruzi* growth. (**A**) rate of infection. (**B**) mean number of amastigotes per infected Vero cell. (**C**) number of trypomastigotes in the culture medium. **-▲-**, control; **-Δ-**, **tp**; **-●- dmtp**;-+-, **C24 b**; **-♦-**, **C49** (at IC_{25} conc.). The values are means of three separate experiment.

As far as is known to date, none of the trypanosomatids studied is capable of completely degrading glucose to CO_2 under aerobic conditions, excreting into the medium as fermented metabolites a great part of their carbon skeleton, which differs depending on the species considered [26]. *T. cruzi* and *L. (V.) peruviana* consume glucose at a high rate, thereby acidifying the culture medium due to incomplete oxidation to acids. ^{1}H-NMR spectra enables us to determine the fermented metabolites excreted by the trypanosomatids during their *in vitro* culture. An example of the types of spectra obtained with fresh (uninoculated) medium is shown in the Fig. (**5I**), and Fig. (**5II**) presents the spectrum given by cell-free medium 4 days after inoculation with the *T. cruzi* strain. Additional peaks, corresponding to the major metabolites produced and excreted during growth, were detected

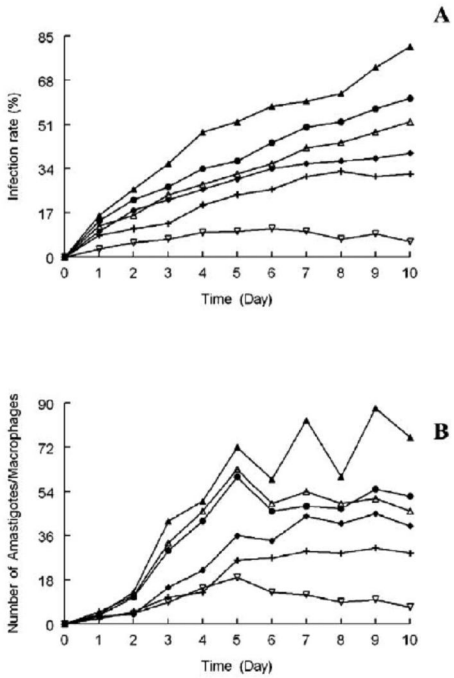

Fig. (4). Effect of 1,2,4-triazole-[1,5-a]pyridimine derivatives on the infection rate and *L. (V.) peruviana* growth. (**A**) rate of infection. (**B**) mean number of amastigotes per infected J774 A.2 macrophage cells. **-▲-**, control; **-Δ-**, **tp**; **-●- dmtp**;-+-, **C24 b**; **-♦-**, **C49; -∇-, C35** (at IC_{25} conc.). The values are means of three separate experiment.

when the last spectrum (Fig. **5II**) was compared with the one made with fresh medium (Fig. **5I**). *T. cruzi* excretes acetate y succinate as majority metabolites, and, in a lower proportion, L-alanine. In the case of promastigotes of *L. (V.) peruviana*, the metabolites were acetate, succinate, L-alanine, and (in a lower proportion) D-lactate (Fig. **6I y 6II**), data that agree well with other authors [27]. We used ^{1}H NMR spectra for the identification and evaluation of the inhibiting effect caused by triazolo-pyrimidine compounds on metabolites excreted by *T. cruzi* and *L. (V.) peruviana*. When the trypanosomatids were treated with the triazolo-pyrimidine compounds (**tp**, **dmtp**, **C24b**, and **C49**, in the case of *T. cruzi* (Fig. **5III-VI**) and **tp**, **dmtp**, **C24b**, **C49**, and **C35** for *L.(V.) peruviana* (Fig. **6III-VII**), the excretion of some of these catabolites was clearly inhibited, at the dosages assayed (IC_{25}).

In the case of *T. cruzi*, acetate was the metabolite most inhibited by the compound **tp** and its complexes **C24b** and **C49** (51, 60 and 62 %, respectively), while the triazolo pyrimidne derivative **dmtp** were the ones that least inhibited acetate production (around 50 %, Fig. **5IV**). The inhibition of acetate excretion can be a direct consequence of the action of these compounds on the enzymes involved in their production (pyruvate dehydrogenase complex or acetate succinate CoA-transferase) or else these compounds act on

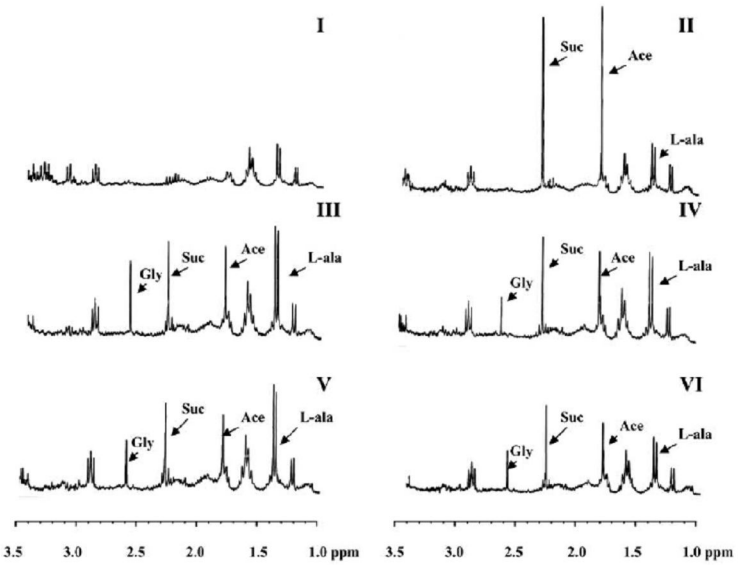

Fig. (5). ^{1}H-NMR spectra epimastigote forms of *T cruzi* treated against 1,2,4-triazolo-[1,5-a]pyridimine derivatives (at a concentration of IC_{25}): (I) fresh medium, (II); Control (untreated); (III) **tp**; (IV) **dmtp**; (V) **C24b** and (VI) **C49**. L-ala, alanine; Ace, acetate; Pyr, pyruvate; Suc, succinate and Gly, glycerol.

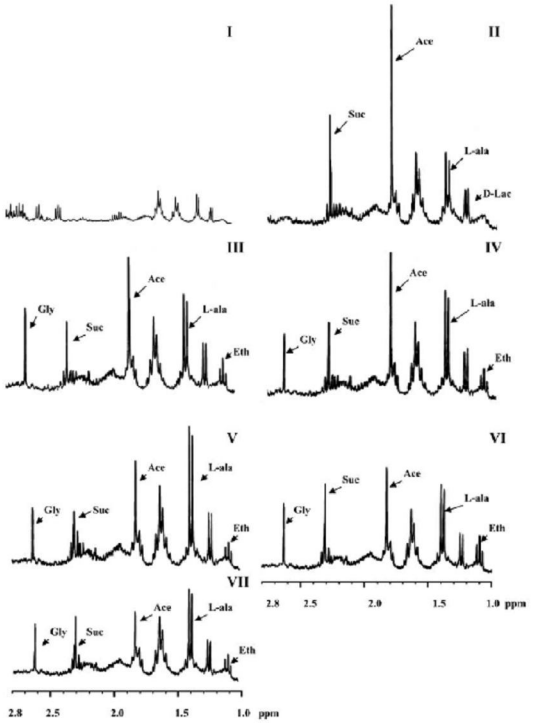

Fig. (6). ^{1}H-NMR spectra promastigote forms of *L. (V.) peruviana* treated against 1,2,4-triazolo-[1,5-a]pyridimine derivatives (at a concentration of IC_{25}): (I) fresh medium, (II); Control (untreated); (III) **tp**; (IV) **dmtp**; (V) **C24b**; (VI) **C49** and (VII) **C35**. L-ala, alanine; Ace, acetate; Pyr, pyruvate; Suc, succinate, Eth, ethanol and Gly, glycerol.

the mitrochondrion and even on the cytoplasmic membrane, causing a loss of functionality [28,29].

In *T. cruzi* and in other trypanosomatids (*Leishmania* spp.), succinate is a another major end product excreted from glucose metabolism, but the pathway leading to its production has been the topic of a long-standing debate [30, 31]. The main role of the succinate (succinic fermentation) is probably to maintain the glycosomal redox balance, by providing two glycosomal oxidoreductase enzymes that allow reoxidation of NADH, produced by glyceraldehydes-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers the significant advantage of requiring only half of the phosphoenolpiruvate produced to maintain the NAD^+/NADH balance. The remaining PEP is converted into acetate, L-lactate, L-alanine, and/or ethanol, depending on the species. The excretion of succinate, like that of acetate, is inhibited by the compound **tp** and its complexes **C24b** and **C49** (49, 50, and 50 %, respectively). The inhibition of acetate and succinate excretion explains the observed increase in L-alanine production and the appearance of a new peak that was not present in the spectrum found for *T. cruzi* cultured without the addition of triazolo-pyrimidine compounds; this peak has also been identified as glycerol.

In *L. (V.) peruviana* cultivated *in vitro*, these products are greatly effective and inhibit the excretion of the end-products of glucose metabolism, **tp**, and its derivatives **C24b**, **C35**, and **C49**; they are more effective (Fig. **6 III-V**) mainly on acetate and succinate, while the compound **C35** reaches the highest inhibition values on acetate and succinate, 70 and 46 %, respectively, at a concentration of IC_{25} (Fig. **6VI**). As occurred with *T. cruzi*, the triazolo-pyrimidine compound **dmtp** showed the least inhibition (Fig. **6IV**), and, as in *T. cruzi*, in *L. (V.) peruviana*, when both acetate as well as succinate production was inhibited, the excretion of the rest of the excreted metabolites augmented, even with the appearance of ethanol and glycerol in the medium at highly significant levels (Fig. **6**).

Morphological alterations visible under electron microscopy (TEM) were frequent in *T. cruzi* epimastigotes treated as compared to controls cells (Fig. **7**). Treatment with **tp** (Figs. **7B** and **C**) caused death in some cases and showed distorted parasite bodies, many becoming irrecognizable, strongly electrodense, and completely deformed (Fig. **7B**). The supernatant appeared with abundant remains of parasite degradation. Other frequent cell alterations included the separation of nuclear membranes (Fig. **7C**). Similar results were found with the parasites treated with **dmtp** (Fig. **7D**), also showing this separation of the nuclear membranes in addition to other changes such as the alteration of the cytoplasmic membrane and the disorganization of the subpellicular microtubules. Mitochondria and kinetoplasts were also affected, appearing swollen and many remains of dead parasites appeared in the supernatant. **C24b** was highly effective against *T. cruzi* epimastigotes, as shown in Fig.**7E**, where parasites had strongly vacuolated cytoplasm, empty vacuoles, and lipidic vacuoles, enormous reservosomes and abundant endoplasmic reticulum. **C49** and **C24b** caused similar alterations, with intense vacuolization and swelling of mitochondrion and kinetoplast (Fig. **7D**).

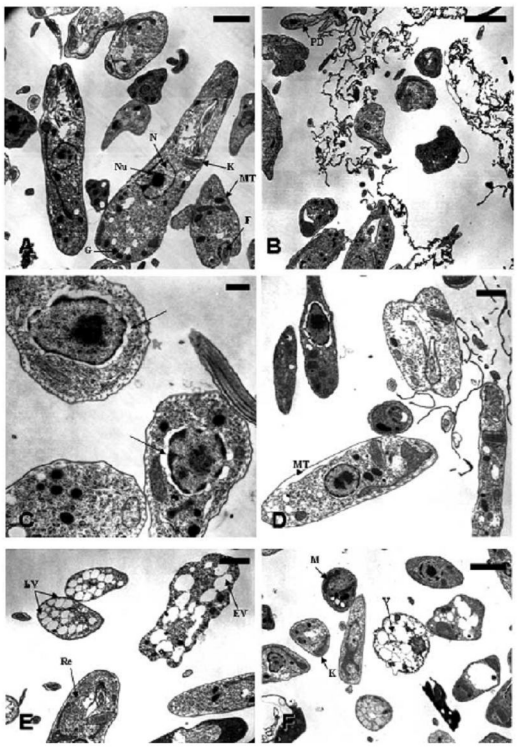

Fig. (7). Ultraestructural alterations by TEM in *T. cruzi* treated with 1,2,4-triazolo-[1,5-a]pyridimine derivatives. **(A)** Control parasite, Bar=1.59μ **(B** and **C)** Epimatigote forms treated with **tp,** Bar=2.33 and 0.583μ respectively**. (D)** Epimatigote forms treated with **dmtp**, Bar=1.0μ. **(E)** Epimatigote forms treated with **C24b,** Bar=1.0μ. **(F)** Epimatigote forms treated with **C49,** Bar=1.59μ**.** (N) nucleus, (Nu) nucleolus, (K) kinetoplast, (M) mitochondrion, (F) flagellum, (V) vacuoles, (G) glicosomes, (MT) microtubules. (Rs) restos de dye parasites, (PD) parasites deformados, (MN) membranes nucleares (arrow). (EV) empty vacuoles, (LV) lipidic vacuoles, (R) reservosomes and (RE) endoplasmic reticulum.

The same products were assayed against *L. (V.) peruviana* (Figs. **8**). Treatment with **tp** caused death in the most of the parasites and many cell remains were present in the supernatant (Fig. **8B**). Other parasites appeared with swollen kinetoplast and separation between the nuclear membranes were frequent (Fig. **8C**). **Dmtp** caused death in some parasites and grave alterations in others (Fig. **8D**). These alterations included a striking distortion of the parasite body and of the subpellicular microtubules as well as the swelling of the mitochondrion and intense vacuolization. **C24b** was less effective than in the case of *T. cruzi* but also provoked many alterations such as kinetoplast swelling, intense cytoplasmic vacuolization, and separation of the two nuclear membranes (Fig. **8E**). **C49** caused the most ultrastructural alterations in *L. (V.) peruviana* promastigotes (Fig. **8F**). Most of the parasites died and some were distorted with swelling to the point

of bursting or had shrunken cell size. The complex **C35** proved the most effective of all the ones tested, the images (Fig. **8G** and **H**) reflecting practically complete destruction of the cells, the presence of cell remains indicating grave alterations in the parasites.

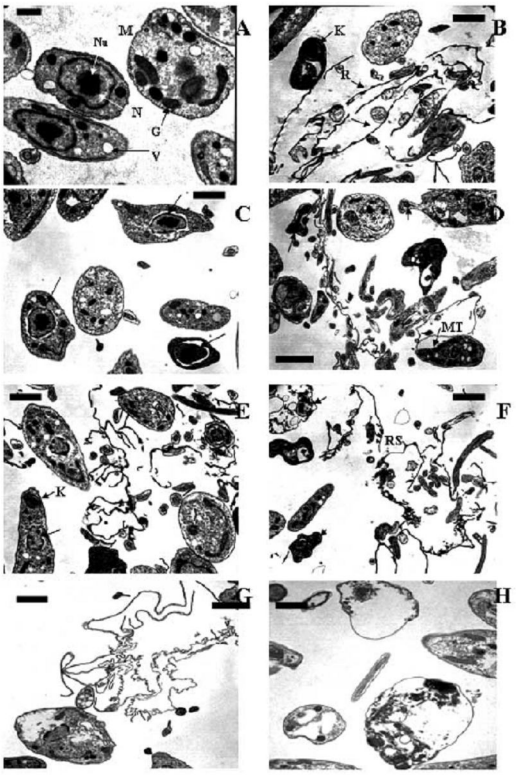

Fig. (8). Ultrastructural alterations by TEM in *L. (V.) peruviana* treated with 1,2,4-triazole-[1,5-a]pyrimidine derivatives. **(A)** Control parasites, Bar= 0.583µ. **(B** and **C)** Parasites treated with **tp,** Bar=1.0µ. **(D)** *L. (V.) peruviana* treated with **dmtp**, Bar=1.59µ. **(E)** Parasites treated with **C24b**, Bar=1.0µ. **(F)** *L. (V.) peruviana* treated with **C49**, Bar=1.0µ. **(G** and **H))** *L. (V.) peruviana* treated with **C35,** Bar=1.0 and 1.59µ respectively. (N) nucleus, (Nu) nucleolus, (K) kinetoplast, (V) vacuoles, (M) mitochondrion, (G) glycosomes. (CR) cell remains, (MT) microtubules, distortion of the parasite body (arrow), (NM) nuclear membranes (arrow). swollen parasite (arrow) and small parasites (arrowhead).

CONCLUSION

In conclusion, these triazolo-pyrimidine compounds clearly inhibit *in vitro* epimastigote forms of *T. cruzi* and promastigotes of *L. (V.) peruviana*. Of all the compounds assayed, those that presented the ligand **tp** were most active, and within these the most effect ones had phenanthroline as the auxiliary ligand—two of these being perchlorate of copper (**C24b** and **C49**) and the other copper nitrate (**C35**). The presence of the nitrate ion may confer stronger power against the parasites, although it also surely makes the compound more toxic. These three compounds are not only less toxic against mammal cells, but also are more effective against the parasite forms present in the vector and also against the forms present in the definitive host. Thus, these clearly affect the infection capacity of the parasites as well as the replication capacity of the amastigotes inside the cells, and consequently cause an evident reduction in the number of amastigotes and in the case of *T. cruzi* a reduction in the number of trypomastigotes. These three compounds alter the energy metabolism of the parasites; one alteration could be at the level of the NAD^+/NADH balance of the parasites, affecting mainly the excreted metabolites. Another of the potential action mechanisms is at the level of the membranes of the organelles, either by direct action on the microtubules or by their disorganization, all of this leading to a vacuolization, degradation, and ultimately cell death.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

tp	=	1,2,4-Triazolo-[1,5-a]pyrimidine
dmtp	=	5,7-Dimethyl-1,2,4-triazolo-[1,5-a]pyrimidine
C24b	=	$[Cu(H_2O)(phen)(tp)_2](ClO_4)_2 \cdot H_2O$
C33c	=	$[Cu(H_2O)(phen)(dmtp)_2](ClO_4)_2$
C35	=	$Cu(NO_3)(H_2O)(phen)(tp)](NO_3)$
C38	=	$[Cu(H_2O)_2(en)(tp)_2](ClO_4)_2$
C41	=	$[Cu(H_2O)_2(en)(dmtp)_2](ClO_4)_2$
C49	=	$[Cu_2(OH)(H_2O)_{2.5}(tp)_5](ClO_4)_3(H_2O)_{1.5}$
en	=	Ethylenediamine
phen	=	1,10-Phenanthroline
DMSO	=	Dimethyl sulfoxide
NNN medium	=	Novy-Nicolle-McNeal culture medium
MEM	=	Minimal Essential Medium
MTL	=	Medium Trypanosome Liquid
RPMI	=	Roswell Park Memorial Institute
IC	=	Inhibitory concentration
^{1}H-NMR	=	Nuclear magnetic resonance spectroscopy of protons

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Antileishmaniasis Activity of Flavonoids from *Consolida oliveriana*

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Antileishmaniasis Activity of Flavonoids from *Consolida oliveriana*

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A set of flavonoids from *Consolida oliveriana*, kaempferol (**1**), quercetin (**2**), trifolin (**3**), and acetyl hyperoside (**5**) and their *O*-acetyl derivatives (**1a**, **2a**, **3a**), and octa-*O*-acetylhyperoside (**4**) showed leishmanicidal activity against promastigote as well as amastigote forms of *Leishmania* spp. The cellular proliferation, metabolic, and ultrastructural studies showed that the acetylated compounds **2a**, **3a**, and **4** were highly active against *Leishmania (V.) peruviana,* while **2a** as well as **4** were effective against *Leishmania (V.) braziliensis*. These compounds were not cytotoxic and are effective at similar concentrations up to or lower than the reference drugs (pentostam and glucantim).

In humans, *Leishmania* spp. cause a variety of clinical diseases due to the ability of the organism to proliferate in deep tissue or close to the skin surface at low temperatures. The varied manifestations of the disease have been used by the World Health Organization as the basis for classifying leishmaniasis into four clinical forms: (a) visceral, (b) mucocutaneous, (c) cutaneous diffuse or disseminated, and (d) cutaneous. Certain species of the parasite have been associated with the different clinical forms of the disease; that is, the *Leishmania donovani* complex causes visceral leishmaniasis, while the *Leishmania tropica* complex is known to induce cutaneous lesions in the Old World and *Leishmania mexicana L. (V.) peruviana* does so in the New World. Some 25% of the mucous cutaneous forms cause mucocutaneous and cutaneous diffuse leishmaniasis in several countries of Latin America, the pathogen being *Leishmania (V.) braziliensis*.[1]

Historically, the chemotherapy of leishmaniasis has been based on the use of toxic heavy metals, particularly antimony compounds. Whenever these kinds of drugs are no longer effective, some others are used, including pentamidine and amphotericin B. These chemicals have to be injected, and clinical care or hospitalization during treatment may be necessary due to possible side effects;[2,3] thus other treatments are needed. Folk medicine is very often a valid source for researchers looking for bioactive substances potentially useful against many diseases. Extracts from medicinal plants or compounds derived from them are a valuable source of new medicinal agents for treating Leishmaniasis[4] and other diseases.[5] The range of families and species from which potentially active leishmanicidal substances can be extracted is very broad,[6] for example, *Bixa orellana* (Achiote), *Polypodium calaguala* (Calaguala), *Sida rhombifolia* (Escobilla), *Psidium guajava* (Guayaba), *Plantago major* (Llantén), *Ficus dendrocida* (Matapalo), *Piper angustifolium* (Matico), *Musa paradisiacal* (Plátano), *Lupinus tauris* (Tauri), *Solanum nigra* (Yerba mora), and *Lepidium peruvianum* (Maca).[7−12] The leishmanicidal effect may reside in its phytochemical component such as flavonoids and, specifically, quercetin, which is a strong candidate in the combination therapy against the infection and the anemia associated with VL.[13]

Part of our group (Tenerife group) has been working on flavonoids derived from the aerial parts of *Consolida oliveriana* (DC) Schrod, a species used medicinally in parts of Anatolia (Turkey). A large number of publications have dealt with the diterpenoid alkaloids of other *Consolida* species, but reports on the flavonoid content of members of this genus are scarce.[14−19]

Most of the studies directed toward the detection of secondary plant metabolites with leishmanicidal activity have used the promastigote form of the parasite because it is easier to maintain under *in vitro* conditions. However, since the promastigote is not the developed form of the parasite in vertebrate hosts, evaluations made with promastigotes have only an indicative value of the possible leishmanicidal activity of the metabolite tested. As a result, a preliminary evaluation using promastigotes needs to be complemented with an evaluation using intracellular amastigotes in macrophages. At the same time, an evaluation of the possible cytotoxicity of the metabolite should be carried out using nonparasitized macrophages, in order to establish whether the *in vitro* activity of the metabolite is due to its general cytotoxic activity or whether it is selectively active against the *Leishmania* parasite.[20]

We investigated the inhibitory effects of flavonoid compounds derived from aerial parts of *C. oliveriana* (DC) Schrod, on the extracellular promastigote and the intracellular amastigote stages of *L. (V.) peruviana* and *L. (V.) braziliensis*, causal agents of both cutaneous and mucocutaneous leishmaniasis. In addition, we studied the cytotoxic effects of these compounds against a cell line of macrophages, analyzing the mechanism by which these molecules act.

Results and Discussion

The importance of phytotherapy as a guide in the search for new antileishmanial drugs becomes evident in a single search of specialized literature. Thus, the strategy for discovering new drugs is to investigate natural products from medicinal plants.[21−23] Dozens of potential new compounds from nature have been found in the past 5 years, especially in the rainforests of South America and Africa, only concerning Leishmaniasis.[24] Plant-derived active principles and their semisynthetic and synthetic analogues have served as a major path to new chemotherapeutic compounds.[23,25,26] Furthermore, the leads obtained from the search for natural products with antileishmanial activity give new impetus for developing valuable synthetic compounds.[27]

Flavonoids are found in abundance in diets rich in fruits, vegetables, and plant-derived beverages and appear to have anticancer, antimicrobial, and antiparasitic properties.[28−30] Our Tenerife group is searching for bioactive substances potentially useful against many diseases, and recently they have shown that flavonoid acetates derived from flavonoids extracted from *C. oliveriana* exhibited significant impact on the growth of three human cell lines: HL-60, U937, and SK-MEL-1.[19]

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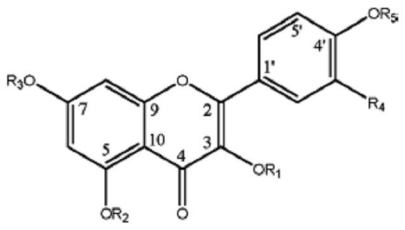

Figure 1. Chemical structure of flavonoid compounds.

Table 1. In Vitro Activity of Flavonoids on Promastigotes of *L. (V.) peruviana* and *L. (V.) braziliensis*[a]

compound	IC_{50} (μM) *L. (V.) peruviana*	IC_{50} (μM) *L. (V.) braziliensis*	toxicity IC_{50} on J774.2 macrophages cells (μM)[b]
pentostam	11.32	9.56	12.44
glucatim	15.33	25.61	15.20
1	71.29	53.65	53.67
1a	53.32	68.56	15.56
2	60.04	30.49	125.44
2a	11.18	46.78	109.23
3	53.34	52.46	161.32
3a	10.53	8.72	148.71
4	7.35	6.21	122.31
5	86.95	51.60	61.32

[a] IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at the concentrations used (0.1, 1, 10, 25, 50, and 100 μM) at 72 h culture. Note: Average of three separate determinations. [b] J774.2 macrophages cells at 72 h of culture.

Here, we evaluate whether the inhibitory effect on cell growth of four flavonoids (**1**, **2**, **3**, and **5**) obtained by natural means from *C. oliveriana* and their acetylated products (**1a**, **2a**, **3a**, and **4**)[19] (Figure 1) is active against intra- and extracellular forms of *L. (V.) peruviana* and *L. (V.) braziliensis*. The results are displayed in Table 1, using pentostam and glucatim as the reference drugs and including toxicity values against the J774.2 macrophage cells. After 72 h of exposure against *L. (V.) peruviana*, three of the acetylated compounds tested (**2a**, **3a**, and **4**) registered IC_{50} values lower than the reference drugs' IC_{50} (pentostam 11.32 and glucantim 15.33 μM), i.e., an IC_{50} of 7.35 μM for **4**, 11.18 μM for **2a**, and 10.53 μM for **3a**. Therefore, the toxicity values were very low when tested on J774.2 macrophage cells. In addition to being slightly toxic, these three compounds gave IC_{50} values on J774.2 macrophage cells of about 10- to 15-fold higher than those for the reference drugs. Against *L. (V.) braziliensis*, two of these acetylated compounds (**4** and **3a**) inhibited cell growth at a IC_{50} of 6.21 and 8.72 μM, respectively, this being significantly lower than the IC_{50} of the reference drugs. The rest of the compounds had an IC_{50} higher than the reference drugs and in some cases were toxic for the macrophages. These results indicate that the acetylated compounds performed better than the phenolic analogues. It appears that the kaempferol derivatives possessing a monosubstituted B-ring are more active than the quercetin analogues.

It has been shown that acetylation of certain flavonoids increases the antiproliferative activities of the parent compounds against HL-60 and other cell lines. For example, **3a** induced cell death in human leukemia cells.[26] This greater efficiency may possibly be due to acetylation, which facilitates the compound absorption, leading to greater effectiveness.[31] Little information is available on the leishmanicidal activity of flavonoids for comparison with our results; however, recently it has been possible to establish the activity of some of these compounds, showing them to be effective against promastigote development of both *L. (L.) mexicana*[32] and *L. (L.) donovani*,[33] with IC_{50s} values similar to those found by us. In a study by Tasdemir et al.,[34] quercetin was demonstrated to be highly effective against *L. (L.) donovani* and *T. cruzi*, at doses slightly lower than ours. These discrepancies presumably derive from the use of different methods and different life-cycle stages of the parasites.[35]

The products **2a**, **3a**, and **4** were selected because they had the greatest inhibitory effect on the *in vitro* growth of *L. (V.) peruviana* and *L. (V.) braziliensis* and had less toxic effects on macrophage cells, using the IC_{25} of each product as the test dosage.

When 1×10^5 J774.2 macrophage cells were incubated for 2 days and then infected with 1×10^6 promastigote forms of *L. (V.) peruviana* and *L. (V.) braziliensis* (Figure 2), the parasites invaded the cells and underwent morphological conversion to amastigotes from the first 3 h postinfection with a steadily increasing number of cells invaded until the last day of the assay (day 10). The values for infected cells (control experiment) reached 95% in the case of *L. (V.) peruviana* and 97% in the case of *L. (V.) braziliensis*. Nevertheless, in the amastigote-cell assay with the flavonoid compounds added simultaneously to the infection of macrophages cells with promastigote forms, the treatment significantly reduced the infection rate with respect to the control. That is, in the case of *L. (V.) peruviana* the infection rate reached 55%, 40%, and 59% for the compounds **4**, **2a**, and **3a**, respectively, on day 10 (Figure 2A). Meanwhile, in the infected *in vitro* cultures of *L. (V.) braziliensis* the results were 46% and 67% for compounds **4** and **3a** (Figure 2B). The addition of **4**, **2a**, and **3a** had a similar effect with a markedly lower amastigote number of *L.(V.) peruviana* per infected cell with respect to the control at day 10, i.e., reaching a value of roughly 72% (Figure 2C). When the cultured parasite was *L. (V.) braziliensis*, compound **4** decreased the amastigote number by as much as 60%, while compound **3a** reached 90% (Figure 2D).

Most studies on activity assays of new compounds use parasite forms that develop in vectors (promastigote forms in the case of *Leishmania* spp.),[20] for the ease of working with these forms *in vitro*. However, in the present study we have included the effect of the flavonoid compounds on the forms that are developed in the host (amastigotes) to determine the effects in humans. For this objective, we selected the products that had the greatest inhibitory effect on the *in vitro* growth of *L. (V.) peruviana* (**2a**, **3a**, and **4**) and *L. (V.) braziliensis* (**3a** and **4**) and had less toxic effects on J774.2 macrophage cells, using the IC_{25} of each product at the test dosage. The infective capacity on macrophage cells by *L. (V.) peruviana* and *L. (V.) braziliensis* significantly decreased when acetylated flavonoids were added. This infective capacity declined from the early hours of culture, these compounds altering the invasive capacity of the parasites. Until now, the mechanism by which the parasite's invasive capacity is lost was not known. It was found that these compounds decrease the parasite's multiplication rate into the macrophages. The acetylated compounds (**2a**, **3a**, and **4**) are highly effective not only against the extracellular forms of the parasite (promastigote forms) but also against intracellular forms (amastigote forms).

Despite the good results achieved in some cases, only a few compounds of natural origin are under clinical evaluation, as most of them have been disapproved because of their high toxicity.[36] It should be noted that these antiprotozoal agents are basically cytotoxic and act selectively against the parasites. Currently, publications on antiparasitic agents include activity evaluations against mammals and/or human cell lines.[33,37] Following this pattern, we have determined the toxicity of our compounds against a human cell line, noting that compounds **2a**, **3a**, and **4** showed little toxicity for J774.2 macrophage cells, with IC_{50}'s of around 10 to 15 times higher than those of the reference drugs, confirming a selective activity of these compounds against *L. (V.) peruviana*. These data are consistent with findings in the case of *L. (V.) braziliensis*, given that compounds **3a** and **4** proved extremely effective and with very low toxicity, opening the possibility of using

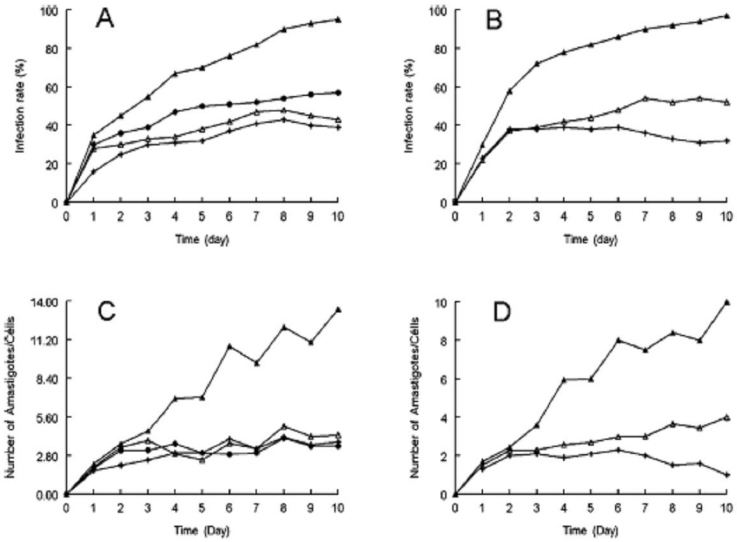

Figure 2. Effect of flavonoid compounds on the infection rate and growth of *Leishmania (V.) peruviana* (A) and *L. (V.) braziliensis* (B). (C) Mean number of amastigotes of *L. (V.) peruviana* and (D) for *L. (V.) braziliensis* per infected J774.2 macrophage cells. −▲−, control; −●−, **2a**; −+−, **3a**; and −△−, **4** (at IC_{25} conc). The values are means of three separate experiments.

these compounds in the treatment of both cutaneous and mucocutaneous leishmaniasis.[38]

The *in vitro* culture of trypanosomatids depends on the available carbon sources (mainly glucose) present in their culture medium for their energy metabolism.[39] None of the trypanosomatids studied are capable of completely degrading glucose to CO_2 under aerobic conditions, excreting a great part of their carbon skeleton into the medium as fermented metabolites, this difference depending on the species considered.[39] *L. (V.) peruviana* and *L. (V.) braziliensis* consume glucose at a high rate, thereby acidifying the culture medium due to incomplete acid oxidation. Using ^{1}H NMR spectra, we determined the fermented metabolites excreted by the parasites during their *in vitro* culture, and we identified and evaluated the inhibiting effect caused by the flavonoid compounds over final metabolite excretion in promastigote forms of *L. (V.) peruviana* and *L. (V.) braziliensis* cultured *in vitro* in MTL medium for 96 h (Figures 3 and 4). Figure 3B corresponds to the spectrum given by cell-free medium 96 h after inoculation with the promastigote forms of *L. (V.) peruviana.* Additional peaks, corresponding to the major metabolites produced and excreted during growth, were detected when the last spectrum (Figure 3B) was compared with that found with fresh medium (Figure 3A). *L. (V.) peruviana* excreted acetate and succinate as majority metabolites and L-alanine in a lower proportion. When the trypanosomatids were treated with the flavonoid compounds (**2a**, **3a**, and **4**; Figure 3C, D, and E), the excretion of some of these catabolites was clearly inhibited at the dosage tested (IC_{25}). Acetate and succinate were decreased by around 30 to 40%, while with compound **4** the acetate decreased up to 67%. L-Alanine also increased, and new excreted metabolites such as pyruvate and glycerol appeared. In the case of *L. (V.) braziliensis,* when the cell-free medium spectrum was compared (Figure 4B) to that of the parasite-free medium (Figure 4A), the excretion of acetate and succinate as major metabolites was found together with L-alanine in a lower proportion. When compounds **3a** (Figure 4C) and **4** (Figure 4D) were added to the cultures, a clear inhibition was detected in the peaks corresponding to acetate and succinate, while the peaks corresponding to L-alanine and D-lactate were higher, and peaks corresponding to pyruvate and glycerol appeared.

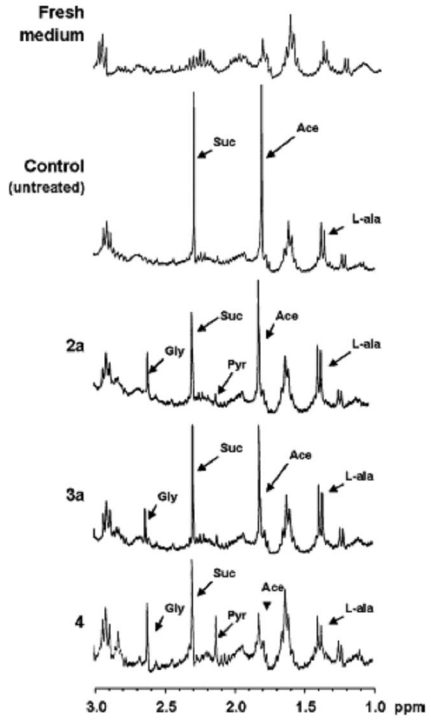

Figure 3. ^{1}H NMR study of the production of metabolites excreted by the promastigote forms of *L. (V.) peruviana* treated against flavonoid compounds (at a concentration of IC_{25}). (Ace) acetate; (Suc) succinate; (L-ala) L-alanine; (Pyr) pyruvate, and (Gly) glycerol.

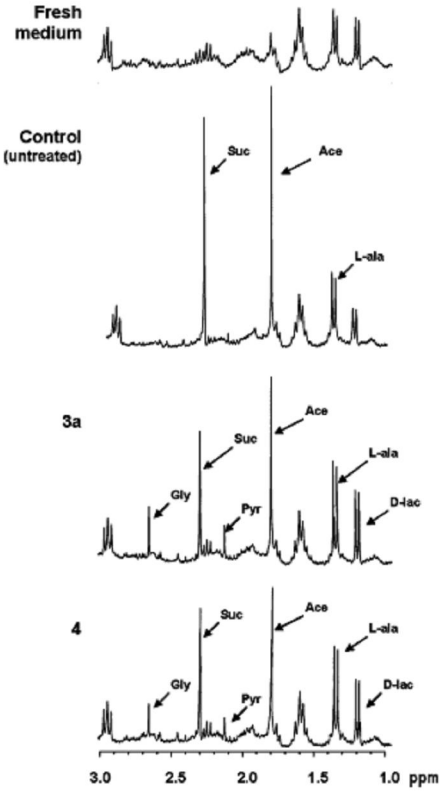

Figure 4. ^{1}H NMR study of the production of metabolites excreted by the promastigote forms of *L. (V.) braziliensis* treated against flavonoid compounds (at a concentration of IC_{25}). (Ace) acetate; (Suc) succinate; (L-ala) L-alanine; (Pyr) pyruvate (Gly) glycerol, and (D-lac) D-lactate.

The excretion of some of these catabolites (acetate and succinate) was clearly inhibited at the dosages assayed (IC_{25}). In the catabolism of glucose in *Leishmania* spp., pyruvate is located at metabolic branching points, leading to several excreted end products, such as acetate, L-alanine, ethanol, and L-lactate.[40] Acetate is a major end product formed in the mitochondrion and excreted by simple diffusion across the mitochondrial and cytoplasmic membranes. The inhibition of acetate excretion may be a direct consequence of the action of these flavonoids on the enzymes involved in their production (pyruvate dehydrogenase complex or acetate-succinate CoA-transferase), or else these compounds act on the mitochondrion and even on the cytoplasmic membrane, causing a loss of functionality. Succinate is another major end product excreted from glucose metabolism, but the pathway leading to its production has been the topic of a long-standing debate.[41] The controversy concerns the relevance of the NADH-dependent fumarate reductase activity detected in most trypanosomatids, for which the contribution in succinate production has not been clearly demonstrated. The main role of the succinate (succinic fermentation) is probably to maintain the glycosomal redox balance, by providing two glycosomal oxidoreductase enzymes that allow reoxidation of NADH, produced by glyceraldehydes-3-phosphate dehydrogenase in the glycolytic pathway. Succinic fermentation offers the significant advantage of requiring only half of the phosphoenol pyruvate (PEP) produced to maintain the NAD^+/NADH balance. The remaining PEP is converted into acetate, L-lactate, L-alanine, and/or ethanol, depending on the species. The inhibition of acetate and succinate excretion explains the observed increase in L-alanine and D-lactate production and the appearance of two new peaks identified as glycerol and pyruvate.

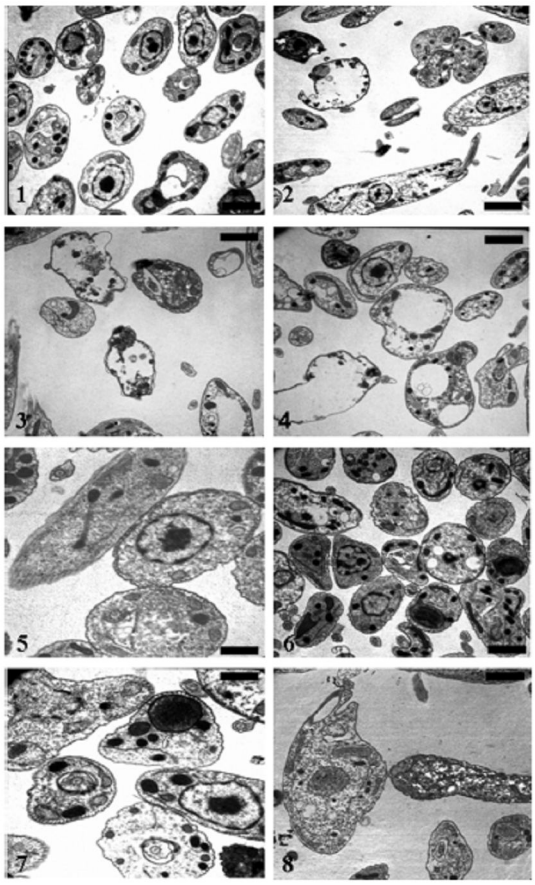

Figure 5. Ultrastructural alterations by TEM in *L. (V.) peruviana* and *L. (V.) braziliensis* treated with flavonoids compounds. (1) Control of *L. (V.) peruviana*, bar = 1.0 μm. (2 and 3) Promastigotes of *L. (V.) peruviana* treated with **2a**, bar = 1.0 μm. (4) Promastigotes of *L. (V.) peruviana* treated with **3a**, bar = 1.0 μm. (5) Control of *L. (V.) braziliensis*, bar = 1.59 μm. (6) Promastigotes of *L. (V.) braziliensis* treated with **3a**, bar = 1.0 μm. (7 and 8) Promastigotes of *L. (V.) braziliensis* treated with **4**, bar = 1.59 μm. (N) nucleus; (Nu) nucleolus; (CM) cytoplasmatic membrane; (MT) microtubules; (G) glycosomes; (R) reservosomes; (M) mitochondrion; (K) kinetoplast; (V) vacuole; and (LV) lipidic vacuoles.

Morphological alterations of *L. (V.) peruviana* treated with **2a** and **3a** were examined by transmission electron microscopy (TEM). The parasites were sensitive mainly to the treatment with these compounds at IC_{25}, and a considerable number of dead parasites were observed. Several alterations could be detected when compared with the images corresponding to control cells (Figure 5-1). Compound **2a** induced death with distortion of the parasite body and disruption of the cytoplasm, which appeared almost empty in some parasites but with many glycosomes throughout (Figure 5-2 and 3). Meanwhile, **3a** induced intense vacuolization in the parasites (Figure 5-4), and empty vacuoles and lipidic vacuoles were visible. Glycosomes were abundant in the altered parasites, while other parasites appeared to be dead.

The treatment with **3a** and **4** at IC_{25} for 96 h induced alterations in *L. (V.) braziliensis*, which can be observed when comparing the images made with untreated parasites (Figure 5-5). The treatment with **3a** (Figure 5-6) disrupted the cytoplasm, which in the majority of the parasites had little electrodensity and abundant vacuoles. The mitochondrion and kinetoplast were also affected, both organelles appearing swollen. Some parasites had abundant lipidic vacuoles. *L. (V.) braziliensis* treated with **4** (Figure 5-7 and 8) and showed marked alterations. Figure 5-7 shows an undulating cytoplasmic membrane and microtubules in disarray. The kinetoplasts were intensely swollen, glycosomes were abundant, and some parasites showed vacuolization. The rest of the compounds were highly toxic, but it was not possible to study them by TEM.

In general, several morphological alterations were noted in *L. (V.) peruviana* and *L. (V.) braziliensis* when additional compounds were added to the cultures. There was an intense vacuolization as well as disruption of the cytoplasmic, nuclear, and mitochondrial membranes, suggesting a possible effect on tubuline, a protein known to be affected by flavonoids.[42] This disruption of the membranes may in turn account for the strong inhibition of the acetate that is synthesized inside the mitochondria and then diffused to the cytoplasm and outside the cell.

In conclusion, our study demonstrates that several acetylated flavonoids derived from *C. oliveriana* (**2a**, **3a**, and **4**) were very active *in vitro* against both the extracellular as well as the intracellular forms of *L. (V.) peruviana* and *L. (V.) braziliensis* (**3a** and **4**). These compounds are not toxic to the host cells and are effective at concentrations similar to or lower than the reference drugs used in the present study. These flavonoids have promising leishmanicidal properties. The data from transmission electronic microscopy and nuclear magnetic resonance raise the possibility that the action (or part of the action) could be at the level of the parasite membranes. The potent leishmanicidal activities described here for flavonoids represent an exciting advance in the search for new antiprotozoal agents.

Experimental Section

General Experimental Procedures. The ^1H and ^{13}C NMR spectra were measured using a Bruker AMX-400 or a Bruker MAX-500 instrument. FAB5 and exact mass measurements were determined using a Micromass Autospec instrument at 70 eV. ESIITMS data were obtained by tandem electrospray ion trap mass spectrometry (LCQ Deca XP Plus; ThermoFinnigan; San Jose, CA). Column chromatography was performed over Sephadex LH-20 Pharmacia (ref 17-0090-01; Upsala, Sweden), silica gel 60 (Merck 230−400 mesh; Darmstadt, Germany, and analytical TLC, Merck Kieselgel 60 F 254. HPLC separations were performed on a JASCO Pu-980 series pumping system equipped with a JASCO UV-975 ultraviolet detector and with a Waters Kromasil 5 (5 mm × 250 mm) column. A Macherey-Nagel VP 250/10 nucleodur Sphinx RP 5 mm column (Düren, Germany) was used for HPLC-RP chromatography; chromatograms were visualized under UV light at 255 and 366 nm and/or sprayed with oleum followed by heating. All solvents were distilled before use; the purity of all compounds was 99.0% as judged by HPLC. Stock solutions of 10 mM flavonoids were made in DMSO, and aliquots were frozen at −20 °C. The metabolite excretion ^1H NMR spectra were obtained at 300 MHz on a Bruker AM-300 spectrometer, which was operated at pulse in Fourier transformation with quadrature detection. The temperature of the probe was maintained at 27 °C. The acquisition parameters were as follows: pulses of 90° in radius and a wavelength of 3287.5 Hz, 8 s recycle time, and 160 accumulations. The chemical displacements were expressed as parts per million (ppm) using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the reference signal. The ultrastructural alterations were examined with an EMCIO Zeiss transmission electron microscope.

Parasite Strain, Culture. *L. (V.) peruviana* (MHOM/PE/1984/LC26) and *L. (V.) brazilensis* (MHOM/BR /1975/M2904) were cultured *in vitro* in MTL medium plus 10% inactivated fetal bovine serum kept in an air atmosphere at 28 °C, in Roux flasks (Corning, USA) of 75 cm^2 in surface area, according to the methodology described by González et al.[22]

Plant Material. Aerial parts of *C. oliveriana* were collected and identified near Pazarkik in eastern Turkey at an altitude of 980 m by Prof. Julian Molero Briones, Department of Botany, Faculty of Pharmacy, University of Barcelona (Spain), where a voucher specimen (BCF-37810) has been deposited.

Extraction and Isolation. Dried and powdered aerial parts of *C. oliveriana* (2.23 kg) were defatted with hexanes (6 L) for one month and subsequently extracted repeatedly with 80% EtOH (7 L) at room temperature for two weeks. The extract was filtered and concentrated at reduced pressure. The remaining aqueous layer was exhaustively extracted with *n*-BuOH to give, after removal of the solvent, 36 g of a brown viscous residue. The aqueous layer was concentrated and filtered through a column of Amberlite XAD-2 resin (8 × 40 cm) to remove the polar compounds, while the flavonoids remaining on the column were eluted with MeOH (see below). The viscous *n*-BuOH extract (10 g) was fractionated on a 50 × 8 cm column packed with Sephadex LH-20 and eluted with hexanes−CH_2Cl_2−MeOH, 1:1:2, 15 500-mL fractions (S1−S15) being collected. Fractions S1−S15 contained mainly alkaloids contaminated by material exhibiting no UV absorption, and fractions S4−S8 contained mixtures of glycosides. Fractions S9 and S10 and fractions S11−S13, after recrystallization from MeOH−EtAc, gave quercetin (**2**, 556 mg) and kaempferol (**1**, 472 mg), respectively. Fractions S4−S8 were chromatographed over a 40 × 4 cm Sephadex LH-20 column using hexane−CH_2Cl_2−MeOH (1:1:1), the elution being monitored by TLC analysis. This resulted in three fractions: A (430 mg), B (70 mg), and C (55 mg). Fraction B afforded 90 mg of trifolin (**3**) after recrystallization from MeOH. Rechromatography of fraction A over silica gel using 40 mL of hexanes−EtOAc mixtures of increasing polarity rendered, from fractions 64−77 (hexanes−EtOAc, 2:8), 24 mg of 6″-*O*-acetylhyperoside after further purification over Sephadex LH-20 (hexanes−MeOH−CH_2Cl_2, 2:1:1). Fractions 86−90 (*n*-hexane−EtOHc, 1:9) yielded 21 mg of 6″-*O*-acetylhyperoside (**5**).

The material eluted from the Amberlite XAD-2 column with MeOH was further purified over a 50 × 8 cm column packed with Sephadex LH-20 and eluted with CH_2Cl_2−MeOH (1:1), six fractions (J1−J6) of 500 mL each being collected. Fractions J1−J3 containing mainly alkaloids and other components exhibiting no UV absorption were combined with fractions S1−S3. Fractions J4−J6 (2 g) were subjected to gel filtration on Sephadex LH-20 using 42 200-mL fractions of H_2O−MeOH (1:1). Fractions 28−31 yielded 45 mg of trifolin (**3**) and fractions 33−38 18 mg of octa-*O*-acetylhyperoside (**4**).

General Method for Acetylation. Dry phenolic material was dissolved in the minimum volume of pyridine. Twice the amount of acetic anhydride was added, and the solution was allowed to stand overnight at ambient temperature. The mixture was diluted with H_2O and extracted three times with EtOAc. The extract was evaporated under vacuum, and the residue containing the polyacetate was further purified by column chromatography over silica gel using hexanes−EtOAc as the eluent. Mass spectra of the polyacetylated compounds, all gums, are listed below.

Tetra-*O*-acetylkaempferol (1a): EIMS m/z = 412 ($M^+ - C_2H_3O_2$, 23), 370 ($M^+ - 2\ C_2H_3O_2$, 57) 286 ($M^+ - 4\ C_2H_3O_2$, 100).

Penta-*O*-acetylquercetin (2a): HREIMS m/z = 513.0997, calcd for $C_{25}H_{20}O_2 + H^+$ 513.1033.

Hepta-*O*-acetyltrifolin (3a): HRFABMS m/z = 743.1784, calcd for $C_{35}H_{34}O_{18} + H^+$ 743.1823.

The compound purity of 99% was determined by HPLC.

Promastigote Assay. The compounds obtained (Figure 1) were dissolved in DMSO (Panreac, Barcelona, Spain) at a concentration of 0.1% and were afterward assayed as nontoxic and without inhibitory effects on the parasite growth, according to Luque et al.[43] The compounds were dissolved in the culture medium, and the dosages used were 100, 50, 25, 10, and 1 μM. The effect of each compound against promastigote forms, as well as the concentrations, was evaluated at 24, 48, and 72 h using a Neubauer hemocytometric chamber. The leishmanicidal effect is expressed as IC_{50} values, i.e., the concentration required to give 50% inhibition, calculated by linear-regression analysis from the K_c values at the concentrations employed.

Cell Culture and Cytotoxicity Tests. Macrophage line J774.2 (ECACC number 91051511) cells was obtained from a tumor in a female BALB/c rat in 1968. Macrophages were kept in the laboratory by cryopreservation in liquid N_2 and then by successive subcultures in RPMI medium. For the cytotoxicity test, macrophages were placed in 25 mL cone-based bottles (Sterling) and centrifuged at 1500 rpm for 5

min. The culture medium was removed, and minimal essential medium (MEM; Gibco) supplemented with 10% inactivated fetal bovine serum (adjusted to pH 7.2) was added to a final concentration of 10^5 cells/mL. This cell suspension was distributed in a culture tray (24 wells) at a rate of 100 μL/well and incubated for 2 days at 37 °C in a humid atmosphere enriched with 5% CO_2. The medium was removed, and the fresh medium was added together with the product to be studied (at a concentration of 100, 50, 25, 10, and 1 μM). The cultures were incubated for 72 h. The vital stain trypan blue (0.1% in phosphate buffer) was used to determine cell viability. The number of dead cells was recorded, the percentage of viability was calculated in comparison to that of the control culture, and the IC_{50} was calculated by linear-regression analysis from the K_e values at the concentrations employed.

Amastigote Assay. J774.2 macrophage cells were grown in MEM medium in a humidified 95% air−5% CO_2 atmosphere at 37 °C. Cells were seeded at a density of 1×10^5 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultivated for 2 days. Afterward the cells were infected *in vitro* with promastigote forms of *L. (V.) peruviana* and *L. (V.) brazilensis*, at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and were incubated for 6 h at 37 °C in 5% CO_2. The nonphagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h.

The drug activity was determined from the percentage of infected cells and the number of amastigotes per infected cell in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed in randomly chosen microscopic fields. Values are the means of four separate determinations.

Metabolite Excretion. Cultures of *L. (V.) peruviana* and *L. (V.) braziliensis* promastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} of the drugs (except for control cultures). After incubation for 96 h at 28 °C, the cells were centrifuged at 1500 rpm for 10 min. The supernatants were collected to determine excreted metabolites by nuclear magnetic resonance spectroscopy (^{1}H NMR) as previously described by Sánchez-Moreno et al.[44] The chemical displacements were expressed in parts per million (ppm), using sodium 2,2 dimethyl-2-silapentane-5-sulfonate as the reference signal. The chemical displacements used to identify the respective metabolites were consistent with those described by Fernandez-Becerra et al.[45]

Ultrastructural Alterations. *L. (V.) peruviana* and *L. (V.) braziliensis*, at a density of 5×10^6 cells/mL, were cultured in MTL medium plus 10% inactivated fetal bovine serum kept in an air atmosphere at 28 °C, in Roux flasks (Corning, USA) of 75 cm^2 in surface area, containing the drugs at IC_{25} concentration. After 96 h, the cultures were centrifuged at 1500 rpm for 10 min, and the pellets were washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Pellets were prepared for transmission-electron microscopy following the technique of Luque et al.[43]

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Granada, 04/06/09

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To: The Editor
Open Natural Products Journal

Dear Editor

We are enclosing our manuscript entiled "**Activity and mode of action of flavonoids compounds against intracellular and extracellular forms of *Trypanosoma cruzi***" by Boutaleb-Charki et al., that we wish to submit for publication in The Open Natural Products Journal.

This work reports the *in vitro* activity of a number of flavonoids (kaempferol, quercetin, trifolin, and acetyl hyperoside) and their acetylated products: kaempferol acetate, quercetin acetate, trifolin acetate, and acetyl hyperoside acetate) isolated from the aerial parts of plant *Consolida oliveriana*. against epimastigote, amastigote and metacyclic forms of *T. cruzi*. These flavonoid derivatives decreased the ability of metacyclic forms to invade mammalian cells, their intracellular replications and their transformation in trypomastigotes, with no toxicity to the host cells. The cells treated with these compounds presented severe damage in their ultrastructure. Thus is, the flavons have promising anti-trypanocide properties and represent an exciting advance in the search for new antiprotozoal agents.

We hope that this manuscript will be found acceptable for publication in your journal.

Yours sincerely,

C. Marín

Activity and mode of action of flavonoids compounds against intracellular and extracellular forms of *Trypanosoma cruzi*

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Abstract

This study investigates the in vitro activity of a number of flavonoids (kaempferol, quercetin, trifolin, and acetyl hyperoside) and their acetylated products: kaempferol acetate, quercetin acetate, trifolin acetate, and acetyl hyperoside acetate) isolated from the aerial parts of plant *Consolida oliveriana* against epimastigote, amastigote and metacyclic forms of *T. cruzi*, their cytotoxic against a host Vero-cell line and analyse the possible mechanism by which these molecules act. Acetylated compounds were potent *T. cruzi* epimastigote growth inhibitors with activity levels similar to those of benznidazole, used as the reference drug. These compounds, at the dosage IC_{25}, decreased the ability of metacyclic forms to invade mammalian cells, their intracellular replications and transformation in trypomastigotes, with no toxicity to the host cells. The cells treated presented severe damage in their ultrastructure: intense vacuolization,

and appearance of lysosomes as well as other residual bodies. The mitochondrial section appeared larger in size, with a swollen matrix. In addition, these compounds changed the excretion of end metabolites, primarily affecting acetate and succinate excretion, possibly by directly influencing certain enzymes or their synthesis. The potent tripanocidal activities of the flavons described here represent an exciting advance in the search for new antiprotozoal agents.

Keywords: Flavonoids compounds, *Consolida oliveriana*, antitripanocidal agents, mode of action.

INTRODUCTION

Chagas disease is a complex zoonosis caused by *Trypanosoma cruzi*. It constitutes a permanent threat for almost a fourth of the total population of Latin America, estimated at 28 million people in the year 2006, which are under risk of infection, taking into account the geographic distribution of the vector insects and the multiple reservoirs involved in the diverse transmission cycles. The disease is found in Mexico as well as all of Central and South America, where the clinical manifestations and epidemiological characteristics are highly variable in the different zones where the disease is endemic[1].

It is important to take into account that migratory movements from rural to urban zones has changed the epidemiological characteristics of Chagas disease. It is estimated that around 70% of the Latin American population lives in urban areas, whereas the figures from the 1930s showed 70% in rural areas. Thus, the infection that had been primarily rural has become urban and transmissible by blood transfusion. Furthermore, transmission is not limited to the countries in which the disease is endemic. In fact, it has extended to other countries such as Canada, the United States, and Spain, where cases of transmission of *T. cruzi* through blood products have been documented [2].

Drugs currently used to treat Chagas disease are nitro-heterocyclic nitrofurane compounds: nifurtimox (Lampit® Bayer) and benznidazole, a derivative of nitroimidazol (Rochagan®, Radanil®, Roche), the anti-*T. cruzi* activities of which were empirically described three decades ago [3]. Nifurtimox and benznidazol show strong activity, which has been well documented in the acute phase (c. 80% cures in patients

treated). However, in the indeterminate phase and in the chronic form, the results are less conclusive. Also, there is information indicating that the effectiveness of these drugs varies according to the geographical area, probably due to differences in the drug susceptibility of different *T. cruzi* strains. In addition, the common side-effects, including anorexia, vomiting, peripheral polyeuropathy, and allergic dermopathy, can in some cases require the interruption of the treatment [4].

The reasons for the great differences in the anti-parasite effectiveness of the nitro-heterocyclic compounds in the acute and chronic phases is not yet clear, but may be related to inadequate pharmocokinetic properties of the drugs in treating chronic infections. This has led to the conclusion that new drugs with a different pharmacological profile need to be developed.

One strategy to discover new drug is to investigate natural products from plants used medicina1ly [5]. *Consolida*, a highly specialized genus of *Ranunculaceae* closely allied to *Delphinium* with its centre of diversity in Anatolia, is, like its relatives, a rich source of alkaloids. Previous studies of the aerial parts of *Consolida oliveriana* (DC) Schrod., a species used medicinally in parts of Anatolia, have concentrated on the isolation of its constituent alkaloids [6]. Many publications have dealt with the diterpenoid alkaloids of other *Consolida* species but reports on the flavonoid content of members of this genus are scarse[7]. The biological action of those compounds is less known. There are a few reports on their plant defensive and pharmacologica1 properties, including their effects on *T. cruzi* epimastigote forms and anti-leishmanial properties, but their neurotoxic effects are unknown [8,9].

As part of the ongoing research by the Tenerife group on flavonoids in the genus *Delphinium* and related genera, we have investigated the most polar fraction of the ethanol extract of the aerial parts of *C. oliveriana*. In our study, we investigate the activity of a number of these flavonoids against epimastigote, amastigote, and metacyclic forms of *T. cruzi*. In addition, we examine the cytotoxic effects of these compounds against a host Vero-cell line and we analyse the possible mechanism by which these molecules act.

MATERIALS AND METHODS

Parasite Strain, Culture and in vitro Studies

The Maracay strain of *T. cruzi* was isolated at the Institute of Malariology and Environmental Health in Maracay (Venezuela). Epimastigote forms were obtained in biphasic blood-agar NNN medium (Novy-Nicolle-McNea1) supplemented with MEM and 20% inactivated feta1 bovine serum and afterwards reseeded in a monophasic culture (MTL), following the method of [10].

Plant material

Aerial parts of the plant *Consolida oliveriana* (DC) Schrod. were collected and processed as done elsewhere [11]. The compounds obtained (Fig. **1**) were dissolved in dimethyl sulfoxide (Panreac, Barcelona, Spain) at a concentration of 0.1 %, after being assayed as nontoxic and without inhibitory effects on the parasite growth, according to existing methods [10]. The compounds were dissolved in the culture medium, and the dosages used were: 100, 50, 25, 10 and l μM. The effect of each compound against epimastigote forms, as well as the concentrations, was evaluated at 24, 48 and 72 h using a Neubauer hemocytometric chamber and the trypanocidal effect is expressed as IC_{50} values, i.e the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at the concentrations employed.

Cell Culture and Cytotoxicity Tests

Vero cells (F1ow) were grown in Minimal Essential Médium (MEM; Gibco) supplemented with l0% inactivated fetal calf serum and adjusted to pH 7.2, in a humidified 95% air-5% CO_2 atmosphere at 37°C for 2 days. The test for cytotoxicity, cells were placed in 25 ml colie-based bottles (Sterling), and centrifuged at 100 x **g** for 5 min. The cu1ture medium was removed, and fresh médium was added to a fina1 concentration of 1 x 10^5 cells/ml. This cell suspension was distributed in a culture tray (with 24 wells) at a rate of 100 μl/well and incubated for 2 days at 37°C in humid atmosphere enriched with 5% CO_2. The medium was removed, and the fresh medium was added together with the product to be studied (at a concentration 100, 50, 25, l0 and 1 μM). The cu1tures were incubated for 72 h. The vita1 stain trypan blue (0.1% in phosphate buffer) was used to determine cell viability. The number of dead cells was recorded, and the percent viability was ca1culated in comparison to that of the control

cu1ture, and the IC_{50} ca1culated by linear regression ana1ysis from the Kc va1ues at the concentrations employed.

Transformation of epimastigote to metacyclic forms

To induce metacyclogenesis, parasites were cu1tured at 28°C in modified Grace's medium (Gibco) for 12 days according to the methods described by [12]. Twelve days after cu1tivation at 28°C, metacyc1ic forms were counted in order to infect Vero ce11s. The proportion of metacyc1ic forms was around 40% at this stage.

Amastigote- Vero Cell Assay

Vero cells fueron cultivadas in MEM medium in a humidified 95% air-5% CO_2 atmosphere at 37°C. Cells were seeded at a density of 1 x 10^5 cells/ well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultivated for 2 days. After were infected in vitro with *T. cruzi* metacyclic forms at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection, and were incubated for 6 h at 37°C in a 5% CO_2. The non-phagocytosed parasites and the drugs were removed by washing and then the infected cultures were grown for 8 days in fresh medium. Fresh cu1ture medium was added every 48 h.

The drug activity was determined from the percentage of infected cells and the number of amastigotes per Vero cells infected in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The percentage of infected Vero cells and the mean number of amastigotes per infected cell were determined by analyzing more than 100 host cells distributed in randomly chosen microscopic fields. Va1ues are the means of four separate determinations. The number of trypomastigotes in the medium was determined as described previously [13].

Ultrastructural alterations

The parasites, at a density of 5×10^6 cells/mL, were cultured in their corresponding medium, containing the drugs at la concentración de IC_{25}. After 72 h, the cultures were centrifuged at 400 x ***g*** for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) *p*-formaldehyde-glutaraldehyde in 0.05M cacodylate buffer

(pH 7.4) for 2 h at 4 ◦C. Pellets were prepared for transmission-electron microscopy following the technique of [10].

Metabolite Excretion

Cu1tures of epimastigote forms of *T cruzi* (initia1 concentration 5 x 10^5 cells/ml) received de IC_{25} of the flavonoids conpounds (except for control cultures). After incubation for 72 h at 28 ° C, the cells were centrifuged at 400 x **g** for l0 min. The supernatants were collected to determine excreted metabolites by nuclear magnetic resonance spectroscopy (^{1}H-NMR) as previously described by [14]. The chemical displacements were expressed in parts per million (ppm), using sodium 2,2dimethyl-2-silapentane-5-su1fonate as the reference signal. The chemica1 disp1acements used to identify the respective metabo1ites were consistent with those described by [14].

RESULTS AND DISCUSSION

Plant-derived active principles and their semi-synthetic and synthetic analogues have served as a major route to new chemotherapy compounds [15,16]. Current conventional chemotherapy treatments are very expensive, toxic, and less effective in treating the disease. The use of natural products for the treatment of protozoal infections (*Leishmania* spp. and *T. cruzi*) is well known and has been documented since ancient times [5,9]. Flavonoids are found in abundance in diets rich in fruits, vegetables and plant-derived beverages and appear to have anti-cancer, anti-microbial and anti-parasitic properties [11]. Here, we evaluate the anti-protozoan properties of a number of flavonoids obtained from natural sources and their acetylated derivatives.

The inhibitory effect of eight flavonoids compounds on the in vitro growth of *T. cruzi* epimastigotes was measured at different times following established procedures (see Materials and Methods). The results are displayed in **Table 1** for benznidazole used as the reference drug and including toxicity values against Vero cells. The control epimastigote cells were cultured in the presence of dimethyl sulfoxide at the same concentration as that added to the cultures with the flavonoid compounds. Of the eight compounds assayed, four (**1, 2, 3 and 5**) were obtained in a natural way from the plant *Consolida oliveriana* and the other four by acetylation of these natural products (**1a, 2a, 3a and 4**) and these latter four acetylated compounds proved the most active against *T.*

cruzi epimastigotes. After 72 h of exposure, the compounds **1a** y **4** showed IC_{50} values of 9.87 and 13.22 μM, respectively, close to those of benznidazole (referente drugs). These four acetylated compounds tested proved much less toxic than benznidazole against Vero cells. It bears mentioning that compounds **1**a and **4** exhibited an inhibitory concentration of 102.5 and 188.5 μM after 72 h of culture, respectively. This is 8 and 14 times higher than the corresponding value measured for benznidazole (13.6 μM), respectively. The acetylation of certain flavonoids has been shown to increase the antiproliferative activities of the parent compounds against HL-60 and other cell lines. For example, **3a** caused cell death in human leukaemia cells [15]. This greater effectiveness may be due to acetylation facilitating absorption of the product.

In most studies on activity assays of new compounds against parasites, forms that develop in vectors are used (epimastigote forms in the case of *T. cruzi*) [10], for the ease of working with these forms *in vitro*; however, in this study, we have included the effect of these compounds on the forms that are developed in the host (amastigotes and trypomastigotes), the study of which is of great importance, given that the final aim is to determine the effects in the definitive host. For this type of work and studies on the action mechanism, we selected the products that had the greatest inhibitory effect on the *in vitro* growth of *T. cruzi* (**1a, 2a, 3a and 4**) and that at the same time had less toxic effect on Vero cells, using the IC_{25} of each product as the test dosage.

Figure **(2a)** illustrates *T. cruzi* propagation in Vero cell (with and without co-addition of the **1a, 2a, 3a and 4** compounds). When $1x10^5$ Vero cells were incubated for 2 days and then infected with $1x10^6$ metacyclic forms, the parasites invaded the cells and underwent morphologic conversión to amastigotes within 1 day after infection. On days 4, 6, 8 and 10, the rate of host-cell infection increased to 15, 46, 80 and 90%, respectively. When flavonoids compounds (**1a, 2a, 3a and 4**) were added simultaneously to the infection of Vero cells with *T. cruzi* metacyclic forms (IC_{25} concentration), the infection rate significantly decreased with respect to the control, reaching a 32 and 38% for the compounds **1a** and **3a,** respectively on day 10. In the control experiments, the average number of amastigotes per infected cell increased to 85.6 on day 6, descreasing to 50 on day 10 (Figure **2b**). The compounds **4, 1a and 3a** inhibited *T. cruzi* amastigote replication in Vero cells in vitro. Thus, the addition of a concentration equivalent to the IC_{25} of these flavonoids compounds produces a markedly lowered the amastigote number per infected cell, reaching 60, 56 y 42 %, respectively, reduction in amastigote number respect to control for cultures day 10.

The decrease in the average amastigote number on day 7 for the control experiment coincided with the increase in trypomastigote numbers in the médium. This behavior is due to the rupture of the Vero cells with the subsequent release of amastigotes and futre transformation into trypomastigotes. The number of trypomastigotes in the médium was 1.8 x 10^6 parasites on day 10. While, the three acetilados flavonoids compounds (**4, 1a and 3a**) gave 88, 71 and 59 % reduction in trypomastigote numbers, respectively (Figure **2c**).

Alterations in the excretion of different metabolites, as well as inhibition of enzyme activity in the major metabolic pathways by which these organisms gain energy, are data of great use for elucidating the toxic activity in this field. The epimastigote forms of *T. cruzi* depend entirely on glycolysis for obtaining energy, as also happens in other trypanosomatids. The special compartmentalization presented by these organisms in the glycolytic pathway enables them to direct this process more efficiently than can a conventional eukaryotic cell [17]. The ability to utilize a fast and effective form of sugar is of unquestionable value in adapting and colonizing different hosts, especially given that these organisms lack a conventional system to store carbohydrates [18]. As far as it is known to date, none of the trypanosomatids studied can completely degrade glucose to CO_2 under aerobic conditions, excreting into the medium as fermented metabolites a great part of their carbon skeleton, which differs depending on the species considered [19]. *Trypanosoma cruzi* consumes glucose at a high rate, thereby acidifying the culture medium due to incomplete oxidation to acids.

Therefore, we have considered of interest the use of ^{1}H NMR spectra for the identification and evaluation of the inhibiting effect caused by the compounds in metabolites excreted by *T. cruzi*. An example of the types of spectra obtained with Graces medium is shown in the different frames of Figure (**3a**) displays the spectrum obtained with fresh (uninoculated) medium, and Figure (**3b**) corresponds to the spectrum given by cell-free medium 4 days after inoculation with the *T. cruzi* strain. Additional peaks, corresponding to the major metabolites produced and excreted during growth, were detected when the last spectrum (Figure **3b**) was compared with that one obtained with fresh medium (Figure **3a**). The metabolites excreted were mainly acetate, succinate, and L-alanine. When the flagellates were treated with four compounds, the excretion of some of these catabolites was clearly inhibited, mainly in the cases of **4**, which inhibited mainly the production of acetate and succinate by 34 and 35 %, respectively, at the dosage assayed (IC_{25}) (Figure **3c**). Meanwhile, **1a** caused inhibitions

of 32 and 20 % in the production of these two metabolites (Figure **3d**) whereas **3a** inhibited acetate and succinate production by 20 and 22 %, respectively, but pyruvate production increased significantly (Figure **3e**), respectively, order of effectiveness in metabolite inhibition being **4** > **1a** > **3a**. The effect on the excretion products could be due to the action that the compounds tested exerted on enzymes involved in their degradation pattern or to a loss in functionality of the mitochondrion, the organelle where succinate and acetate are formed by these parasites, since glycolysis is compartmentalized [14]. This hypothesis is reinforced by electron-microscope data. The main role of the production of these metabolites was to maintain the redox balance [20,21], as its production was inhibited. Parasites develop alternative strategies to subsist, opening other fermentative pathways by which they produce L-alanine, ethanol, glycerol, and even pyruvate, thereby maintaining the redox balance.

Morphological alterations of *T. cruzi* epimastigote treated with compounds **1a, 2a, 3a** and **4** were examined by transmission electron microscopy (TEM). All the compounds assayed at IC_{25} for 72 h induced lysis or alterations of the parasites (Figs**. 4-A** to **I**) as compared to control cells (Fig. **1-A**).

The treatment with **4** (Figs. **4-B** and **C**) caused death in some parasites and distortion of the parasite body, swelling, and flattening. The compound shortened cell length and severely distorted cell shape (Fig. **4-B**). Frequent cells alterations included the disruption of the cytoplasm with marked alterations of reservosomes (arrow heads, Figs **4-B** and **C**) and strong cytoplasmic vacuolisation. Glycosomes were abundant and swelling appeared in some cases (Fig. **4-C**). The kinetoplast was also altered (Fig. **1-C**), with distortion of the DNA helicoidal structure.

Parasites treated with **2a** showed great alterations (Figs. **4-D** and **E**). Most of the cells died at this concentration and many of cytoplasmic organelles appeared free in the culture (Fig**. 4-D**), by the rupture of the parasites. Epimastigotes were smaller and the distortion of the nucleus, cytoplasm, and plasma membrane was clear (Fig**. 4-E**). The treatment with **1a** (Figs. **4-E** and **F**) caused different alterations. Most of the parasites showed complete alteration in shape. Other parasites presented alterations in shape, becoming almost unrecognizable, with electrodense cytoplasm that hampered the identification of the cytoplasmic organelles (Figs. **4-F** and **G**). In these parasites the acidocalcisomes and endoplasmic reticulum were abundant (Fig. **4-F**). Dead parasites were also detected. Nuclei could not be found in some cells and appeared disrupted in

others (Fig**. 4-G**). Concentric structures, myelin-like figures, appeared in some parasites and vacuolization was frequent. The mitochondrion and kinetoplast were also affected by the treatment with **1a**, (Fig. **4-G**) appearing swollen. Glycosomes were normal in some parasites but in others appeared less electrodense.

The product **3a** caused no alterations in the parasite shape but many cells were dead (Figs. **4-H** and **I**). The most frequent alterations were the swelling of the mitochondria and disruption of kinetoplastic DNA (Fig**. 4-H**) together with the formation of autophagic vesicles and progressive vacuolization of the cytoplasm (Figs. **4H** and **I**). Some parasites showed abundant endoplasmic reticulum or ribosomes in the cytoplasm (Fig. **4-H**) while in other parasites the cytoplasm was almost empty, containing only the degraded remains of the nucleus and cytoplasmic organelles (Fig. **4-I**).

This is the first study made on the trypanocide activity of aceylate flavonoid compounds obtained from *C. oliveriana*. Our results show that the compounds tested here were highly active *in vitro* against both the extracellular as well as the intracellular forms of *T. cruzi*. These compounds (mainly acetylated derivatives obtained in a natural way from the plant) are not toxic to the host cells and are effective at concentrations similar to or lower than the reference drug used in the present study. The *in vitro* growth rate of *T. cruzi* was depressed, its capacity to infect cells was diminished and the multiplication of the amastigotes was greatly reduced.

In conclusion, our study provides data that these flavonoid derivatives have promising anti-trypanocide properties. These could have implications for other intracellular pathogens or phylogenetically related parasites, as shown for *Leishmania* spp. The potent trypanocidal activities of the plant-derived flavons described here represent an exciting advance in the search for new antiprotozoal agents.

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Leyends

Figure 1. Chemical structure of flavonoids compounds

Figure 2.- Effect of acetilated flavonoids compounds on the infection rate and *T. cruzi* growth. (**A**) rate of infection. (**B**) mean number of amastigotes per infected Vero cell. (**C**) number of trypomastigotes in the culture medium. -▲-, control; -+-, **1a**; -●-, **2a**; -♦-, **3a;** -Δ-, **4** (at IC_{25} conc.). The values are means of three separate experiment.

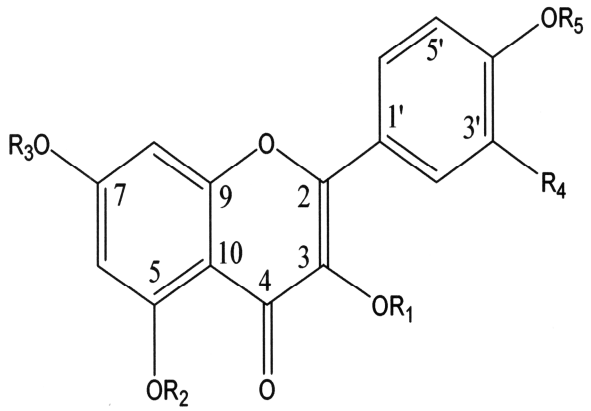
Figure 3. ^{1}H-NMR spectra epimastigote forms of *T cruzi* treated against flavonoids compounds (at a concentration of IC_{25}): (**I**) fresh medium, (**II**); Control (untreated); (**III**) **1a**; (**IV**) **3a**; (**V**) **4** and (**VI**) **2a**. L-ala, alanine; Ace, acetate; Pyr, pyruvate; Suc, succinate and Gly, glycerol.

Figure 4. Ultraestructural alterations by TEM in *T. cruzi* treated with acetilated flavonoids compounds (at a concentration of IC_{25}). **(A)** Control parasite, 12000 x (bar= 0.583 μ). **(B** and **C)** Epimatigote forms of T. cruzi treated with **4,** 12000 and 7000 x (bar=0.583 and 1.00 μ respectively) **. (D** and **E)** Epimatigote forms treated with **2a,** 4400 x (bar=1.59 μ). **(F** and **G)** Epimatigote forms treated with **1a,** 4400 x (bar=1.59 μ). **(H** and **I)** Epimatigote forms treated with **3a,** 7000 x (bar=1.00 μ). (N) nucleus, (Nu) nucleolus, (K) kinetoplast, (M) mitochondrion, (F) flagellum, (V) vacuoles, (G) glicosomes,. (R) reservosomes, (C) cytoplasmic organelles, (Mi) myelin-like figures and (A) acidocalcisomes.

Table 1. In vitro activity of flavonoids compounds on *Trypanosoma cruzi* epimastigote

Compounds	IC_{50} (μM)	Toxicity IC_{50} on Vero cells (μM)[a]
Benznidazole	15.83	13.56
1	321.43	122.51
1a	9.87	102.48
2	52.02	143.71
2a	38.77	152.68
3	112.00	86.13
3a	35.47	117.97
4	13.22	188.46
5	102.21	45.90

[a]On Vero cells at 72 h of culture. IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the K_c values at the employed concentrations (0.1,1,10, 25, 50 and 100 μM) at 72 h culture
Note: Average of three separate determinations.



1.- Kaempferol: R_1= R_2= R_3= R_4= R_5 = H

1a.- Kaempferol acetate: R_1= R_2= R_3= R_5= Ac, R_4 = H

2.- Quercetin: R_1= R_2= R_3= R_5 = H, R_4= OH

2a.- Quercetin acetate : R_1= R_2= R_3= R_5 = Ac, R_4= OAc

3.- Trifolin: R_1= Gal; R_2= R_3= R_4= R_5 = H

3a.- Trifolin acetate: R_1= Gal Ac; R_2= R_3= R_5= Ac, R_4 = H

4.- Hyperoside acetate : R_1= GalAc; R_2= R_3= R_5 = Ac, R_4= OAc

5.- 6" Acetyl hyperoside: R_1= Gal 6"Ac; R_2= R_3= R_5 = H, R_4= OH

Gal = -β-galactopyranosyl.

Figure 1. Chemical structure of flavonoids compounds

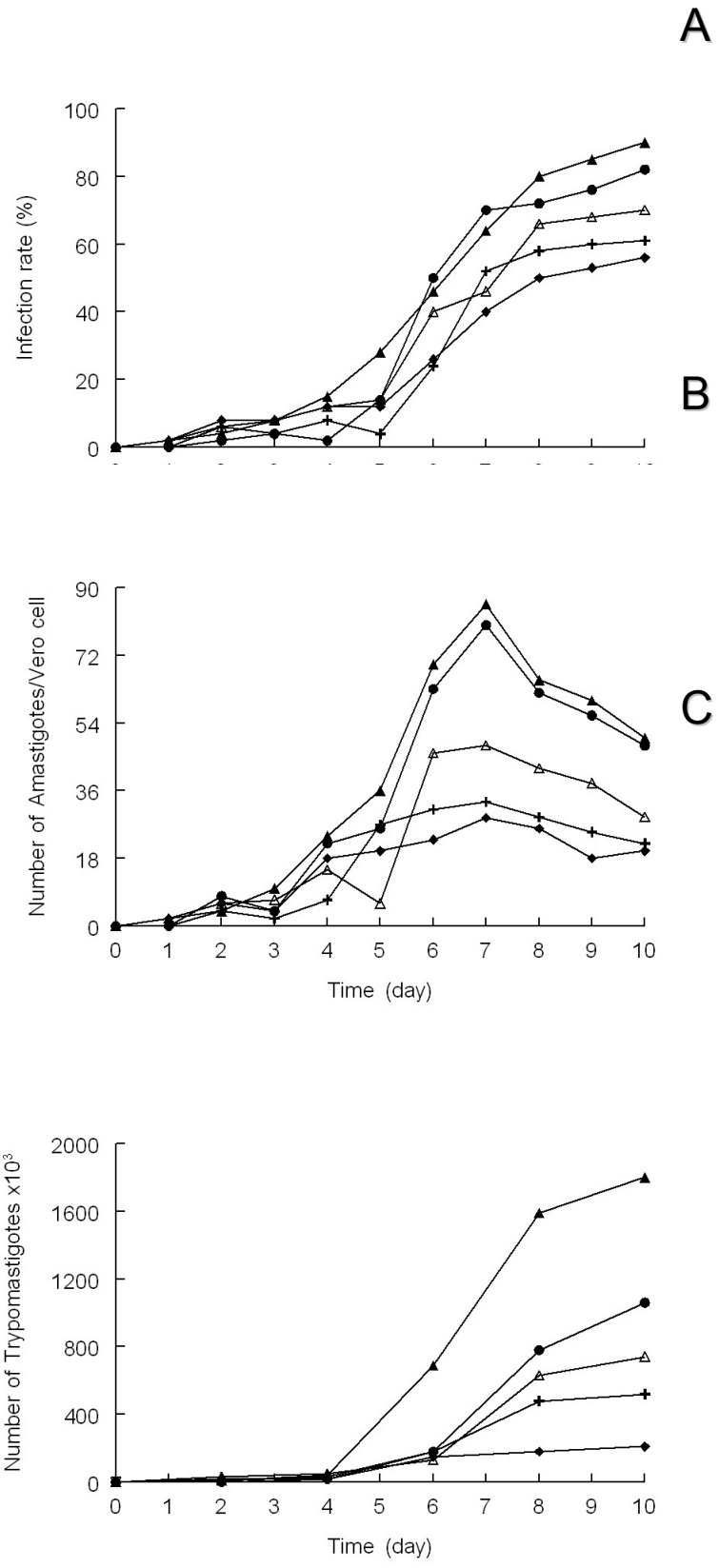
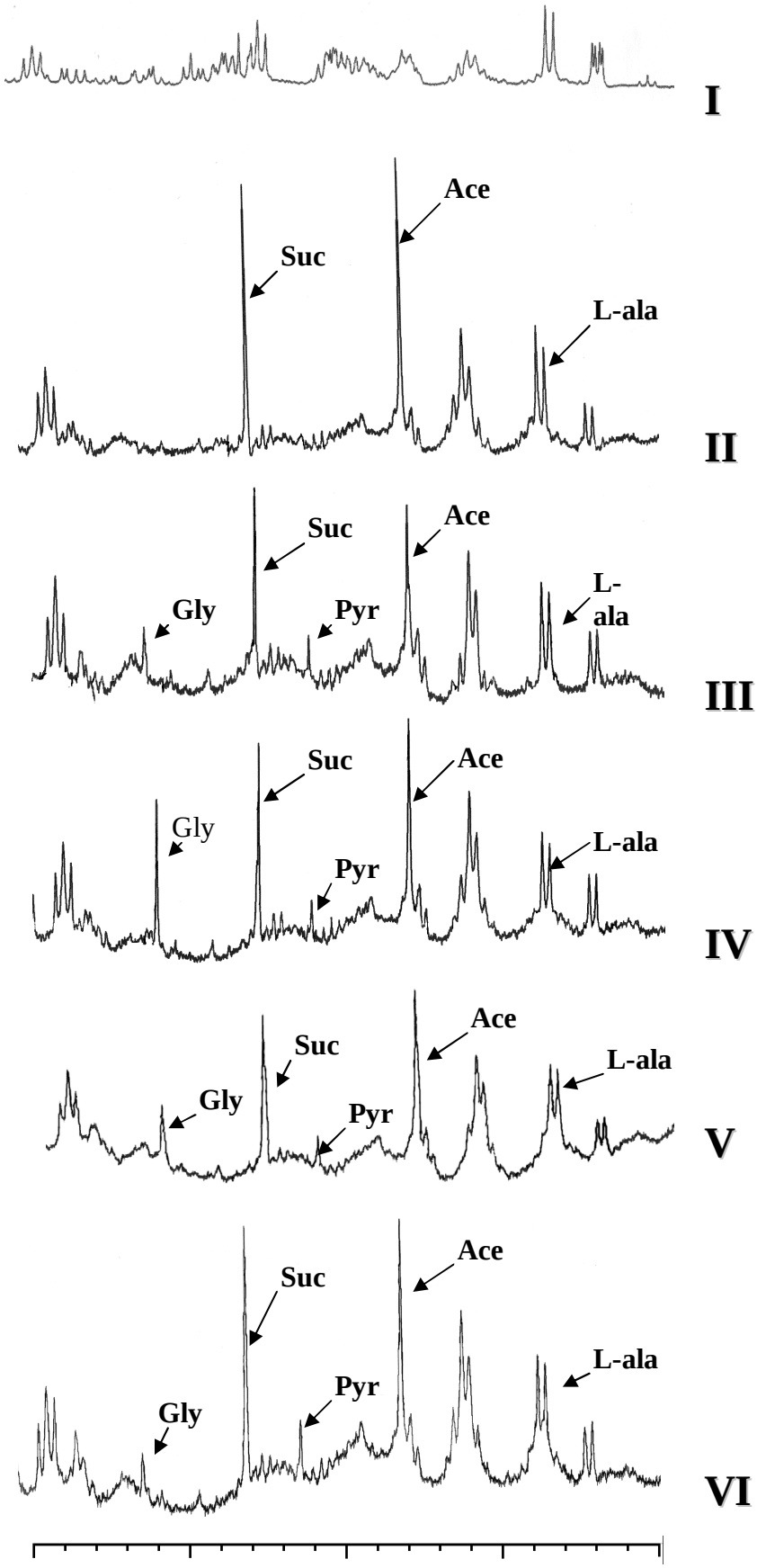

A
B
C
Infection rate (%)
100
80
60
40
20
0
Number of Amastigotes/Vero cell
90
72
54
36
18
0
0 1 2 3 4 5 6 7 8 9 10
Time (day)
Number of Trypomastigotes x10³
2000
1600
1200
800
400
0
0 1 2 3 4 5 6 7 8 9 10
Time (day)

Figure 2


I
Ace
Suc
L-ala
II
Suc
Ace
Gly
Pyr
L-
ala
III
Suc
Ace
Gly
L-ala
Pyr
IV
Ace
Suc
L-ala
Gly
Pyr
V
Suc
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Gly
Pyr
L-ala
VI
3.0
2.5
2.0
1.5
1.0 ppm

Figure 3.

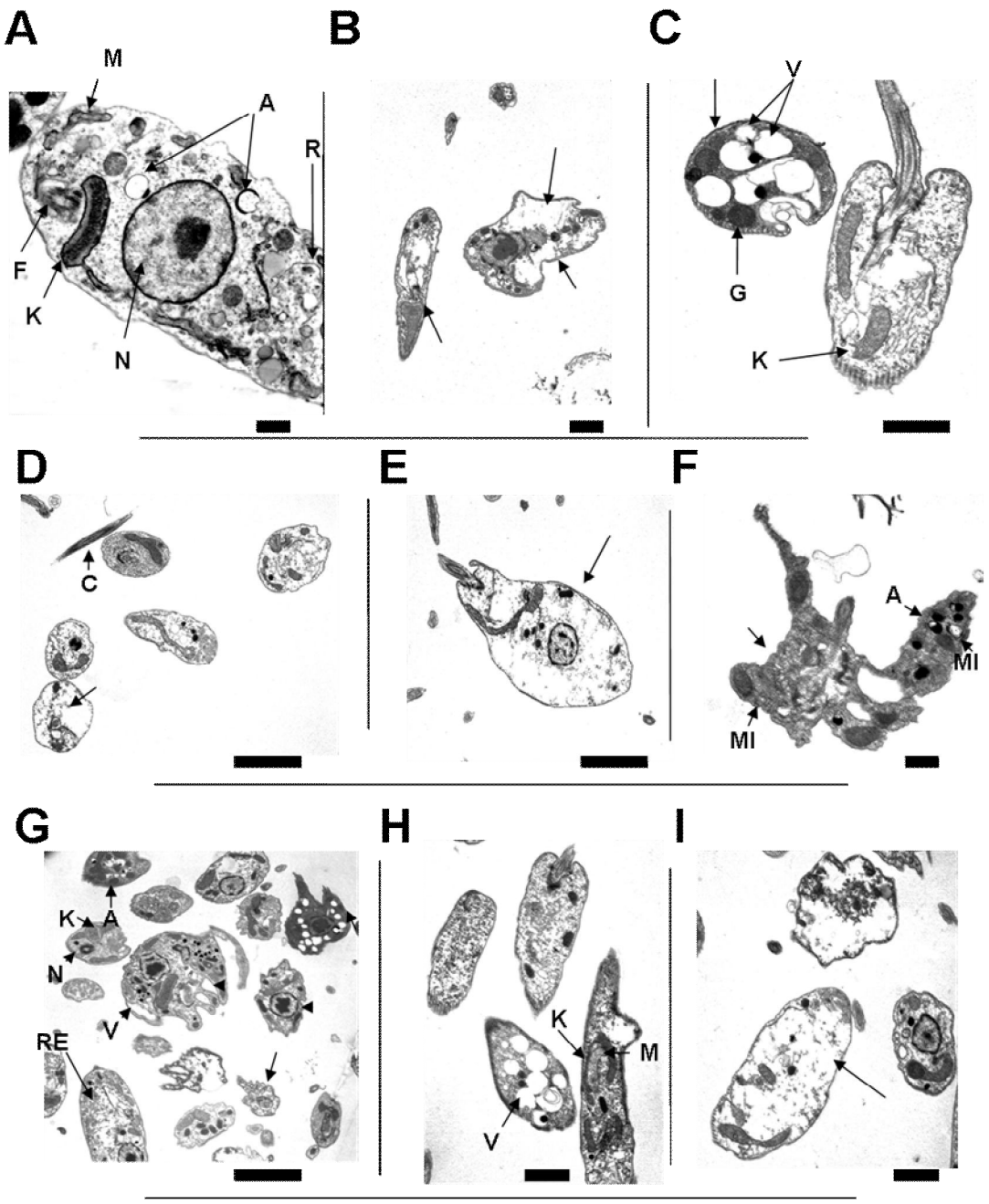

Figure 4

V-DISCUSIÓN

Las enfermedades tropicales causadas por parásitos protozoarios (enfermedad de Chagas y leishmaniasis) afectan a millares de personas principalmente en los países menos desarrollados suponiendo un grave problema de salud pública mundial en expansión por los flujos migratorios y que se agrava ante la inexistencia de un tratamiento eficaz. Estas patologías, también llamadas “enfermedades extremadamente olvidadas”, han sido relegadas por las empresas farmacéuticas dado que las poblaciones afectadas no representan un retorno lucrativo suficiente que justifique la inversión en investigación y desarrollo. La crisis de falta de medicamentos alcanza la actual proporción no por falta de conocimiento científico, ni por la brecha que existe entre la investigación básica y la preclínica, sino por la carencia de políticas públicas para I+D de medicamentos de interés nacional de los países en desarrollo, como el desinterés del mercado por el bajo interés económico que estos pacientes representan para la industria.

Así, la búsqueda de nuevos fármacos recae casi exclusivamente en la investigación pública financiada por gobiernos y organizaciones sin ánimo de lucro. En los últimos años, la tendencia respecto al desarrollo de nuevos fármacos está cambiando de un “screening” aleatorio hacia un diseño más racional o dirigido hacia enzimas o moléculas “dianas”. Las investigaciones realizadas en las dos últimas décadas en bioquímica y biología molecular en tripanosomátidos, han revelado algunos "blancos" terapéuticos potenciales.

Por ello, el primer objetivo de esta memoria fue determinar los efectos de distintos compuestos de nueva síntesis y extraidos de plantas medicinales sobre el crecimiento de los parásitos in vitro.

En total se han ensayados **48** compuestos: **40** de ellos son sintetizados y 8 de origen natural.

En un primer paso, se determinó el efecto de 6 compuestos **1,4-Bis (alkylamino) benzo[g]ftalazinas** complejante de Cu(II) y Zn(II) (Serie Moon 1-6), sobre el cultivo in vitro de epimastigotas de *T. cruzi*, fundamentandonos en la capacidad complejante de estos compuestos y la consiguiente actividad tripanocida.

Es conocida la actividad inhibitoria frente a este parásito de derivados quinolicos, que contienen cadenas poliaminicas y que estan relacionados con los compuestos de la serie Moon (Salmon-Chemin y col., 2000), objeto de este estudio. De nuestros resultados se desprende el marcado efecto inhibidor de algunos de ellos (1-3), incluso superior al efecto inhibidor del crecimiento del parásito que produce el benznidazol (BZN), utilizado como droga de referencia. Estos compuestos no presentan toxicidad frente a células Vero. El grado de toxicidad de cada uno de los compuestos, es crucial para el rendimiento final del producto, buscamos sustancias con altos porcentajes de inhibición a dosis poco tóxicas para las células del hospedador.

El compuesto Moon-1 es el más activo frente al forma epimastigotas de *T. cruzi*, pero sin embargo es el de mayor toxicidad frente a la células de mamífero, aunque su IC_{50} es menor que la del BZN. Los compuestos Moon-2 y 3 son ligeramente menos activos que el compuesto Moon-1, pero significativamente menos tóxicos, lo que puede estar relacionado con el modo de complejación de los iones metálicos sobre estos compuestos.

El desarrollo de un modelo experimental, diseñado en nuestro laboratorio, nos ha permitido investigar la posible inhibición de la capacidad de invasión celular, así como la de su posterior multiplicación. Determinándose la capacidad tripanocida de estos compuestos no solo sobre las formas extra celulares del parásito sino sobre las formas intracelulares. Cuando al cultivo de células Vero se le añade simultáneamente formas tripomastigotas metacíclicas y los 3 derivados que mostraron una mayor actividad se observa un descenso significativo del índice de infección, con respecto al ensayo control al día 8º de experimentación. Igualmente se inhibio la multiplicación intracelular (formas amastigotas), y consiguientemente, el número de tripomastigotas liberados de las células infectadas se vio reducido.

Es un hecho conocido, que la supervivencia de parásitos protozoarios está próximamente relacionada con la capacidad de determinadas enzimas para evadir el daño originado por radicales libres tóxicos procedentes de sus huéspedes. Concretamente, la Superóxido dismutasa de hierro (FeSOD), que es la enzima normalmente asociada con tripanosomátidos, juega un papel relevante como defensa antioxidante de parásitos que originan la enfermedad de Chagas (*Trypanosoma cruzi*), ó la Leishmaniosis (Castillo y col; 2007). Considerando el papel predominante de los

grupos prostéticos en dichos procesos (Lavault y col, 2005), la complejación competitiva del ión metálico de la FeSOD puede ser una eficaz vía de desactivación de su efecto antioxidante. Por tanto, aquellos compuestos capaces de inhibir la acción protectora de la FeSOD por complejación competitiva de su ión metálico pueden ser buenas dianas de actividad antiparasitaria que afectarían tanto al crecimiento como a la supervivencia de células parasitarias.

Estos resultados nos llevaron a evaluar el efecto inhibidor de los compuestos Moon-1, 2 y 3 sobre la actividad de la enzima FeSOD, a fin de probar sus potenciales como competidores de los iones metálicos de la enzima. Los compuestos 1,2 y 3 han mostrado unos valores significativos de inhibición de la actividad FeSOD. La FeSOD, a dosis de 25 μg/ml, es inhibida al 100% por el compuesto 2, mientras que el compuesto 1 alcanza un 75% y el 3 un 53% a la misma dosis. Lo que nos permite apuntar que uno de los posible mecanismos de acción de estos compuestos sea el bloqueo de los iones de metal de la enzima, dando lugar a una reducción de su actividad.

La importancia de esta actividad anti-FeSOD parasitaria, radica en que estos compuestos no son efectivos frente a la CuZn-SOD de los eritrocitos humanos. Reforzando la teoría del potencial interes de estos derivados de los alkylaminobenzo[g]ftalazinas estudiados en este trabajo.

Todos estos resultados son novedosos puesto que es la primera vez que estos compuestos son ensayados y demostrada su capacidad tripanocída.

Siguiendo la colaboración con el Departamento de Química Orgánica (Universidad Complutense de Madrid) y el Instituto de Química Medica (CSIC. Madrid), se estudiarón cuatro nuevos compuestos 1,4Bis(alkylamino) benzo[g]phthalazines, Serie J: 1-4.

Después de 72 horas de interacción producto-parásito, todos los compuestos mostraron una mayor actividad que el BZN frente a formas epimatigotas de *T. cruzi*. Los 4 compuestos ensayados fueron mucho menos tóxicos frente a células Vero que la droga de referencia, destacando que el compuesto monosustituido J-3 presenta una citoxicidad 16 veces mas baja que el BZN.

El estudio de la inhibición de la capacidad de invasión celular parasitaria y la posterior multiplicación intracelular sobre un cultivo de células Vero al adicionarle de forma simultánea los 4 productos de la Serie J y con las formas tripomastigotas metacíclicas, mostró que la tasa de infección decreció significativamente en más de un 50% en comparación con el ensayo control. Destaca especialmente el comportamiento de los compuesto 2, 3 y 4, que provocaron una bajada del índice de infección del 81, 84, y 74%, respectivamente.

De la misma manera, estos nuevos compuestos afectaron al número de amastigotas por célula infectada. Los compuestos 1 y 4 produjeron una inhibición alrededor de 28 y 10% respectivamente. Frente a los compuestos 2 y 3 que disminuyeron notablemente el número de amastigotas intracelulares, esto es, el 55 y el 44%, respectivamente. Igualmente, el efecto sobre el número de tripomastigotas liberados al medio de cultivo fue más evidente en presencia de J-2 y J-3, resultando un valor mayor al 50%.

Con el mismo interés mostrado para la Serie Moon, decidimos estudiar el potencial de la Serie J como competidores de los iones metálicos de la enzima Fe-SOD. Estos compuestos también mostraron un marcado efecto inhibidor del enzima. Así, a la dosis mayor, los compuestos 2, 3 y 4 bloquearon la actividad enzimática (100%, 100% y 94%, respectivamente). Y a la dosis menor ensayada, la inhibición del compuesto 4 fue igualmente alta (80%), mientras que, para los compuestos 1 y 3 el efecto osciló entorno del 50%.

Estos resultados podrían ser interpretados considerando que la alta actividad del compuesto 2 frente a epimastigotas de *T. cruzi* obtenidas in vitro no es sólo debido al bloqueo del ión metálico de la SOD, sino que también pueden estar involucrados otros mecanismos de acción en las actividades tripanosomáticas observadas.

A estos interesantes resultados hay que unirles que tienen una acción selectiva para la Fe-SOD y que la SOD humana (Cu-ZnSOD) es practicamente insensible a su efecto. Por lo cual, estos resultados confirman el potencial interés antiparasitario de los derivados de alkylaminobenzo [g] ftalazina estudiados hasta la fecha.

Estudios previos indican que los derivados 1, 2, 4-triazolo-[1,5-a]pirimidínicos (tp y dmtp) como ligandos y etilendiamina (en) o 1,10-fenantrolina (fen) como ligandos auxiliares son drogas prometedoras quimioterapeuticas para el tratamiento de las enfermedades causadas por los miembros de la familia Tripanosomatidae (Magán. y col. 2004, 2005). Algunos de estos compuestos pueden considerarse miméticos de las purinas y debido a esta similitud, diversos autores los han estudiado como agentes quimioterapéuticos, vasodilatadores e inhibidores de microorganismos desde los años 90 (Fischer, 1993). En este trabajo en colaboración del Departamento de Química Inorgánica (Universidad de Granada), hemos evaluado la actividad de los compuestos de triazolo-pirimidinas y varios derivados complejados por cobre (II) contra *T. cruzi y L. peruviana*.

De los compuestos ensayados, 8 de ellos y frente a epimastigotes de *T. cruzi*, mostraron valores de IC_{50} muy próximos a los del BZN, e incluso en algunos casos presentaron valores de IC_{50} significativamente inferiores a los de la droga de referencia.

De los 8 compuestos activos, los compuestos TP, DMTP, C24b y C49 eran mucho menos tóxicos contra las células Vero que el BZN. El resto de los compuestos mostraron una citotoxicidad muy alta frente a las células Vero.

Al igual que con *T. cruzi*, los complejos C24b y C49 son muy eficaces en la inhibición del crecimiento de las formas promastigotas de *L. peruviana* cultivadas in vitro. Los valores de IC_{50} de estos dos compuestos, son inferiores a las de las dos drogas de referencia utilizadas (Glucantime y Pentostan). Estos dos complejos, también, han mostrado una baja citotoxicidad frente a macrófagos J-774.2, la IC_{50} es siete veces superior que la de las drogas de referencia. Los dos derivados de triazolo-pirimidina TP y DMTP también han resultado eficaces en la inhibición del crecimiento de *L.peruviana* y mostraron una baja citotoxicidad. Sin embargo, el mas eficaz fue el complejo C35 frente al crecimiento de *L .peruviana* (IC_{50} <0,01 μM) y necesita una concentración alrededor de 100 veces mayor para afectar a los macrófagos.

En la mayoría de los estudios sobre ensayos de actividad de nuevos compuestos contra los parásitos, se utilizan las formas que se desarrollan en los vectores (epimastigotas en el caso de *T. cruzi* y promastigotas de *L. peruviana*), por la facilidad

de trabajar con estas formas in vitro, en este estudio hemos incluido el efecto de estos compuestos sobre las formas que se desarrollan en el hospedador (amastigotas y tripomastigotas en el caso de *T. cruzi*). Hemos seleccionado los productos (TP, DMTP, C24b y C49 para *T. cruzi* y estos mismos compuestos más el C35 en el caso de *L.peruviana*) que mostraron el mayor efecto inhibidor sobre el crecimiento de los parásitos in vitro y que, al mismo tiempo, tenían el menor efecto tóxico. Utilizando el IC_{25} de cada producto como dosis de prueba.

Por lo que se conoce hasta la fecha, ninguno de los tripanosomátidos estudiado es capaz de degradar la glucosa completamente a CO_2 en condiciones aeróbicas, excretando al medio de cultivo metabolitos fermentativos de su esqueleto de carbono, según las necesidades de las especies consideradas (Bringaud, y col., 2006). *T. cruzi* y *L.peruviana* consumen glucosa a un porcentaje alto, lo que explica la acidificación del medio de cultivo. La ^{1}H-RMN (Nuclear Magnetic Resonance Spectroscopy of Protons) nos permite determinar los metabolitos fermentativos excretados por los tripanosomátidos durante su cultivo in vitro.

T. cruzi excreta acetato y succinato como metabolitos mayoritarios y L-alanina como metabolito minoritario. En el caso de promastigotas de *L.peruviana*, los metabolitos mayoritariamente excretados son acetato, succinato, L-alanina y en un menor proporción D-lactato; datos que están de acuerdo con los obtenidos por otros autores (Ginger, M. 2005). Hemos usado los espectros de ^{1}H RMN para identificar y evaluar el efecto inhibidor de los compuestos triazolo-pirimidina en los metabolitos excretados por *T. cruzi* y *L. peruviana*. Cuando los tripanosomátidos fueron tratados con los compuestos triazolo-pirimidina TP, DMTP, C24b, y C49, en el caso de *T. cruzi* y tp, dmtp, C24b, C49, y C35 para *L. peruviana*, la excreción de algunos de los metabolitos fue claramente inhibida, a la dosis ensayada.

En el caso de *T. cruzi*, el acetato es el metabolito más inhibido por TP, C24b y C49, esta inhibición de la excreción de acetato puede ser una consecuencia directa de la acción de estos compuestos sobre las enzimas que intervienen en su producción (complejo piruvato-deshidrogenasa o acetato succinato –CoA transferasa), o bien porque estos compuestos actúan sobre la mitocondria e incluso en la membrana

citoplasmática, causando una perdida de funcionamiento (Hannaert, V. y col 2003; Van HeIlemond, J.J. y col; 2005).

En *T. cruzi* como en otros tripanosomátidos (*Leishmanias pp.*), el succinato es un producto final del metabolismo de la glucosa, pero su producción, ha sido el tema de un prolongado debate (Turrens, J., 1999; Tielens, A.G. y Van Hellemond, J.J, 998). La fermentación del succínico ofrece la gran ventaja de requerir solo la mitad de la producción del fosfoenolpiruvato para mantener el equilibrio NAD^+/NADH.

La excreción de succinato, al igual que de acetato, es inhibida por el tp y sus complejos C24b y C49, esta inhibición podría explicarnos el aumento observado de la producción de L-alanina y la aparición de un nuevo pico que no estaba presente en el espectro encontrada para *T. cruzi* cultivadas sin adiciones, este pico ha sido identificado como el glicerol.

En el cultivo de *L. peruviana*, los productos probados son muy eficaces e inhiben la excreción de los productos finales del metabolismo de la glucosa. Aunque es nuevamente el compuesto C35 el que produce los valores de inhibición más altos en la producción del acetato y succinato. Al igual que en *T. cruzi*, en *L. peruviana*, la inhibición del acetato, así como la del succinato, la excreción del resto de los metabolitos excretados, ha aumentado, incluso con la aparición del etanol y el glicerol en el medio, con niveles muy importantes.

En general todos los compuestos ensayados alteran morfológicamente las estructuras de los parásitos: separación de la membrana nuclear, alteración de la membrana citoplasmática y desorganización de la subpelicula de los microtúbulos, la mitocondria y kinetoplasto se ven igualmente afectados. El compuesto C35 es nuevamente el compuesto más eficaz frente a *L. peruviana* puesto de manifiesto por la destrucción prácticamente completa de los parásitos, la presencia de restos de células indicando graves alteraciones en las células.

Los extractos de plantas medicinales o compuestos derivados de ellas son un recurso prometedor de nuevos agentes medicinales para el tratamiento de las tripanosomiasis y otras enfermedades (Rocha y col., 2005; Carvalho y Ferreia, 2001).

Los efectos antitripanosomátidos pueden residir en sus componentes fotoquímicos, tales como: flavonoides y, específicamente, quercetin, que es un buen candidato en combinación terapeúticas contra la infeccion y la anemia asociada a leishmaniasis visceral.

Los flavonoides se encuentran abundantemente en dietas ricas en fruta, hortalizas, y en zumos y parece tener propiedades anticancerígenos, antimicrobianas y antiparasitarias (Taleb-Contini y col 2004; Grael y col., 2005 y OPS/WHO/NTD/IDM 2006).

Desde hace años, el grupo de Tenerife del Departamento de Química (Universidad de la Laguna) buscan sustancias potencialmente bioactivas útiles frente muchas enfermedades, y recientemente han mostrado que los derivados acetilados de los flavonoides extraidos de *Consolida oliveriana* muestran un impacto significativo sobre el crecimiento de varias líneas celulares humanas (Diaz y col. 2008).

Continuando con nuestra línea de trabajo, se ha investigado la principal fracción del extracto etanólico de las partes aéreas de *C. oliveriana*. En nuestro estudio hemos investigado la actividad de un número de estos flavonoides frente a las formas epimastigotas, amastigotas y trypomastigotas de T. cruzi, así como sobre los estadíos extracelular promastigota e intracelular amastigota de *L. peruviana* y *L. braziliensis*.

Se ha determinado el efecto inhibitorio de 8 compuestos flavonoides en el cultivo in vitro de epimastigotas de *T. cruzi*. De estos compuestos, cuatro (1, 2, 3 y 5) obtenidos de forma natural y los otros cuatro por acetilación de estos productos naturales (1a, 2a, 3a y 4). Siendo estos últimos los que mostraron una mayor actividad inhibitoria. Los compuestos 1a y 4 mostraron unos valores de IC50 similares a los del BZN, mientras que, 2a y 3a tiene una IC50 ligeramente mayor a la del BZN. Sin embargo todos los compuestos acetilados son significativamente menos toxicos que la droga de referencia.

En el caso de las leishmanias, los compuestos acetilados (2a, 3a, y 4) vuelven a ser los más efectivos sobre el crecimiento de las formas extracelulares, ademas de ser muy poco toxicos, 10 a15 veces menos tóxicos que las drogas de referencia (Glucantime

y Pentostan). El resto de los compuestos presentaron una IC50 superior a las drogas de referencia y en algunos casos fueron tóxicos para los macrófagos.

Parece ser que los derivados del kaempferol que poseen un anillo β monosustituido son más activos que los análogos del quercetin. Es conocido que la acetilación de determinados flavonoides incrementa las actividades antiproliferativas de los compuestos originarios frente a diversas líneas celulares, esta afirmación concuerda con los resultados obtenidos por nosotros (Torres y col. 2008). La acetilación parece que facilita la absorción de los compuestos resultando en una mayor efectividad (Manach y col. 2004).

No existen datos que nos permitan comparar nuestros resultados con los de otros autores, sin embargo y frente a otras especies de leishmanias (*L. mexican* y *L. donovani*) se ha demostrado que algunos de estos compuestos tienen unos valores de IC50 similares a los encontrados por nosotros (Suarez y col. 2003 y Weniger y col. 2001). En un estudio realizado por Tasdemir y col (2006) demostrarón la alta efectividad del quercetin frente a *L. donovani* y *T. cruzi*, a dosis ligeramente menores que las nuestras. Presumiblemente, estas discrepacias pueden ser derivadas del uso de diferentes métodos y diferentes estadíos del ciclo de vida de los parásitos (Lopez-Posadas y col. 2008).

En la mayoría de los estudios de actividad de nuevos compuestos antiparasitarios se usan las formas que se desarrollan en los vectores (epimastigotas en el caso de *T. cruzi* y promastigotas en el caso de Leishmanias) (Luque y col. 2000) como hemos mencionado por su facilidad de trabajo in vitro. Sin embargo en este estudio hemos incluido el efecto de estos compuestos sobre las formas que se desarrollan en el hospedador (amastigotas y tripomastigotas), el estudio de ellas, es de gran importancia, dado que el objetivo final es determinar el efecto sobre el hospedador humano.

Para este tipo de ensayos del mecanismo de acción, hemos seleccionado aquellos compuestos con mayor efecto inhibitorio en el crecimiento in vitro y que al mismo tiempo tuvieran el menor efecto toxico sobre las líneas celulares de mamíferos, usando el IC25 de cada producto como dosis de ensayo.

En el caso de *T. cruzi* seleccionamos los compuestos acetilados: 1a, 2a, 3a y 4; y para *L. peruviana*: 2a, 3a y 4 y, para *L. braziliensis*: 3a y el compuesto 4. El tanto por

ciento de infectación de células vero por *T. cruzi* descendio significativamente con respecto al control, cuando se le añadio al cultivo los compuestos acetilados 1a y 3a. Tambien, la replicación de amastigotas en las células Vero se vio inhibida por acción de los compuestos 1a, 3a y 4. Hecho que se comprueba con el descenso del número de tripomastigotes liberados al medio de cultivo como consecuencia de la disminución del número de amastigotas.

Los compuestos acetilados seleccionados mermaron el poder de infección sobre los macrófagos por parte de las dos leishmanias estudiadas. Esta inhibición se puso de manifiesto desde las primeras horas de cultivo, lo que nos indica que estos compuestos alteran la capacidad invasiva de los parásitos, aunque hasta ahora, no conocemos las causas que impiden el mecanismo de entrada de los parásitos al interior de las células hospedadora. Además interfieren en la replicación intracelular. Por todo ello los compuestos 2a, 3a y 4 son muy efectivos no solo frente a las forma extracelulares del parásito sino también frente a las formas intracelulares.

A pesar de los buenos resultados obtenidos con los compuestos de origen natural, solo uno pocos están en la fase de evaluación clínica, ya que la mayoría de ellos son descartados por su alta toxicidad (Osorio y col. 2008). Hay que tener en cuenta que estos agentes antiprotozoarios son básicamente citotóxicos, pero actúan de forma selectivamente contra los parásitos. Actualmente, las publicaciones sobre agentes antiparasitarios incluyen evaluaciones de la actividad frente a líneas celulares de mamíferos y/o humano (Weniger y col. 2001 y Barrett, y col. 2003). Siguiendo este modelo, hemos determinado la toxicidad de nuestros compuestos seleccionados frente a una línea celular humana, y ninguno de ellos manifestan toxicidad. Estos resultados abren la posibilidad de su uso para el tratamiento tanto de la leishmaniasis cutánea como mucocutánea (Labraña y col., 2002).

La alteraciones en la excrección de diferentes metabolitos (acetato y succinato, principalmente), así como la inhibición de la actividad enzimática en las principales rutas metabólicas por las que estos organismos obtienen energía, son datos de gran utilidad para elucidar la actividad tóxica en este área. El efecto inhibidor sobre los productos excretados por *T. cruzi* podría ser debido a la acción que los compuestos ensayados ejercen sobre las enzimas involucradas en la degradación de los azucares o

por la perdida de la funcionalidad de la mitocondria, orgánulo donde el acetato y succinato son formados por estos parásitos, ya que la glucolisis esta compartimentalizada (Sánchez-Moreno y col 1992). Esta hipótesis esta reforzada por los datos de microscopía electrónica, donde se observa que la mitocondria y el kinetoplasto se ven desorganizados como consecuencia de la acción de estos compuestos. La inhibición en la excreción del acetato puede ser una consecuencia directa de las acción de estos flavonoides sobre las enzimas involucradas en su producción (complejo piruvato deshidrogenada o acetato-succinato CoA-transferasa), o que estos compuestos actúen sobre la mitocondria e incluso sobre la membrana citoplasmática provocando una perdida de funcionalidad. Efectos similares son observados en el caso de las leishmanias.

Estos resultados muestran que los flavonoides acetilados derivados de *C. oliveriana* no son tóxicos para las células hospedadoras, además son sumamente efectivos a concentraciones similares o menores que las dosis usadas con las drogas de referencia, y nos abren la posibilidad de su uso como tripanocidas y leishmanicidas. Los datos de microscopía electrónica de transmisión y ^{1}HRMN indican la posibilidad de que su modo de acción (o parte de él) podría localizarse a nivel de las membranas del parásito. Estas potentes actividades tripanocidas y leishmanicidas descritas para los flavonoides suponen una gran avance en la busquedad de nuevos agentes antiprotozarios.

VI-CONCLUSIONES

1ª.- Se han determinado los efectos antiparasitario de 40 compuestos de nueva síntesis y de 8 compuestos extraídos de plantas sobre el crecimiento in vitro de tripanosomátidos.

2ª.- De los 6 compuestos 1,4-Bis (alkylamino) benzo[g]ftalazinas complejante de Cu(II) y Zn(II) (**Serie Moon 1-6**), 3 de ellos (Moon 1, 2 y 3), muestran un efecto tripanocida sobre las formas epimastigotas de *T. cruzi*, superior al efecto ejercido por el benznidazol (BZN), utilizado como droga de referencia. No presentando toxicidad frente a células Vero.

3ª Estos 3 derivados Moon ocasionaron un descenso significativo del índice de infección, una inhibición multiplicación intracelular del parásito (formas amastigotas) y su posterior transformación en tripomastigotas.

4ª El posible mecanismo de acción de estos compuestos es la inhibición selectiva de la acción protectora de la FeSOD parasitaria.

5ª El estudio de cuatro nuevos compuestos 1,4-Bis (alkylamino) benzo[g]ftalazinas**, Serie J,** mostraron una mayor actividad inhibitoria que el BZN frente a formas epimatigotas de *T. cruzi* y menor toxicidad frente a células Vero que la droga de referencia.

6ª Al igual que los compuestos de la serie Moon, estos presentaron una marcada inhibición de la capacidad infectiva, multiplicación intracelular y transformación en formas tripomastigotas.

7ª De la misma forma son inhibidores selectivos de la FeSOD del parásito.

8ª Como continuación de nuestro estudio, se ha evaluado la actividad de 30 compuestos de triazolo-pirimidinas y varios derivados complejados por cobre (II) contra *T. cruzi y L. peruviana*.

9ª Cuatro de ellos (TP, DMTP, C24b y C49) presentaron valores significativamente inferiores a los de la droga de referencia frente a *T. cruzi*.

10ª Al igual que con *T. cruzi*, los complejos C24b y C49 son muy eficaces en la inhibición del crecimiento de las formas promastigotas de *L. peruviana* cultivadas in vitro. Los valores de IC_{50} de estos dos compuestos, son inferiores a las de las dos drogas de referencia utilizadas (Glucantime y Pentostan). No presentando toxicidad frente a macrófagos J-774.2.

11ª El complejo triazolo-pirimidinico C35 fue el más eficaz frente al crecimiento de *L.peruviana,* necesitando una concentración 100 veces mayor para afectar a los macrófagos.

12ª Los compuestos C24b, C49 y C35 presentan una marcada inhibición de la capacidad infectiva y multiplicación intracelular de *L. peruviana*.

13ª Mediante ^{1}H RMN se ha evaluado el efecto inhibidor de los triazolo-pirimidinas sobre los metabolitos excretados por *T. cruzi* y *L.peruviana*, siendo el acetato y succinato, los metabolitos más inhibidos en ambos parásitos.

14ª Esta inhibición puede ser una consecuencia directa de la acción de estos compuestos sobre las enzimas que intervienen en su producción, o bien, porque estos compuestos actúan sobre la mitocondria e incluso a nivel de la membrana citoplasmática, causando una perdida de funcionalidad.

15ª De los 8 flavonoides obtenidos de la fracción etanólica de las partes aéreas de *Consolida oliveriana* frente a las formas epimastigotas, amastigotas y trypomastigotas de *T. cruzi*, así como sobre los estadíos extracelular promastigota e intracelular amastigota de *L. peruviana* y *L. braziliensis*, los compuestos acetilados resultaron ser los más efectivos.

16ª Los derivados del kaempferol que poseen un anillo β monosustituido son más activos que los análogos del quercetin.

17ª Los flavonoides acetilados mermaron el poder de infección de *T. cruzi* sobre células Vero y sobre los macrófagos por parte de las dos leishmanias estudiadas.

18ª El efecto inhibidor de estos flavonoides sobre los metabolitos excretados por los parásitos sería debido a la acción que estos compuestos ejercen sobre las enzimas involucradas en la degradación de los azúcares o por la perdida de la funcionalidad de la mitocondria, orgánulo donde se produce el acetato y succinato.

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