Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

ESCOLA NACIONAL DE SAÚDE PÚBLICA
SERGIO AROUCA
ENSP

"Avaliação não-clínica da segurança do antimoniato de meglumina em roedores: toxicocinética, toxicidade para o desenvolvimento e efeitos sobre citocromos P450 hepáticos"

por

Deise Riba Coelho

Tese apresentada com vistas à obtenção do título de Doutor em Ciências na área de Saúde Pública.

Orientador: Prof. Dr. Francisco José Roma Paumgartten

Rio de Janeiro, abril de 2015.

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apresentada por

Deise Riba Coelho

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RESUMO

Antimoniais pentavalentes são considerados medicamentos de primeira linha no tratamento das diferentes formas de leishmaniose. O perfil de segurança dos medicamentos à base de antimônio (Sb), entretanto, ainda não foi completamente elucidado. O objetivo deste conjunto de estudos que constam desta tese foi fornecer informações adicionais sobre a segurança de um curso de tratamento com o antimoniato de meglumina (AM). O primeiro estudo investigou o acúmulo e eliminação do Sb do sangue e órgãos de ratos machos adultos tratados com uma dose diária de AM, por um período de 21 dias consecutivos. Foi observado que o antimônio é lentamente eliminado. O segundo estudo avaliou o desenvolvimento pós-natal da prole nascida e amamentada por ratas tratadas na gestação e lactação até o desmame com AM. A transferência de Sb através da placenta e via leite materno para a prole foi determinada. Os resultados mostraram, em geral, que o desenvolvimento pós-natal e a fertilidade dos ratos expostos não foram alterados. Os dados também sugerem que o Sb passa facilmente para o leite e está presente nesta matriz biológica em uma forma química que o torna bem absorvido pelos lactentes. Além disso, nós também investigamos se as atividades das enzimas citocromo P450 hepáticas (CYP), que participam do metabolismo de endo- e xenobióticos, foram alteradas pelo tratamento. Os resultados mostraram que um curso de tratamento de 24 dias com AM causou um consistente declínio das atividades de CYP1A no fígado de camundongos SW e DBA-2, e uma diminuição nas atividades de CYP2B9/10 nas fêmeas de SW, mas não em DBA-2 de ambos os sexos. Não foram observadas outras alterações de atividade de CYPs (CYP2A5, 2E1, 3A11). Em síntese, os estudos aqui reunidos mostram que, após tratamento com antimoniais pentavalentes, níveis residuais de Sb persistem no fígado e outros órgãos por longo tempo. Os níveis de Sb são muito baixos no cérebro o que é consistente com a ausência de efeitos sobre o desenvolvimento neuromotor. O Sb é transferido para o leite materno e encontra-se nesta matriz biológica em forma que torna este metalóide biodisponível por via oral. Os resultados também mostraram que o acúmulo de Sb residual no fígado, exceto por discreto efeito depressor sobre CYP1A, não altera a atividade de monooxigenases. Este conjunto de dados amplia a base de dados disponível para avaliar a cinética de eliminação do Sb e a segurança do tratamento da leishmaniose com antimoniais pentavalentes.

ABSTRACT

Pentavalent antimony compounds are considered as first choice drugs to treat different clinical manifestations of leishmaniasis. The safety profile of antimony-based anti-leishmanial drugs, however, has not been entirely elucidated so far. The objective of the set of experimental studies presented in this thesis was to provide additional information on the safety of a course of treatment with meglumine antimoniate (MA). The first study was an investigation of the accumulation and clearance of antimony (Sb) in the blood and organs of adult male rats treated with a 21-day course of MA. It was observed that residual Sb is slowly eliminated from rat's organs and blood. The second study evaluated the postnatal development of the offspring born to and nursed by rats treated during gestation and lactation until weaning with MA. The transfer of Sb via placenta and mothers' milk to the offspring was determined as well. Results showed that offspring postnatal development and fertility remained virtually unaltered after treatment with MA. Data suggested that Sb is transferred into breast milk and is present there in a chemical form that makes this metalloid bioavailable to suckling pups. Furthermore, we investigated whether activities of liver cytochrome P450 enzymes that take part in the metabolism of endogenous and exogenous substances were altered after a course of treatment with MA. It was found that a 24-d course of treatment with MA caused a consistent decline in CYP1A activity in the mouse liver. A decrease of CYP2B9/10 activity was noted in SW females but not in SW males and in DBA-2 of either sex. No other change of CYP activity (CYP2A5, 2E1, 3A11) was noted. In summary, data presented here showed that, after treatment with pentavalent antimonial drugs, residual levels of Sb remain in the liver and other tissues for a long time. Sb levels are low in the brain a finding that is consistent with the absence of effects of MA on the neuromotor development. Sb was transferred into breast milk and is found in this biological matrix in a chemical form that makes this metalloid bioavailable by the oral route. Results also showed that accumulation of residual levels of Sb in the liver, except for a mild depression of CYP1A, did not alter the activity of monooxygenases. The comprehensive set of experimental data presented adds to the database currently available for assessing the Sb elimination kinetics and the safety of pentavalent antimonial drugs used to treat leishmaniases.

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LISTA DE SIGLAS E ABREVIATURAS

µg – micrograma/micrograms

ALT - alanina aminotransferase/ Serum alanine

AST – aspartato aminotransferase/ aspartate aminotransferase

BROD – benziloxiresorufina-*O*-desbenzilase/ benziloxy - resorufin-*O*- deethylase

Cd – cádmio/ cadmium

Co – cobalto/ cobalt

COH – cumarina hidroxilase/ Coumarin hydroxylase

CYP – citocromo P 450/ cytochrome P450

d- dia/day

END – eritromicina desmetilase/ Erythromycin-*N*-demethylase

EROD - etoxiresorufina-*O*-desetilase/ ethoxy-resorufin–*O*-deethylase

g – gramas/ grams

GD- dia gestacional/ gestation day

GSH – glutationa / glutathione

h – hora/hour

HG- geração de hidreto/ hydride generation

HO- hemeoxigenase/ heme-oxygenase

ICP-MS – espectrometria de massas com plasma indutivamente acoplado/ inductively coupled plasma-mass spectrometry

im – intramuscular/ intramuscular

ip-intra peritoneal/ intra peritoneal

iv – intravenoso/ intravenous

kg – quilograma/ kilogram

L – litro/ liter

LC – leishmaniose cutânea/ cutaneous leishmaniases

LMC – leishmaniose mucocutânea/mucocutaneous leishmaniases

LMF – fração microssomal hepática/ Liver microsomal fraction

LV – leishmaniose visceral/visceral leishmaniases

MA- antimoniato de meglumina/ meglumine antimoniate

mg – miligrama/ miligram

min – minuto/ minute

ml – mililitros/ milliliters

ng – nanogramas / nanograms

PND- dia pós-natal/ post-natal day

PNPH – paranitrofenol hidroxilase/ p-Nitrophenol-hydroxylase

Sb – antimônio/ antimony

Sb^{+3}/Sb^{III}/Sb^{3+} - antimônio trivalente/ trivalent antimony

Sb^{+5} / Sb^{V}/ Sb^{5+}– antimônio pentavalente/ pentavalent antimony

sc- subcutâneo/ subcutaneous

SSG – estibogluconato de sódio/ sodium stibogluconate

WHO – World Health Organization

wt – weight

I. INTRODUÇÃO GERAL

I.1 Leishmaniose

I.1.1 - Um breve histórico

Leishmanioses são doenças crônicas com manifestações cutâneas, cutâneo-mucosas ou viscerais causadas por protozoários do gênero *Leishmania* que são transmitidas pela picada de flebotomíneos. No hospedeiro vertebrado, os parasitos invadem ou são fagocitados por macrófagos onde se mutiplicam por mitose. Diferentes espécies de protozoários do gênero *Leishmania* causam uma variedade de manifestações clínicas (Herwaldt, 1999).

A primeira observação de protozoários do gênero *Leishmania* foi feita por Cunningham em 1885 na Índia. Sabe-se hoje que o pesquisador examinando material obtido de pacientes com Kala-azar observara a *Leishmania major*, no estágio amastigota, parasitando um macrófago. Em 1898, Borovsky, um pesquisador russo, descreveu que lesões cutâneas denominadas de Botão do Oriente eram causadas por protozoários e não por bactérias, como se acreditava até então. Em 1903, J. H. Wright , reinterpretando as observações histológicas de Cunningham, chegou às mesmas conclusões que Borovsky ao estudar um caso de úlcera tropical em Boston, e, em 1906, Max Lühe redenominou como *Leishmania tropica* a espécie descrita em 1903 por Wright como *Helcosoma tropica*. (Hart, 1985; Rath, Trivelin *et al.*, 2003).

Os agentes etiológicos da leishmaniose visceral foram descritos simultaneamente em 1903, em Londres, Inglaterra, por W. B. Leishman e, em Madras, Índia, por C. Donovan. Neste mesmo ano, Ross denominou *Leishmania donovani* os parasitos viscerais, tendo recebido os créditos por ter criado o gênero *Leishmania*. Já em 1904, pesquisadores como Leishman, Chistoohers e Mesnil notaram a grande similaridade entre os protozoários associados às manifestações clínicas das formas cutâneas e viscerais da doença (Hart, 1985).

Em 1908, Nicole, observando que na região do mediterrâneo o Kala-Azar atingia mais as crianças denominou a espécie aí encontrada *Leishmania infantum*. Desta forma Nicole fez as primeiras constatações de que havia diferenças morfológicas entre os protozoários que causavam leishmaniose dependendo da região geográfica em que a doença ocorria (Rath, Trivelin *et al.*, 2003).

Em 1909, Lindenberg fez os primeiros registros da occorrência de leishmaniose cutânea na América do Sul. Este autor verificou que, em lesões cutâneas e nasofaríngeas, apresentadas por trabalhadores das matas do interior de São Paulo, havia protozoários com as características típicas das leishmânias. Posteriormente, em 1911, Gaspar Vianna caracterizou o tipo de protozoário causador desta doença no Brasil, denominando-a de *Leishmania brasiliensis*. Coube, portanto a Gaspar Vianna a descrição do agente etiológico da então chamada "úlcera de Baurú". Em seguida, em 1913, Bates relatou o primeiro caso de leishmaniose mucocutânea (*apud* Falcão, 1962; Oumeish, 1999; Basano e Camargo, 2004).

No Brasil, o primeiro relato de leishmaniose visceral ocorreu em 1934, quando protozoários do gênero *Leishmania* foram detectados em cortes histológicos do fígado, de pessoas falecidas com suspeita de febre amarela (*apud* Gontijo e Melo, 2004).

I.1.2 - O parasito e seu vetor

Os protozoários das diversas espécies que pertencem ao gênero *Leishmania* são morfologicamente semelhantes e têm um ciclo de vida que alterna o hospedeiro invertebrado (flebotomíneo) com o hospedeiro vertebrado. As leishmânias fazem parte da família Trypanosomatidae de protozoários flagelados possuindo dois estágios de desenvolvimento: o amastigota e o promastigota (Ashford, 2000; Dostalova e Volf, 2012).

O amastigota possui formato arredondado ou oval, e não apresenta flagelo aparente. Esta fase é intracelular e está presente no hospedeiro vertebrado, no interior de monócitos e macrófagos, onde se multiplica por divisão binária até causarem o rompimento da célula que os aloja. Os parasitos

se disseminam pela via linfática ou sanguínea (Ashford, 2000; Basano e Camargo, 2004).

A forma amastigota, após ser sugada pelo flebotomíneo (apenas a fêmea suga o sangue de vertebrados) se aloja no intestino do inseto e se transforma em poucas horas em promastigota, forma alongada que exibe um longo flagelo. Este acessório é utilizado para propulsão e para fixação do parasito nas microvilosidades do intestino do inseto, impedindo que ele seja excretado antes de finalizar o seu desenvolvimento. No intestino há divisão assexuada do parasito que migra para a probóscide do vetor invertebrado. O hospedeiro vertebrado é infectado com a forma promastigota junto com a saliva do flebotomíneo. Há alguns anos tornou-se evidente que a saliva deste inseto possui substâncias vasodilatadoras e é crucial para o estabelecimento da infecção (Ashford, 2000; Basano e Camargo, 2004; Reithinger, Dujardin *et al.*, 2007).

Algumas espécies de mamíferos são hospedeiros conhecidos de protozoários do gênero *Leishmania*, e se infectam na natureza. Os cães são os principais reservatórios de importância médica, principalmente das espécies que possuem afinidade pelas vísceras. As Leishmanias também podem infectar roedores, marsupiais e primatas. É importante ressaltar que as leishmanioses podem ter surgido em novas regiões devido ao transporte de cães infectados, e também em virtude de mudanças climáticas e expansão geográfica dos flebotomíneos (Palatnik-De-Sousa e Day, 2011).

Os flebotomíneos são encontrados em regiões tropicais ou subtropicais. São insetos pequenos que possuem pernas longas e corpo piloso. Apresentam geralmente coloração parda, e por isso são popularmente conhecidos como “mosquito palha”. Outros nomes vulgares comuns são birigui, cangalha, cangalhinha e outros. Estes insetos são mais ativos e realizam a hematofagia predominantemente ao entardecer e à noite (Basano e Camargo, 2004; Dostalova e Volf, 2012).

Para efetivo controle das leishmanioses, a Organização Mundial da Saúde (OMS) recomenda o tratamento dos pacientes humanos, aliado a cuidados na região peridomiciliar voltados ao controle dos insetos vetores (o

que inclui inseticidas, uso de mosquiteiros, repelentes, telas nas janelas, etc), entre outras medidas (Basano e Camargo, 2004; Palatnik-De-Sousa e Day, 2011).

I.1.3 - Manifestações clínicas

As leishmanioses possuem diversas manifestações clínicas sendo as principais a leishmaniose visceral (LV) e a leishmaniose cutânea (LC), que também pode ser chamada de leishmaniose tegumentar americana (LTA), quando ocorre nas Américas. As leishmanioses cutâneas possuem variações, sendo que as principais delas são a leishmaniose mucocutânea (LMC) e a leishmaniose cutânea difusa (LCD) (Brasil, 2006; Brasil, 2010).

No Brasil, a LTA é causada principalmente pelas espécies: *L. braziliensis*, *L. guyanensis*, *L. amazonenses*, e a LV predominantemente pela *L. chagasi* (Brasil, 2006; Brasil, 2010).

A *L. donovani* ocorre na India e na África Central, enquanto a *L. infantun* é encontrada principalmente no Oriente Médio, Ásia Central, China e Mediterrâneo (Palatnik-De-Sousa e Day, 2011).

A LV tem um período de incubação de 2 a 6 meses, mas este pode variar de semanas até anos. Os pacientes apresentam mal-estar e febre inicial, perda de peso e uma hepato-esplenomegalia notável. Anemia, caquexia e neutropenia, com supressão progressiva da resposta imunológica, também são sinais e sintomas frequentes (Palatnik-De-Sousa e Day, 2011; Griensven e Diro, 2012).

A *L. infantun* infecta em geral crianças de 1 a 4 anos e a *L. donovani* jovens de 5 a 15 anos. A infecção de crianças e jovens manifesta-se inicialmente por febre alta e vômitos, anorexia, perda de peso e palidez. Dois picos diários de febre são sinais característicos durante os quais a temperatura pode atingir mais de 40 ºC. No primeiro mês, a espleno-megalia aparece gradualmente, mas é evidente. Há indícios de que pode haver transmissão vertical congênita. Se não for prontamente tratada, a LV pode levar à morte do paciente. Entre jovens, a LV é fatal em 75% a 85% dos casos não tratados, enquanto entre os adultos este percentual sobe para 90% (Wittner e Tanowitz, 2000).

Os sintomas iniciais da LC aparecem semanas após a picada do vetor, com o surgimento de uma vesícula vermelha com prurido no local da picada onde o parasito foi inoculado. Esta vesícula pode aumentar gradativamente, e posteriormente secar assumindo o aspecto de uma úlcera com margens endurecidas no local da picada, mas geralmente não evolui para outras complicações mais graves. A vesícula pode também ter cura espontânea (Wittner e Tanowitz, 2000; Goto e Lauletta Lindoso, 2012).

A LMC causada pela *L. mexicana* frequentemente aparece como lesões cutâneas na orelha que podem ser auto-limitantes. Ocasionalmente, entretanto, o quadro clínico dos pacientes pode evoluir para a forma disseminada ou difusa da doença. A LMC associada à *L. brasiliensis,* por outro lado, pode dar origem a lesões destrutivas na naso-orofaringe, que resultam de metástases de lesões mais superficiais. Nesses casos, pode aparecer uma única úlcera ou diversas delas espalhadas pelo corpo. O acometimento das mucosas ocorre na maioria dos casos, e em 30% deles pode haver mutilação de boca, nariz, palato, e outros locais (Wittner e Tanowitz, 2000).

I.1.4 – Epidemiologia e distribuição geográfica

A leishmaniose é doença prevalente em regiões tropicais e subtropicais. A LV ocorre em aproximadamente 70 países, de 5 continentes, incluindo as Américas, a Europa (Mediterrâneo), Oriente Médio, África Ocidental e Ásia Central. Aproximadamente 90% dos casos registrados nas Américas ocorrem no Brasil. Alguns poucos países (India, Bangladesh, Nepal, Sudão, Etiópia e Brasil) concentram cerca de 90% dos casos globais (Harhay, Olliaro *et al.*, 2011; Alvar, Velez *et al.*, 2012; Griensven e Diro, 2012).

As leishmanioses costumavam atingir predominantemente áreas rurais, porém nos últimos anos há cada vez mais casos registrados em regiões urbanas e peri-urbanas, no Brasil e em outros países. No nosso país, até 2005, a maioria dos casos ocorria na região Nordeste, porém, atualmente a doença vem crescendo em outras regiões (Harhay, Olliaro *et al.*, 2011).

A LC ocorre em 82 países, porém a maior concentração de casos ocorre em apenas alguns destes, tais como: Brasil, Afeganistão, Irã, Peru, Arábia Saudita,

Colombia, Etiópia, Costa Rica, Sudão e Siria (Alvar, Velez *et al.*, 2012; Goto e Lauletta Lindoso, 2012).

Ao se tentar estimar o número de casos que ocorrem em cada localidade, depara-se com o problema da subnotificação desta doença negligenciada típica de países, regiões e populações pobres. Muitos autores acreditam que os casos registrados não correspondem ao número real de pessoas afetadas (Alvar, Velez *et al.*, 2012).

No Brasil, a partir dos anos 2000, houve uma expansão e urbanização da LV. O fato do ciclo do parasita, antes quase exclusivo de áreas rurais, passar a ocorrer também em áreas urbanas e peri-urbanas pode se dever a migrações da população, adaptações do vetor a essas regiões, problemas com saneamento básico, entre outros. Embora a LC esteja presente em quase todos os estados do país, a sua ocorrência não é homogêneamente distribuída entre as regiões geográficas (Basano e Camargo, 2004; Gontijo e Melo, 2004).

Ilustra a expansão da LV o fato de, nos anos 80, terem sido detectados casos de LC em 19 unidades federadas, enquanto em 2003, havia registros de casos em todo o país, sendo mais significativa a contribuição da Região Norte, seguida da Região Nordeste e, logo após, a Centro-Oeste. Entre 2000 e 2009, houve registro de 24684 casos de LV. Em 2009 foram notificados 3894 casos de LV, dentre os quais 91% eram casos novos, enquanto a LC teve 23399 casos confirmados, sendo 94,1% de casos novos, e entre estes, apenas 6,2% exibiam a forma mucosa (Brasil, Saúde *et al.*, 2010; Pelissari, Cechinel *et al.*, 2011).

I.1.5 - Tratamento

O tratamento de primeira escolha para as leishmanioses, desde a década de 1940, é feito com medicamentos à base de antimônio pentavalente: o antimoniato de meglumina (Glucantime®) e o estibogluconato de sódio (Pentostam®). Os antimoniais pentavalentes, embora eficazes, exibem algumas desvantagens como: administração apenas por via parenteral; tratamento de longa duração (cursos de tratamento); dor no local da injeção e incômodos frequentes e toxicidade, incluindo fadiga, dores no corpo, cardiotoxicidade, pancreatite, entre outros. Entre os medicamentos alternativos, caso o paciente não responda ao tratamento primário, estão a anfotericina B e pentamidina, ambos também apresentam acentuada toxicidade. Porém, até o momento, não foi

possível encontrar nenhum outro medicamento com uma melhor relação custo/benefício e toxicidade/eficácia terapêutica (Herwaldt, 1999; Tiuman, Santos *et al.*, 2011).

A LC, apesar de eventualmente curar espontaneamente em alguns meses, deve ser tratada para acelerar o desaparecimento da lesão e evitar a disseminação ou até mesmo a evolução para a forma mucocutânea. A LV, todavia, pode levar à morte do paciente se não for tratada, sendo recomendado que se inicie o tratamento logo após o diagnóstico (Murray, Berman *et al.*, 2005).

De acordo com o Ministério da Saúde, no Brasil o medicamento de primeira escolha para o tratamento das leishmanioses (cutânea e visceral) é o antimoniato de meglumina. A dose administrada deve ser calculada em miligramas de antimônio pentavalente – componente ativo – por quilo de peso corporal do paciente por dia (mg Sb^{+5}/kg/dia) até o máximo de 03 ampolas por dia para adultos. O antimoniato de meglumina é o único medicamento que possui o Sb^{+5} como componente ativo registrado no Brasil (Brasil, Saúde *et al.*, 2010).

Segundo o Ministério da Saúde, no caso da LC, indica-se uma dose de 10 a 20 mg Sb^{+5}/kg/dia, por via intravenosa ou intramuscular, durante 20 dias consecutivos. Já para a forma mucosa, é recomendado 20 mg Sb^{+5}/kg/dia por 30 dias consecutivos. Quando se trata da LV, a dose deve ser de 20 mg Sb^{+5}/kg/dia por 20 dias consecutivos, podendo chegar a 30 dias e nunca ultrapassando 40 dias, respeitando-se a dose máxima de 3 ampolas por dia (Brasil, Saúde *et al.*, 2010; Pelissari, Cechinel *et al.*, 2011).

I.2 Antimoniais

I.2.1 - Histórico

Paracelsus, médico e alquimista do século XVI, foi um entusiasta do uso de compostos de antimônio na terapêutica, recomendando o uso de preparações à base de antimônio para tratar feridas, úlceras, lepra e outras doenças de pele. Em 1604, compostos de antimônio foram preconizados para tratar sífilis, dores no peito, febres, e outras condições. Na primeira metade do século 17, os compostos de antimônio eram prescritos para a corte francesa e na década de 1660, o Parlamento de Paris respaldou o uso medicinal do tartarato de antimônio e potássio e outros compostos de antimônio. Na

época, o uso médico de compostos de antimônio adquiriu grande notoriedade (Sneader, 2005).

O tartarato de antimônio e potássio (também conhecido como tártaro emético) foi descrito em 1631 e passou a ser conhecido como tártaro emético porque produzia em doses relativamente baixas (da ordem de 65 mg) vômitos e sudorese. O tártaro emético foi prescrito para febre antes da introdução dos antipiréticos modernos que surgiram apenas no final do século dezenove. Na medicina popular, o tártaro emético era também empregado por aplicação externa, misturado com banha ou emplastro. A aplicação tópica produzia sensação de queimação, seguida do aparecimento de características de erupção pustular, sendo na época considerado como benéfico para o tratamento de úlceras severas (Sneader, 2005).

Em virtude da toxicidade, a popularidade dos medicamentos à base de antimônio teve acentuada queda nos séculos XVIII e XIX. Entretanto, no início do século XX, após demonstração de que a solução de trióxido de arsênio (metalóide próximo ao Sb na tabela periódica) era capaz de matar tripanossomas em camundongos infectados experimentalmente, ressurgiu o interesse nos medicamentos à base de antimônio. Verificou-se também que injeções intravenosas de tartarato de antimônio eram capazes de curar o gado infectado com tripanossomas (Sneader, 2005).

A eficácia do tártaro emético para tratar doenças causadas por tripanossomas levou médicos e pesquisadores a tentar utiliza-lo contra outras enfermidades, incluindo doenças que desfiguravam e dizimavam pessoas no Brasil e no mundo. Na época, uma das doenças mais temidas no Brasil era a leishmaniose. No início do século XX, Gaspar Vianna, um jovem médico e patologista paraense que trabalhava no Instituto de Manguinhos, fez importantes estudos sobre o agente etiológico desta protozoose e também experimentou com sucesso o uso de compostos antimoniais para tratá-la. Gaspar Vianna foi o primeiro a verificar que o tártaro emético por via parenteral era capaz de curar a leishmaniose, doença para qual até então não se conhecia tratamento eficaz (Falcão, 1962).

Na primeira década do século passado, a leishmaniose tegumentar era doença comum no Brasil, acometendo particularmente os trabalhadores que atuavam nas zonas de colonização, regiões pioneiras, onde havia processo acelerado de desmatamento, modificando a paisagem natural e desorganizando os ecossistemas locais. Foi este o

caso de muitos operários que trabalhavam na construção de uma rodovia em Bauru, no estado de São Paulo, e foram acometidos por doença cutânea caracterizada pelo aparecimento de ulcerações. Constatou-se que tais lesões estavam associadas à infecção por um protozoário (do gênero *Leishmania*) sendo então a doença denominada Úlcera de Bauru. Esta afecção foi inicialmente atribuída (erroneamente) a um agente etiológico já conhecido na Europa: a leishmaniose cutânea, causada pela *Leishmania tropica* (Albuquerque, 1995; Albuquerque e Maciel, 1995; Da Silva, 2005).

Após o aparecimento de numerosos casos da Úlcera de Baurú em São Paulo, a doença causava transtornos também em outras regiões do país, Gaspar Vianna decidiu se dedicar à investigação da sua etiologia e possível tratamento. Em 1911, Gaspar Vianna descreveu uma nova espécie de Leishmania, a *Leishmania brasiliensis*, (nome científico que ainda hoje é utilizado) como o agente etiológico da Ulcera de Baurú, a leishmaniose cutânea do novo mundo (*apud* Falcão, 1962; Fonseca Filho, 1962.; Moraes, 1968; Gachelin, 2005).

Após esta descoberta, tentou Gaspar Vianna encontrar a cura para esta moléstia. Medicamentos que já eram utilizados no tratamento de outras doenças parasitárias começaram a ser aplicados nas lesões cutâneas causadas pela leishmaniose sem que obtivesse êxito (Gachelin, 2005).

Inicialmente Gaspar Vianna tentou utilizar arsenicais para tratar a leishmaniose tegumentar, uma vez que estes compostos haviam sido relativamente eficazes contra outras doenças infecciosas, como a sífilis e doença do sono (ou tripanossomíase africana humana) (Albuquerque, 1995; Albuquerque e Maciel, 1995).

Gaspar Vianna, entretanto, não obteve resultados animadores com os arsenicais 606 e 914 em lesões mucosas ou cutâneo-mucosas. Por uma dedução racional, considerando os bons resultados obtidos com uso de antimoniais em certas tripanossomoses, Gaspar Vianna decidiu experimentar o tártaro emético. Como os antimoniais estavam em desuso por conta da alta toxicidade, Gaspar Vianna injetou o tártaro emético com cautela por via intravenosa em pacientes com leishmaniose observando cicatrização rápida e definitiva das lesões causadas por leishmanias (Falcão, 1962; Albuquerque e Maciel, 1995).

O anúncio oficial da descoberta do tratamento e da cura da leishmaniose tegumentar se deu em 1912 quando Gaspar Vianna comunicou os resultados dos seus

experimentos no VII Congresso Brasileiro de Medicina e Cirurgia, realizado em Belo Horizonte, em abril de 1912. Gaspar Vianna informou ter observado a cura de vários casos de leishmaniose cutânea, sendo um caso com lesões na mucosa nasal e bucal. Relatou ainda ter aplicado diversos medicamentos que eram utilizados para tratar o Botão do Oriente (como o Bayer 606 ou Salvarsan), não obtendo sucesso na cura das lesões, o que o levou a concluir que estes, diferentemente do tártaro emético, não seriam tratamentos de escolha para o tipo de leishmaniose que ocorria no Brasil. As observações de Gaspar Vianna constituem um marco na re-introdução dos antimoniais no arsenal terapêutico moderno e no uso destes compostos para tratamento de leishmanioses (Vianna, 1912a; b; Greenwood, 2008).

O tártaro emético (à base de antimônio trivalente) foi o primeiro medicamento comprovadamente eficaz usado para tratar as leishmanioses, sendo ainda hoje seus sucessores, os antimoniais pentavalentes, medicamentos de primeira escolha (de acordo com o Ministério da Saúde) para o tratamento desta parasitose. O tartarato de antimônio e potássio (ou tártaro emético) foi possivelmente o composto de antimônio com maior importância comercial já utilizado. Esta substância já foi utilizada como emético para tratar pacientes intoxicados com uma variedade de compostos. O uso como agente emético foi abandonado, porém ainda é empregado como tal quando é adicionado a certos raticidas, para tornar estes últimos menos perigosos caso venham a ser ingeridos acidentalmente por animais de estimação (Clarkson, 2001; Da Silva, 2005).

Em junho de 1914, Gaspar Vianna publicou o seu artigo “Sobre o tratamento da leishmaniose tegumentar” em que comenta que as evidências levaram-no a crer que a melhor forma de combater dada doença era por via sistêmica (circulatória), pois as lesões mucosas podem ocorrer a grandes distâncias do local de inoculação do parasito. Ele cita que os medicamentos que tinham o arsênio como componente ativo não resultavam em melhora satisfatória quando se tratava dos tipos de leishmaniose unicamente mucosas ou mucocutâneas. Vianna explica que lesões antigas demoram mais para atingir a cura, e que o tratamento com tártaro emético, como qualquer outro tratamento, também exibia inconvenientes tais como dores articulares, musculares, cefaléia, entre outros. Enfatizou, porém, que este medicamento usado com cautela podia ser considerado como seguro e por isso poderia ser usado em crianças e idosos com

mais de 60 anos. Reafirmou também a necessidade de injeções repetidas ao longo de dias para obtenção da cura (Vianna, 1914).

Em 1916, Otavio Torres discorreu sobre o uso do tártaro emético, que começou a ser empregado na forma de injeções intravenosas no início da década de 1910, após as observações pioneiras feitas pelo Gaspar Vianna. Segundo ele, os pacientes se queixavam de dores no local da aplicação do medicamento. Acreditava-se, então, que estas dores eram causadas pela ação cáustica, coagulante e desidratante do tártaro emético. De acordo com este médico, estudioso de casos de leishmaniose, o modo de ação do tártaro emético seria muito semelhante ao dos arsenicais, já que se acreditava na época que ambos os metalóides, administrados repetidamente, acumulavam-se no organismo dos pacientes. Esta suspeita vem sendo confirmada através de estudos em animais, e também por alguns estudos realizados em humanos. Atualmente acredita-se que a maioria dos efeitos adversos relacionados ao tratamento com antimoniais pentavalentes aparece no final do tratamento, o que está de acordo com a hipótese de que a toxicidade deste medicamento se deve a acumulação do antimônio nos tecidos (Torres, 1916; Marsden, 1985).

De acordo com os recursos disponíveis nas primeiras décadas do século passado, era possível observar clinicamente a intolerância ao tártaro emético, sendo a tosse um dos efeitos colaterais mais frequentemente observados. Geralmente a tosse era seca, mas caso a dose fosse mais forte para determinado paciente, apareciam náuseas e vômitos, que eram ou não precedidos por tosse. Outros efeitos adversos comuns eram mialgias e artralgias. A febre tóxica ou medicamentosa causada pelo tártaro emético, na maioria das vezes em que ocorria, começava a ser observada no final do tratamento, provavelmente devido a sua lenta eliminação e consequente acúmulo no organismo (Torres, 1916).

O tartarato de antimônio e potássio tinha outros inconvenientes, além de seus sérios efeitos adversos, como o fato de ser tratamento de longa duração. Estes fatos estimularam pesquisas voltadas para o desenvolvimento de análogos mais seguros e menos irritantes. Hans Schmidt, na Alemanha, descobriu o método de sintetizar compostos orgânicos antimoniais, que culminou na fabricação de vários medicamentos. Schmidt desenvolveu um método para síntese de compostos orgânicos de antimônio, no qual o antimônio é o pentavalente e não o trivalente como, ocorria no tártaro emético.

Este método permitiu a preparação de uma gama de compostos antimoniais que vieram a ser mais eficazes e seguros para leishmaniose do que o tartarato de antimônio e potássio. Embora estes compostos de antimônio pentavalente ainda retenham em parte o potencial tóxico daqueles com antimônio trivalente, as novas preparações possuíam uma importante vantagem sobre as demais, pois não causavam náuseas frequentes. (Sneader, 2005; Greenwood, 2008).

O primeiro composto sintetizado por Hans Schimdt que demonstrou ser promissor na terapêutica foi o estibenil. Ele foi introduzido na clínica em 1915 e aparentou ser menos tóxico que o tártaro emético. Este medicamento, então recém-descoberto, obteve sucesso no tratamento do calazar em crianças. Logo, porém, surgiu o estibosan (Sneader, 2005; Greenwood, 2008). No final da década de 1920, foi sintetizado um derivado do estibosan, o neoestibosan. Estes compostos representaram um progresso, mas foram ofuscados pelo uso da estibamina, particularmente na India. Em 1928, estibamina foi usada intensamente na terapêutica e tornou-se o tratamento de escolha para leishmaniose por vários anos. Em 1925, todavia, ensaios clínicos com outro antimonial, o neostam (estibamina glucosida), ocorreram na Inglaterra (Greenwood, 2008).

Todos os antimoniais pentavalentes, embora efetivos (a mortalidade do kala-azar foi reduzida de 90% para 5%) são instáveis em solução aquosa. Em virtude deste inconveniente, o grupo de Schimdt desenvolveu o solustiban, no qual o antimônio pentavalente foi complexado com açúcar. Ensaios clínicos realizados a partir de 1935 indicaram que não só este medicamento era de mais fácil administração, como apresentava menor toxicidade. Então, em 1937 a Bayer passou a comercializar o solustiban como uma solução estável pronta para ser utilizada em uma administração intravenosa (Greenwood, 2008).

Nesta linha de trabalho, Schmidt também sintetizou o estibogluconato de sódio, um antimonial pentavalente no qual o ácido glucônico ($C_6H_{12}O_7$) substitui o ácido tartárico. Este medicamento foi submetido a testes laboratoriais em 1937 (Sneader, 2005).

Em 1946, no Sudão, estava pronto para ensaios clínicos o gluconato de antimônio e sódio (Pentostam®), substância bem semelhante ao solustiban. Na época,

1948, na Itália, o antimoniato de meglumina estava em fase de investigação clínica (Glucantime®) (Greenwood, 2008).

O antimoniato de meglumina foi introduzido no mercado no final da década de 1940. Este antimonial pentavalente foi sintetizado substituindo o ácido tartárico por um amino-açúcar derivado da glicose. Hoje o antimoniato de meglumina é considerado um dos medicamentos mais eficazes no tratamento de todas as formas de leishmaniose, dentre aqueles disponíveis no mercado (Sneader, 2005).

Portanto, existem atualmente duas formulações de antimônio pentavalentes complexados com carboidratos: o estibogluconato de sódio (mais utilizado em ex-colônias britânicas) e o antimoniato de meglumina. Aparentemente, não há diferenças quanto à eficácia e segurança entre estes dois compostos orgânicos de antimônio pentavalente. O antimoniato de meglumina é o único antimonial pentavalente disponível no Brasil, onde não é vendido em farmácias comerciais, mas adquirido e distribuído via SUS pelo Ministério da Saúde (Moraes, 2008).

Na época em que o antimoniato de meglumina entrou em uso clínico, os estudos realizados, e principalmente os exigidos, antes da aprovação de medicamentos novos para comercialização, eram muito limitados, quando comparados com a abrangente comprovação de eficácia e segurança, incluindo sofisticados estudos não clínicos e clínicos, que as agências regulatórias exigem hoje das indústrias farmacêuticas.

O registro do antimoniato de meglumina no Brasil ocorreu em 1947 e antecedeu a lei básica de vigilância sanitária, a Lei 6360, sancionada em 23 de setembro de 1976, que ainda vigora e regula, em linhas gerais, o registro de medicamentos no nosso país. Segundo esta Lei (artigo 16, inciso III), para o registro de produto farmacêutico novo é necessário que "*... sejam oferecidas amplas informações sobre a sua composição e o seu uso, para avaliação de sua natureza e determinação do grau de segurança e eficácia necessários*". Portanto, apesar de sua comprovada eficácia e das décadas de uso no tratamento das leishmanioses, ainda hoje existem dúvidas sobre alguns aspectos da segurança na utilização dos antimoniais pentavalentes como medicamento. Como os métodos atuais de investigação são bem mais sofisticados do que os que existiam quando o antimoniato de meglumina foi introduzido, uma avaliação mais profunda dos possíveis efeitos adversos do tratamento com este composto é viável.

I.2.2 - Antimonial Pentavalente

O antimônio compartilha propriedades tanto de metais como de não metais sendo portanto classificado no grupo dos metalóides. Este metalóide é considerado um elemento-traço, pois é encontrado em quantidades muito pequenas no ambiente natural. O antimônio é similar ao arsênio, inclusive em seus estados de oxidação, compartilhando com este outro metalóide algumas características químicas e toxicológicas. Possui 4 estados de oxidação, sendo as formas +3 e +5 as mais abundantes, e a espécie +3 a mais estável (De Boeck, Kirsch-Volders *et al.*, 2003; Zangi e Filella, 2012).

Como mencionado anteriormente, os antimoniais pentavalentes são usados há mais de 70 anos no tratamento das leishmanioses. Entretanto, a despeito deste fato, ainda hoje pouco se sabe sobre sua estrutura química, seu mecanismo de ação e sobre os métodos industriais para sua preparação (Haldar, Sen *et al.*, 2011).

Em relação ao mecanismo de ação leishmanicida, algumas hipóteses foram levantadas. Prevalece hoje a hipótese de que o antimônio pentavalente seja o que se chama de pró-droga. No organismo, o antimônio pentavalente sofreria bio-redução a trivalente, que seria a espécie mais ativa contra o parasita e a mais tóxica para o hospedeiro. Entretanto, o exato local e o mecanismo de catálise da redução ainda não foram completamente elucidados. Foi também sugerido que o antimônio interfere com vias metabólicas do parasita (Rath, Trivelin *et al.*, 2003; Haldar, Sen *et al.*, 2011).

O que se sabe é que o antimoniato de meglumina causa uma rápida regressão dos sinais clínicos e hematológicos em todos os tipos de leishmaniose e também impede que o parasita continue a se multiplicar. Todavia, em virtude dos efeitos indesejáveis, o tratamento exige um acompanhamento com exames laboratoriais dos pacientes. Dentre os efeitos adversos mais comuns pode-se citar as mialgias, dores abdominais, dores musculares principalmente no local da aplicação, náuseas, vômitos, diarreia, dor de cabeça, anorexia, fadiga, urticária, alterações no fígado e cardíacas (por exemplo, as arritmias). As alterações cardíacas parecem ser dose-relacionadas, e pacientes ao serem tratados com antimoniato de meglumina devem fazer exames cardíacos (eletrocardiogramas) como monitoramento de rotina, uma vez que a cardiotoxicidade é a causa mais frequente de mortes durante o tratamento. Particularmente no caso da administração por via endovenosa pode ocorrer edema e flebite. Tem sido relatado que

a maioria dos efeitos adversos são relacionados com a dose administrada e a duração do tratamento dos pacientes. O tratamento é via de regra contínuo, por 20 ou 30 dias consecutivos, o que é necessário para a eficácia (Ribeiro, Drummond *et al.*, 1999; Rath, Trivelin *et al.*, 2003; Neves, Caldas *et al.*, 2009; Oliveira, Schubach *et al.*, 2011).

Foi relatado que o tratamento com antimonial pentavalente pode acarretar nefrotoxicidade e insuficiência renal, quando o medicamento é empregado em altas doses (Veiga, Rosa *et al.*, 1985; Cuce, Belda *et al.*, 1990; Oliveira, Lima *et al.*, 2012).

Como anteriormente mencionado, a espécie trivalente do antimônio parece ser a principal responsável pelos principais efeitos tóxicos do medicamento. O antimônio tende a se acumular em tecidos mais vascularizados, pois a espécie trivalente concentra-se no interior dos eritrócitos em relação ao plasma, estando presente em quantidade considerável no sangue. A excreção em humanos ocorre preferencialmente por via renal (Rath, Trivelin *et al.*, 2003).

I.3 Justificativa

Apesar de o antimônio ser o componente ativo de medicamentos utilizados contra leishmaniose há mais de 70 anos, há muitos aspectos ainda obscuros sobre a segurança destes compostos leishmanicidas. Ainda não se conhece completamente, por exemplo, a farmacocinética, acumulação em tecidos, especiação, os efeitos do tratamento sobre a biotransformação de xenobióticos, possíveis interações medicamentosas, e a toxicidade reprodutiva e para o desenvolvimento.

Uma dos razões que explicariam a persistência destas lacunas na base de dados toxicológicos sobre os antimoniais pentavalentes é o fato da leishmaniose ser uma das doenças mais negligenciadas, segundo a OMS. Como as leishmanioses são doenças mais prevalentes em países em desenvolvimento, e afetam populações pobres que não tem recursos para adquirir medicamentos, não há mercado e portanto investimentos por parte das indústrias para desenvolver novos medicamentos leishmanicidas ou investigar e aperfeiçoar os já existentes.

Os estudos experimentais em animais podem ajudar na compreensão de diversos aspectos da segurança dos antimoniais que não seriam éticos ou viáveis de serem estudados diretamente em humanos. Assim, apesar das diferenças entre o homem e os roedores, esses animais de laboratório são úteis para identificação de perigo, estudos cinéticos e investigações de modo de ação.

Os estudos que serão aqui apresentados buscaram reduzir as lacunas existentes, aumentando o nosso conhecimento sobre a cinética, distribuição em tecidos, efeitos sobre enzimas citocromo P450 hepáticas e toxicidade reprodutiva resultantes de cursos de tratamento com antimoniais pentavalentes.

I.4 Objetivos

O objetivo geral desse trabalho foi a obtenção de dados toxicológicos não-clínicos e fármaco/tóxico-cinéticos importantes para avaliação da segurança no uso de antimoniais como tratamento de duração prolongada, com doses repetidas.

Objetivos específicos foram avaliar:

- O acúmulo do antimônio residual em órgãos e sua presença no sangue total, inclusive após o término do tratamento.
- Efeitos adversos do tratamento materno com o antimonial pentavalente, ao longo de toda a gestação (exposição transplacentária), e período de amamentação (exposição via leite materno), sobre o desenvolvimento somático e neurocomportamental da prole exposta, assim como efeitos em longo prazo sobre a fertilidade e desempenho reprodutivo na vida adulta.
- A passagem transplacentária do antimônio, e transferência para leite materno e deste para os filhotes amamentados.
- Possíveis alterações causadas pelo tratamento prolongado sobre a atividade de enzimas hepáticas citocromo P450 responsáveis pelo metabolismo de xenobióticos.

II. APRESENTAÇÃO DOS ARTIGOS

Artigo 1

O primeiro artigo ("*Tissue distribution of residual antimony in rats treated with multiple doses of meglumine antimoniate*") foi publicado no periódico Memórias do Instituto Oswaldo Cruz em Junho de 2014 - Vol 109(4), páginas 420 a 427. Neste estudo foram avaliadas as concentrações de antimônio em tecidos de ratos machos 24 horas e três semanas após o fim do tratamento de 21 dias. Determinamos também os níveis de antimônio no sangue destes ratos durante e após o fim do tratamento, até no máximo 105 dias após o fim da exposição.

Artigo 2

O segundo ("*Effects of in utero and lactational exposure to* Sb^{V} *on rat neurobehavioral development and fertility*") foi publicado no peródico Reproductive Toxicology em Dezembro de 2014 – Vol 50, páginas 98 a 107. Este estudo avaliou o desenvolvimento somático e neurocomportamental, e a fertilidade, após longa exposição compreendendo a gestação e lactação até o desmame. Investigamos também a passagem do antimônio através da placenta, e da mãe para o leite e através deste para os filhotes durante a amamentação.

Artigo 3

O terceiro artigo ainda não foi publicado, e por isso será apresentada a versão preliminar a ser submetida. O título provisório deste trabalho é: *"Effects of repeat meglumine antimoniate administration on mouse liver cytochrome P450 activities*". Este estudo avaliou se o tratamento em doses repetidas com antimoniato de meglumina altera a atividade de enzimas citocromo P450 que tem importante papel no metabolismo hepático de xenobióticos.

III. ARTIGO 1

Running title: Sb in tissues from MA-treated rats

Tissue distribution of residual antimony in rats treated with multiple doses of meglumine antimoniate.

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III.1 Summary

Meglumine antimoniate (MA) and sodium stibogluconate are pentavalent antimony (Sb^V) drugs used since the mid-1940s. Notwithstanding the fact that they are first-choice drugs for the treatment of leishmaniases, there are gaps in our knowledge of their toxicological profile, mode of action and kinetics. Little is known about the distribution of antimony in tissues after Sb^V administration. In this study, we evaluated the Sb content of tissues from male rats 24 h and 3 weeks after a 21-day course of treatment with MA (300 mg Sb^V/kg body wt/d, *sc*). Sb concentrations in the blood and organs were determined by inductively coupled plasma-mass spectrometry (ICP-MS). In rats, as with in humans, the Sb blood levels after MA dosing can be described by a two-compartment model with a fast ($t_{1/2}$=0.6 h) and a slow ($t_{1/2}$>> 24 h) elimination phase. The spleen was the organ that accumulated the highest amount of Sb, while bone and thyroid ranked second in descending order of tissues according to Sb levels (spleen>> bone, thyroid, kidneys> liver, epididymis, lungs, adrenals> prostate> thymus, pancreas, heart, small intestines> skeletal muscle, testes, stomach> brain). The pathophysiological consequences of Sb accumulation in the thyroid and Sb speciation in the liver, thyroid, spleen and bone warrant further studies.

Key terms: Pentavalent antimonials, thyroid, liver, leishmaniases, Glucantime, pharmacokinetics.

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III.2 Introduction

Although it is a metalloid for which no natural biological function has been identified so far, antimony has a long history of medicinal uses. In the XVI century, Paracelsus, a famous alchemist and physician and one of the pioneers of iatrochemistry, was especially fond of antimony and prescribed medicines based on its salts for a number of morbid conditions (Haldar et al. 2011). During the following two centuries, antimony-based drugs became the center of a dispute between Galenic school doctors and iatrochemists, and the medical use of antimony was banned in France and other countries. In the early-20th century, antimony-based drugs made a remarkable return to physicians' therapeutic armamentarium thanks to their efficacy in treating some parasitic diseases. In 1912, Gaspar Vianna reported that he had achieved a complete clinical cure for muco-cutaneous leishmaniasis with a course of intravenous injections of tartar emetic (antimony potassium tartrate) (Vianna 1912). A few years later, in Italy, Di Cristina and Caronia (1915) successfully treated children afflicted with visceral leishmaniasis by injecting repeated doses of tartar emetic (Di Cristina & Caronia 1915). Shortly thereafter, in Sudan, after confirming previous reports that intravenous injections of tartar emetic could cure cutaneous ('Oriental sore') and visceral (kala-Azar) forms of leishmaniases, John Christopherson noticed that this antimonial drug was also effective against both urinary and intestinal schistosomiases (Christopherson 1918; Christopherson 1923). Since then and until the advent of praziquantel in the 1970s, trivalent antimonial drugs remained as one of the most effective therapeutic approaches for schistosomiasis.

As far as leishmaniasis therapy is concerned, tartar emetic and other Sb^{III}-based drugs were replaced by sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®), less toxic Sb^{V} drugs that were introduced in the market in mid 1940s (Haldar et al. 2011).

The effective dosing schedules for antimony-based drugs in leishmaniasis and schistosomiasis were established decades before their complex kinetics were partially elucidated. The first kinetic investigations showed that patients excreted most of the antimony via urine within a few hours of injection of Sb^{III} or Sb^{V} drugs (Goodwin & Page 1943; Otto et al. 1947). A clear picture of Sb kinetics, however, came to light only in 1988, when Chulay et al. (1988) reported that most of the Sb administered by a single

intramuscular injection of Sb^V is rapidly eliminated ($t_{½}$ =2.02 h) so that only residual concentrations are found in the blood 24 h after drug administration. During a 30-day course of injections of Sb^V spaced 24 h apart, however, these nadir Sb blood levels gradually rose. According to Chulay et al. (1988), their data on Sb blood levels could be described by a two-compartment kinetic model the slow elimination phase of which had a half-life of 76 h. Based on the foregoing information, the authors speculated that the slow elimination phase was related to the *in vivo* conversion of Sb^V into Sb^{III}, a bio-reduction that, according to them, could contribute to the toxicity often noted in long-term Sb^V therapy. Further studies in humans and Rhesus monkeys used more sensitive analytical methods, suggesting that Sb elimination could be even slower, with a terminal elimination half-life longer than 30 days (Miekeley et al. 2002; Friedrich et al. 2012). Moreover, data on speciation of Sb in monkeys' plasma 1 and 9 days after a 21-day treatment course with meglumine antimoniate indicated that the proportion of Sb^{III} in nadir plasma levels of Sb markedly increased with time during the slow elimination phase, a finding that is consistent with the hypothesis that Sb^{III} becomes a major Sb species during the terminal elimination phase (Friedrich et al. 2012).

Despite the recent advances in the knowledge of antimonial drug kinetics, little is known about the distribution of Sb into tissues of individuals treated with Sb^{III} or Sb^V compounds. The same holds true for organ distribution of Sb following exposures through occupationally and environmentally relevant routes (*i.e.*, oral, dermal or inhalation routes).

Molokhia and Smith (1969) measured (by neutron activation analysis) the Sb content of tissues of *Schistosoma mansoni*-infected mice at different time intervals (0.5, 8, 24 h, 2, 4, 7 and 15 days) after a single *ip* injection of a Sb^{III} drug (tartar emetic or sodium antimony *2,3* mesodimercapto-succinate/ Astiban®). The authors found the highest levels of Sb in the liver and spleen, followed by alimentary tract organs (colon, duodenum and stomach), 30 minutes after treatment. Levels of Sb were similarly low in all tissues of mice euthanized on post-treatment day 4 and thereafter.

Recently, Borborema et al. (2013) determined the proportion (%) of injected radioactive Sb (^{122}Sb and ^{124}Sb radioisotopes produced in neutron-irradiated Glucantime®) in tissues of *Leishmania infantum chagasi*-infected (BALB/c) mice treated with an *ip* injection of MA. Mice treated with Sb^V were euthanized at post-

treatment time intervals ranging from 3 minutes to 3 days. The highest % of Sb injected activities (IA) was found in the liver 30 minutes after the MA injection (61 and 47.5% in non-infected and infected mice, respectively). According to the authors, measurable activities of Sb radioisotopes were also detected in spleen, intestines, stomach and kidneys, while no accumulation of radioactive Sb was noted in the brain, lungs, heart or uterus.

The foregoing studies shed some light on the tissue distribution of Sb after single doses of Sb^{III} or Sb^{V} drugs. Two additional studies determined Sb levels in organs of mice and rats exposed to potassium antimony tartrate (APT) for longer periods. Poon et al. (1998) exposed rats to APT orally (drinking water) for 90 days, and described that levels of Sb in tissues (measured by inductively coupled plasma emission spectrometry, ICP) were dose-related and followed a descending order of concentrations from liver and spleen to brain and adipose tissue (red blood cells>> spleen, liver> kidneys> brain, fat> plasma). Dieter et al. (1991) exposed B6C3F1 mice and F344 rats to APT through the drinking water for 14 days, and by *ip* injections every other day (a dosing schedule intended to minimize local mesenteric inflammation) for 90 days. The authors found dose-related concentrations of residual Sb in the blood, liver, kidney, spleen, and heart of rats, and in the liver and spleen of mice.

As far as the authors are aware, except for a previous study from our laboratory in Rhesus monkeys infected with *Leishmania braziliensis*, residual levels of Sb in different tissues after a treatment course with Sb^{V} drugs had not been investigated yet. This study was undertaken to provide data on the kinetics and tissue distribution of Sb in rats treated with a 21-day course of meglumine antimoniate (MA).

III.3 Materials and Methods

III.3.1 Animals

Male Wistar rats from the Oswaldo Cruz Foundation breeding stock were used in this study. Upon arriving at the laboratory animal quarters, approximately 80 day-old rats were individually housed in standard plastic cages with stainless steel cover lids and pinewood shavings as bedding. Animals were kept under controlled environmental

conditions (12 h light:12 h dark cycle, lights on from 8:00 to 20:00 h; temperature 22±1°C; relative humidity approximately 70%) throughout the study. All rats were given free access to a pelleted diet for rats and mice (CR1 Nuvital, Nuvilab Ltd., Curitiba, PR, Brazil) and tap water. Experiments were conducted in accordance with Brazilian animal protection and welfare laws, and the study protocol was cleared by the Ethics Committee on the Use of Laboratory Animals of Oswaldo Cruz Foundation (CEUA-FIOCRUZ).

III.3.2 Treatment

Meglumine antimoniate (MA; Glucantime®, Sanofi-Aventis Farmacêutica Ltd, Suzano, SP, Brazil) was administered by intravenous injections (penis vein) or by subcutaneous injections on the back skin of the rat. MA is a poorly characterized drug that is produced by the reaction of Sb^V with N-methyl-D-glucamine. Evidence has been provided that up to 4 N-methyl—D-glucamine hydroxyls are coordinated with each antimony atom (Roberts et al, 1998). According to the manufacturer, each ampoule (5 mL) of Glucantime® contains 425 mg *N*-methyl meglumine antimoniate/mL, or 85 mg Sb^V/mL. Total Sb, Sb^V and Sb^{III} concentrations were determined in the Glucantime® lot used in this investigation and also in ampoules of additional lots. Levels of total Sb in ampoules from the lot used in this study was 90.1 mg/mL while the concentration of Sb^{III} (measured by HG-ICP-MS as described by Miekeley et al, 2002) was 3.2 mg/mL, or 3.5% total Sb, while the concentration of Sb^v ([Sb-total] – [Sb^{III}] was 86.9 mg/mL.

The injected doses were 75 mg Sb^V/kg body wt (single dose, *iv*) or 300 mg Sb^V/kg body wt/d (*sc*), and injection volumes were 0.88 mL/kg body or 3.5 mL/kg body wt/d, respectively. A vehicle-only treated control group (N=6) received subcutaneous injections (1.76 mL/kg body wt/d) of the vehicle (potassium metabisulfite, 1.6 mg/mL, and sodium sulfite, 0.18 mg/mL). In a preliminary test, six animals were injected intravenously with a single dose of MA (75 mg Sb^V/mg/kg body wt) to evaluate the fast elimination phase of Sb kinetics in the male rat. In a subsequent experiment, twelve rats were treated by the subcutaneous route with a dose of MA as high as 300 mg Sb^V/kg body wt/d for 21 days. Half of the MA-treated rats were euthanized 24-h after the last dose of MA while the remaining animals were euthanized 21 days later. A third experiment (6 MA-treated and 3 vehicle-control rats) was performed to evaluate the

extent to which residual Sb blood levels declined after the end of a 21-d course of treatment with MA (300 mg Sb^V/kg body wt/d, *s.c.*) when a longer post-treatment time interval (105 days) was examined. Rats were euthanized by carbon dioxide inhalation.

III.3.3 Antimony Determination in Biological Matrices

Levels of Sb in biological matrices (whole blood, plasma and tissues) were determined by inductively coupled plasma mass spectrometry (ICP-MS) as described in detail elsewhere (Miekeley et al. 2002; Friedrich et al. 2012).

An ELAN DRC II (PerkinElmer Sciex, USA) instrument equipped with a Meinhard nebulizer and a cyclonic spray chamber (Glass Expansion, Australia) was used. Antimony measured isotopes were ^{121}Sb and ^{123}Sb and ^{103}Rh were employed as internal standards. To determine total Sb by solution nebulization ICP-MS, whole blood and plasma samples were analyzed after digestions with 2-fold sub-boiled distilled HNO_3 and adequate dilution (1:10 or 1:100) with deionized water (18 MΩ cm minimum resistivity, MilliQ, Millipore, USA). Tissue samples were lyophilized and wet-ashed with HNO_3-H_2O_2 in closed polyethylene tubes essentially as previously reported (Miekeley et al. 2002). The diluted digest was then analyzed by ICP-MS in the quantitative external calibration method.

The accuracy of the method was checked by the analysis of a reference material (bovine whole blood provided by the Adolpho Lutz Institute, Sao Paulo, SP, Brazil) and a tissue (liver) from a control individual). The Sb concentration in these samples was below the limit of detection. The samples were spiked with 10 µg/L and 60 µg/L of Sb and recoveries were between 96.0 and 99.7% (10 µg/L) and between 101.9 and 102.8% (60 µg/L), respectively. The RSD was below 5%. The repeatability (calculated as $r= t_{(n-1,\ 1-\alpha)}.\sqrt{2}.s$) was 1.8 µg/L and 10.9 µg/L for the spikes of 10 µg/L and 60 µg/L, respectively. The limits of detection (LOD) of the method were 0.5 ng Sb/g for plasma and whole blood, and 1 ng Sb/g for tissues, while limits of quantification (LOQ) were 1.7 ng Sb/g for plasma and whole blood and 3.3 ng Sb/g for tissues.

III.3.4 Blood Sampling

Venous blood samples (0.5 mL) were taken from the tail vein prior to MA treatment (d 0) and immediately before injections on treatment days 1, 5, 9, 13, 18, and on post-treatment days 1, 4, 8, 12, 16 and 21. Na-heparin was used as anticoagulant and plasma was separated by centrifugation (2400 × g for 15 min). Whole blood and plasma samples were distributed into polyethylene tubes and kept at –20°C until further use.

III.3.5 Statistical Analysis

Data were evaluated by one-way analysis of variance (ANOVA) and Dunnett's test, by the Student t test, or, when results did not fit a normal distribution, by the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test. In any case, a difference was considered significant for $P<0.05$. Descriptive statistics, statistical inference tests, and linear regression were performed using SPSS (version 11) or a Graph Pad Prism 45 software.

III.4 Results

III.4.1 Blood levels of Sb after single and multiple doses of MA

Figure 1A depicts the time course of changes in whole blood levels of Sb after a single intravenous (bolus) injection of MA (75 mg Sb^V/kg body wt) given to male rats. The sharp fall in Sb blood concentrations ($t_{1/2}$= 0.6 h) indicates that almost all Sb given as MA was cleared from the body within 6 to 12 h of drug injection. Nonetheless, a closer look at Sb terminal elimination phase (Figure 1A insert) reveals that, after attaining concentrations as low as 2 μg/g or less within 6 h of administration, further elimination proceeds very slowly so that 24 h after an intravenous injection of MA, nadir levels of Sb are found in the blood. Similar fast elimination phases of Sb with very low residual levels 24 h after MA administration were also observed when a Sb^V drug is given to rats by the s.c. route (Miranda et al, 2006). In this study, we did not examine the fast elimination following sc administration, but Figure 2 shows the increase in nadir blood levels of Sb (measured 24 h after a previous injection) during the treatment

course period and the slow decline of Sb concentrations thereafter. As shown in Figure 2, upon repeated administration (by the subcutaneous route) of 24 h spaced doses of MA (300 mg Sb^V/kg body wt/d, sc), nadir levels of Sb steadily rose so that at the end of a 21-day course of treatment, Sb attained levels as high as 35-40 μg/g in whole blood. Blood levels of Sb in rats euthanized one day after the last dose of MA (on day 22) did not differ from the levels of this metalloid in the blood of rats euthanized 21 days later (on day 42). In a subsequent experiment, six rats were treated with MA (300 mg Sb^V/kg body wt/d, sc) for 21 days, and their Sb blood levels were measured on the day following the last dose of MA (day 22) and 105 days later (day 126). The results showed that after almost three months post-treatment, Sb blood levels fell modestly from 51.0 ± 7.3 μg/g (day 22) to 35.6 ± 4.6 μg/g (day 126). Sb levels in whole blood of control (untreated and vehicle only-treated) rats remained undetected or close to the LOQ.

III.4.2 Residual levels of Sb in tissues after a 21-day course of treatment with MA

Tissue concentrations of Sb were determined in rats killed 24 h after the last dose of MA and in a second group of animals killed 21 days after treatment discontinuation (Table 1). Figure 3 shows the distribution of Sb in the spleen, kidneys, femur, thyroid, liver, epididymis, lungs and adrenals, all of which presented Sb levels higher than 5 μg/g at the end of treatment. The spleen ranked first among the tissues with the highest levels of Sb at the end of treatment. Although declining markedly over a three-week post-treatment period, levels of Sb in the spleen were still the highest among all tissues on day 42 (Table 1). The kidneys, bones (femur) and thyroid gland ranked second in a descending order of Sb content in tissues at the end of MA administration period (Table 1). In contrast to the bones and thyroid, the Sb levels which exhibited a small reduction, kidney levels showed a drastic decline during the three post-treatment weeks. The liver, epididymis, lungs and adrenals showed intermediate levels of Sb at the end of treatment (Table 1). On day 42, levels of Sb in the liver and epididymis were markedly lower than the levels on the day after the last dose of MA, while levels in lungs and adrenals were modestly reduced. Figure 4

presents tissues that showed the lowest concentrations of Sb (<5 μg/g) after a course of treatment with MA. The tissues with low (<5 μg/g) Sb concentrations on day 22 that exhibited a further reduction of metalloid content within the next three weeks were as follows: prostate, thymus, small intestine, skeletal muscle, testes, and stomach. The tissues which did not exhibit a discernible change in Sb levels between day 22 and 42 were pancreas, heart, and brain (Table 1).

Whole blood and plasma Sb 105 days after the end of treatment with MA are shown in Table 2. The huge difference between whole blood and plasma concentrations of Sb is consistent with the notion that at this terminal elimination phase red blood cells account for almost all Sb contained in the blood. It is of note that levels of antimony in the blood and plasma of untreated and vehicle-only treated rats were extremely low or below the quantification and/or detection limits.

III.5 Discussion

Data provided by this study indicated that, in male rats treated with MA, a decline of Sb levels in the blood can be described by a two-compartment kinetic model with fast ($t_{1/2}$=0.6 h) and slow elimination phases. Similar bi-exponential declines in Sb blood concentration over time had been described for pregnant and non-pregnant female rats treated with MA (Miranda et al. 2006), and also for dogs (Tassi et al. 1994), mice (Nieto et al. 2003), hamsters (Radwan et al. 2007), non-human primates (Friedrich et al. 2012), and humans (Chulay et al. 1988; Miekeley et al. 2002) treated with Sb^{V} drugs by parenteral routes.

There is a paucity of data on the distribution of Sb into different tissues and on their elimination from the body after exposure to organic antimony compounds or even to inorganic antimony. As mentioned in the Introduction of this article, Borborema et al. (2013) has recently described the distribution of labeled Sb (^{122}Sb, ^{124}Sb) into some BALB/c mouse tissues at different time intervals after a single intraperitoneal injection of MA. A study by Molokhia and Smith (1969) also reported the distribution of Sb into a variety of mouse tissues following a single intraperitoneal administration of Sb^{III} schistosomicidal drugs. The foregoing studies described the distribution of Sb after single injections of antimonial drugs. There are only a few studies on the tissue

distribution of residual Sb after prolonged exposures through drinking water or repeated injections of Sb^{III} organic compounds (e.g., tartar emetic) (Dieter et al. 1991; Poon et al. 1998). As far as the authors are aware, except for a report on Sb levels in organs from Rhesus monkeys treated with MA (Friedrich et al. 2012), no study has provided data on Sb distribution into tissues after a course of treatment with Sb^{V} drugs.

The marked disproportion between Sb levels in whole blood and in plasma 105 days after the end of treatment of with MA (Table 2) is consistent with the hypothesis that, during the terminal slow elimination phase, Sb is found inside red blood cells with very little in plasma. Friedrich et al. (2012) reported that, in monkeys that received intramuscular injections of MA, the ratio of the Sb concentration in the plasma to the Sb concentration in red blood cell ($[Sb]_{plasma}/[Sb]_{RBC}$) was greater than 1 (> 1) but progressively diminished with time in the fast elimination phase (*e.g.*, 6 and 12 h post-dosing). An inverse ratio (< 1), however, was noted in the slow elimination phase (*e.g.*, 24-h and longer post-treatment time intervals). Along the same line, a study by Quiroz et al. (2009) found that the levels of Sb-total in the blood of workers occupationally exposed to Sb in the air (vehicle emissions) were higher in the RBCs than in the plasma. In a recent study, Quiroz et al. (2013) spiked human blood samples (in vitro) with Sb^{III} (APT) and Sb^{V} [$KSb(OH)_6$] and demonstrated that both species penetrate the RBC membrane and leave the cell cytoplasm with time.

Many authors believe that some of the Sb^{V} that penetrates the erythrocyte is intracellularly reduced to Sb^{III}, a form that is retained within the cell by forming complexes with organic ligands, such as glutathione (Haldar et al. 2011). A hypothesis has also been suggested that, while the initial rapid elimination (via urine) is governed by a major pool of Sb (or Sb^{V} in the case of MA and SSG) that remained in the extracellular medium (including plasma), the slow terminal phase is governed by an intracellular Sb pool, the mobilization of which is slow (Friedrich et al. 2012).

The spleen was the organ that ranked first in a descending order of tissues according to Sb residual content after treatment with MA (Figure 3). The marked accumulation of Sb in the spleen is possibly explained by some of the organ's functions, such as to hold a reserve of blood and to remove senescent erythrocytes (Mebius & Kraal 2005). Moreover, erythrophagocytosis by the spleen and liver macrophages, and the scavenging of hemoglobin (and haptoglobin-bound hemoglobin) from the

circulation by splenic macrophages play a key role in iron recycling (Mebius & Kraal 2005). Antimony speciation and the fate (metabolomics) within the splenic and liver tissues, however, are still obscure questions. Spleen has been reported to be one of the tissues with the highest residual concentrations of Sb in mice treated with a single injection of Sb^{III} (Molokhia & Smith 1969) or Sb^{V} (Borborema et al. 2013) drugs, and in rats and mice exposed by the oral or *ip* route to Sb^{III} (tartar emetic) for 90 days (Dieter et al. 1991; Poon et al. 1998).

Among all rat tissues examined in this study, the brain (whole brain) had the lowest residual levels of Sb. Friedrich et al. (2012) also found that, in monkeys treated with MA, the CNS (central nervous system) structures (frontal and occipital lobes, parietal and temporal lobes, mesencephalon, medulla oblongata and cerebellum) were the tissues that exhibited the lowest levels of Sb. The brain also had the lowest residual levels of antimony in rodents treated with a single (Molokhia & Smith 1969) or multiple doses of tartar emetic (Dieter et al. 1991; Poon et al. 1998). These findings in rodents and non-human primates are consistent with the hypothesis that the blood-brain barrier prevents the penetration of Sb^{III} and Sb^{V} into the brain.

A remarkable finding of this study was that the thyroid gland of rats treated with MA accumulated a high content of Sb and that no decline of Sb concentrations occurred in the organ over three post-treatment weeks. In the rat, the levels of Sb in the thyroid gland were higher than the levels in the liver and comparable to levels found in the bones (Figure 3). Along the same line, Friedrich et al. (2012) had reported that, in monkeys treated with a low and a standard MA dosage regimen, the thyroid was the analyzed tissue with the highest content of Sb. Although the marked accumulation of Sb by the thyroid during treatment with Sb^{III} or Sb^{V} drugs remained almost unnoticed in the medical literature, it had already been noted in a few older studies with Sb^{III} compounds. Brady et al. (1945), for instance, dogs infected with *Dirofilaria immitis* were treated with antimony tartrate (labeled with ^{124}Sb), and found that while the liver ranked first in Sb content, combined thyroid and parathyroid were the tissues with the second largest accumulation of radioactive Sb. Kramer (1950) treated male rabbits with tartar emetic (once a day for 21 days *i.v.*) and noted that Sb concentrations in the thyroid were appreciably higher than those in any other tissue (kidneys, muscle and spleen) with the exception of the liver. According to Kramer (1950), Sb accumulation in the

rabbit thyroid was not accompanied by changes of gland function or histology. In human volunteers who received sodium antimony mercapto-succinate (labeled with ^{124}Sb) by the *iv* route, Abdallah and Saif (1962) noted that the highest radioactivity was recorded in the liver, followed by that in the thyroid and in the heart. Poon et al. (1998) did not determine Sb levels in the gland of rats exposed to APT, but they reported some treatment-related histological abnormalities, such as reduced follicle size, increased epithelial height, and nuclear vesiculation, all of which are morphological changes that have been interpreted as reflecting a mild adaptive change of thyroid function of minor or no toxicological importance (Lynch et al. 1999). At any rate, localization of Sb forms within the thyroid tissue and possible influences of Sb accumulation on gland function deserve further studies.

The Sb levels in epididymis and prostate fell markedly over the three post-treatment weeks so that residual levels of Sb in all male reproductive organs (epididymis, prostate and testes) were consistently low three weeks after the end of treatment with MA. The low residual levels of Sb in male reproductive organs is consistent with our previous findings, suggesting that treatment of rats during gestation and lactation periods with MA (doses up to 300 mg Sb^V/kg body wt/d, *sc*) did not affect offspring sperm parameters and male fertility in adulthood (Coelho 2010).

Stomach, small intestines and pancreas were among the rat organs that presented the lowest residual content of Sb (<5μg/g) after a 21-day course of treatment with MA. Friedrich et al. (2012) did not measure Sb content in the small intestines, but found that stomach, colon and pancreas were among the analyzed monkey tissues that had the lowest concentrations of Sb 55 and 95 days after a 21-day treatment with MA. Molokhia and Smith (1969), however, found that colon, duodenum and stomach were, following liver and spleen, the murine tissues with the most elevated Sb levels 0.5 h after a single intraperitoneal injection of tartar emetic. Borborema et al. (2013) reported that, in mice treated with a single *i.p.* injection of irradiated MA (^{122}Sb, ^{124}Sb), % of injected activity (IA) in the small intestines fell from nearly 13% (3 min, non-infected mice) to less than 2% within 24 h of dosing, while in the large intestines % IA was approximately 1% at 0.5 h, rose to approximately 16% at 2 h and fell to approximately 2% at 24 h after drug administration. The authors interpreted the foregoing findings as

reflecting a primary elimination of Sb through hepatobiliary excretion after liver processing.

It should be noted, however, that both Borborema et al. (2013) and Molokhia and Smith (1969) injected a Sb^{V} and a Sb^{III} drug, respectively, into the peritoneal cavity, where liver, pancreas, stomach, and small and large intestines are located. Under those conditions, the Sb levels determined by the authors may eventually reflect not only the Sb that reached the tissue indirectly via systemic circulation but also the Sb that was absorbed directly by the tissue at the site of injection. Moreover, Borborema et al. (2013) interpretation that during the fast elimination phase (within 12 h of the injection) Sb from MA is cleared primarily through hepatobiliary excretion is at odds with data provided by most studies. In fact, results from several studies are consistent with the notion that during rapid elimination phase, Sb^{III} is excreted via bile and to a lesser extent via urine, whereas the reverse holds true for Sb^{V}. Bailly et al. (1991), for instance, demonstrated that after a single *iv* administration of Sb^{III} (APT) to rats, about the same percentage (50%) of the administered Sb was excreted in the urine and feces, whereas after *ip* injection, about four times more Sb was excreted in the feces than in the urine. The hepatobiliary transport of Sb^{III} is GSH-dependent. Along this line, it was described that the intravenous administration of APT increased up to 50-fold the biliary excretion of non-protein thiols (mainly GSH) by the rat (Gyurasics et al. 1992; Gregus et al. 1998). Transport of Sb^{V} from MA into the bile apparently requires its reduction to Sb^{III} and the intracellular reduction of Sb^{V} is promoted by GSH and other thiols found in the cytosol (Ferreira et al. 2003). Based on the foregoing, it seems plausible to think that hepatobiliary excretion plays a major role in the elimination of residual Sb (mainly as Sb^{III}) during the slow terminal elimination phase after a course of treatment with Sb^{V} drugs. Nonetheless, Borborema et al. (2013) hypothesis that Sb^{V} is excreted primarily in the bile during the rapid elimination phase needs to be substantiated by experimental data.

In this study, the bone was the tissue with the second highest Sb concentration after spleen and next to thyroid, while the skeletal muscle ranked among rat tissues with the lowest levels of Sb. In monkeys treated with MA, both bone (femur) and skeletal muscle were among the tissues classified by Friedrich et al. (2012) as having accumulated “intermediate” levels of Sb. As far as the authors are aware, these are the

only two studies that measured Sb residual levels in bone and muscle after a treatment course with MA.

In conclusion, the decline of Sb blood levels with time after a parenteral injection of MA can be described by a two-compartment model, with a fast elimination phase the half-life of which was 0.6 h and a very slow terminal elimination phase, the half-life of which is longer than 24 h. A course of treatment of rats with 24 h spaced doses of MA, therefore, results in a gradual increase of nadir Sb levels in blood, a kinetic behavior similar to that described for humans, non-human primates, dogs and mice. This kinetic similarity with humans makes the rat a suitable model for studies of Sb^V distribution in tissues and toxicity. Furthermore, data from this study also showed that during the terminal elimination phase, the highest residual concentrations were found in the spleen, bones, thyroid gland and the liver. The levels in the kidneys were high at the end of treatment but decline sharply within three post-treatment weeks, a fall that is consistent with the notion renal excretion plays a major role in the clearance of Sb in the fast elimination phase. Sb residual levels were particularly low in the brain. The pathophysiological consequences of Sb accumulation in the thyroid gland and the localization of Sb forms within the liver, thyroid, spleen, and bones warrant further studies.

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III.7 References

Abdallah A, Saif M 1962. Trace studies with antimony 124 in man. In Wolstenholme GEW, O'Connor M (eds), Bilharziasis, (Ciba Foundation Symposium), 287-309, London: Churchill.

Bailly R, Lauwerys R, Buchet JP, Mahieu P, Konings J 1991. Experimental and human studies on antimony metabolism: their relevance for the biological monitoring of workers exposed to inorganic antimony. *Br J Ind Med. 48* (2): 93-7.

Borborema SE, Osso JA Jr, de Andrade HF Jr, do Nascimento N 2013. Biodistribution of meglumine antimoniate in healthy and *Leishmania (Leishmania) infantum* chagasi-infected BALB/c mice. *Mem Inst Oswaldo Cruz.108* (5): 623-30.

Brady FJ, Lawton AH, Cowie DB, Andrews HL, Ness AT, Ogden GE 1945. Localization of trivalent radioactive antimony following intravenous administration to dogs infected with *Dirofilaria immitis*. *Am.J.Trop Med 25*: 103-107.

Christopherson JB 1918. The successful use of antimony in Bilharziosis. *The Lancet*, Sept 7, 325-327.

Christopherson JB 1923. The curative dose of antimony tartrate in schistosomiasis (Bilharzia disease). *Br Med J. 2* (3287):1254-5.

Chulay JD, Fleckenstein L, Smith DH 1988. Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. *Trans R Soc Trop Med Hyg. 82*(1): 69-72

Coelho DR 2010. Desenvolvimento somático, neurocomportamental e fertilidade da prole de ratos exposta ao antimoniato de meglumina pela via transplacentária e leite materno. MSc Dissertation, National School of Public Health, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, 72 pp.

Di Cristina G, Caronia G 1915. Sulla terapia della leishmaniosi interna. *Bulletin de la Société de Pathologie Éxotique 8*: 63-65

Dieter MP, Jameson CW, Elwell MR, Lodge JW, Hejtmancik M, Grumbein SL, Ryan M, Peters AC 1991. Comparative toxicity and tissue distribution of antimony

potassium tartrate in rats and mice dosed by drinking water or intraperitoneal injection. *J Toxicol Environ Health. 34*(1): 51-82.

Ferreira C dos S, Martins PS, Demicheli C, Brochu C, Ouellette M, Frézard F 2003. Thiol-induced reduction of antimony(V) into antimony(III): a comparative study with trypanothione, cysteinyl-glycine, cysteine and glutathione. *Biometals. 16*(3): 441-6

Friedrich K, Vieira FA, Porrozzi R, Marchevsky RS, Miekeley N, Grimaldi G Jr, Paumgartten FJ 2012. Disposition of antimony in rhesus monkeys infected with Leishmania braziliensis and treated with meglumine antimoniate. *J Toxicol Environ Health A.75*(2): 63-75

Goodwin LG, Page JE 1943. A study of the excretion of organic antimonials using a polarographic procedure. *Biochem J. 37*(2):198-209

Gregus Z, Gyurasics A, Koszorús L 1998. Interactions between selenium and group Va-metalloids (arsenic, antimony and bismuth) in the biliary excretion. *Environ Toxicol Pharmacol. 5*(2):89-99.

Gyurasics A, Koszorús L, Varga F, Gregus Z 1992. Increased biliary excretion of glutathione is generated by the glutathione-dependent hepatobiliary transport of antimony and bismuth. *Biochem Pharmacol.44* (7):1275-81.

Haldar AK, Sen P, Roy S 2011. Use of antimony in the treatment of leishmaniasis: current status and future directions. *Mol Biol Int.* 2011:571242

Kramer L B 1950. Antimony concentration in the thyroid gland and its effect upon metabolic rate and serum cholesterol level. *Bulletin of the Johns Hopkins Hospital 86*: 179-181

Lynch BS, Capen CC, Nestmann ER, Veenstra G, Deyo JA 1999. Review of subchronic/chronic toxicity of antimony potassium tartrate. *Regul Toxicol Pharmacol. 30*(1): 9-17.

Mebius RE, Kraal G 2005. Structure and function of the spleen. *Nat Rev Immunol. 5*(8): 606-16.

Miekeley N, Mortari SR, Schubach AO 2002. Monitoring of total antimony and its species by ICP-MS and on-line ion chromatography in biological samples from patients treated for leishmaniasis. *Anal Bioanal Chem. 372*(3): 495-502

Miranda ES, Miekeley N, De-Carvalho RR, Paumgartten FJ 2006. Developmental toxicity of meglumine antimoniate and transplacental transfer of antimony in the rat. *Reprod Toxicol. 21*(3): 292-300.

Molokhia MM, Smith H 1969. Tissue distribution of trivalent antimony in mice infected with *Schistosoma mansoni*. *Bull World Health Organ. 40*(1): 123-8.

Nieto J, Alvar J, Mullen AB, Carter KC, Rodríguez C, San Andrés MI, San Andrés MD, Baillie AJ, González F 2003. Pharmacokinetics, toxicities, and efficacies of sodium stibogluconate formulations after intravenous administration in animals. *Antimicrob Agents Chemother. 47*(9): 2781-7.

Otto GF, Maren TH, Brown HW 1947. Blood levels and excretion rates of antimony in persons receiving trivalent and pentavalent antimonials. Am J Hyg. 46(2): 193-211.

Poon R, Chu I, Lecavalier P, Valli VE, Foster W, Gupta S, Thomas B 1998. Effects of antimony on rats following 90-day exposure via drinking water. *Food Chem Toxicol. 36* (1): 21-35.

Quiroz W, De Gregori I, Basilio P, Bravo M, Pinto M, Lobos MG 2009. Heavy weight vehicle traffic and its relationship with antimony content in human blood. J Environ Monit. 11(5):1051-5.

Quiroz W, Aguilar L, Barría M, Veneciano J, Martínez D, Bravo M, Lobos MG, Mercado L 2013. Sb(V) and Sb(III) distribution in human erythrocytes: speciation methodology and the influence of temperature, time and anticoagulants. Talanta. 115:902-10.

Radwan MA, Al Jaser MH, Al Rayes ZR 2007. The effects of induced diabetes and cutaneous Leishmania infection on the pharmacokinetics of antimony in hamsters. *Ann Trop Med Parasitol. 101*(2):133-42

Roberts WL, McMurray WJ, Rainey PM 1998. Characterization of the antimonial antileishmanial agent meglumine antimonate (glucantime). *Antimicrob Agents Chemother*. 42(5):1076-82

Tassi P, Ormas P, Madonna M, Carli S, Belloli C, De Natale G, Ceci L, Marcotrigiano GO 1994. Pharmacokinetics of N-methylglucamine antimoniate after intravenous, intramuscular and subcutaneous administration in the dog. *Res Vet Sci*. *56*(2): 144-50

Vianna, G 1912. Tratamento da leishmaniose tegumentar por injeções intravenosas de tártaro emético. *Anais do VII Congresso Brasileiro de Medicina e Cirurgia 4*: 426-8.

III.8 Tables

Table 1. Concentrations of antimony (μg/g) in rat tissues 24-h and 21-d after a 21-d treatment with meglumine antimoniate (MA; 300 mg Sb^V/kg bw/d, *sc*).

Tissue	Time after the last injection of MA	
	1 day	21 days
Spleen	148.0±14	81.9±4.6*
Kidneys	31.1±1.9	5.4±0.4*
Femur	28.2±0.7	18.3±0.8*
Thyroid gland	25.2±4.5	18.0±1.2
Liver	13.8±1.3	3.2±0.2 *
Epididymis	10.9±0.7	1.2±0.1 *
Lungs	10.2±0.8	7.7±0.7*
Adrenals	7.4±0.5	5.9±0.5
Prostate	3.2±0.5	1.0±0.2*
Thymus	3.1±0.2	1.6±0.2*
Small intestines	2.7±0.3	1.8±0.3*
Skeletal muscle	2.2±0.4	0.6±0.1*
Testes	2.0±0.1	1.0±0.2*
Stomach	1.7±0.2	1.0±0.1*
Pancreas	3.0±0.4	2.5±0.4
Heart	2.8±0.3	2.7±0.3
Brain	0.6±0.0	0.5±0.0

Values are means ± SD, N= 6 rats/ group. * Differ from Sb concentrations measured 24-h after treatment (Student t test, $P<0.05$).

Table 2. Levels of antimony (Sb) in the whole blood and plasma of male rats 105 days after the end of a 21-day course of treatment with the vehicle-only (controls) or meglumine antimoniate (300 mg Sb^V/kg body wt/d, *sc*).

Treatment	**Vehicle-only** (1.76 mL/kg body wt/d, *sc* x 21 d)	**Meglumine antimoniate** (300 mg Sb^V/ kg body wt/d, *sc* x 21 d).
Whole blood	3 ± 0 ng/g	35,614 ± 4,625 ng/g
Plasma	< LOQ^+	21 ± 12 ng/g

Data are shown as the means ± SD of Sb concentrations (wet wt) determined by ICP-MS. $^+$ Levels below the limit of quantification (LOQ) of the method (1.7 ng Sb/g). Vehicle-only group, N=6; MA-treated group, N=6.

III.9 Figures

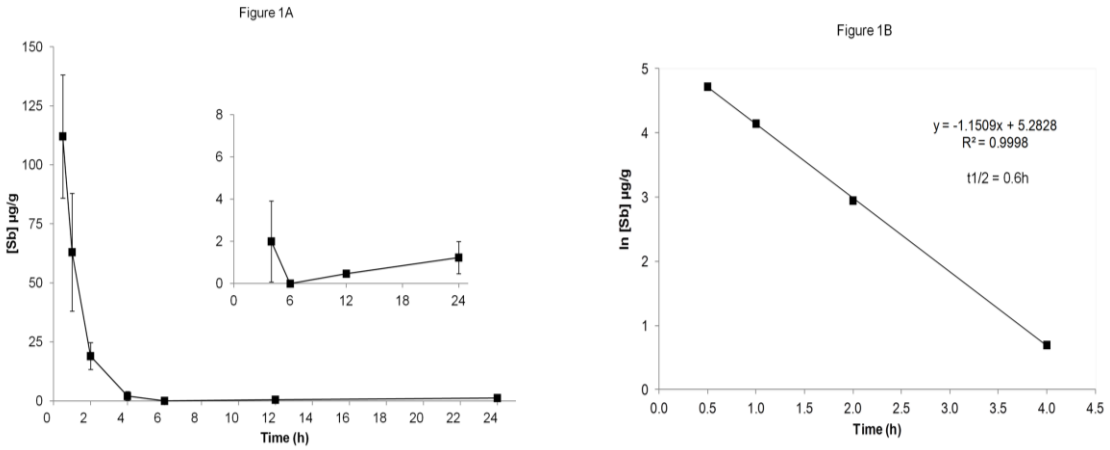

Figure 1. **A**- Time course of antimony (Sb) concentrations (μg/g) in the blood (whole blood) of male rats (N=6) treated intravenously with a single dose (75 mg Sb^V/kg body wt) of meglumine antimoniate. Insert: A magnified view of nadir Sb levels at post-injection intervals longer than 6 h (terminal elimination phase). **B**- Linear plot of decline in Sb blood levels during the fast elimination phase. Data are shown as natural logarithm (ln) of Sb concentration (μg/g) in the blood versus time after MA administration.

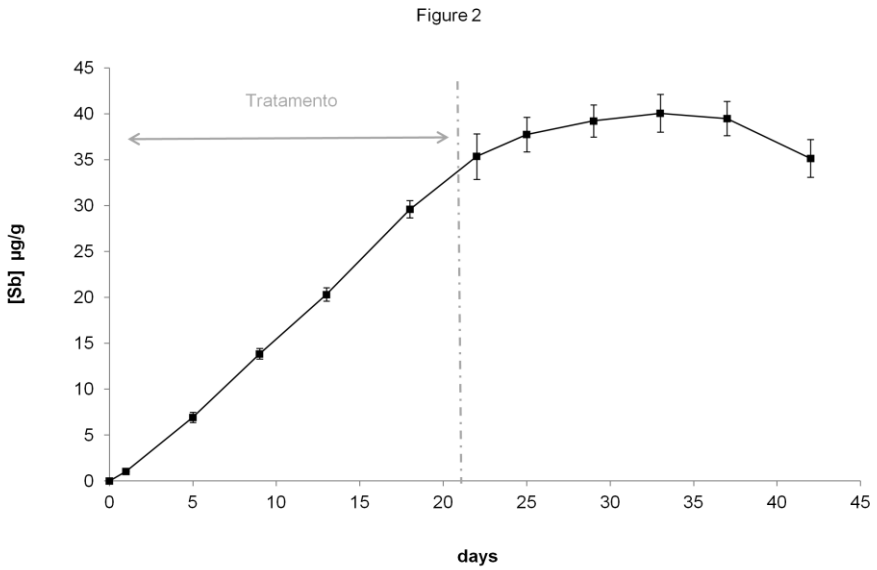

Figure 2. Time course of nadir concentrations of antimony (µg/g) in the blood from male rats (N=6) treated with meglumine antimoniate (300 mg Sb^V /kg body wt, *sc*) during 21 consecutive days. Blood samples were taken from the tail vein 24 h after a prior MA administration. Levels of Sb in the blood of untreated controls (N=3) and of rats treated with the vehicle only (N=6) (not shown) were below the limit of quantification of the method.

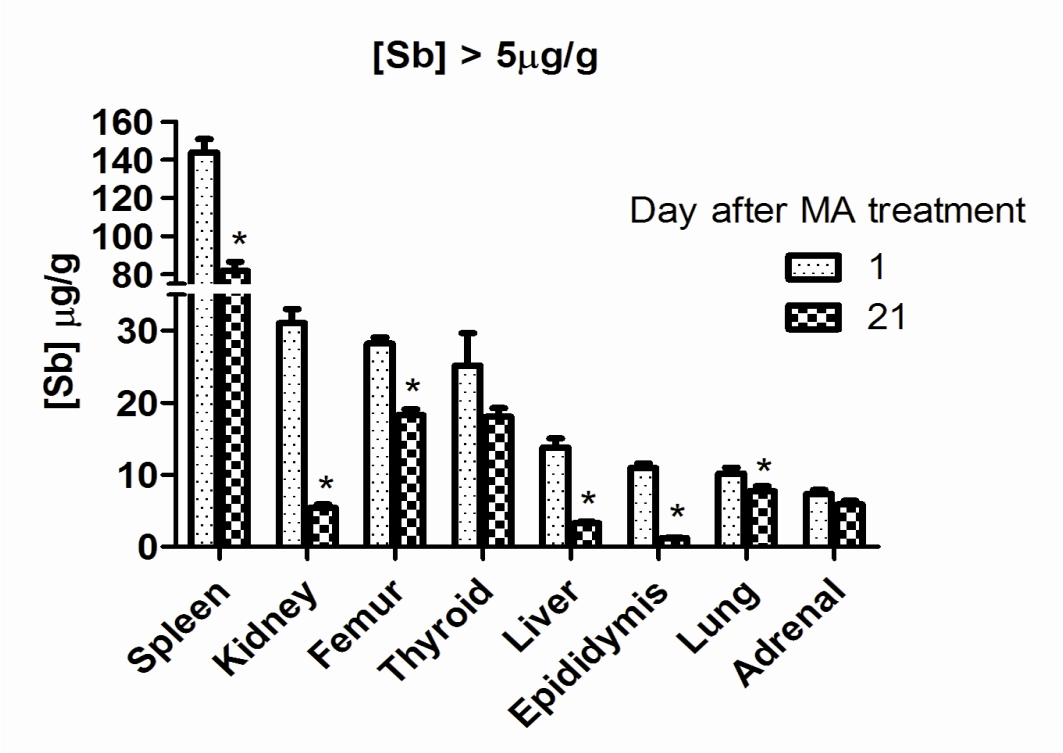

Figure 3. Tissues the residual antimony levels of which were higher than 5 μg/g. Sb content (μg/g, dry wt) was determined by ICP-MS in rats killed 24 h (N=6) and 21 days (N=6) after a 21-d treatment with meglumine antimoniate (300 mg Sb^V/kg body wt/d, *sc*). An asterisk indicates a decrease (Student t test, $P<0.05$) of Sb concentration within 3 weeks of the end of MA administration.

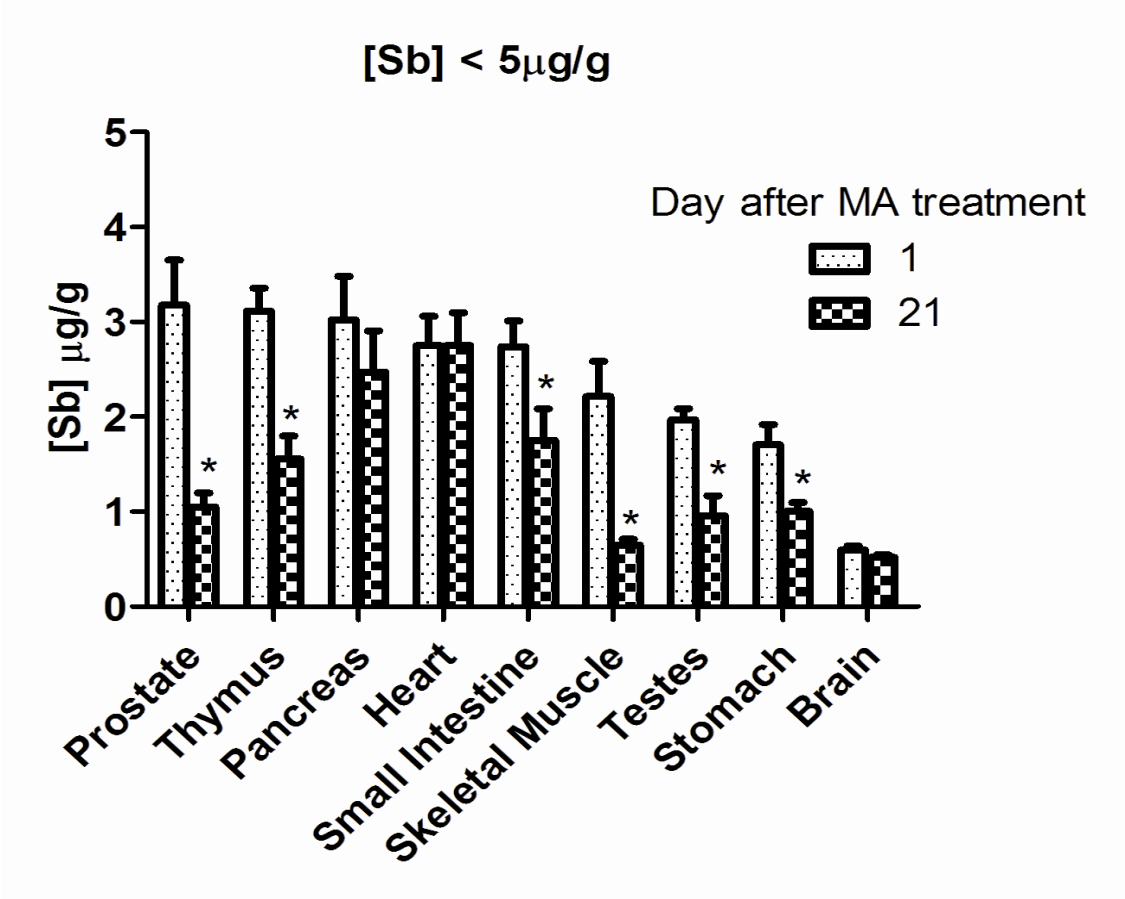

Figure 4. Tissues the residual antimony levels of which were lower than 5 μg/g. Sb content (μg/g, dry wt) was determined by ICP-MS in rats killed 24 h (N=6) and 21 days (N =6) after a 21-d treatment with meglumine antimoniate (300 mg Sb^V/kg body wt/d, *sc*). An asterisk indicates a decrease (Student t test, $P<0.05$) of Sb concentration within 3 weeks of the end of MA administration.

IV. ARTIGO 2

Effects of *in utero* and lactational exposure to Sb^V on rat neurobehavioral development and fertility

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Running title: Developmental exposure to Sb^V and post-natal maturation of rats

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Abbreviations: MA: meglumine antimoniate; SSB, sodium stibogluconate; GD: gestation day; PND: post-natal day; ICP-MS: inductively coupled plasma-mass spectrometry; HG: hydride generation.

IV.1 Abstract

Meglumine antimoniate (MA) is a pentavalent antimony drug used to treat leishmaniases. We investigated the neurobehavioral development, sexual maturation and fertility of the offspring of MA-treated rats. Dams were administered MA (0, 75, 150, 300 mg Sb^V/kg body wt/d, sc) from gestation day 0, throughout parturition and lactation, until weaning. At the highest dose, MA reduced the birth weight and the number of viable newborns. In the male offspring, MA did not impair development (somatic, reflex maturation, weight gain, puberty onset, open field test), sperm count, or reproductive performance. Except for a minor effect on body weight gain and vertical exploration in the open field, MA also did not affect the development of female offspring. Measurements of the Sb levels (ICP-MS) in the blood of MA-treated female rats and their offspring demonstrated that Sb is transferred to the fetuses via the placenta and to the suckling pups via milk.

Key words: pentavalent antimonial drugs, developmental toxicity, leishmaniasis, kinetics, maternal milk, reproductive toxicity, metalloids, antimony.

IV.2 Introduction

The pentavalent antimony (Sb^V) drugs sodium stibogluconate (SSB) and meglumine antimoniate (MA) are the mainstay of treatment of leishmaniases, a group of phlebotomine (sandfly)-transmitted diseases caused by protozoa of the genus *Leishmania* [1-5]. Cutaneous leishmaniasis, the most prevalent form, appears as skin ulcers that usually heal within a few months, leaving scars. The other clinical forms are as follows: diffuse-cutaneous leishmaniasis, characterized by disseminated and chronic skin lesions; muco-cutaneous leishmaniasis, which partially or totally destroy the mucous membranes and the surrounding tissues of the nose, mouth and throat, leaving facial disfigurations; and visceral leishmaniasis, a severe and life-threatening infectious disease. The clinical manifestations of visceral leishmaniasis, also known as kala-azar, include high fever, weight loss, asthenia, immunosuppression, enlargement of the spleen and liver, and anemia. If untreated, patients with visceral leishmaniasis may die [1-4].

SSB (Pentostam®) is used in English-speaking countries, while MA (Glucantime®) is the preferred Sb^V drug in Brazil and most other countries where the disease is endemic. SSB and MA are assumed to be equivalent in terms of efficacy and safety [1-4].

Leishmaniases typically affect underprivileged people living in poor regions of the tropics and have been listed by World Health Organization among the most neglected diseases [4].

The safety of the Sb^V drugs was not thoroughly evaluated prior to their introduction into clinical practice in the mid-1940s [5], and since then, major gaps in their safety profile have remained unfilled. One of these gaps in the toxicity data for the Sb^V drugs is the lack of pre-clinical studies on the reproductive toxicity and kinetics during pregnancy and lactation. In regions where leishmaniases are endemic, pregnant and nursing women and their very young children are at risk of acquiring *Leishmania* infections, including the visceral form, which requires an expeditious treatment [4]. Data regarding the reproductive and developmental toxicities of the Sb^V drugs could help physicians choose the best therapeutic option for pregnant or nursing women and infants with leishmaniases.

There is also a paucity of data on the transplacental transfer of the Sb forms and on their passage into breast milk. The effective dosing schedules of Sb^V drugs in

leishmaniases were established empirically decades before their kinetics began to be elucidated in 1988 [6]. The standard Sb^V dosage regimen recommended for cutaneous leishmaniasis is a 20-d course of treatment with daily doses of 20 mg Sb^V/kg body weight, administered either by *im* or *iv* injections. For the visceral and muco-cutaneous leishmaniases, the recommended courses of treatment are longer, *i.e.*, 28 and 30 days, respectively [4,7].

In humans [6,8], monkeys [9], dogs [10], mice [11], hamsters [12] and rats [13,14], the decreases in the Sb blood levels with time after Sb^V drug injection fit a two-compartment (or even multi-compartment) kinetic model. The slow elimination phase of the bi-exponential decline of the Sb blood levels has a half-life longer than 24 h. This fact explains the therapeutic and toxic effects resulting from the repeated administration of doses administered at 24-h intervals (generally a three-week course of treatment) [6,9,13]. Most of the Sb given as MA is rapidly eliminated (via urine) within 6 to 12 h after drug injection; therefore, very low (nadir) levels are found in the blood 24 h after dosing. Nonetheless, there is a gradual increase in residual Sb levels in blood during a course of treatment involving dosing of the Sb^V–containing drugs at 24-h intervals. The therapeutic and toxic effects are apparently related to the residual levels of Sb, and the magnitude of these effects depends on both the Sb^V drug daily dose and the length of the treatment course. During the slow elimination phase, Sb is found predominantly inside the red blood cells, with very little found in the plasma [8,9]. Many authors believe that some of the Sb^V forms that penetrate erythrocytes and other cells are reduced intracellularly to Sb^{III}, an antimony form that appears to be retained within the cell by forming complexes with organic molecules, such as glutathione (GSH) [1]. It has also been suggested that, while the rapid elimination phase depends on the major pool of Sb (Sb^V in the case of Sb^V drugs) found in the extracellular medium and plasma, the slow elimination phase involves an intracellular pool of Sb, the mobilization of which is slow [9].

In this study, we investigated the effects of maternal treatment with an Sb^V drug (MA) during pregnancy and lactation on the somatic and neurobehavioral development of the offspring and their reproductive performance when sexually mature. Furthermore, the transfer of Sb via the placenta to the fetuses and via maternal milk to the suckling pups was evaluated.

IV.3 Materials and methods

IV.3.1 Animals

Male and nulliparous female Wistar rats, approximately 80 days old, from the FIOCRUZ central animal house breeding stock were used in the study. The animals were individually housed in standard plastic cages with stainless steel covers and pinewood shavings as bedding and maintained under controlled environmental conditions (12-h light:12-h dark photoperiod, lights on from 8:00 to 20:00 h; room temperature 21±2°C; relative air humidity approximately 70%). A commercial pellet diet for rats and mice (CR1 Nuvital, Nuvilab Ltd, Curitiba, PR, Brazil) and filtered tap water were available *ad libitum* throughout the experiment. Experiments were conducted in accordance with Brazilian animal protection and welfare legislation, and the study protocol was approved by the Ethical Committee on the Use of Laboratory Animals of FIOCRUZ (CEUA-FIOCRUZ, permit number: L0016/08).

IV.3.2 Mating procedure

Mating was accomplished by placing two females into the cage of one male for 2 h at the end of the dark period. The day on which copulation was confirmed by the presence of spermatozoa in the vaginal smear was designated as day 0 of pregnancy (GD 0).

IV.3.3 Treatment

Meglumine antimoniate (MA; Glucantime®, Sanofi-Aventis Farmacêutica Ltd, Suzano, SP, Brazil) was administered by subcutaneous injections in the skin of the rat's back. According to the manufacturer, each ampoule of Glucantime® (5 mL) contains 425 mg of *N*-methyl meglumine antimoniate/mL, or 85 mg of Sb^{V}/mL. The concentrations of total Sb, Sb^{V} and Sb^{III} were measured in the lot of Glucantime® used in this study and in ampoules from additional lots. The concentration of total Sb in the ampoules from the lot used in the experiments was 90.1 mg/mL, while the concentration of Sb^{III} (determined by HG-ICP-MS, as described by Miekeley et al. [8]) was 3.2 mg/mL, or 3.5% of the total Sb. Therefore, the calculated concentration of Sb^{V} ([Sb-

total]–[Sb^{III}]) in the ampoules used in this investigation was 86.9 mg/mL. The injected volumes were adjusted to obtain the administered dose of MA (0, 75, 150, 300 mg Sb^{V}/kg body wt/d, sc). A vehicle-only control group (dose 0) received subcutaneous injections (1.76 mg/mL kg body wt/d) of the vehicle (potassium metabisulfite 1.6 mg/mL and sodium sulfite 0.18 mg/mL).

The dams were treated with daily (24-h intervals) sc injections of MA or its vehicle from pregnancy day 0 (GD 0), throughout gestation, spontaneous parturition and lactation, until post-natal day 21 (PND 21), when the offspring were weaned, *i.e.*, for 42 consecutive days. The day on which pups were born was designated as post-natal day 1 (PND 1) (Experiment I, Table 1).

Dams were weighed daily before MA (or the vehicle-only) injections and observed in their cages for approximately 30-40 min for any clinical sign of toxicity and/or behavioral abnormalities. After euthanasia on PND 31, the major abdominal and thoracic organs were examined for macroscopically visible abnormalities. The liver, spleen, uterus, kidneys and heart were weighed, and the number of implantation sites in the uterus was determined by the method of Salewski.

The rats were euthanized by carbon dioxide inhalation, narcosis and decapitation.

IV.3.4 Evaluation of somatic and neurobehavioral development of the offspring

Beginning on GD 20, the dams' cages were inspected twice a day for spontaneous deliveries. On the day of birth (PND 1), newborns were examined for litter size, stillbirths and live births and were sexed, weighed and individually marked (India ink tattoo).

To evaluate landmarks of somatic maturation and reflex acquisition, the dams were removed and placed into a holding cage while the pups were examined. Pups younger than 14 days were kept in a warm environment (37 °C). The somatic maturation landmarks were the days on which the following were observed:

Incisor eruption: eruption of the upper and lower incisors through the gums.

Fur development: downy hair was first detected.

Ear unfolding: any detachment of the external pinnae of both ears.

Eye opening: total separation of the upper and lower eyelids and complete opening of both eyes.

Vaginal opening: discontinuity of the skin and appearance of a definite aperture in the vaginal area.

Testes descent: testicular descent (both testes), confirmed by scrotum palpation.

Preputial separation: separation of the prepuce from the glans penis, assessed by attempting to retract the prepuce. The day of complete preputial separation was the endpoint used in the analysis.

The neurological reflexes of the pups were tested as follows.

Surface righting: The pup was placed supine on a flat surface, and the time for it to turn over to rest in the prone position with all four feet on the ground was determined. A response was positive when a pup had turned over to the prone position within 30 s.

Cliff avoidance: The pup was placed on the edge of a bench with its nose and forefeet just over the edge. The time for the pup to move away from the cliff was recorded. A response was considered positive if this movement occurred in less than 60 s.

Negative geotaxis: The pup was placed head downwards on a 40° slope, and the time for it to turn 180° to face up the slope was recorded. A response was considered positive if this movement occurred in less than 60 s.

Auditory startle response: A spring-loaded metal rat trap held above and behind the pup was closed. A positive response was indicated by a whole-body startle in response to this stimulus.

Palmar grasp: A forepaw was gently stroked with a paper clip, and the digit flexing response was observed. The day of disappearance of the flexing response was recorded.

Free-fall righting: The pup was dropped, back downwards, from a height of 30 cm onto a cotton-wool pad. Turning in mid-air and landing on all fours was considered a positive response. The criterion for reflex acquisition was two perfect landings out of the three tests on one day.

The age at which pups started to be examined (somatic landmarks) or tested (reflexes) for developmental landmarks was as follows: PND 1: ear unfolding, fur development surface righting; palmar grasp, and negative geotaxis; PND 2: cliff avoidance; PND 6: incisor eruption; PND 12: eye opening, free fall righting, and

auditory startle response; and PND 14: testes descent, PND 30 preputial separation, and vaginal opening.

IV.3.5 Determination of estrus cycling onset

Beginning on the day after VO was first detected, daily vaginal lavage was collected between 8 and 10 a.m. until a typical estrus phase was diagnosed, or for 15 consecutive days. A vaginal smear was prepared by introducing a drop of distilled water into the vagina and applying the lavage fluid to a clean glass slide. The vaginal smear was freshly examined under a light microscope using a low magnification (×100). The estrus phase was confirmed when the vaginal smear showed over 50% cornified epithelial cells. On the day that estrus was first detected (FO), the pup's age and body weight was recorded.

IV.3.6 Open field test

The open-field test apparatus was a circular arena made of wood with a diameter of 80 cm and walls that were 30 cm high. The floor was divided into areas as follows: a central floor (30 cm in diameter) plus two additional concentric circles divided by 6 radii. The apparatus was kept in a quiet room and illuminated by 3 centrally positioned lights (60 W each) hanging 150 cm above the floor level. Each rat was placed individually in the central circle and observed for a period of 6 minutes. The aspects of behavior scored were as follows: latency (time to leave the central circle), locomotor activity (number of floor subdivisions traversed), rearing up (number of times the animal stood on its two hind legs), grooming (number of grooming behavior episodes), and fecal boli (number of fetal boli left on the arena floor). The arena was cleaned with ethanol solution (70%) and allowed to dry after testing each rat. Open field tests were performed when pups were 25 and 60 days old.

IV.3.7 Blood and maternal milk collection for Sb analysis

Blood samples (>0.2 mL) were collected by decapitation after CO_2-induced deep narcosis. To collect maternal milk, dams were separated from their pups for approximately 16 h. The dam was restrained manually while the nipples were gently squeezed between the thumb and the forefinger. The ejected milk was collected into

2.0-mL Eppendorf tubes that were tightly closed and stored in the freezer until further use.

IV.3.8 Determination of Sb levels in blood, milk and liver tissue.

Sb levels in the blood and milk were determined in Experiments II and III (Table 1), whereas concentrations in the liver tissue were measured in Experiment I (Table 1). An ELAN DRC II (PerkinElmer Sciex, USA) ICP mass spectrometer equipped with a Meinhard nebulizer and a cyclonic spray chamber (Glass Expansion, Australia) was used to determine Sb levels. Antimony was measured at the *m/z* ratios of ^{121}Sb and ^{123}Sb isotopes, and Rh *m/z*=103 was employed as an internal standard. To determine the total Sb by solution nebulization ICP-MS, the whole blood and milk samples were analyzed after digestion with HNO_3 (Merck, Darmstadt, Germany), purification by two cycles of sub-boiling distillation and an appropriate dilution with MilliQ (Millipore, Bedford, MA, USA) purified water. The tissue samples were lyophilized and wet-ashed with HNO_3-H_2O_2 in closed polyethylene tubes as previously reported [8,13]. The diluted digest was then analyzed by ICP-MS using the quantitative external calibration method. The limits of detection (LOD) of the method were 0.5 ng Sb/g for whole blood and milk and 1.0 ng Sb/g for liver tissue, while the limits of quantification (LOQ) were 1.7 ng Sb/g for whole blood and milk and 3.3 ng Sb/g for liver tissue.

The accuracy of the method was checked by the analysis of a reference material (bovine whole blood provided by Adolfo Lutz Institute) and of a tissue sample (liver) of a control individual. The Sb concentration in these samples was below the limit of detection. The reference samples were then spiked with 10 µg L^{-1} and 60 µg L^{-1} of Sb before preparing as described. The recoveries were between 96.0% and 99.7% (spike of 10 µg L^{-1}) and between 101.9% and 102.8% (spike of 60 µg L^{-1}). The RSD was below 5% for both concentration ranges.

IV.3.9 Spermatid (testes) and sperm (cauda epididymis) counting

Male offspring were euthanized on PNDs 100-110. Major thoracic and abdominal organs were examined for externally visible abnormalities. The liver and male reproductive organs were weighed. A piece of the liver was removed and kept for further determination of Sb concentrations. Sperm parameters were determined as follows. After removing the tunica albuginea, the testes were minced and homogenized

in 10 mL of 0.9% NaCl containing 0.5% Triton X-100. Similarly, the cauda of one epididymis was cut into small pieces, minced and homogenized. After a 10-fold dilution, the number of homogenization-resistant spermatids of each testis and the number of spermatozoa in the cauda epididymis were counted using a Neubauer chamber [15]. The daily sperm production rate (DSP) and the sperm transit time (STT) were calculated as follows: DSP= No of spermatids per testis / 6.1 d, and STT= No of sperm in epididymis / DSP."

IV.3.10 Fertility test

To evaluate the reproductive capabilities of the F_1 animals, when the offspring of the treated dams (F_0) reached sexual maturity (*i.e.*, 90 to 110 days of age), one male and two females of the same dose level but different litters (brother-sister matings were avoided) were mated as described elsewhere (section 2.2). The mating procedure was repeated daily until the two females became "sperm-positive" or, alternatively, for fifteen consecutive mating sessions. On GD 21, the F_1 dams were weighed and euthanized by CO_2 inhalation narcosis and subsequent decapitation. The gravid uteri were weighed with their contents. The implantation sites [16], resorptions and live and dead fetuses were counted. All live fetuses (F_2) were weighed, sexed and examined for external morphological abnormalities. The abdominal and thoracic organs of the F_1 dams were examined macroscopically, and their livers were weighed.

IV.3.11 Statistical analyses

Wherever applicable, the litter was the statistical unit of analysis. The data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's *post-hoc* test. For data that were not normally distributed, the Kruskal–Wallis test was used, followed by the Mann–Whitney U test. Proportions were compared using the chi-square test or Fisher's exact test. The statistical evaluations were performed using the SPSS® program, and differences were considered significant when $P<0.05$. Data on the day of appearance of developmental landmarks are shown as the group median value and range (minimum-maximum), whereas other data are presented as means±SD.

IV.4 Results and discussion

IV.4.1 Effects of MA dosage regimens on pregnant and nursing rats

All control and MA-treated dams survived to the scheduled euthanasia on day 10 after weaning. The MA-treated rats showed no clinical signs of toxicity, and no treatment-related abnormalities in the maternal organs were noted at the necropsy. As shown in Figure 1, the body weight gain of the MA-treated dams during the pregnancy and lactation periods did not differ from that of the vehicle-treated dams. Therefore, the continued administration of MA throughout pregnancy and lactation did not cause discernible adverse effects on the pregnant and nursing rats.

IV.4.2 Effects of maternal treatment with MA on pregnancy outcome

The average number of implants in the uteri (evaluated at necropsy 31 days after parturition) of the control female rats did not differ from those in the uteri of MA-treated females at any dose level (Table 2). This finding suggests that the maternal treatment with MA starting on GD 0 did not enhance the occurrence of pre- and/or peri-implantation gestational losses. However, at the highest dose regimen tested (300 mg Sb^{v}/kg body wt/d), the continued treatment with MA throughout the pregnancy caused a nearly 30% reduction in the number of live newborns per litter on PND 1 and an approximately 13% decrease in the pup body weight at birth (Table 2). When male and female offspring were analyzed separately, the birth weight of the males did not differ among dose groups, whereas the birth weight of the females prenatally exposed to MA was lower (approximately 17% reduction in body weight) than that of the control females. Because no maternal toxicity was noted at any dose level, these findings suggest that treatment with the highest dose of MA (300 mg Sb^{V}/kg body wt/d) selectively impaired the embryo/fetal growth and fetal and/or neonatal viability. The results from a previous study by Miranda et al. [14] were also consistent with the notion that MA is a selective, albeit weak, developmental toxicant. Miranda et al. [14] found that MA (300 mg Sb^{V}/kg body wt/d, sc) administered to rats on GD 0-20 increased embryo lethality, reduced fetal body weight at term and augmented the incidence of some soft-tissue and skeletal variations in the absence of any sign of maternal toxicity.

IV.4.3 Effects of treatment with MA during pregnancy and lactation on the somatic and neurobehavioral development of the offspring

The continuation of maternal treatment with MA after parturition throughout lactation did not adversely affect the survival of the offspring during the post-natal period through weaning (PND 21) or thereafter until scheduled euthanasia.

On reaching sexual maturity, the body weights of the female rats exposed to MA during pregnancy and lactation remained lower than those of the vehicle control group (PND 60, all dose levels, approximately 10% lower body weights). Treatment of the dams with MA, however, had no significant effect on the birth weight and post-natal body weight gain of the male pups (Figure 2).

The acquisition of landmarks of somatic and reflex maturation by offspring of either sex was unaffected by the maternal treatment with MA (Table 3). An open field test on PND 25 (four days after weaning) showed no effect of the maternal treatment on the behavior of either male or female pups (data not shown). A second open field test performed approximately one month later (PND 60) detected a decreased frequency of "rearing up" behavior (vertical exploration) in the female offspring of dams treated with the highest dose of MA (300 mg Sb^{v}/kg body wt/d; Table 4). The decrease in vertical exploration and a non-significant reduction in motor activity (number of squares crossed) suggested that females exposed to MA *in utero* and throughout lactation were less active in the open field arena than females of the vehicle control group. However, MA had no effect on the performance of male offspring in the open field test (Table 4).

IV.4.4 Effects of maternal treatment with MA on puberty onset and the reproductive capabilities of the offspring.

Maternal exposure to MA from pregnancy day 0 through weaning (PND 21) did not alter the day of vaginal opening and first estrus in the females or the day of preputial separation in the males (Table 5). Moreover, the body weights of the pups on the day of appearance of the puberty-onset landmarks did not differ between the MA-treated and control groups (Table 5). These findings indicated that maternal treatment with MA during pregnancy and lactation did not affect puberty onset in the male or female offspring.

Similarly, the data showed that exposure to Sb during pre- and post-natal development had no effect on the reproductive performance in adulthood. The mating (*i.e.*, proportion of females impregnated by male rats) and pregnancy (*i.e.*, ratio of pregnant to sperm-positive females) indices were high and equivalent for all dose groups. In addition, the average number of daily cohabitation sessions required for successful copulation (precoital time) did not differ between MA dose groups (Means±SD, vehicle control: 2.94±1.79; 75 mg Sb^V/kg body wt/d: 2.86±1.87; 150 mg Sb^V/kg body wt/d: 2.96±1.75; 300 mg Sb^V/kg body wt/d: 3.00±2.17; ANOVA, $P>0.05$). Furthermore, there were no discernible effects of the pre- and post-natal exposure to Sb on pregnancy outcome. As shown in Table 6, there were no differences between the control and MA-treated groups for the number of implantation sites per litter, live fetuses per litter (litter size), resorptions per litter, fetal body weights or sex ratio. A dose-dependent decrease in maternal body weight gain (after subtracting the gravid uterus weight on GD 21), accompanied by a reduction in liver weight (dams' absolute liver weights), was the only effect observed. The mode by which developmental exposure of female rats to Sb leads to this weight-gain deficit during pregnancy is unclear. It should be noted, however, that female rats accumulate fat and energy reserves during pregnancy to cope with the increased energy demands for milk production after delivery. The observed maternal weight-gain deficit during pregnancy might therefore eventually have a negative influence on milk production and the successful rearing of the offspring [17].

The necropsy of the adult males (PND100-110) revealed no alterations in the reproductive organ (prostate, testes, epididymis, and seminal vesicle) weights, except for a slight decrease in the drained seminal vesicle weight in the animals exposed to the highest MA dose (Table 7). The body weights and liver weights of the males exposed to MA *in utero* and during lactation were lower than the body weights and liver weights of the control males (Table 7). The sperm parameters (spermatids and spermatozoa counts, daily sperm production and sperm transit time) were unaffected by the prenatal and lactational exposure to MA (Table 7). The results therefore indicated that the sexual organ weights of the adult males and females and sperm parameters in males remained unaltered in the offspring of the MA-treated dams. All males and females exposed to Sb successfully mated and yielded progeny. Moreover, except for small reductions in maternal body weight gain and absolute liver weights at term pregnancy, there were no

discernible effects of the prenatal and lactational exposure to Sb on the reproductive performance of the adult rats.

IV.4.5 Levels of Sb in term fetuses, maternal milk and pup blood after treatment with MA during pregnancy and lactation

To examine the extent to which Sb was transferred to fetuses via the placenta, the dams were treated subcutaneously with 300 mg Sb^{v}/kg body wt/d or vehicle only during pregnancy (GD 0-20). At the time of the Cesarean-section (C-section; GD 21, nearly 24 h after last MA injection), maternal and fetal blood samples were collected, and the Sb concentrations were determined. The data shown in Table 8 and those obtained in a previous study of the developmental toxicity of MA [14] indicated that Sb is transferred through the placenta, and at full-term pregnancy, the fetal blood concentrations reach values 30% to 44% of those found in the maternal blood. Taken together, the data from these two studies consistently indicated that Sb levels in the fetal blood as high as 12.00 µg/g [14] and 15.87 µg/g (Table 8) are associated with prenatal growth retardation (lower female offspring body weight at birth) and decreased fetal/neonatal viability.

Two additional groups of pregnant rats continued to receive MA (300 mg Sb^{v}/kg body wt/d, sc) or vehicle only during lactation until PND 13. As expected from a longer course of treatment with MA, the Sb blood levels in the dams treated until PND 13 and their offspring were higher than levels in the blood of the dams and their fetuses on GD 21. Specifically, the ratio of offspring (fetuses or pups) to maternal Sb blood levels rose from 0.44±0.07 (mean±SD) at full-term pregnancy (GD 21) to 0.62±0.05 at mid-lactation (PND 13) (Table 8). Appreciable amounts of Sb were found in the dam milk collected 48 h after the last injection of MA. Because the concentrations of Sb in the blood of the suckling pups on PND 13 (36.38±3.85 µg/g) were markedly higher than the levels recorded in blood of the full-term fetuses (15.87±1.58 µg/g), it is reasonable to conclude that Sb transferred via milk accounted for this increase in the pups' metalloid body burden during post-natal growth. Together, these results indicated that when a lactating rat is treated with MA, Sb is transferred to her milk, where it is in a chemical form that is absorbable orally.

This interpretation was further supported by two experiments in which lactating dams received a 14-day course of treatment with MA (0 or 300 mg Sb^{V}/kg body wt/d,

sc) on PND 1-15 or, alternatively, on PND 5-19, but were not treated during pregnancy. As shown in Table 8, the levels of Sb in the pups' blood were equivalent to those found in their dams' blood on PND 15 (treatment on PND 1-15) and on PND 20 (treatment on PND 5-19). These findings were also consistent with the concept that Sb passes into the milk of the MA-treated dams and is present there in a chemical form that is orally bio-available to suckling pups.

Although both groups of dams had similar levels of Sb in their blood (Table 8), the levels of Sb in the milk extracted on PND 15 (5.07±3.18 µg/g) were nearly 5-fold the levels in the milk drawn on PND 19 (1.00±0.69 µg/g). In the former group (PND 15), however, the lactating females were milked 24 h after the last dose of MA, while the latter group (PND 19) was milked 48 h after the drug injection and 24 h after the pups had been removed from their dam's cage (Table 8). Previous kinetic studies have shown that most of the Sb contained in a single dose of MA is eliminated from the maternal organism during the first 12 h after injection (fast elimination phase); therefore, only residual levels are found in the blood afterwards (slow elimination phase) [13]. One can expect therefore that the milk produced by the mammary glands during the first 24 h after MA injection (including the initial period of fast elimination phase) contains more Sb than the milk that was produced between 24 h and 48 h after the injection, a period that corresponds to the terminal slow elimination phase only.

An additional group of rats was exposed to Sb via milk on PND 5-19 (maternal dose: 300 mg Sb^V/kg body wt/d sc) and euthanized on PND 90. These rats exposed during lactation had residual blood levels of Sb as high as 2.099±0.76 µg/g at this time, nearly 70 days after weaning.

To the best of our knowledge, the passage of Sb to milk and from it to suckling infants has not been previously described. A case report by Berman et al. [18] provided some data on the levels of Sb in the milk extracted from a nursing woman with cutaneous leishmaniasis who had been treated with intravenous injections of the Sb^V drug Pentostam®. Berman et al. [18] noted that the serum levels of Sb declined to nadir levels within 6 h after dosing, with an initial half-life of 2 h, while the Sb levels in the milk achieved a peak at 4 h, subsequently declining with a half-life of approximately 6 h. To avoid potential toxicity to the baby, the patient was instructed to bottle-feed her infant during the Pentostam® therapy; therefore, no data on the passage of Sb from the breast milk to the child were available. In a separate experiment, Berman et al. [18] administered Sb orally (300 mg/kg body wt) to hamsters and found no Sb in their blood.

Based on these results, the authors concluded that Sb cannot be absorbed from the oral route and speculated that during Sb^V drug therapy "*breast-feeding should be safe, as the infant is very unlikely to be exposed to detectable or toxic levels of antimony*" [18]. In fact, soluble Sb salts administered orally are poorly absorbed and, at high concentrations, are reported to irritate GI mucosa, causing vomiting, abdominal cramps, and diarrhea [19, 20]. Sb^V drugs (MA and stibogluconate) have poor oral bioavailability and are not effective by this route of administration. However, the data from the present study suggest that Sb in the milk is in a chemical form (*e.g.*, bound to milk proteins) that is well tolerated and favors oral absorption of the metalloid by suckling pups. The speciation of Sb in maternal milk warrants further investigation. The identification of the chemical forms of Sb in the milk of MA-treated dams may be informative for the further development of Sb^V drug formulations that are bioavailable and effective by the oral route.

IV.4.6 Residual levels of Sb in the liver of adult rats that had been exposed via the placenta and maternal milk

The data from this study indicated that the elimination of Sb transferred to the offspring proceeds very slowly. As shown in Table 9, nearly 90 days after the end of the maternal treatment with MA, the liver levels of Sb in the offspring of the treated dams were approximately 20-fold (for the dams treated with 75 mg Sb^V/kg body wt/d), 60-fold (for 150 mg Sb^V/kg body wt/d), and 140-fold (for the 300 mg Sb^V/kg body wt/d) the levels found in the offspring of dams treated with the vehicle only.

The residual concentrations of Sb in the livers of the females were approximately half the concentrations found in the livers of the males at the same age for all dose groups (Table 9). However, it should be noted that the female livers used for Sb quantification were removed from pregnant rats at the time of the C-sections (fertility tests) that were performed on GD 21. Because a marked maternal liver enlargement occurs during rat pregnancy (50% to 60% increase compared with pre-conception hepatic size), the Sb concentrations in the female rat liver were reduced due to this pregnancy-associated liver hypertrophy [21, 22].

IV.5 Conclusions

The overall findings of this study indicated that Sb transferred via the placenta impaired prenatal growth (detected in female offspring) and fetal and/or neonatal viability at the highest dosage regimen tested (300 mg Sb^V/kg body wt/d).

Although the body burden of Sb markedly increased during suckling, except for slight decreases in the body weight gain rates in both male and female pups and a slight reduction in exploratory behavior in the females, no detrimental effects of treatment on neurobehavioral development were observed. Moreover, pre- and post-natal exposure to Sb had no effect on the sexual maturation, male fertility or reproductive performance of the exposed offspring in adulthood. Additional remarkable findings of this study were that appreciable amounts of Sb were transferred via the maternal milk to the suckling pups and that the residual levels of Sb in the livers and blood of exposed offspring remained markedly augmented for a long time (>2 months) after the end of exposure.

Finally, it should be highlighted that the doses of MA used in this toxicity study were much higher than the doses used to treat patients with leishmaniasis. As a consequence, the nadir blood levels of Sb achieved in rats were much higher than those that are found in the blood of patients after a course of treatment with Sb^V drugs. For example, in patients treated with Sb^V drugs (10 mg Sb/kg body wt/d, im), Chulay et al [6] found blood levels of Sb as high as 0.19-0.33 μg/mL 24 h after the 30^{th} dose, whereas Sb blood levels in pregnant rats treated with MA (300 mg Sb^V/kg body wt/d, sc) 24 h after the 20^{th} dose were 36.25±3.41 μg/g (Table 8), i.e., at least 100-fold the blood levels reported in humans. Notwithstanding the fact that only slight to mild toxic effects were noted in rats at rather high levels of exposure, transfer of Sb via placenta to the embryo/fetus and via milk to suckling pups, and the long persistence of Sb in body tissues, are causes for deep concern.

IV.6 Acknowledgments

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dedicate this article to late Prof. Norbert Fritz Miekeley, an analytical chemist who made important contributions to the study of antimony drug kinetics.

IV.7 Highlights

- Sb^V drugs administered to pregnant rats decrease the birth weights and the numbers of viable newborns.
- Sb from Sb^V leishmanicidal drugs is transferred via the placenta to the fetuses.
- Sb of Sb^V leishmanicidal drugs is transferred via the maternal milk to the suckling pups.
- Sb transferred via the placenta and milk does not affect rat reproductive performance.
- Sb^V administered to lactating rats causes only minor weight gain deficits in the offspring

IV.8 References

[1] Haldar AK, Sen P, Roy S (2011). Use of antimony in the treatment of leishmaniasis: current status and future directions. Mol Biol Int. 2011:571242

[2] Monge-Maillo B, López-Vélez R (2013) Therapeutic options for visceral leishmaniasis. Drugs. 73(17):1863-88

[3] McGwire BS, Satoskar AR.(2014) Leishmaniasis: clinical syndromes and treatment. QJM. 107(1):7-14.

[4] WHO (2010) Control of the leishmaniases. WHO Thechnical Report Series, no. 949. World Health Organization, Geneva. Switzerland. 222p.

[5] Mishra J, Saxena A, Singh S (2007). Chemotherapy of leishmaniasis: past, present and future. Curr Med Chem. 14(10):1153-69

[6] Chulay JD, Fleckenstein L, Smith DH (1988). Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. Trans R Soc Trop Med Hyg. 82(1):69-72.

[7] Berman JD (1996). Treatment of New World cutaneous and mucosal leishmaniases. Clin Dermatol. 14(5):519-22.

[8] Miekeley N, Mortari SR, Schubach AO (2002). Monitoring of total antimony and its species by ICP-MS and on-line ion chromatography in biological samples from patients treated for leishmaniasis. *Anal Bioanal Chem. 372*(3): 495-502

[9] Friedrich K, Vieira FA, Porrozzi R, Marchevsky RS, Miekeley N, Grimaldi G Jr, Paumgartten FJ (2012). Disposition of antimony in rhesus monkeys infected with *Leishmania braziliensis* and treated with meglumine antimoniate. *J Toxicol Environ Health A.75*(2): 63-75

[10] Tassi P, Ormas P, Madonna M, Carli S, Belloli C, De Natale G, Ceci L, Marcotrigiano GO (1994). Pharmacokinetics of N-methylglucamine antimoniate after intravenous, intramuscular and subcutaneous administration in the dog. Res Vet Sci. 56(2):144-50

[11] Nieto J, Alvar J, Mullen AB, Carter KC, Rodríguez C, San Andrés MI, San Andrés MD, Baillie AJ, González F (2003). Pharmacokinetics, toxicities, and

efficacies of sodium stibogluconate formulations after intravenous administration in animals. Antimicrob Agents Chemother. 47(9):2781-7.

[12] Radwan MA, Al Jaser MH, Al Rayes ZR (2007). The effects of induced diabetes and cutaneous Leishmania infection on the pharmacokinetics of antimony in hamsters. Ann Trop Med Parasitol. 101(2):133-42.

[13] Coelho DR, Miranda ES, Saint'Pierre TD, Paumgartten FJR (2014) Tissue distribution of residual antimony in rats treated with multiple doses of meglumine antimoniate. Mem Inst Oswaldo Cruz, 109(4): 420-7.

[14] Miranda ES, Miekeley N, De-Carvalho RR, Paumgartten FJ 2006. Developmental toxicity of meglumine antimoniate and transplacental transfer of antimony in the rat. *Reprod Toxicol. 21*(3): 292-300.

[15] Amann RP (1986). Detection of alterations in testicular and epididymal function in laboratory animals. Environ Health Perspect. 70:149-58.

[16] Salewski E. (1964) Färbemethoden zum makrokospischen Nachweis von Implantationsstellem am Uterus der Ratte Naunyn-Schmiedeberg's Arch Exp Pathol Pharmakol, 281:367

[17] Johnstone LE, Higuchi T (2001). Food intake and leptin during pregnancy and lactation. Prog Brain Res. 133:215-27.

[18] Berman JD, Melby PC, Neva FA (1989). Concentration of Pentostam in human breast milk. Trans R Soc Trop Med Hyg. 83(6): 784-5.

[19] Elinder CG, Friberg L (1986). Antimony. In: Friberg L, Nordberg GF, Vouk VB, eds. Handbook on the toxicology of metals. 2nd ed. New York: Elsevier; 1986. pp. 26-42.

[20] Dieter MP, Jameson CW, Elwell MR, Lodge JW, Hejtmancik M, Grumbein SL, Ryan M, Peters AC (1991). Comparative toxicity and tissue distribution of antimony potassium tartrate in rats and mice dosed by drinking water or intraperitoneal injection. J Toxicol Environ Health. 34(1):51-82.

[21] Bokelmann O, Scheringer W (1932). Gewicht und Stickstoffgehalt der Schwangerschaftsleber und ihre Beziehungen zur Frage der Arbeitshypertrophie. Archiv für Gynäkologie 150 (1):247-256.

[22] Harkness ML, Harkness RD (1956). The collagen content of the liver in pregnancy and lactation. J Physiol. 134(1): 135-8.

IV.9 Tables

Table 1. Study of the effects of prenatal and lactational exposure to Sb^V on rat development: Number of treated dams per group, treatment periods and endpoints evaluated in the experiments.

Experiment	Treated dams	Endpoints			
	MA dose (mg Sb^V/kg. body wt/d, sc)	N	Treatment	Maternal	Offspring
I	0	14	GD 0 – PND 21	Body wt, signs of toxicity.	Birth weight, body weight gain, survival, developmental landmarks, puberty onset landmarks, open field test (PND 25 and 60), fertility test (females C-section on PND 100), male reproductive organ weight and sperm parameters (PND 100-110), Sb levels in the liver
	75	16			
	150	16			
	300	16			
IIA	0	4	GD 0 - 21	Sb levels in the blood	Sb levels in fetal blood
	300	5			
IIB	0	4	GD 0 – PND 13	Sb levels in the blood and milk**	Sb levels in pups' blood
	300	5			
IIIA	0	5	PND 1 - 15	Sb levels in the blood and milk*	Sb levels in pups' blood
	300	6			
IIIB	0	3	PND 5 - 19	Sb levels in the blood and milk**	Sb levels in pups' blood on PND 20 (N=23) and 90 (N=5)
	300	6			

MA: meglumine antimoniate; GD: gestation day, PND postnatal day; * blood and milk collected 24 h after the last dosing; ** blood and milk collected 48 h after last dosing.

Table 2. Pregnancy outcome of rats treated with meglumine antimoniate (0, 75, 150 and 300 mg Sb^V/kg body wt/d, sc) from gestation day 1 (GD 1) onwards (Experiment I).

Treatment	Meglumine Antimoniate (mg Sb^V/kg body wt/d)			
	0	75	150	300
Litters (N)	14	16	16	16
Number of implants (N)	11.8±2.3	12.7±2.8	11.2±2.2	11.9±2.7
Litter size at birth (N)	10.5 ±3.1	11.4 ±2.4	10.1 ±2.3	8.7±2.2
Live pups on PND 1 (N)	10.3±3.0	10.1±3.4	10.1±2.3	7.0 ±2.8*
Dead pups on PND 1 (N)	0.2±0.6	1.3 ±2.7	0	1.7 ±2.8
Sex ratio	1.1 (0.14-11)	1.0 (0.5-2.0)	0.83 (0.25-1.6)	1.0 (0.29-3.5)
Pup body wt on PND 1 (g)	7.1±1.1	6.7±0.8	6.7 ±0.6	6.0 ±0.5*

The data are shown as the mean±SD or (sex ratio) median and range (minimum-maximum). The litter was the statistical unit of analysis. The means were compared by ANOVA and Dunnett's test; the sex ratio were compared using the Kruskal-Wallis test. An asterisk indicates differences ($P<0.05$) between the MA-treated groups and the vehicle controls.

Table 3. Developmental landmarks in the offspring of rats treated with meglumine antimoniate (0, 75, 150 and 300 mg Sb^V/kg body wt/d, sc) during pregnancy and lactation until weaning (PND 21). The data are post-natal days on which the somatic landmarks appeared or reflexes were acquired (Experiment I).

Treatment	Meglumine antimoniate (mg Sb^V/kg body wt/d)			
	0	75	150	300
Litters (N)	14	16	16	16
Physical landmarks				
Ear unfolding	3 (2-4)	3 (2-4)	3 (2-4)	3 (2-4)
Fur development	6 (6-9)	7 (6-9)	6 (5-9)	6 (6-9)
Incisor eruption	12 (11–15.5)	12 (10-13)	12 (11-13)	12 (10-13)
Eye opening	16 (15–17.5)	16 (14-18)	16 (16-17)	16 (15-17)
Testes descent	15.75 (15–17.5)	16 (14-18)	16 (14-17)	15.75 (14.5-17)
Reflex maturation				
Surface righting	2 (1–3.5)	2 (1-4)	2 (1-5)	1.5 (1-3)
Cliff avoidance	5.75 (4–7)	6 (3-11.5)	5.5 (4-8)	5.5 (3-7)
Negative geotaxis	5.25 (4.5-9)	6 (4.5-8)	6 (4-9.5)	6 (4-10.5)
Palmar grasp	8 (7-10)	8 (6-10)	7.25 (6-11)	8 (4-9.5)
Auditory startle response	13 (12-14)	13 (12-14)	13 (12-14)	13 (12.5-14)
Free-fall righting	18 (16-19)	18 (15.5-20)	18 (16-20)	17.75 (16-20)

The data are shown as the median and range (minimum-maximum) of the day on which landmarks appeared or reflexes were acquired. The litter was the statistical unit of analysis. The median days were compared using Kruskal-Wallis test and there were no significant differences among groups ($P>0.05$). Palmar grasp is the only reflex that was present at birth and disappears with post-natal maturation. The days on which preputial separation and vaginal opening occurred are in a separate table (puberty onset). Because the statistical analyses showed no differences between the males and females, the table shows the combined data for both sexes (except for "testes descent").

Table 4. Performance of the male and female offspring of rats treated with meglumine antimoniate (0, 75, 150 and 300 mg Sb^V/kg body wt/d, sc) during the pregnancy and lactation periods on the open field test on PND 60 (Experiment I).

Treatment	Meglumine antimoniate (mg Sb^V/kg body wt/d)			
	0	75	150	300
Litters (N)	14	16	16	16
Female offspring				
Rearing up (N)	57.4±16.5	52.1±11.0	47.6±11.2	40.6±11.6*
Motor activity (N)	161.6±19.2	158.9±20.4	155.2±26.7	151.4±27.7
Latency (s)	22.0±9.3	19.1±8.8	20.1±6.8	19.2±9.6
Grooming (N)	1.2±0.6	1.5±0.7	1.2±0.5	1.2±0.9
Fecal boli (N)	1.4±1.5	1.6±1.4	1.6±1.3	1.3±1.3
Male offspring				
Rearing up (N)	38.5±11.3	40.0±8.7	37.0±12.9	36.0±14.5
Motor activity (N)	136.6±18.0	142.1±20.9	130.2±34.1	142.5±40.4
Latency (s)	18.7±8.2	16.8±8.8	17.3±9.8	17.3±10.4
Grooming (N)	1.2±0.6	1.0±0.5	0.9±0.3	1.2±1.0
Fecal boli (N)	2.3±0.9	3.2±1.7	2.3±1.5	2.0±1.8

The data are shown as the mean±SD and were analyzed using ANOVA with Dunnett's post-hoc test. Values that differ ($P<0.05$) from those for the vehicle-control group are indicated by an asterisk (*). The litter was the statistical unit of analysis. Latency: time to leave the central circle of the OF arena.

Table 5. Appearance of landmarks of puberty onset in the offspring of female rats treated with meglumine antimoniate (0, 75, 150 and 300 mg Sb^V/kg body wt/d, sc) during pregnancy and lactation until weaning on PND 21 (Experiment I).

Treatment	Meglumine antimoniate (mg Sb^V/kg body wt/d)			
	0	75	150	300
Litters (N)	14	16	16	16
Female offspring				
Vagina opening, VO (d)	35.3 (33.5-40)	36 (33-40)	36.75 (33-40)	36 (31-40)
Body wt at VO (g)	121.4±15.9	114.3±15.9	117.9±14.2	112.5±18.3
First estrous, FO (d)	38 (35-41)	38 (37-43)	39 (35–42)	38 (36-41)
Body wt at FO (g)	134.0±15.8	127.5±12.5	129.5±11.5	126.7±11.8
Male offspring				
Preputial separation, PS (d)	38 (35-41)	40 (35-42)	38.75 (36.5-41.5)	38.5 (35-42)
Body wt at PS (g)	150.7±20.3	153.4±22.3	148.1±12.9	144.7±14.6

The days at which VO, FO and PS occurred are shown as the median and range (minimum-maximum), and the body weights are the mean±SD. The medians were compared using the Kruskal-Wallis test, while body wt means were analyzed using ANOVA. No significant differences among the treatment groups were detected ($P>0.05$).

Table 6. Maternal weight gain and C-section data of the reproductive capability test for the offspring (F_1) of rats treated with meglumine antimoniate (0, 75, 150 and 300 mg Sb^V/kg body wt/d, sc) during pregnancy and lactation (Experiment I).

Treatment	Meglumine antimoniate (mg Sb^V/kg body wt/d)			
	0	75	150	300
Litters (N)	14	16	16	16
Maternal body wt gain (Δ g)				
GD 21 – GD 0	121.0±24.9	113.1±20.5	109.1±25.5*	106.0±15.9*
GD 21 (minus uterus wt) – GD 0	44.3±14.7	41.7±12.1	39.7±14.6	37.0±9.5*
Maternal liver				
absolute wt (g)	15.4±1.4	13.7±1.2*	13.9±1.5*	13 1±1.3*
relative wt (%)	5.5±0.4	5.2±0.5	5.1±0.4	5.0±0.5
Live fetuses per litter (N)	10.9±3.1	10.2±2.9	10.0±2.9	9.6±2.4
Implantation sites per litter (N)	11.9±3.1	11.0±2.8	11.0±2.7	10.6±2.4
Resorptions per litter (N)	1.0±0.9	0.8±1.0	1.1±1.1	1.0±1.1
Sex ratio	1.17 (0.0–3.0)	1.00 (0.0-4.5)	1.00 (0.0–6.0)	0.83 (0.0–4.0)
Fetal body weight (g)	4.9±0.2	5.0±0.2	4.9±0.2	5.0±0.1

The data are shown as the mean±SD or (sex ratio) median and range (minimum-maximum). The litter was the statistical unit of analysis. The means were compared by ANOVA and Dunnett's test; the sex ratio were compared using the Kruskal-Wallis test. An asterisk indicates differences ($P<0.05$) between the MA-treated groups and the vehicle controls. GD: gestation day.

Table 7. Liver and reproductive organ weights and sperm parameters of adult male rats (approx. 100 day-old) exposed to meglumine antimoniate (0, 75, 150 and 300 mg Sb^V/kg body wt/d, sc) during gestation and through maternal milk until weaning on PND 21 (Experiment I).

	Meglumine antimoniate (mg Sb^V kg body wt/d)			
	0	75	150	300
Litters (N)	14	16	16	16
Body wt (g)	433.3±26.3	408.4±23.9*	420.8±22.7	402.8±24.9*
Liver absolute wt (g)	16.2±1.7	14.3±1.8*	15.2±1.6	14.4±1.5*
Liver relative wt (%)	3.7±0.3	3.5±0.3	3.6±0.3	3.6±0.3
Prostate wt (g)	0.41±0.08	0.64±1.03	0.39±0.04	0.38±0.08
Testes wt (g)				
right	1.78±0.12	1.72±0.17	1.80±0.12	1.76±0.19
left	1.78±0.14	1.74±0.17	1.80±0.12	1.74±0.25
Epididymis wt (g)				
right	0.59±0.04	0.57±0.05	0.60±0.04	0.59±0.07
left	0.58±0.04	0.57±0.05	0.59±0.03	0.58±0.07
Seminal vesicle (g)	1.62±0.18	1.63±0.20	1.66±0.20	1.51±0.25
Seminal vesicle drained (g)	0.80±0.08	0.77±0.11	0.76±0.10	0.70±0.10*
Spermatids per testis ($x10^6$), (N)	158.2±40.6	163.9±44.2	148.3±45.6	141.0±35.8
Spermatids per gram of testis ($x10^6$), (N)	89.9±24.3	93.5±24.6	83.9±27.7	83.4±24.8
Sperm number in epididymis ($x10^6$), (N)	154.2±56.0	160.4±51.2	141.4±56.8	132.1±53.4
Daily sperm production ($x10^6$/testis/day)	25.9±6.7	26.9±7.3	24.3±7.5	23.1±5.9
Sperm transit time (d)	5.8±1.1	5.8±1.0	5.7±1.0	5.6±1.4

The data are shown as the mean±SD and were analyzed using ANOVA followed by Dunnett's test. An asterisk indicates significant differences ($P<0.05$) between the MA-treated and vehicle groups.

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Table 8. Antimony concentrations (µg/g) in the blood (dams, term fetuses and pups) and milk from rats exposed to meglumine antimoniate (MA, 0 or 300 mg Sb^V/kg body wt/d, sc) during pregnancy and lactation until PND13 (Experiment II) or during lactation only (Experiment IIII).

Experiment	Treatment	MA (mg Sb^V /kg body wt/d)	
		0	300
IIA	GD 0 - GD21		
	Maternal blood	0.002±0.001	36.250±3.412*
	Fetal blood		
	Males + Females	0.016±0.011	15.868±1.580*
	Males	0.015±0.017	15.710±2.579*
	Females	0.016±0.010	16.025±2.706*
IIB	GD 0 - PND 13		
	Maternal blood	0.005±0.003	60.050±6.069*
	Milk (48 h)	0.026±0.035	0.478±0.169*
	Pups' blood		
	Males + Females	0.009±0.005	36.378±3.846*
	Males	0.009±0.005	35.615±3.899*
	Females	0.009±0.005	37.141±4.593*
IIIA	PND 1 - 15		
	Maternal blood	0.004 ±0.011	18.297±4.163*
	Milk (24 h)	0.002±0.003	5.072±3.180*
	Pups' blood	0.037±0.041	14.140±2.138*
IIIB	PND 5 -19		
	Maternal blood	0.024±0.021	19.151±1.767*
	Milk (48 h)	0.003±0.006	1.008±0.693*
	Pups' blood PND 20	0.443±0000	25.925±3.990*
	PND 90	-	2.099±0.766

The data are shown as the mean±SD. Asterisks (*) indicate that the mean differs (Student t test; $P<0.05$) from that of the vehicle control group. Exp II: the pups' blood and maternal blood and milk were drawn 24 h and 48 h after last injection of MA, respectively. Exp IIIA: Treatment on PND 5-15: maternal blood and milk and pup blood were collected 24 h after the last MA injection. Exp IIIB: Treatment on PND 5-19: maternal blood and milk were collected 48 h after the last MA injection while pups' blood was taken 24 h and 90 d (distinct groups of pups) after the end of treatment, respectively.

Table 9. Antimony (Sb) concentration (µg/g) in the liver of adult (approximately 100 day-old) male and (pregnant) female offspring (F1) of rats treated with meglumine antimoniate (0, 75, 150 and 300 mg Sb^V/kg body wt/d, sc) during pregnancy and lactation (Experiment I).

Treatment	Meglumine antimoniate (mg Sb^V/kg body wt/d)			
Liver Sb levels (µg/g)	0	75	150	300
Litters (N)	5	4	6	7
Males + females	0.01±0.01	0.24±0.19*	0.63±0.40*	1.42±0.88*
Males	0.01±0.00	0.31±0.26*	0.79±0.49*	1.97±0.96*
Females	0.01±0.01	0.19±0.12*	0.42±0.14*	0.88±0.28*

The data are shown as the mean±SD. The data were analyzed using the Kruskal-Wallis test and Mann-Whitney U test. Asterisks (*) indicate that the value differs ($P<0.05$) from that of the control group. The males and females were approximately the same age at the time of euthanasia (PND 100-110). The female livers were removed at the C-section.

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IV.10 Figures

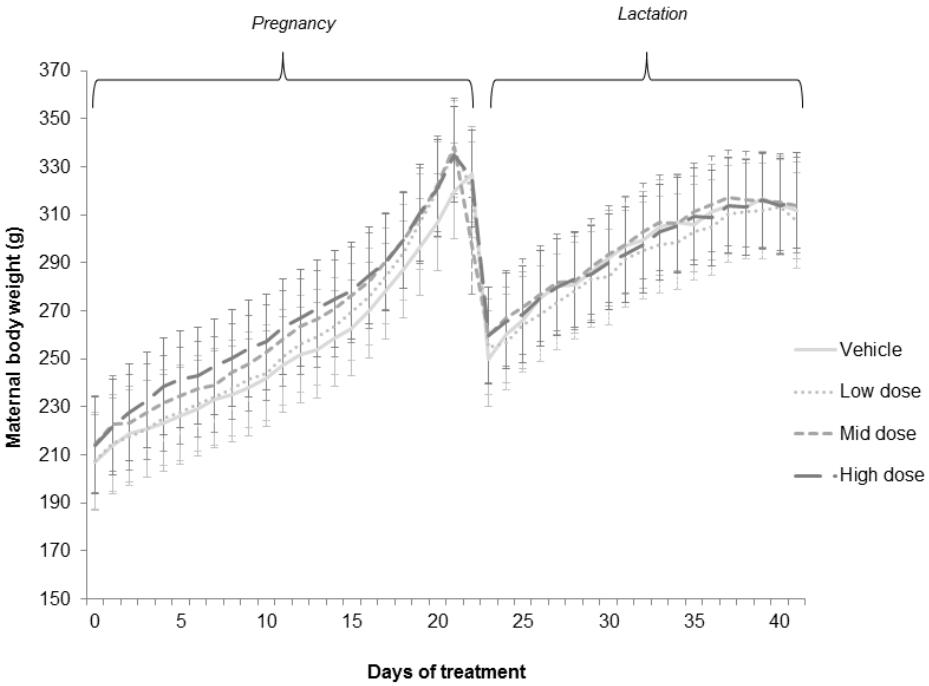

Figure 1. Body weight (g, mean±SD) gain of female rats treated with subcutaneous injections of meglumine antimoniate (MA: 0, 75, 150, 300 mg Sb^V/kg body wt/d, sc) once a day starting on gestation day 0 (GD 0) and continuing throughout parturition and lactation until weaning on post-natal day 21 (PND 21). The number (N) of treated rats per group was 14 for the vehicle- control (0 mg Sb^V/kg body wt/d) group and 16 for the remaining dose groups. The group means did not differ at any time point (ANOVA, $P>0.05$).

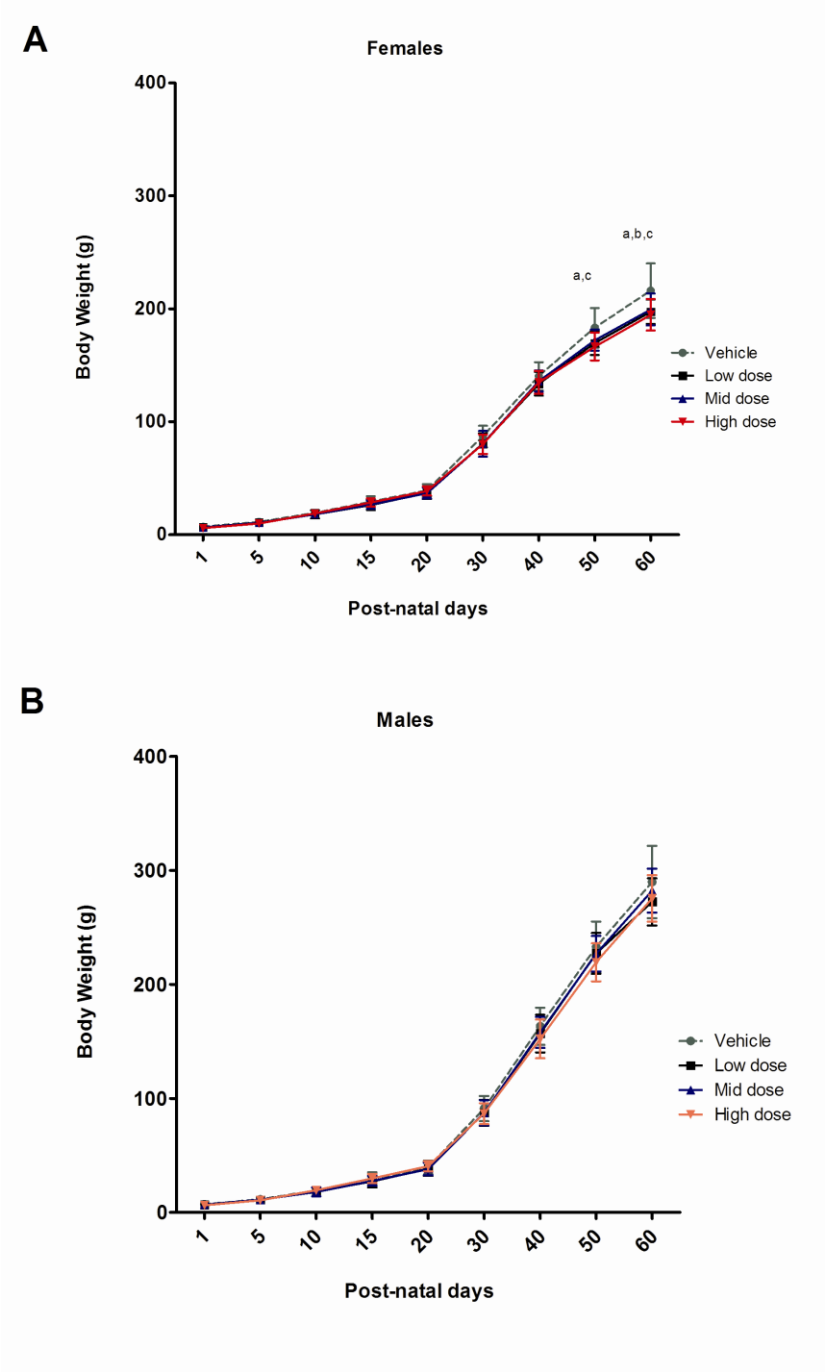

Figure 2. Body weight (g, mean±SD) gain of the male and female offspring of rats treated with subcutaneous injections of meglumine antimoniate (MA; 0, 75, 150, 300 mg Sb^V/kg body wt/d, sc; *i.e.*, vehicle, low, mid and high dose) during pregnancy and lactation until weaning (PND 21). The data were analyzed by ANOVA followed by Dunnett's test. Differences ($P<0.05$) from the vehicle are indicated by letters a (≠ low), b (≠ mid) and c (≠ high).

V. ARTIGO 3

Effects of repeated meglumine antimoniate administration on mouse liver cytochrome P450 activities

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Running title: Meglumine antimoniate and mouse CYP activities

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V.1 Abstract

Pentavalent antimonial (Sb^{5+}) drugs such as meglumine antimoniate (MA) are the mainstay treatment of leishmaniases in developing countries. The effects of these compounds on drug metabolizing enzymes have not been characterized and their potential pharmacokinetic interactions with other drugs are therefore unknown. The present study investigated whether treatment with MA (300 mg Sb^{5+}/kg bw/day, subcutaneously) for 24 days affected the activities of cytochrome P450 (CYP)1A (ethoxyresorufin-*O*-deethylase), CYP2A5 (coumarin 7-hydroxylase), CYP2E1 (p-nitrophenol-hydroxylase), CYP2B9/10 (benzyloxi-resorufin-*O*-debenzylase), or CYP3A11 (erythromycin-*N*-demethylase) in the livers of Swiss Webster and DBA-2 male and female mice. The results showed that CYP2A5-, CYP2E1-, and CYP3A11-catalyzed reactions were unaffected by MA treatment. A decrease in CYP2B9/10 activity was noted in DBA-2 females (but not males) and was not observed in Swiss Webster males or females. However, repeated MA administration reduced mouse liver CYP1A activity. Since *in vitro* treatment of liver microsomes failed to depress CYP1A activity, this effect may require intact cells.

Keywords: Leishmaniasis, CYP1A, CYP2A5, heme oxygenase, pharmacokinetic interactions, liver monooxygenases.

V.2 Introduction

The medical use of antimony (Sb) compounds dates back to ancient times. Paracelsus, the famous XVI century physician and alchemist, was particularly fond of these compounds and recommended them for a variety of illnesses and disorders. Antimony-based drugs re-emerged in modern therapeutics in 1912 when Gaspar Vianna noted that repeated injections of tartar emetic, a trivalent antimony (Sb^{3+}) compound, led to the complete healing of skin ulcers in patients with cutaneous forms of leishmaniasis.[1] In 1915, Di Cristina and Caronia[2] reported that tartar emetic was effective in treating visceral leishmaniasis (or kala-azar) a clinical form of protozoan disease that, if untreated, may be fatal. Shortly afterwards (1923), John Cristopherson[3] discovered that antimony tartrate was also active against urinary and intestinal schistosomiases. Until the introduction of praziquantel in the 1970s, Sb^{3+} drugs provided some of the most effective therapies for this disease, which is caused by trematode worms. Pharmacological treatment of leishmaniases relied almost exclusively on the use of Sb^{3+} compounds until the mid-1940s, when they were replaced by the less toxic Sb^{5+} drugs, sodium stibogluconate (SSG; Pentostam®) and meglumine antimoniate (MA; Glucantime®). MA and SSB are considered equivalent in terms of their clinical safety and efficacy. MA is the first-line antileishmanial drug in Brazil, Latin America, and in some French-speaking African countries, while SSB is generally preferred in English-speaking countries.

Leishmaniasis is listed by the World Health Organization as one of the most neglected diseases. It is therefore unsurprising that some major knowledge gaps remain in relation to the safety profile of antileishmanial Sb^{5+} drugs, despite their being in clinical use for over half-a-century. For example, little is known about the impact of a course of Sb^{5+} treatment on the activity of liver drug-metabolizing enzymes and thus on the potential pharmacokinetic interactions of MA or SSB with other drugs.

In fact, a small number of clinical and experimental findings suggest that Sb^{5+} drugs may alter the activity of some drug-metabolizing enzymes. Hepburn et al[4,5] noted that a 20-day course of treatment with SSB (20 mg Sb^{5+}kg bw/day, intravenously) markedly delayed the elimination of caffeine from the blood in five patients with cutaneous leishmaniasis. Caffeine clearance in humans and rodents is known to be governed by the activity of the cytochrome P450 (CYP) 1A enzyme subfamily. In addition, rats exposed to potassium antimony tartrate (Sb^{3+}) via drinking water (500 ppm) for 90 days

showed enhanced liver activities of ethoxyresorufin–*O*-deethylase (EROD, a marker for CYP1A) and glutathione *S*-transferase.[6]

To date, the most striking indication that Sb compounds could alter CYP-mediated drug metabolism has come from an experimental study published over thirty years ago. Drummond and Kappas (1981)[7] demonstrated that a single dose (10 mg Sb/kg bw, subcutaneously) of Sb^{3+}-containing parasiticidal drugs (antimony potassium tartrate and antimony sodium dimercaptosuccinate the latter known as 'Astiban') resulted in a strong induction of heme oxygenase (HO) activity, a decline in total CYP and microsomal heme contents, and marked depression of CYP-mediated activities (aniline hydroxylase and ethyl-morphine demethylase) in the rat liver. However, the authors found no alteration of HO activity, total CYP content, or monooxygenase activities after administration of a single dose of the Sb^{5+}-containing drug, SSB (10 mg Sb/kg bw, subcutaneously) to rats.

Currently, most researchers consider Sb^{5+} compounds to be prodrugs that are reduced to the active leishmanicidal Sb^{3+} form intracellularly.[8,9,10,11,12] Moreover, Sb^{5+} drugs exhibit unique kinetics. In primates and rodents, the decrease in blood antimony concentrations over time following injection of Sb^{5+}-containing drugs fits a two-compartment or multi-compartment model, the slow elimination phase of which has a half-life longer than 24 h.[13,14,15] Both the therapeutic and toxic effects of Sb^{5+} drugs apparently depend on the accumulation of residual antimony that takes place during courses of 20-days or longer, with injections at 24-h intervals. In view of these findings, an hypothesis was advanced that the terminal (nadir) phase of antimony elimination is governed by a slowly mobilized intracellular pool of Sb^{3+}.[13] If so, the progressive accumulation of residual Sb in hepatocytes during a 21-day course of treatment with Sb^{5+} would be expected to reduce CYP activities, as proposed by Drummond and Kappas.[7] No further work, however, has been undertaken to test whether the induction of HO, the degradation of heme, and the consequent decline in monooxygenase activities reported by these authors correlated with Sb accumulation in liver cells.

In contrast to most other CYP forms, the activity of murine CYP2A5 (homologous to human CYP2A6), has been shown to be induced by a number of inflammatory and/or toxic conditions, which are commonly associated with liver injury, endoplasmic reticulum stress, and HO-1 over-expression.[16,17,18] Additionally, metals (Cd^{2+}, Co, Sn) and some metalloids (As^{3+}) were reported to induce HO in the liver and/or kidney[19] and

some metals such as Co, Cd, and others were demonstrated to induce CYP2A5 activity in the mouse liver.

Based on these observations, we investigated whether a course of treatment with MA would lead to changes in the activities of CYP monooxygenases in the mouse liver. An ancillary hypothesis tested in this study was that residual Sb in the liver would enhance CYP2A5 activity in a similar manner to that reported previously for other metals, hepatotoxins, and HO inducers. In this context, we also evaluated whether MA treatment altered HO-1 expression or markers of liver tissue injury: serum alanine transferase (ALT) activity, serum aspartate aminotransferase (AST) activity, and liver glutathione (GSH) levels.

V.3 Material and Methods

V.3.1 Animals

Male and female Swiss Webster (SW) and DBA-2 mice were obtained from the Oswaldo Cruz Foundation breeding stock, aged 8-10 weeks. Upon arrival at our laboratory, 5-6 mice of the same sex and strain were housed per standard plastic cage with stainless steel coverlids and white *Pinus* wood shavings as bedding. Mice were maintained under controlled environmental conditions (12 h photoperiod with lights on from 8:00 a.m. to 8:00 p.m.; room temperature of 23 ± 2 °C; relative humidity of approximately 70%) and had unlimited access to a commercial rodent diet (Nuvital CR1, Nuvilab®, Curitiba, PR, Brazil) and filtered tap water. All procedures were conducted in accordance with Brazilian animal protection and welfare legislation and international guidelines. The study protocol was cleared by the Ethics Committee on the Use of Animals of the Oswaldo Cruz Foundation (License – LW-31/14).

V.3.2 Treatment

MA (Glucantime®, Sanofi-Aventis Farmacêutica Ltd., Suzano, SP, Brazil) was administered by subcutaneous injections into the mouse back skin. According to the manufacturer, each ampoule (5 mL) of Glucantime® contained 425 mg N-methyl MA/mL, or 85 mg Sb^{5+}/mL. Analysis of ampoules of the same lot used in the study

found levels of total Sb, Sb^{5+}, and Sb^{3+} as high as 90.1 mg/mL, 86.9 mg/mL, and 3.2 mg/mL, respectively. Additional details can be found in our previous publications.[15,20]

The animals were treated at 24-h intervals with subcutaneous injections (mouse back skin) of MA (300 mg Sb^{5+}/kg bw/day) or its vehicle (potassium metabisulfite, 1.6 mg/mL and sodium sulfite, 0.18 mg/mL) for 24 days. The injected volume was 3.5 mL/kg bw/day. The animals were euthanized 24 h after the last injection.

To serve as a positive control of COH induction, a group of SW was treated with $CdCl_2$ (16 µmol/kg bw intraperitoneally, Cd) 6 h prior to euthanasia.

V.3.3 ALT and AST assays

ALT and AST activities were determined by a colorimetric method using a commercially available kit (Bioclin®, Belo Horizonte, MG, Brazil). Absorbance was read at 505 nm in a spectrophotometer, Spectramax Plus® (Molecular Devices, USA).

V.3.4 Determination of residual Sb levels

Levels of Sb in the liver tissue were determined by inductively coupled plasma mass spectrometry (ICP-MS), as described in detail elsewhere.[13,14,15] The limit of detection (LOD) of the method was 1.0 ng Sb/g, while the limit of quantification (LOQ) was 3.3 ng Sb/g.

V.3.5 Measurement of liver monooxygenase activities

The livers were quickly removed from the euthanized mice, freed from fat and other tissues, weighed, and frozen in liquid nitrogen until further use. The liver microsomal fraction (LMF) was prepared as previously described[21] and the microsomal protein concentration was determined by Bradford assay,[22] using bovine serum albumin as the standard.

Ethoxy (EROD) and Benzyloxy (BROD)-resorufin-*O*-dealkylase activities were assayed in 96-well microplates using the method described by Kennedy and Jones,[23] with some modifications, as described by De-Oliveira et al., 2015.[24] Substrate (ethoxy or benzyloxy resorufin) concentration was 5µM and 0.025 mg of microsomal protein was added to each well. The NADPH regenerating system (G6P 5 mM; ß-NADP 0,25 mM; MgCl2 2,5 mM e G6PD 0,5 U/mL) was used. The reaction product (resorufin) was

quantified using a fluorescence plate reader (Spectramax Gemini XS, Molecular Devices, USA) with excitation and emission wavelengths of 530 nm and 590 nm, respectively.

Coumarin 7-hydroxylase (COH) activity was determined as previously described,[25] with modification for microplate.[24] Substrate (coumarin) concentration was 10µM and 0,040 mg of microsomal protein was added to each well. The NADPH regenerating system (G6P 5 mM; ß-NADP 0,25 mM; MgCl2 2,5 mM e G6PD 0,5 U/mL) was used. Umbelliferone (7-hydroxycoumarin) was quantified using a fluorescence plate reader (Spectramax Gemini XS, Molecular Devices, USA), with excitation and emissions wavelengths of 355 nm and 460 nm, respectively.

p-Nitrophenol-hydroxylase (PNPH) activity was assayed using the real-time kinetic method reported by Allis and Robinson (1994).[26] The substrate (0.1 nM p-nitrophenol) and microsomal protein (0.2 mg) were added to quartz cuvettes, where the reaction took place. The NADPH regenerating system (G6P 5 mM; ß-NADP 0,25 mM; e G6PD 1 U/mL) was used. The activity was determined using a spectrophotometer Shimadzu UV1601, and the wavelength was setted in 480 nm.

Erythromycin-*N*-demethylase activity (END) was determined as previously described,[27] with some modifications. Erythromycin (1 mM), microsomal protein (1 mg), NADPH regenerating system (G6P 5 mM; ß-NADP 0,25 mM; MgCl2 2,5 mM e G6PD 0,5 U/mL) were used. The formaldehyde, a product of Nash[28] reaction, was quantified using the absorbance at 412 nm using a spectrophotometer (Spectramax Plus, Molecular Devices, USA).

V.3.6 In vitro inhibition of CYP1A by Sb^{3+} and Sb^{5+}

SW male mice were treated with β-naphthoflavone (βNF), an aryl hydrocarbon receptor (AhR) ligand and inducer of CYP1A1/2. Mice received three intraperitoneal injections of βNF (80 mg/kg bw/day) at 24-h intervals and were euthanized 24 h after the last injection. Hepatic microsomes were prepared from these mice and used to investigate whether Sb^{3+} ($C_4H_4KO_7Sb.0.5\ H_2O$) or Sb^{5+} (K[Sb(OH)]) inhibited the catalytic activity of CYP1A. Inhibition EROD tests were carried out in 96-well plates. Alpha-naphthoflavone (ANF, 10 and 50 µM) was used as a positive control substance.

V.3.7 Determination of GSH levels

GSH levels were measured as described previously[18], with a few modifications to adapt to microplate. Briefly, the liver was removed immediately after euthanasia, washed in ice-cold phosphate-buffered saline (PBS), dried, and homogenized in 100 mM $NaPO_4$ with 5 mM EDTA. The hepatic homogenate was centrifuged at 10 000 × *g* at 4 °C for 15 min and the supernatant was frozen and kept at -70 °C until further use. After thawing, the supernatant (10 µL) was incubated with 12.5 µL of 25% phosphoric acid and 37 µL of 0.1 M Na_2PO_4 with 5 mM EDTA, pH 8.0, for 10 min at 4 °C. It was centrifuged again at 13 000 × *g* for 10 min at 4 °C, and the resulting supernatant was incubated with 0.1% o-phthaldialdehyde methanol solution and the same basic buffer solution for an additional 10 min at room temperature. GSH concentration was then measured using a spectrofluorometer (Spectramax Gemini XS, Molecular Devices, USA) with excitation and emission wavelengths of 365 nm and 420 nm, respectively, and a band slit width of 3 nm. A GSH standard curve was used to determine the sample GSH concentrations.

V.3.8 Statistical analyses

Data were analyzed by Student's t-test or by the Mann-Whitney U test using Graph Pad Prism software. A difference was considered statistically significant when $P \leq 0.05$.

V.4 Results

V.4.1 Toxicity and residual Sb levels after multiple doses of MA

The 24-day course of treatment with MA (300 mg Sb^{5+}/kg bw/day subcutaneously) did not alter body weight gain, compared to vehicle-controls, nor did it cause death or any other sign of overt toxicity in SW and DBA-2 mice of either sex. As shown in Table 2, repeated administration of MA resulted in an increased liver Sb level; at the end of the treatment period, this was somewhat greater in DBA-2 than in SW mice. Nonetheless, accumulation of residual Sb apparently caused no liver toxicity in either strain. Although the liver weight was slightly decreased in MA-treated SW mice, differences were no longer detected when organ weight data were expressed as relative (%) liver weights (i.e., [liver wt/body wt] × 100). Serum levels of ALT and AST remained unaltered in MA-treated SW and DBA-2 female mice (Table 1).

Concentrations of GSH in the liver tissue of male SW mice were not affected by MA treatment (Table 1).

V.4.2 Effects of MA on liver monooxygenase activities

Liver constitutive activity of COH, a marker for CYP2A5, was 15-20 times higher in the DBA-2 strain than in SW mice. The constitutive activity of COH in male DBA-2 mice was somewhat greater than the activity observed in female DBA-2 mice. As illustrated in Figure 1, a one-injection exposure to cadmium ($CdCl_2$) caused an approximately 2.5-fold enhancement of COH activity. However, liver COH activity remained virtually unaltered after a 24-day course of MA treatment, irrespective of the mouse strain and sex (Figure 1). Liver activities of PNPH (CYP2E1) and END (CYP3A11) were also unaffected by MA treatment in male and female SW mice (Table 1).

The repeated administration of MA decreased the hepatic activity of EROD (a marker for CYP1A) in male and female SW mice and in female DBA-2 mice. EROD activity was also lower in MA-treated DBA-2 males, as compared to the activity recorded in controls, but this difference did not reach statistical significance (Figure 2). A decline of BROD activity (a marker for CYP2B9/10) was noted in female SW mice treated with MA, but no treatment-related change in BROD activity was seen in male SW mice or in male or female DBA-2 mice (Table 1).

Since MA depressed the constitutive activity of CYP1A (EROD) in the mouse liver, we investigated whether MA treatment, or the resulting Sb accumulation, impaired AhR-mediated CYP1A induction. SW mice receiving a 24-day course of treatment with MA and the respective controls were co-treated with a known AhR ligand (βNF, 80 mg/kg bw/day, intraperitoneally) for the last three days prior to euthanasia. As shown in Figure 2, EROD activity was similarly augmented in MA-treated and vehicle-control mice, suggesting that AhR-mediated up-regulation of CYP1A expression was not impaired by MA. It is of note that βNF-induced EROD activity in MA-treated mice was lower than that observed in the control animals (βNF treated only), although this difference did not achieve statistical significance.

V.4.3 In vitro inhibition of CYP1A by Sb^{3+} and Sb^{5+} compounds

A tentative explanation for the MA-associated depression of CYP1A activity is that Sb forms that accumulated within the liver cell would inhibit the catalytic activity of this protein. To test this hypothesis, we exposed mouse liver microsomes to a broad range of concentrations of Sb^{3+} and Sb^{5+} salts *in vitro*. To enrich the level of CYP1A protein, relative to other CYP forms (thereby making EROD a more selective marker of CYP1A1/2 activity in the microsomal fraction), microsomes were prepared from livers of SW mice that had been treated with βNF, a potent inducer of CYP1A. As shown in Figure 3, Sb^{3+} and Sb^{5+} compounds were tested at concentrations as high as 1000 µM but failed to cause any inhibition of EROD, whereas a known inhibitor of CYP1A (ANF, 10-50 µM), caused a pronounced and concentration-dependent inhibition of the microsomal catalytic activity.

V.5 Discussion

Antileishmanial Sb^{5+} drugs (MA and SSB) exhibit unique clinical pharmacokinetics. Following administration of a single dose of MA or SSB intravenously, intra-muscularly, or subcutaneously, almost all of the antimony is eliminated within 6-12 h; only residual (nadir) levels are found in the blood 24 h after dosing. During a course of 21-30 injections spaced at 24-h intervals, however, Sb nadir levels gradually rise so that 24 h after the last daily injection, the residual levels are substantially higher than those observed 24 h after the first injection.[13,14,15,29] . Most researchers consider that the leishmanicidal activity and the toxicity of these compounds depend on this progressive rise in residual Sb levels during a course of treatment. It is believed that the organic moieties of the MA (n-methyl-glucamine) and SSB (gluconate) structures serve as carriers of Sb^{5+} and play no other role in the efficacy and toxicity of these compounds. Studies of the tissue distribution of residual Sb in non-human primates[13] and in rats[15] showed that the liver was the organ that accumulated the highest level of Sb during MA treatment. The liver Sb content was also shown to decline very slowly after treatment discontinuation.[13,15,20.]

Data from the present study showed that a 24-day course of MA treatment did not alter the monooxygenase activities mediated by CYP2A5 (COH), CYP2E1 (PNPH), or CYP3A11 (EMD) in the mouse liver. However, the activity catalyzed by CYP1A1/2 (EROD) was consistently depressed in SW and DBA-2 mice treated with MA. A post-

treatment decline in the activity of CYP2B9/10 was noted only in SW females. No change of BROD was found in SW males or in DBA-2 mice. Taken together, these findings were not consistent with the hypothesis that Sb^{5+} causes generalized depression of liver CYP activities, an effect that would be expected if repeated administration of Sb^{5+} drugs were in fact associated with heme degradation, as suggested by Drummond and Kappas.[7] It should be borne in mind, however, that Drummond and Kappas data indicated that the total liver microsome CYP content was decreased after a single dose of Sb^{3+} (tartar emetic and $SbCl_3$), while no change in total CYP levels was noted after a single dose (82 μmol/kg bw, subcutaneously) of Sb^{5+} or SSB. Moreover, since we investigated the effects of a 24-day course of treatment on CYP activities, rather than acute treatment, it is possible that adaptive alterations might have occurred during the treatment period; acute dosing effects may differ from those elicited by repeated dosing. No effects of MA treatment on GSH levels were observed.

CYP2A5 activity is induced by conditions or chemical exposures that induce HO expression and activity, as well as by endoplasmic reticulum stress and liver injury. Transient elevations of ALT and AST have been reported in patients treated with MA. In non-human primates treated with standard and low-dose regimens, we previously reported dose-related liver histopathology findings (of slight to mild severity) that indicated enhanced hepatocyte necrosis.[30] However, the present study found no indication of liver damage in mice treated with MA. Therefore, these data were not consistent with the hypothesis that repeat MA administration and consequent accumulation of Sb within liver cells would lead to HO-1 induction and up-regulation of CYP2A5 activity.

MA treatment reduced CYP1A activity in the mouse liver, an observation that was consistent with a previous report of reduced caffeine clearance in patients treated with Sb^{5+} drugs.[5] Caffeine clearance depends on an oxidation reaction catalyzed by liver CYP1A1/2 and is often used as an indicator of *in vivo* CYP1A activity.[5] The effective induction of CYP1A activity by βNF, a known AhR ligand, suggested that AhR-mediated regulation of CYP1A1/2 expression was not impaired in MA-treated mice. The notion that Sb^{5+} and/or Sb^{3+} (produced by intracellular reduction of Sb^{5+}) could directly inhibit CYP1A catalytic activity was tested in liver microsomes, but no inhibitory effects were noted at concentrations of up to 1 mM. Future research could investigate whether inhibition of CYP1A protein by Sb^{5+} and/or Sb^{3+} can be observed in intact cell systems.

In conclusion, the results from the present study demonstrated that mouse CYP2A5-, CYP2E1-, and CYP3A11-mediated reactions were unaffected by a 24-day course of treatment with the antileishmanial Sb^{5+} drug, MA. However, repeated administration of MA consistently down-regulated the activity of CYP1A2 and produced a less consistent inhibition of CYP2B9/10. *In vitro* treatment with Sb^{3+} and Sb^{5+} did not alter the CYP1A activity in mouse liver microsomes. The *in vivo* inhibition of CYP1A catalytic activity by Sb^{5+} drugs may involve mechanisms that are not operational in the isolated liver microsomal fraction.

V.6 Acknowledgments

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The authors have no conflict of interest regarding this study.

V.7 References

1. Vianna G. Tratamento da leishmaniose tegumentar por injeções intravenosas de tártaro emético. Anais do VII Congresso Brasileiro de Medicina e Cirurgia 1912; 4: 426–428.

2. Di Cristina G, Caronia G. Sulla terapia della leishmaniosi interna. Bulletin de la Société de Pathologie Éxotique 1915; 8: 63–65.

3. Christopherson JB. The curative dose of antimony tartrate in schistosomiasis (bilharzia disease) British Medical Journal 1923; 3287: 1254–1255.

4. Hepburn NC, Siddique I, Howie AF, Beckett GJ, Hayes PC. Hepatotoxicity of sodium stibogluconate in leishmaniasis. The Lancet 1993; 342: 238-239.

5. Hepburn NC, Siddique I, Howie AF, Beckett GJ, Hayes PC. Hepatotoxicity of sodium stibogluconate therapy for American cutaneous leishmaniasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 1994; 88: 453-455.

6. Poon R, Chu I, Lecavalier P, Valli VE, Foster W, Gupta S, Thomas B. Effects of antimony on rats following 90-day exposure via drinking water. Food and Chemical Toxicology1998; 36: 21-35.

7. Drummond GS, Kappas A. Potent heme-degrading action of antimony and antimony-containing parasiticidal agents. Journal of Experimental Medicine 1981; 153: 245-256.

8. Shaked-Mishan P, Ulrich N, Ephros M, Zilberstein D. Novel Intracellular SbV reducing activity correlates with antimony susceptibility in Leishmania donovani. Journal of Biological Chemisty 2001; 276: 3971-3976.

9. Wyllie S, Cunningham ML, Fairlamb AH. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen Leishmania donovani. Journal of Biological Chemisty 2004; 279: 39925-39932.

10. Baiocco P, Colotti G, Franceschini S, Ilari A. Molecular basis of antimony treatment in leishmaniasis. Journal of Medicinal Chemistry 2009; 52: 2603-2612.

11. Hansen C, Hansen EW, Hansen HR, Gammelgaard B, Stürup S. Reduction of Sb(V) in a human macrophage cell line measured by HPLC-ICP-MS. Biological Trace Element Research 2011; 144: 234-243.

12. López S, Aguilar L, Mercado L, Bravo M, Quiroz W. Sb(V) reactivity with human blood components: redox effects. Public Library of Science One 2015; 10: e0114796.

13. Friedrich K, Vieira FA, Porrozzi R, Marchevsky RS, Miekeley N, Grimaldi G Jr, Paumgartten FJ 2012. Disposition of antimony in rhesus monkeys infected with Leishmania braziliensis and treated with meglumine antimoniate. *J Toxicol Environ Health A.75*(2): 63-75

14. Miekeley N, Mortari SR, Schubach AO. Monitoring of total antimony and its species by ICP-MS and on-line ion chromatography in biological samples from patients treated for leishmaniasis. Analytical and Bioanalytical Chemistry 2002; 372: 495-502.

15. Coelho DR, Miranda ES, Saint'Pierre TD, Paumgartten FJ. Tissue distribution of residual antimony in rats treated with multiple doses of meglumine antimoniate. Memórias do Instituto Oswaldo Cruz 2014; 109: 420-427.

16. Gilmore WJ and Kirby GM. Endoplasmic Reticulum Stress Due to Altered Cellular Redox Status Positively regulates Murine Hepatic CYP2A5 Expression. The Journal of Pharmacology and Experimental Therapeutics 2004; 308:600-608

17. Abu-Bakar A, Hakkola J, Juvonen R, rahnasto-Rilla M, Raunio H, Lang M. Function and Regulation of the CYP2A5/CYP2A6 Genes in Response to Toxic Insults in the Liver 2013; 14:137-150.

18. De-Oliveira AC, Carvalho RS, Paixão FH, Tavares HS, Gueiros LS, Siqueira CM, Paumgartten FJ. Up- and down-modulation of liver cytochrome P450 activities and associated events in two murine malaria models. Malaria Journal 2010; 9: 81.

19. Sunderman FW Jr. Metal induction of heme oxygenase. Annals of the New York Academy of Sciences 1987; 514: 65-80.

20. Coelho DR, De-Carvalho RR, Rocha RC, Saint'Pierre TD, Paumgartten FJ. Effects of in utero and lactational exposure to SbV on rat neurobehavioral development and fertility. Reproductive Toxicology 2014; 50: 98-107.

21. De-Oliveira AC, Fidalgo-Neto AA, Paumgartten FJ. In vitro inhibition of liver monooxygenases by beta-ionone, 1,8-cineole, (-)-menthol and terpineol. Toxicology 1999; 135: 33-34.

22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976; 72: 248–254.

23. Kennedy SW, Jones SP. Simultaneous measurement of cytochrome P4501A catalytic activity and total protein concentration with a fluorescence plate reader. Analytical Biochemistry 1994; 222: 217–223.

24. De-Oliveira AC, Poça KS, Totino PR, Paumgartten FJ. Modulation of cytochrome P450 2A5 activity by lipopolysaccharide: low-dose effects and non-monotonic dose-response relationship. Public Library of Science One 2015; 30; 10: e0117842.

25. van Iersel M, Walters DG, Price RJ, Lovell DP, Lake BG. Sex and strain differences in mouse hepatic microsomal coumarin 7-hydroxylase activity. Food and Chemical Toxicology 1994; 32(4): 387–390.

26. Allis JW, Robinson BL. A kinetic assay for p-nitrophenol hydroxylase in rat liver microsomes. Analytical Biochemistry 1994; 219: 49-52.

27. Carvalho RS, Friedrich K, Oliveira ACAX, Suarez-Kurtz G, Paumgartten FJR. Malária downmodulates mRNA expression. And catalytic of CYP1A2, 2E1 and 3A11 in mouse liver. European Journal of Pharmacology 2009; 616: 265-269.

28. Nash T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochemical Journal. 1953; 55: 416-421.

29. Chulay JD, Fleckenstein L, Smith DH 1988. Pharmacokinetics of antimony during treatment of visceral leishmaniasis with sodium stibogluconate or meglumine antimoniate. *Trans R Soc Trop Med Hyg. 82*(1): 69-72

30. Grimaldi Jr G, Porrozzi R, Friedrich K, Teva A, Marchevsky RS, Vieira F, Miekeley N, Paumgartten FJR. Comparative Efficacies of Two Antimony Regimens to Treat *Leishmania brasiliensis* – Induced Cutaneous Leishmaniasis in Rhesus Macaques (*Macaca mulatta*). Antimicrobial Agents and Chemotherapy 2010; 54:502-505.

V.8 Tables

Table 1. Effects of a 24-day course of treatment with meglumine antimoniate (MA, 300 mg Sb5+/kg bw/day, subcutaneously) in Swiss Webster and DBA-2 mice

	Males		**Females**	
Swiss Webster	**CO**	**MA**	**CO**	**MA**
BROD (pmol/mg protein/min)	23.1 ± 4.30	22.2 ± 5.37	67.71 ± 24.69	32.90 ± 12.82*
PNPH (nmol/mg protein/min)	1.41 ± 0.51	1.58 ± 0.44	1.72 ± 0.65	1.67 ± 0.64
END (nmol/mg protein/min)	2.08 ± 1.02	1.94 ± 0.78	1.77 ± 0.79	1.46 ± 0.53
GSH (nmol/mg protein)	24.16 ± 7.8	25.00 ± 11.31	-	-
ALT (IU/L)	-	-	109.9 ± 19.27	101.6 ± 19.43
AST (IU/L)	-	-	199.6 ± 40.84	204.6 ± 45.27
DBA-2				
BROD (pmol/mg protein/min)	20.79 ± 2.72	18.66 ± 3.71	63.14 ± 29.22	60.52 ± 23.85
ALT (IU/L)	-	-	124.9 ± 37.47	112.9 ± 6.68
AST (IU/L)	-	-	282.7 ± 70.48	241.1 ± 23.58

Data are shown as the mean ± standard deviation. *P < 0.05, Student's t-test.

CO, control group; MA, meglumine antimoniate-treated group; BROD, benzyloxy-resorufin-*O*-debenzylase activity; PNPH, p-nitro-phenol-hydroxylase activity; END, erythromycin-*N*-demethylase activity; GSH liver glutathione level; ALT, serum alanine transferase activity; AST, serum aspartate aminotransferase activity.

Table 2. Levels of antimony in the livers of female Swiss Webster (Swiss) and DBA-2 mice

	CO	MA
SWISS	0.11 ± 0.09	56.51 ± 14.92*
DBA-2	0.10 ± 0.10	118.40 ± 14.54*

Data are shown as the mean ± standard deviation of Sb concentrations (mg/kg dry weight) determined by inductively coupled plasma mass spectrometry. *$P < 0.05$, Student's t-test.

MA, meglumine antimoniate-treated group (300 mg Sb^{5+}/kg body weight/day, subcutaneously); CO, vehicle-treated group.

V.9 Figures

Figure 1

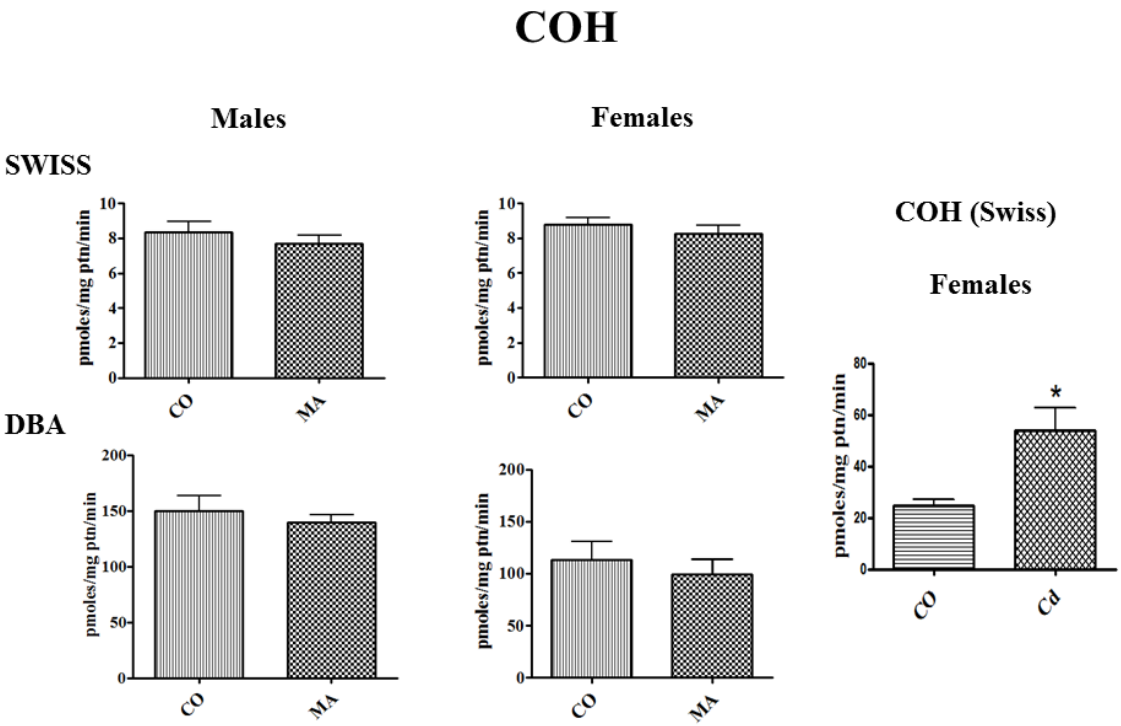

Figure 1. Effects of a 24-day course of treatment with meglumine antimoniate (MA, 300 mg Sb^{5+}/kg bw/day, subcutaneously) or vehicle (CO) on the coumarin 7-hydroxylase (COH) activity of CYP2A5 in the liver of Swiss Webster (Swiss) and DBA-2 (DBA) mice (males and females). Histogram bar heights represent means ± SEM. A positive response to treatment with $CdCl_2$ (16 µmol/kg bw intraperitoneally 6 h prior to euthanasia; Cd) is indicated in the right-hand panel (females). *P < 0.05, Student's t-test.

Figure 2 **EROD**

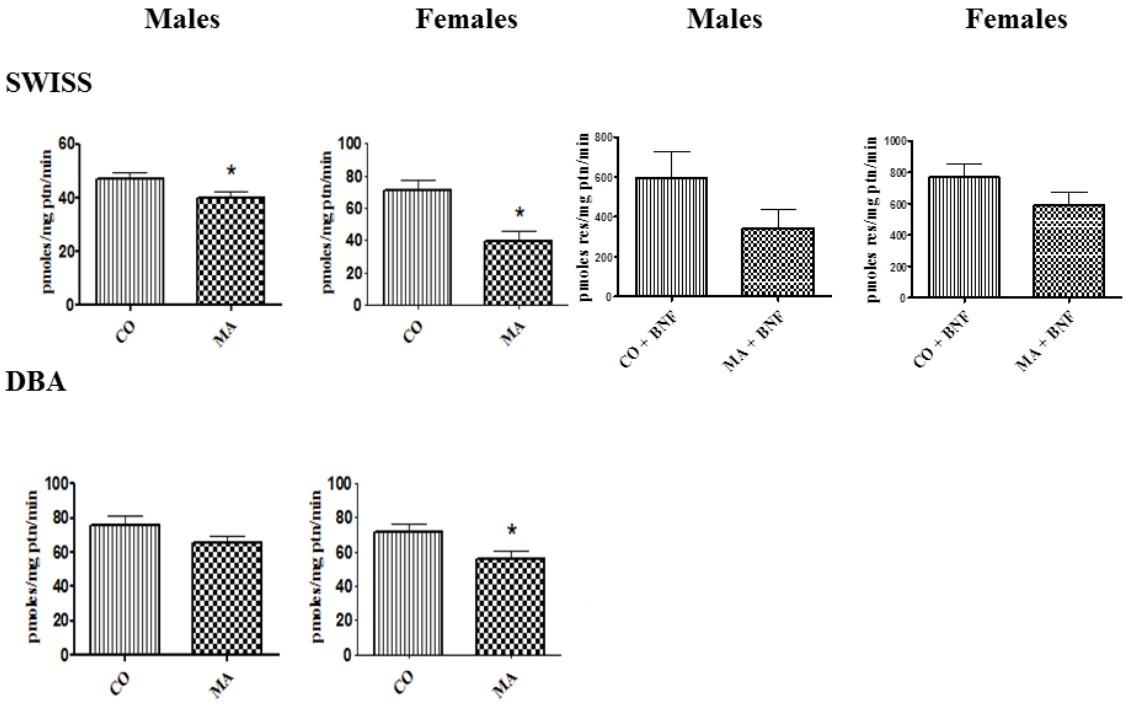

Figure 2. Effects of a 24-day course of treatment with meglumine antimoniate (MA, 300 mg Sb^{5+}/kg bw/day, subcutaneously) on the activity of CYP1A1/2 (ethoxyresorufin-O-deethylase, EROD) in the liver of Swiss Webster and DBA-2 mice. Histogram bar heights are means ± SEM. Histograms on the right (upper part) show the response of vehicle- (CO) and MA-treated mice to β-naphthoflavone (BNF) (80 mg/kg bw/day intraperitoneally for 3 days prior to euthanasia). *P < 0.05, Student's t-test.

Figura 3

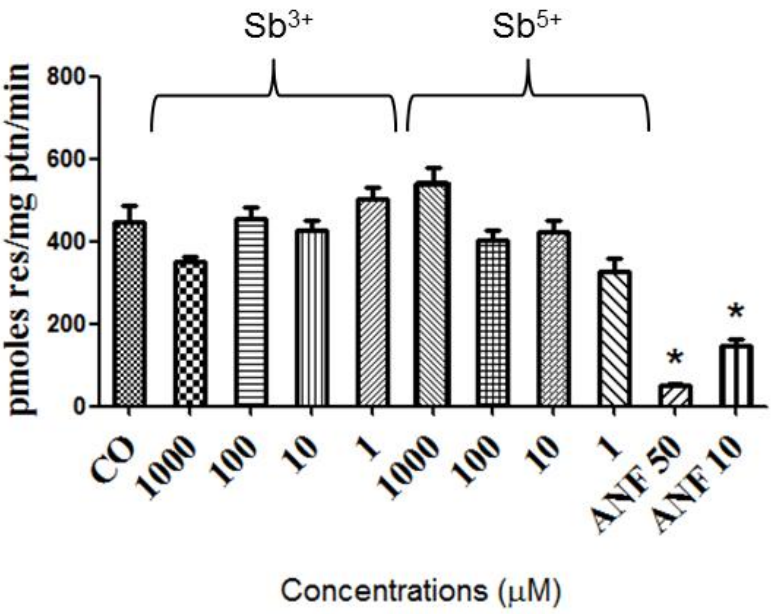

Figure 3. In vitro effects of the indicated concentrations of Sb^{3+} and Sb^{5+} salts or water (CO) on CYP1A activity (ethoxyresorufin-O-deethylase, EROD) in the Swiss Webster mouse liver microsomal fraction. Histogram bar heights are means ± SEM. Bars on the right show the response to α-naphthoflavone (ANF), a known inhibitor of CYP1A. Microsomes were prepared from mice treated with the CYP1A inducing agent, β-naphthoflavone (80 mg/kg bw/day intraperitoneally for 3 days prior to euthanasia).

VI. CONSIDERAÇÕES FINAIS

O conjunto de dados que foi apresentado nesta tese mostrou que o antimônio, após tratamento com doses repetidas com antimoniato de meglumina, persiste no organismo em níveis residuais por um período muito longo. O componente terminal da eliminação em roedores, tal como observado em primatas e no homem, é muito lento.

O acentuado acúmulo de Sb residual na tireóide é consistente com o que havia sido anteriormente notado em primatas não humanos e merece destaque. Embora não tenham sido constatados efeitos (avaliações de desenvolvimento neuromotor e fertilidade) sugestivos de disfunção tireoideana, os mecanismos pelos quais este acúmulo ocorre, e a especiação do antimônio no tecido da glândula, merecem ser investigados em estudos subsequentes.

O desenvolvimento pós-natal da prole das mães tratadas foi pouco afetado, e os efeitos notados ainda que discretos pareceram ser maiores para as fêmeas. Motivo de preocupação, porém, foi a constatação de que o antimônio passa através da placenta e via leite materno para os filhotes expostos até o desmame, e persiste no organismo destes em níveis aumentados, em comparação com controles, até a vida adulta. Essa lenta eliminação recomenda cautela no uso desses compostos quando se tratar de uma gestante ou nutriz, ou ainda lactantes. O fato dos animais não apresentarem alteração no seu desenvolvimento neuromotor é consistente com a observação de que não há presença marcante de antimônio no cérebro dos animais expostos.

O fato dos órgãos reprodutores masculinos não acumularem altas concentrações de Sb por longo período, é consistente com a observação de que a exposição intra-uterina e durante lactação não alterou a fertilidade na vida adulta.

A presença do antimônio no leite materno e absorção do mesmo pelos filhotes que se alimentam deste leite é dado que indica a necessidade de investigar a especiação do antimônio nesta matriz biológica. Ainda existe muito a ser esclarecido em relação a essa passagem do antimônio para o leite e este talvez possa ser considerado uma nova via de excreção deste metaloide. É importante destacar o fato surpreendente do antimônio contido no leite materno ser aparentemente muito bem absorvido por via oral, ao contrário dos sais inorgânicos e orgânicos deste metalóide. Pode-se adiantar a hipótese de que no leite o antimônio está complexado com proteínas (ou outras macromoléculas) o que o torna mais biodisponível para os filhotes. Talvez esse possa ser um caminho

(uma vez esclarecida a especiação) para desenvolver novas formulações de antimoniais bem absorvidas e toleradas por via oral.

Os resultados também mostraram que o antimônio residual acumula-se no fígado, sendo este um dos órgãos que mais acumula o metalóide. Apesar disso, as consequências para as atividades enzimáticas responsáveis pelo metabolismo hepático foram discretas. O efeito observado mais consistente em relação à atividade enzimática foi uma redução na atividade de CYP 1A. Essa depressão pode afetar a metabolização e eliminação de substâncias (e.g. cafeína) que são substrato para essas enzimas durante o período do tratamento com antimoniato de meglumina.

Espera-se que este conjunto de dados possa contribuir para a saúde pública à medida que amplia as informações disponíveis para avaliar a segurança do tratamento prolongado com antimoniato de meglumina, incluindo aspectos que não haviam ainda sido estudados. Apesar de ter sido usado o modelo animal nos trabalhos apresentados aqui, os resultados concordam com a noção de que o uso do antimonial pentavalente no tratamento de leishmanioses se justifica ponderando benefícios e riscos. Embora os resultados apontem para a longa persistência do antimônio residual no organismo, vale lembrar que os estudos aqui mostrados utilizaram uma dose pelo menos 10 vezes superior à maior dose utilizada em humanos, sem que marcantes efeitos tóxicos fossem evidenciados.

VII. CONCLUSÕES

- Após uma única injeção de antimoniato de meglumina em ratos, o Sb é quase que completamente eliminado após 6 h a 12 h.
- Após 12h as quantidades residuais de antimônio são eliminadas lentamente com meia-vida (fase terminal) muito superior a 24 horas.
- Após 21 dias de tratamento os níveis sanguíneos encontrados 24 horas após a última injeção estão próximos àqueles que foram medidos 21 dias e 105 dias após a interrupção das injeções.
- No grupo dos órgãos que exibiram maiores concentrações residuais de Sb estão: baço, fêmur, tireóide, fígado, epidídimo, pulmão e adrenal.
- No grupo dos órgãos que apresentaram menores concentrações de Sb estão: próstata, timo, pâncreas, coração, intestino delgado, músculo esquelético, testículos, estômago e cérebro.
- Apesar da concentração de Sb ter apresentado queda significativa 21 dias após o fim do tratamento, o baço continuou sendo o órgão com maior quantidade deste metalóide.
- Na fase lenta de eliminação (21 dias após a última injeção) os tecidos que apresentaram maiores teores de Sb foram: baço, osso, tireóide e fígado.
- O órgão com menor nível de Sb nos dois momentos coletados foi o cérebro.
- A passagem do Sb através da placenta afetou o crescimento pré-natal e a viabilidade de neonatos nascidos de mães tratadas com a dose de 300 mg Sb^{V}/kg peso corporal /dia.
- A exposição através da placenta e do leite materno levou à discreta diminuição do ganho de peso da prole feminina no dia pós-natal 60, em todas as doses administradas.
- De uma forma geral, a exposição, por via transplacentária e através do leite materno, ao antimoniato de meglumina não afetou o desenvolvimento somático, neurocomportamental, maturação sexual e fertilidade dos filhotes.

- O Sb presente no antimoniato de meglumina é transferido para o leite materno e absorvido pelos filhotes por via oral.
- O Sb presente no fígado e sangue dos filhotes que foram expostos durante gestação e lactação manteve quantidades expressivas, mesmo cerca de dois meses após o fim da exposição.
- Há acúmulo de Sb no fígado de camundongos tratados com doses repetidas do antimoniato de meglumina.
- O tratamento prolongado com o antimoniato de meglumina causa diminuição da atividade de CYP1A no fígado de machos e fêmeas de camundongos SW e em fêmeas da linhagem DBA-2.
- A atividade de CYP2B9/10 no fígado de camundongos SW do sexo feminino também apresentou redução.
- As atividades de CYP2A5, 2E1, 3A e os níveis de glutationa no tecido hepático não foram alterados.

VIII. REFERÊNCIAS

ALBUQUERQUE, M. D., & MACIEL, L. R. ***Resenha histórica das pesquisas em leishmaniose (do inicio do século a década de 1930). A contribuição do Instituto Oswaldo Cruz.*** Rio de Janeiro: Fundação Oswaldo Cruz.: 1995.

ALBUQUERQUE, M. D.; MACIEL, L. R. ***Resenha histórica das pesquisas em leishmaniose (do inicio do século a década de 1930). A contribuição do Instituto Oswaldo Cruz.*** Rio de Janeiro: Fundação Oswaldo Cruz.: 1995.

ALVAR, J. et al. Leishmaniasis Worldwide and Global Estimates of Its Incidence. **Plos One,** v. 7, n. 5, May 31 2012. ISSN 1932-6203. Disponível em: < <Go to ISI>://WOS:000305338500009 >.

ASHFORD, R. W. The leishmaniases as emerging and reemerging zoonoses. **International Journal for Parasitology,** v. 30, n. 12-13, p. 1269-1281, Nov 2000. ISSN 0020-7519. Disponível em: < <Go to ISI>://WOS:000166144900004 >.

BASANO, S. D. A.; CAMARGO, L. M. A. Leishmaniose tegumentar americana: histórico, epidemiologia e perspectivas de controle. **Revista Brasileira de Epidemiologia,** v. 7, n. 3, p. 328-337, 2004.

BRASIL. Ministério da Saúde. Seretaria de Vigilância Sanitária em Saúde. Deparatamento de Vigilância Epidemiológica. **Manual de Vigilância e Controle de Leishmaniose Visceral.** Brasília: Editora do Ministério da Saúde, 2006, 120p.

BRASIL. Ministério da Saúde. Seretaria de Vigilância Sanitária em Saúde. **Manual de Vigilância de Leishmaniose Tegumentar Americana.** Brasília: Editora do Ministério da Saúde, 2010, 2ª edição atualizada, 180p.

BRASIL et al. **Doenças infecciosas e parasitárias : guia de bolso**. Série B. Textos Básicos de Saúde. SAÚDE, M. D.;SAÚDE, S. D. V. E., *et al*. Brasília: Ministério da Saúde 2010.

CLARKSON, T. W. I**norganic and Organometal Pesticides.** In: *PRESS, A.* (Ed.). **H*andbook of Pesticide Toxicology***, v. *2, 2001*.

CUCE, L. C.; BELDA, W.; DIAS, M. C. NEPHROTOXICITY TO GLUCANTIME IN THE TREATMENT OF LEISHMANIASIS. **Revista Do Instituto De Medicina Tropical De Sao Paulo,** v. 32, n. 4, p. 249-251, Jul-Aug 1990. ISSN 0036-4665. Disponível em: < <Go to ISI>://WOS:A1990EB28600003 >.

DA SILVA, L. J. Vianna and the Discovery of Leishmania brasiliensis: the role of Brazilian parasitologists in the identification of Bauru´s ulcer as American leishmaniasis. . **Parassitologia,** v. 47, p. 335-341, 2005.

DE BOECK, M.; KIRSCH-VOLDERS, M.; LISON, D. Cobalt and antimony: genotoxicity and carcinogenicity. **Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis,** v. 533, n. 1-2, p. 135-152, Dec 2003. ISSN 0027-5107. Disponível em: < <Go to ISI>://WOS:000208033800008 >.

DOSTALOVA, A.; VOLF, P. Leishmania development in sand flies: parasite-vector interactions overview. **Parasites & Vectors,** v. 5, p. 12, Dec 2012. ISSN 1756-3305. Disponível em: < <Go to ISI>://WOS:000313217400001 >.

FALCÃO, E. C. ***Opera Omnia de Gaspar Vianna***. TRIBUNAIS, R. D. São Paulo 1962.

FONSECA FILHO, O. D. **Cinquentenário da descoberta por Gaspar Vianna do tratamento das leishmanioses pelos antimoniais.** Boletim da Academia Nacional de Medicina. 133 (7)**:** . p. 1962.

GACHELIN, G. Medical geography and tropical diseases in Brazil, 1880 - 1914. **Parassitologia,** v. 47, p. 265-270, 2005.

GONTIJO, C. M. F.; MELO, M. N. Leishmaniose Visceral no Brasil:

quadro atual, desafios e perspectivas. **Revista Brasileira de Epidemiologia,** v. 7, n. 3, p. 338-349, 2004.

GOTO, H.; LAULETTA LINDOSO, J. A. Cutaneous and Mucocutaneous Leishmaniasis. **Infectious Disease Clinics of North America,** v. 26, n. 2, p. 293-+, Jun 2012. ISSN 0891-5520. Disponível em: < <Go to ISI>://WOS:000305853100008 >.

GREENWOOD, D. ***Antimicrobial drugs: chronicle of a twentieth century medical triunph***. London: Oxford University Press., 2008.

GRIENSVEN, J. V.; DIRO, E. Visceral Leishmaniasis. ***Infectious Disease Clinics of North America,*** v. 26, p. 309-322, 2012.

HALDAR, A. K.; SEN, P.; ROY, S. Use of Antimony in the Treatment of Leishmaniasis:Current Status and Future Directions. **Molecular Biology International,** v. 2011, p. 23 pages, 2011.

HARHAY, M. O. et al. Urban parasitology: visceral leishmaniasis in Brazil. ***Trends in Parasitology,*** v. 27, n. 9, p. 403-409, 2011.

HART, D. T. CUTANEOUS AND VISCERAL LEISHMANIASIS - A HISTORICAL-PERSPECTIVE - CUTANEOUS-LEISHMANIASIS. **Transactions of the Royal Society of Tropical Medicine and Hygiene,** v. 79, n. 5, p. 740-741, 1985 1985. ISSN 0035-9203. Disponível em: < <Go to ISI>://WOS:A1985AUS9600057 >.

HERWALDT, B. L. Leishmaniasis. **Lancet,** v. 354, n. 9185, p. 1191-1199, Oct 2 1999. ISSN 0140-6736. Disponível em: < <Go to ISI>://WOS:000082954100044 >.

MARSDEN, P. D. Pentavalent antimonials: Old drugs for new diseases. **Revista da Sociedade Brasileira de Medicina Tropical,** v. 18, n. 3, p. 187-198, 1985.

MORAES, A. S. ***Dois cientistas brasileiros: Rocha Lima e Gaspar Vianna***. Rio de Janeiro: Tempo Brasileiro, 1968.

MORAES, R. G. ***Parasitologia & imunologia humana***. 5ª. Rio de Janeiro: 2008.

MURRAY, H. W. et al. Advances in leishmaniasis. **Lancet,** v. 366, n. 9496, p. 1561-1577, Oct-Nov 2005. ISSN 0140-6736. Disponível em: < <Go to ISI>://WOS:000232984800026 >.

NEVES, D. B. D.; CALDAS, E. D.; SAMPAIO, R. N. R. Antimony in plasma and skin of patients with cutaneous leishmaniasis - relationship with side effects after treatment with meglumine antimoniate. **Tropical Medicine & International Health,** v. 14, n. 12, p. 1515-1522, Dec 2009. ISSN 1360-2276. Disponível em: < <Go to ISI>://WOS:000271526900013 >.

OLIVEIRA, L. et al. SYSTEMATIC REVIEW OF THE ADVERSE EFFECTS OF CUTANEOUS LEISHMANIASIS TREATMENT IN THE NEW WORLD. **Journal of**

Epidemiology and Community Health, v. 65, p. A454-A454, Aug 2011. ISSN 0143-005X. Disponível em: < <Go to ISI>://WOS:000293901802319 >.

OLIVEIRA, R. A. et al. Renal function evaluation in patients with American Cutaneous Leishmaniasis after specific treatment with pentavalent antimonial. **Bmc Nephrology,** v. 13, Jun 2012. ISSN 1471-2369. Disponível em: < <Go to ISI>://WOS:000309074800001 >.

OUMEISH, O. Y. Cutaneous leishmaniasis: A historical perspective. **Clinics in Dermatology,** v. 17, n. 3, p. 249-254, May-Jun 1999. ISSN 0738-081X. Disponível em: < <Go to ISI>://WOS:000080815600002 >.

PALATNIK-DE-SOUSA, C. B.; DAY, M. J. One Health: The global challenge of epidemic and endemic leishmaniasis. **Parasites & Vectors,** v. 4, Oct 10 2011. ISSN 1756-3305. Disponível em: < <Go to ISI>://WOS:000296919000001 >.

PELISSARI, D. M. et al. **Tratamento da Leishmaniose Visceral e Leishmaniose Tegumentar Americana no Brasil**. SAÚDE, E. D. S. D. Brasília: Secretaria de Vigilância em Saúde. 20**:** 107-110 p. 2011.

RATH, S. et al. Antimonials employed in the treatment of leishmaniaisis: The state of the art. **Quimica Nova,** v. 26, n. 4, p. 550-555, Jul-Aug 2003. ISSN 0100-4042. Disponível em: < <Go to ISI>://WOS:000184605400017 >.

REITHINGER, R. et al. Cutaneous leishmaniasis. **Lancet Infectious Diseases,** v. 7, n. 9, p. 581-596, Sep 2007. ISSN 1473-3099. Disponível em: < <Go to ISI>://WOS:000249091700016 >.

RIBEIRO, A. L. P. et al. Electrocardiographic changes during low-dose, short-term therapy of cutaneous leishmaniasis with the pentavalent antimonial meglumine.

Brazilian Journal of Medical and Biological Research, v. 32, n. 3, p. 297-301, Mar 1999. ISSN 0100-879X. Disponível em: < <Go to ISI>://WOS:000079438000008 >.

SNEADER, W. ***Drug Discovery: a history***. Nova Iorque: 2005.

TIUMAN, T. S. et al. Recent advances in leishmaniasis treatment. **International Journal of Infectious Diseases,** v. 15, n. 8, p. E525-E532, Aug 2011. ISSN 1201-9712. Disponível em: < <Go to ISI>://WOS:000293576600004 >.

TORRES, O. ***Observação de um caso de leishmaniose cutanea: communicação feita À Sociedade Medica dos Hospitaes da Bahia, em 24 de abril de 1915***. Bahia: Besnard Frères 1916.

VEIGA, J. P. R. et al. RENAL-FUNCTION IN PATIENTS WITH MUCOCUTANEOUS LEISHMANIASIS TREATED WITH PENTAVALENT ANTIMONIALS. **Revista Do Instituto De Medicina Tropical De Sao Paulo,** v. 27, n. 6, p. 298-302, Nov-Dec 1985. ISSN 0036-4665. Disponível em: < <Go to ISI>://WOS:A1985A154100002 >.

VIANNA, G. **Tratamento da Leishmaniose Tegumentar por Injeções Intravenosas de Tártaro Emético**. *Belo Horizonte*: *VII Congresso Brasileiro de Medicina e Cirurgia* 4**:** 426-428 p. 1912a.

_____. Tratamento da Leishmaniose Tegumentar por Injeções Intravenosas de Tártaro Emético. **VII Congresso Brasileiro de Medicina e Cirurgia – Sessão da Sociedade Brasileira de Dermatologia, Belo Horizonte. Anais.,** v. 4, n. 3, p. 426-428, 1912b.

_____. Sobre o tratamento da leishmaniose tegumentar. **Anais Paulistas de Medicina e Cirurgia,** v. 2, n. 6, p. 167-169, 1914.

WITTNER, M.; TANOWITZ, H. B. Leishmaniasis in Infants and Children. ***Seminars in Pediatric Infectious Diseases*****,** v. 11, n. 3, p. 196-201, 2000.

ZANGI, R.; FILELLA, M. Transport routes of metalloids into and out of the cell: A review of the current knowledge. **Chemico-Biological Interactions,** v. 197, n. 1, p. 47-57, Apr 2012. ISSN 0009-2797. Disponível em: < <Go to ISI>://WOS:000303972800007 >.

IX. ANEXOS