IN VIVO ANTILEISHMANIAL EFFICACY OF COMBRETASTATIN HETEROANALOGUES

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ABSTRACT

Two combretastatin heteroanalogues, called SAAS-41 and SAAS-59, were selected within a series of 44 compounds previously evaluated in vitro against Leishmania spp, and have now been assayed in vivo on BALB/c mice, infected with amastigotes of L. amazonensis. They were administered by three different routes. The most efficient compound, SAAS-59, administered subcutaneously (50 mg/kg), led to fair reductions of lesion size (-86.4%) and parasitic load (-97.4%), in percentages fairly higher than those observed for the reference drug glucantime at the dose of 100 mg/kg (-49.1% size; -60.1% load). Intrallesional and oral administration of the product at the same dose, also induced significant decreases (-91.4% and -77.3% respectively) of the lesion size, but it did not show significant reductions of the parasitic load.

Keywords: Leishmaniasis, antileishmanial efficacy, glucantime, combretastatin heteroanalogues.

RESUMEN

Dos heteroanálogos de la combretastatina, denominados SAAS-41 y SAAS-59, fueron seleccionados de una serie de 44 compuestos previamente evaluados in vitro contra Leishmania spp, y han sido ahora evaluados in vivo en ratones BALB/c, infectados con amastigotes de L. amazonensis. Los compuestos fueron administrados por tres rutas diferentes. El compuesto más eficiente, SAAS-59, administrado subcutáneamente (50 mg/kg), produjo reducciones importantes del tamaño de la lesión (-86.4%) y de la carga parasitaria (-97.4%), en porcentajes bastante más altos que los observados con la droga de referencia, glucantime, en una dosis de 100 mg/kg (-49.1% tamaño; -60.1% carga). La administración intrallesional y oral...
del producto a la misma dosis produjo disminuciones significativas del tamaño de la lesión (-91,4% y -77,3% respectivamente) pero no produjo reducciones significativas de la carga parasitaria.

Palabras claves: Leishmaniasis, eficacia antileishmania, glucantime, heteroánalógos de la combretastatina.

INTRODUCTION

Leishmaniasis is prevalent throughout the world and endemic in developing countries, affecting around 12 million people in the world and 350 million are at risk (WHO, 1995; WHO, 2000b; OPS, 2002; Singh & Sivakumar; 2004). The currently used drugs for treatment require long course, parenteral administration, not too efficient against the parasite and display a number of adverse side-effects. In addition, significant resistance of several Leishmania strains to these drugs has emerged, mainly due to the intermittent use in patients with tegumentary leishmaniasis (Berman, 1988; Guerin et al., 2002; Singh & Sivakumar, 2004). There also exist a progressive number of patients with visceral leishmaniasis co-infected with HIV who generally displays a poor response to conventional treatment and a frequent recidivism (Montalban et al., 1990; WHO, 2000a; WHO, 2000b; Guerin et al., 2002; Murray et al., 2005). Due to these failures and the appearance of new challenges, there is a real need to find new drugs and other therapeutic strategies for the treatment of this parasitosis.

Combretastatins constitute an interesting group of natural stilbenoids, isolated from the bark of the South African willow tree Combretum caffrum, receiving attention for their antitumor activity produced by their ability to inhibit tubulin polymerization (Pettit et al., 1982; McGown et al., 1988; Cirla & Mann, 2003; Hsieh et al, 2005; Vitale et al., 2007). Not many of these compounds have been described and most of those reported derivatives and analogues have been evaluated as antimitotic agents, looking for ascertaining their potential for development as anticancer drugs (Cushman et al., 1991; Shirai et al., 1994; Croft et al., 1996; Nam, 2003). Our group prepared different families of combretastatins analogues, including several substituted or fused heterocyclic systems, whose activity and toxicity were evaluated leading to encouraging results because of the low cytotoxicity found for many of them (Medarde et al., 1995; Medarde et al., 1998; Medarde et al., 1999).

The in vitro leishmanicidal activity of natural combretastatins and related compounds was first reported by our group. In such paper, we described a number of compounds displaying fair leishmanicidal effects, at concentrations under 10 µg/mL, with the furyl combretastatone 1 and the furyl indolecombretastatin 2, being the most potent within those evaluated compounds (Del Rey et al., 1999).

The continued synthetic chemical research and the analysis of the corresponding in vitro evaluation results led to the final selection of compound 1, the most potent representative of the heterosubstituted series of combretastatin analogues, now called SAAS-41 and of compound 3, the corresponding most representative element of the heterofused series, now called SAAS-59, for evaluating their efficacy, in vivo, against experimental leishmaniasis. Both compounds were previously examined in relation with their physico-chemical properties (molecular weight, number of acceptor and donor H atoms and logP). They were also in agreement with the Lipinski rules (Lipinsky et al., 1997) and ranged within the 90% cutoff of the World Drug
Infection
Mice were inoculated in the left hind footpad with $2 \times 10^6$ amastigotes of *L. amazonensis* (PH8 strain) obtained from golden hamsters. Parasites were injected suspended in 100 µL of PBS. The disease progression was evaluated by weekly measurements of the lesion during five weeks. When the difference of the lesion diameter average was higher than 4 mm in relation to the control hind (right hind), treatment was started.

Experimental drugs
The structures of the SAAS compounds tested are shown in Figure 1. They were synthesized according to the previously reported procedures and their physical and spectral data were also published by us (Del Rey et al., 1999). Chemically, SAAS-41 corresponds to: 1-(5-methylfuran-2-yl)-2-(3,4,5-trimethoxyphenyl)-ethanone, and SAAS-59 corresponds to: 5-methoxy-2-(5-methylfuran-2-yl)-3-(3,4,5-trimethoxyphenyl)-1H-indole.

Experiment design
This is a controlled and randomized experimental study to evaluate the efficacy of two heterocombretastatin derivatives in mice experimentally infected with *Leishmania amazonensis* (IFLA/BR/67/PH8).

Experimental animals
Male and female BALB/c mice bred in the “Instituto de Investigaciones en Ciencias de la Salud (IICS)”, Asunción, Paraguay, were used as experimental murine model.

Experimental groups
The first assay was performed on 6 groups of 10 animals each, treated separately with 25 mg/kg/day of each compound in evaluation, SAAS-41 and SAAS-59, along with one other group of animals treated subcutaneously with 100 mg/kg/day of glucantime and with one untreated control group.

The second experiment was performed similarly, on 6 groups of animals with 16 mice each one, which were treated only with SAAS-59 at two different doses of 25 mg/kg/day and 50 mg/kg/day by oral (OR), subcutaneous (SC) and intralesional (IL) routes. One group of 20 animals was treated with the standard drug (100 mg/kg/day) and other control group of 20 animals too remained untreated.

Treatment
In the first experiment, the treatment started five weeks after parasites inoculation. The reference drug was glucantime (N-methylglucamine or meglumine antimoniate). The drug was dissolved in phosphate buffered saline (PBS) and was administered in regimes of 100 mg/kg weight for 4 weeks by subcutaneous route. SAAS drugs were dissolved in PBS and administered by oral
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(OR), subcutaneous (SC) and intralesional (IL) routes, in doses of 25 mg/kg/day during the same period. Treatment was suspended during the weekend. The non-treated control group received (SC) 50 µL of PBS and 5µL of Tween 80 daily for the same period of time (Fournet et al., 1994).

The second experiment was performed similarly using SAAS-59 that was dissolved in PBS and administered by oral (OR), subcutaneous (SC) and intralesional (IL) routes, in two doses of 25 and 50 mg/kg/day, during a period of only two weeks, without treatment during the weekends. Again, the reference drug was glucantime that was dissolved and administered in similar conditions to the first experiments and the non-treated control group received the same solution above described.

Sacrifice of experimental animals
The animals were sacrificed two weeks after ending the first experiment and one week after the second by cervical dislocation following international recommendations (AVMA, 2007). The lesions of the infected legs were cut and macerated in a homogenizer with 2 mL of the culture medium of the Roswell Park Memorial Institute (RPMI).

Determination of parasitic load
The macerate solution was observed microscopically (40X) and the number of parasites or parasitic load was determined, counting the number of parasites per field. Specifically, smears were prepared and amastigotes were counted at 100X, examining 500 nuclei (Fournet et al., 1994).

Parasite Suppression Index
A corrected parasite suppression index (％) was calculated by the following formula (Ferreira et al., 2002)

\[
\frac{\text{Mean amastigotes in treated mice} \times 100 - 100}{\text{Mean amastigotes in control mice}}
\]

Negative results indicate reduction percentages.

Assay for determination of viable amastigotes
Before the in vivo assays, the determination of amastigotes viability was carried out as follows:

Intracellular forms (amastigotes) of L. amazonensis (PH8 strain), maintained in hamsters by successive passages every two months, were used (Sereno et al., 1997). For parasite maintenance, hamsters infected with L. amazonensis (PH8) were sacrificed taking all the precautions to avoid their suffering. The granulomas of the hamsters’ legs containing the parasites were extracted, placed in a Potter tube containing RPMI medium for grinding and homogenization, then they were centrifuged at 500 rpm during 10 min to eliminate rests of muscles and pus that could exist in the granulomas. The supernatant was extracted, washed with RPMI medium, and centrifuged again at 3,000 rpm during 10 min. The supernatant was eliminated and the pellet counted in a Neubauer chamber to calculate the No. of parasites/mL. The macroplates containing the macrophages with three hours of incubation were taken out of the oven, put in contact with the amastigotes of L. amazonensis (PH 8 strain) in a relation of 1 macrophage: 5 amastigotes (100 µL of RPMI medium + amastigotes). They were incubated at 37°C during 1 to 2 h and then the experimental compounds were added. The heterocombretastatins were weighted and dimethylsulphoxide was used as vehicle up to a concentration of 100 µg/mL. Dilution of 50 and 25 µg/mL and controls were prepared in triplicate following a previously published procedure (Deharo et al., 2000). Then, they were incubated at 37°C during 48 hours and reading was made in an inverted microscope (Olympus). The percentages of alive and dead amastigotes were calculated and results were compared with controls.

Statistical analysis of data
Descriptive statistics was used for the result analysis. To establish significant dif-
ferences between treatments, the ANOVA (Kruskal Wallis) multivariate analysis and student`s T tests were used.

**Ethical approval**
The study protocol was approved by the Ethical Committee of the Instituto de Investigaciones en Ciencias de la Salud before starting the study. The minimal number of animals to yield significant results were used and they were sacrificed by cervical dislocation following international recommendations (AVMA, 2007) in order to avoid them unnecessary suffering.

**RESULTS AND DISCUSSION**

**Table 1** shows the results observed in the first assay for both **SAAS** compounds administered by the three different routes. As it can be seen, at the dose of 25 mg/kg/day compound **SAAS-41**, administered via IL, induced considerable increase of the lesion size and the parasite load in lesions, while the OR and principally the SC administration clearly reduced the parasite load, but only slightly the size of the lesion. On the other hand, compound **SAAS-59**, behaved similarly, or even poorer, when administered by the IL or OR routes, but displayed a substantially good antileishmanial profile in the case of SC administration, attaining to reduce the lesion size by more than 21% and the parasite load by more than 70%. Consequently, aiming to confirm the favorable results observed for **SAAS-59** and to explore, from one side, the actual dose / antileishmanial response dependence and, from the other side, the antileishmanial efficacy of a shorter (2 weeks) treatment, a new experiment was planned for this compound. Infected animals were then treated with 25 and 50 mg/kg/day of **SAAS-59**. The new results confirming those previously found for this compound are shown in **Table 2** and **Figure 2**. Most notably, it should be noted that, at the dose of 50 mg/kg/day and after the short treatment of two weeks, the results of the SC administration of **SAAS-59** compared most positively with respect to those of glucantime at a double dose. In fact, remarkable and significant reductions of both, the lesion size (-86.4%; p<0.001) and the parasite load in the lesion (-97.4%; p<0.0001) were observed, with a fairly better therapeutic profile than the standard drug. Nevertheless, IL and OR

**Table 1. Efficacy of SAAS-41 and SAAS-59 on BALB/c mice experimentally infected with amastigotes of *L. amazonensis***

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Route</th>
<th>Lesion weight X ± SD (mg)</th>
<th>Lesion weight change (%)</th>
<th>Parasites in lesion X ± SD (x 10^8)</th>
<th>Parasites suppression index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAAS-41</td>
<td>25</td>
<td>OR</td>
<td>261 ± 151</td>
<td>- 7.1</td>
<td>6.9 ± 4.5</td>
<td>- 44.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>241 ± 84</td>
<td>- 14.3</td>
<td>8.2 ± 4.6</td>
<td>- 39.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL</td>
<td>427 ± 136</td>
<td>+ 52.0</td>
<td>10 ± 0.5</td>
<td>+ 41.8</td>
</tr>
<tr>
<td>SAAS-59</td>
<td>25</td>
<td>OR</td>
<td>381 ± 186</td>
<td>+ 35.7</td>
<td>8.4 ± 3.6</td>
<td>- 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SC</td>
<td>221 ± 94</td>
<td>- 21.4</td>
<td>4.9 ± 3.5</td>
<td>- 70.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL</td>
<td>512 ± 193</td>
<td>+ 82.1</td>
<td>10.0 ± 0.4</td>
<td>+ 59.6</td>
</tr>
<tr>
<td>Glucantime</td>
<td>100</td>
<td>SC</td>
<td>211 ± 56</td>
<td>- 25.0</td>
<td>83.0 ± 2.7</td>
<td>- 54.7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>SC</td>
<td>0.281 ± 0.15</td>
<td>--</td>
<td>83.0 ± 2.5</td>
<td>--</td>
</tr>
</tbody>
</table>

OR: Oral, SC: Subcutaneous, IL: Intraleesional X± SD: average ± standard deviation (n = 10, treatment for 4 weeks except weekends)
administration of **SAAS-59**, though inducing an important reduction of the lesion size (-91.4% and -77%, respectively), were not beneficial as antileishmanial, because administration through these routes increased conside-rably the parasitic load in lesions.

As it is stated above, the furylindole derivative **SAAS-59**, when administered via SC against murine leishmaniasis, after two weeks of treatment showed considerable dose/response dependent decreases of the number of parasites in lesions (97% and 50%, at the respective doses of 50 and 25 mg/kg/day). Both doses also reduced lesion sizes remarkably. Nevertheless, due to that OR administration substantially changed the therapeutic profile, it was deduced that a poor absorption/distribution or a rapid metabolization of **SAAS-59** would occur. Consequently, further ADME related aspects were analyzed by using online pre-ADMET prediction facilities (PreADMET, 2002) to confirm that, though intestinal absorption of the compound can be fairly ensured (>94%), the plasma protein binding (PPB) is too high (>90%) to ensure its ar
Table 3. Citotoxicity of SAAS-59 on macrophages and leishmanicidal effect on L. amazonensis amastigotes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µg/ml)</th>
<th>Viable amastigotes/100 macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAAS-59</td>
<td>100</td>
<td>31 ± 5.13</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>45 ± 3.46</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>74 ± 4.01</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>99.5 ± 0.71</td>
</tr>
</tbody>
</table>

X ± SD: average ± standard deviation

rival and liberation into the lesion in enough concentration. Deeper studies are needed to correct this fact and to design appropriate structure modifications and/or formulation helps in order to retain the antileishmanial efficacy after oral administration.

Two possible explanations have been suggested for the in vivo activities of combretastatins: oxidative activation to a quinine intermediate likely to bind to protein thiols and possibly to nucleic acids and stimulation of oxidative stress by enhancing superoxide/hydrogen peroxide production (Folkes et al., 2007). However, the mechanism of action of the furylindolic derivative is yet unknown though a selective inhibition of several enzymes and/or functions of the Leishmania parasite has been suggested. Because of the antimitotic characteristics of this indole (Cushman et al., 1991; Shirai et al., 1994) and its demonstrated activity on Leishmania spp. parasites (Sereno et al., 1997; Del Rey et al., 1999), it would be possible that the inhibition route is the usual for other antineoplastics that have shown their activity on these parasites (Chulay et al., 1988; Croft, 1988).

These results and the low toxicity of SAAS-59 encourage us to continue the experiments using other administration routes because of the easy absorption of combretastatins at the skin level (unpublished data), thus opening the possibility of administering this compound by topical application, as an ointment, in future assays. Besides, future additional studies should be focused on the in vivo evaluation of other molecules of this family that have shown leishmanicidal activity in vitro in previously published works of our group (Del Rey et al., 1999).

The compound studied showed moderate toxicity for amastigotes of Leishmania amazonensis at 25 µg/ml. Interesting in vitro and in vivo activities were observed at the intermediate concentration (50 µg/mL) of the furylindolic derivative.

CONCLUSION

The remarkable leishmanicidal action by subcutaneous route plus the clear chances of improving its action by oral route and its low proven toxicity in the in vivo assays shown by this compound make it a potential leading compound for the treatment of leishmaniasis acute infection. However, along with the search of options of higher activity in relation to its structure and absorption, the ongoing assays about its mechanism of action will provide important knowledge to improve its potential role for a future use in the treatment of cutaneous leishmaniasis.

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