Short communication

Synergistic effect of Ir-(COT)-pentamidine alizarin red and pentamidine, amphotericin B, and paromomycin on *Leishmania donovani*

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The treatment of various human infections caused by parasitic organisms of the genus *Leishmania* is still based on pentavalent antimonials, an empirical treatment developed more than 50 years ago (Croft, 1988, Herwaldt and Berman, 1992, Olliaro and Bryceson, 1993). Pentamidine is a molecule of high interest for curing early stages of African trypanosomiasis and both visceral and mucocutaneous leishmaniasis, refractory to pentavalent antimonials which is increasing in all areas of endemicity (Sands et al., 1985, Olliaro and Bryceson, 1993). The combination between a metallic structure and an organic moiety is one of the strategies in finding new antileishmanial drugs since it was demonstrated that organometallic compounds were less toxic and usually more active (Farrell et al., 1984, Gayral et

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In a previous study, we investigated the in vitro antileishmanial activity of 15 organometallic complexes of pentamidine on *Leishmania donovani* DD8 promastigotes (Mbongo et al., 1997). The most promising compound selected from this primary screening was Ir-(COT)-pentamidine alizarin red. IC₅₀ and IC₉₀ values of Ir-(COT)-pentamidine alizarin red on *L. donovani* DD8 promastigotes were found at 3.1 and 11.2 μM, respectively, whereas the corresponding values for pentamidine isethionate were 7.7 and > 50 μM, respectively (Mbongo et al., 1997).

In this study, the compound is evaluated in vitro on macrophages infected by *L. donovani* LV9 and a research of synergy with glucantime, pentamidine, amphotericin B and paromomycin was carried out on promastigote and amastigote forms. Furthermore, Ir-(COT)-pentamidine alizarin red was compared to pentamidine isethionate on the *L. donovani* LV9/BALB/C mouse model.

Pentamidine isethionate was kindly supplied by Roger Bellon, (Neuilly sur Seine, France); amphotericin B, paromomycin and alizarin red were purchased from Sigma (Saint Quentin Fallavier, France). The organometallic compound, also named P2368 had the following general formula:

\[
[M(I)(Y)(L)]^{2-} + 2X^- \quad \text{or} \quad X^{2-}
\]

where *M* is Iridium, *I* is coordination index indicating that the compound is a square planner complex, *Y* is 1,3,5-cyclooctatetraene (COT), *L* is pentamidine, and *X* is alizarin red.

The formula of the compound is presented in Fig. 1. The ability of the compound to reach the intracellular forms was assessed on mouse peritoneal macrophages infected by *L. donovani* LV9 (MHOM/ET/67/L82) amastigotes. This strain was provided by Dr S.L. Croft (London School of Hygiene and Tropical Medicine). Uninfected mouse macrophages were used for cytotoxicity assays over a 5-day period according to the Mossman method (Mossman, 1983).

![Fig. 1. Chemical structure of Ir-(COT)-pentamidine alizarin red (compound P2368).](image-url)
Leishmanicidal activities against amastigotes were performed on infected-mouse macrophages following the method of Neal and Croft (1984). The toxicity of the compound to uninfected macrophages was verified in order to determine the range of drug concentrations to be used. Ir-(COT)-pentamidine alizarin red had similar toxicity to pentamidine isethionate toward the uninfected macrophages (IC₅₀ at 35 and 30 μM, respectively). The organometallic compound was at least twice as active as pentamidine isethionate on the L. donovani LV9 amastigotes in macrophages with IC₅₀, respectively, at 6 and 15 μM. The maximum tolerated concentration by infected macrophages were 25 μM for the organometallic compound and 20 μM for pentamidine isethionate.

The organometallic compound was more active than Platinum complexes since Pt(II)-pentamidine displayed only 31% of amastigotes inhibition at 30 μM on L. donovani LV9 (Croft et al., 1992). Compound P2368 is therefore more active in vitro than pentamidine isethionate against L. donovani intracellular amastigotes and its toxicity on macrophages is identical to that of pentamidine.

The synergistic effect with other drugs was determined both on promastigote forms of L. donovani (MHOM/IN/80/DD8) originated from the WHO strain collection in London School of Hygiene and Tropical Medicine and amastigote forms of L. donovani LV9 in mouse peritoneal macrophages.

Promastigotes were cultivated in Hepes (25 mM) buffered RPMI 1640 medium enriched with fetal calf serum 10% and 1000 IU penicillin/ml at 27°C. The screening was performed in flat-bottomed 24-well tissue culture plastic trays with a well size of 17.8 (diameter) × 16 mm (depth), Falcon No 3047, maintained at 27°C in an atmosphere of air 95%-CO₂ 5%. Promastigote forms from a logarithmic phase culture were suspended to 10⁶ cells/ml. Each well was filled with 1 ml of the parasite suspension, and plates were incubated at 27°C for 1 h before drug addition. The compounds to be tested alone or in combination were dissolved in dimethylsulphoxide or sterile water and diluted in medium before to be added in each well under a 10 μl volume. Dimethylsulphoxide had no effect on the parasite growth up to 2% v/v. Serial dilutions (2-fold) of the tested drugs were performed and each concentration or combination was screened in triplicate. The checkerboard broth dilution technique was used to analyze the effect of the combination of Ir-(COT)-pentamidine alizarin red and pentamidine, amphotericin B and paromomycin on promastigote growth. The combination with glucantime was not carried out since IC₅₀ of glucantime on promastigotes was superior to 100 μM. The viability of promastigotes was checked using the MTT colorimetric method (Mossman, 1983). The inhibition of growth is given in inhibitory concentration 50% (IC₅₀) after a 3-day incubation period.

IC₅₀ of pentamidine isethionate was 7.5 μM (Table 1). Alizarin red was inactive at 50 μM.

Fig. 2, Fig. 3 and Fig. 4 show a synergistic effect 3 days after exposure to P2368 combined with pentamidine, amphotericin B and paromomycin. It is of interest that P2368 and pentamidine were synergistic suggesting that these compounds have two distinct mechanisms of action. This result corroborates a previous study carried out on a similar compound, Ir-(COD)-pentamidine tetraphenylborate, that showed that
pentamidine was not released from the complex, which seems to act itself by its own mechanism (Mbongo et al., in press). The IC₅₀ obtained on L. donovani LV9 amastigotes in macrophages were 3.5 μM for P2368, 14.7 μM for glucantime, 14.9 μM for pentamidine isethionate, 0.08 μM for amphotericin B and 26.2 μM for paromomycin. No significant synergistic effect was observed when P2368 was combined with these drugs on amastigotes in macrophages due probably to low penetration into macrophages in relation to drugs interaction. The in vitro results justified an in vivo evaluation.

Female BALB/c mice were infected with 10⁷ amastigotes of L. donovani LV9 by the retro-orbital sinus. The mice were randomly divided into groups of 10, one week after infection. Ir-(COD)-pentamidine alizarin red in DMSO and pentamidine isethionate in sterile water were given by the subcutaneous route or intraperitoneally at doses up to 232 mg/kg (50 mg pentamidine equivalent/kg) and 87 mg/kg respectively. Mice were dosed once per day for 5 consecutive days and sacrificed 3 days after the completion of treatment. Drug activity was estimated by counting the number of amastigotes/500 liver cells in Giemsa stained impression smears prepared from the weighed livers of treated and untreated mice (Neal and Croft, 1984).

P2368 was partially active at 37.5 μmol/kg per day x 5 (48% of parasite suppression) whereas pentamidine isethionate was toxic at this dose. Lower dose of pentamidine (18.7 μmol/kg per day x 5) was poorly active (11% of parasite suppression) when compared with reference drugs.

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (i.p.)¹</th>
<th>Staubert count × 10⁸ (± S.E.M.)</th>
<th>Percent of parasites suppression (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ir-(COT) -pentamidine</td>
<td>230 x 2b</td>
<td>150 x 2b</td>
<td>–</td>
</tr>
<tr>
<td>alizarin red</td>
<td>75.5 x 5</td>
<td>37.5 x 5</td>
<td>88.5 ± 17.3</td>
</tr>
<tr>
<td>Pentacarinat²</td>
<td>89 x 1b</td>
<td>150 x 1b</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>22 x 5b</td>
<td>37.5 x 5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>11 x 5</td>
<td>18.7 x 5</td>
<td>151.6 ± 45.1</td>
</tr>
<tr>
<td>Glucantime²</td>
<td>200 x 5</td>
<td>546 x 5</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>55 x 5</td>
<td>150 x 5</td>
<td>152.6 ± 31.2</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>Excipient only</td>
<td>170.1 ± 47.1</td>
</tr>
</tbody>
</table>

230 mg/kg of Ir-(COT)-pentamidine alizarin red corresponds to 50 mg pentamidine equivalent/kg.

¹ n = 10 Mice per group.

² Toxic dose which provokes death of mice.

³ Treatment with 0.2 ml excipient per day for 5 days.
suppression) and not toxic. The in vivo evaluation demonstrated the advantage of P2368 comparatively to pentamidine. In conclusion, the fact that synergism was observed between Ir-(COT)-pentamidine alizarin red and pentamidine isethionate suggests two possibilities: the existence of two different transport systems or two different targets for these compounds. The complex has therefore a specific action.
Fig. 4. Isobologram demonstrating synergistic action of compound P2368 and paromomycin acting alone and simultaneously on L. donovani DD8 promastigotes. The points show the IC$_{50}$ when compound P2368 paromomycin are used in combination.

which is different from that of pentamidine. Further studies will be directed to understand the absence of synergistic effect on infected macrophages.

One advantage of such complexes is their long half-life in mammal serum, which is responsible for an extended antiparasitic effect, even after administration of a single dose (Dreyfuss et al., 1993).

References


