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In vitro interaction between paromomycin sulphate and four drugs with leishmanicidal activity against three New World *Leishmania* species

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Objectives: To evaluate *in vitro* interactions between paromomycin sulphate and the antileishmanial drugs meglumine antimoniate, amphotericin B, miltefosine and azithromycin against intracellular *Leishmania* (*Leishmania*) *infantum chagasi*, *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* amastigotes in peritoneal mouse macrophages.

Methods: First, drug susceptibility was assessed in 3, 5 and 7 day assays, followed by drug interaction assays with a modified fixed-ratio method. An overall mean sum fractional inhibitory concentration ($\sum$FIC) was calculated for each combination and each *Leishmania* species. The nature of the interactions was classified as synergistic if the mean $\sum$FIC was $\leq$0.5, indifferent if the mean $\sum$FIC was >0.5–4.0 and antagonistic if the mean $\sum$FIC was >4.0.

Results: *In vitro* synergism was observed for the combinations of paromomycin plus miltefosine [at 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively)] and paromomycin plus amphotericin B (at the IC_{90} level) against *L.* (*L.*) *amazonensis*, paromomycin plus meglumine antimoniate (at the IC_{50} and IC_{90} levels) and paromomycin plus amphotericin B (at the IC_{50} level) against *L.* (*V.*) *braziliensis*, and paromomycin plus miltefosine, paromomycin plus amphotericin B (both at the IC_{90} level) and paromomycin plus azithromycin (at the IC_{50} level) against *L.* (*L*) *infantum chagasi*.

Conclusions: This work provides a preclinical dataset that supports future studies on multidrug treatment schedules against New World leishmaniasis.

Keywords: leishmaniasis, drug combinations, treatments, drug susceptibility tests

Introduction

The current options to treat cutaneous and visceral forms of leishmaniasis include pentavalent antimonials, amphotericin B (as deoxycholate or liposomal formulations), miltefosine and the aminoglycoside paromomycin.[1] Although this therapeutic arsenal is more promising than that which was available many decades ago, it is far from satisfactory due to its many disadvantages. Resistance to pentavalent antimonials, which have been the first-line drugs to treat cutaneous and visceral leishmaniasis, is now widespread in the Indian subcontinent.[2] New drug formulations such as amphotericin B, and its lipid formulations, and miltefosine have shown efficacy in treating leishmaniasis, but their high cost, parenteral administration, long term-therapy and potentially severe side effects limit the use of these drugs.[3,4]

In this context, drug combinations are strategic alternatives for antileishmanial treatment. Possible favourable outcomes of multidrug therapy include drug synergism and increased efficacies, reduced dosages and treatment durations with subsequent decreases in toxicities and costs and delays or prevention of drug resistance. Treatment schedules with drug combinations have been tested mainly against Old World visceral leishmaniasis, both in experimental models and in humans.[5–8]

Here we report on the *in vitro* interactions between paromomycin and four other drugs with leishmanicidal activity against three *Leishmania* species of medical relevance in the Americas. This study aimed to construct a database for a rational approach to the identification of useful paromomycin combinations in the treatment of New World cutaneous and visceral leishmaniasis. The choice of paromomycin as the fixed drug was based on the

following factors: reported clinical antileishmanial activity of this drug against cutaneous and visceral leishmaniasis caused by Old World *Leishmania* species;[4,5,7–9] the scarce reports of this drug in Latin America; and the feasibility of topical formulations of this drug for the treatment of cutaneous leishmaniasis, caused by New World species, that might be used in combination with systemic drugs.[10,11]

Materials and methods

Leishmania strains and the culture of promastigote and amastigote-like forms

Leishmania (*Leishmania*) *amazonensis* strain IFLA/BR/1967/PH-8, *Leishmania* (*Viannia*) *braziliensis* strain MHOM/BR/75/M2903 and *Leishmania* (*Leishmania*) *infantum chagasi* strain MHOM/BR/70/BH46 were used throughout the study. The strains were maintained by successive passages in golden hamsters (*Mesocricetus auratus*) and parasites were harvested from the spleens [*L.* (*L.*) *infantum chagasi*] or skin lesions [*L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis*] of infected animals.

Drugs

Miltefosine and paromomycin sulphate were kindly supplied by Zentaris GmbH (Frankfurt, Germany) and Antibioticos (Milan, Italy), respectively. Amphotericin B deoxycholate (Anforicin®) and azithromycin dehydrate (Zitromax®) were purchased from Cristalia (Itapira, Brazil) and Pfizer (Bedford, USA). Meglumine antimoniate (Glucantime®, Sanofi-Aventis Farmacêutica Ltd, Suzano, Brazil) was provided by the Brazilian Ministry of Health.

Peritoneal macrophage preparation, infection procedures and determination of 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}, respectively) of individual drugs

The determination of IC_{50} and IC_{90} values of individual drugs was performed using the amastigote–macrophage assay.[12] Incubation time and concentration ranges of azithromycin, meglumine antimoniate and miltefosine evaluated for each species of *Leishmania* were defined from previous studies.[12–14] Incubation time and the concentration ranges for amphotericin B and paromomycin were determined in preparation for this assay. The plates were incubated with drugs for 3, 5 and 7 days at 37°C in 5% CO_2. Further medium changes with fresh drugs were performed after 3 days in the 5 day assays and after 3 and 5 days in the 7 day assays. The assay results were analysed if at least 80% of the macrophages in the control wells were infected (assay quality control).

The results were obtained as ratios of the proportion of infection (amastigotes/100 macrophages) between the treated and non-treated cultures and were expressed as IC_{50} and IC_{90} as calculated by linear regression analysis (MiniTab 13.0) or linear interpolation (Microsoft Office Excel 2003) according to Huber and Koella.[15]

In vitro drug interaction assays

The interactions between the drugs were assessed using a modified fixed-ratio isobologram method according to Fivelman *et al.*[16] Predetermined IC_{50} values were used to decide the maximum concentrations of the individual drugs after ensuring that the IC_{50} values fell near the midpoint of a six-point 2-fold dilution series. The optimal time of action of the drugs in combination was chosen to establish a common time for the use of the two related drugs without prejudice to the actions of either.

The fractional inhibitory concentrations (FICs) and the sum FICs ($\sum$FICs) were calculated as follows: FIC of paromomycin sulphate = IC_{50} or IC_{90} of paromomycin sulphate in combination/IC_{50} or IC_{90} of paromomycin sulphate alone. The same was applied to the partner drug. $\sum$FIC was calculated as FIC of paromomycin sulphate + FIC of partner drug. FICs and $\sum$FICs were calculated for all fixed-ratio solutions. Overall mean $\sum$FICs were used to classify the nature of the interactions as synergistic if the mean $\sum$FIC was $\leq$0.5, antagonistic if the mean $\sum$FIC was >4.0 and indifferent (additive) if the mean $\sum$FIC was >0.5–4.0, according to Odds.[17]

Ethics

This study was conducted under a license approved by the Committee on Animal Research of the Oswaldo Cruz Foundation, Brazil (Protocol P321/06; License L0024/8).

Results

In vitro susceptibility of intracellular amastigotes to individual drugs

The individual IC_{50} and IC_{90} values that were calculated for different periods of drug exposure are given in Table 1 as μM and μg/mL concentrations. All *Leishmania* isolates were susceptible to the drugs. No toxicity towards macrophages was observed at the drug concentrations used in this study, based on morphological observations.

In vitro drug interactions against intracellular amastigotes in peritoneal macrophages

Interactions between paromomycin sulphate and other drugs were assessed with a modified fixed-ratio method, and the data were analysed at IC_{50} and IC_{90} levels. Table 2 shows the mean $\sum$FICs for three independent experiments at each level. For the associations between paromomycin sulphate and miltefosine and between paromomycin and amphotericin B against *L.* (*L.*) *infantum chagasi*, it was not possible to determine the FIC values at the IC_{50} level because the lowest concentrations in the drug combinations used inhibited >50% of growing parasites. Synergism was observed between paromomycin and miltefosine at the IC_{50} and IC_{90} levels and between paromomycin and amphotericin B at the IC_{90} level against *L.* (*L.*) *amazonensis*. For *L.* (*V.*) *braziliensis*, synergism was observed between paromomycin and meglumine antimoniate at the IC_{50} and IC_{90} levels and between paromomycin and amphotericin B at the IC_{50} level. For *L.* (*L.*) *infantum chagasi*, synergism was verified between paromomycin and miltefosine and amphotericin B, both at the IC_{90} level, and between paromomycin and azithromycin at the IC_{50} level.

Discussion

For the three tested species, there was no constant nature of interaction according to the IC_{50} and IC_{90} levels for some of the combinations. This was true for paromomycin plus amphotericin B against *L* (*L.*) *amazonensis* (synergy only at the IC_{90} level), paromomycin plus amphotericin B against *L.* (*V.*) *braziliensis* (synergy only at the IC_{50} level) and paromomycin plus miltefosine or paromomycin plus amphotericin B (both showed synergy only at the IC_{90} level) and paromomycin plus azithromycin (synergy only at the IC_{50} level) against *L.* (*L*) *infantum chagasi*. These results suggest that the modes of action of drug combinations against *Leishmania* spp. may depend on their concentrations. The same situation was

Table 1. Activity of individual drugs against intracellular amastigotes expressed as IC_{50} and IC_{90}

		L. (*L.*) *amazonensis*				*L.* (*V.*) *braziliensis*				*L.* (*L.*) *infantum chagasi*			
		IC_{50}		IC_{90}		IC_{50}		IC_{90}		IC_{50}		IC_{90}	
Drugs	Days	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL
Meglumine antimoniate, mol. wt 121.75	3	1365.9	166.3 (99.6–232.9)	2726.1	331.9 (189.7–474.1)	330.2	40.2 (34.6–45.8)	682.5	83.1 (71.3–94.8)	1416.0	172.4 (127.9–216.8)	2955.2	359.8 (279.1–440.5)
	5	1433.3	174.5 (130.9–218.2)	2822.2	343.6 (299.1–398.0)	307.2	37.4 (28.5–46.3)	568.4	69.2 (52.0–86.4)	1122.8	136.7 (85.0–188.0)	2457.5	299.2 (217.4–381.0)
	7	886.2	107.1[a]	3766.7	458.6[a]	178.2	21.7 (12.5–31.0)	544.6	66.3 (49.7–82.9)	398.4	8.5[a]	1362.6	165.9[a]
Miltefosine, mol. wt 407.57	3	3.2[b]	1.3[b] (0.7–1.9)	7.9[b]	3.2[b] (2.2–4.2)	5.4[b]	2.2[b] (1.5–2.9)	13.9[b]	5.7[b] (4.7–6.7)	4.4[b]	1.8[b] (0.7–3.0)	12.4[b]	5.0[b] (3.3–6.8)
	5	ND	ND	0.8	0.3[a]	2.0	0.8[a]	9.8	4.0[a]	6.9	2.8 (1.6–3.9)	13.5	5.5 (3.8–7.1)
	7	ND	ND	2.3	0.9[a]	0.8	0.3 (0.2–0.8)	2.2	0.9 (0.5–1.2)	ND	ND	1.5	0.6[a]
Paromomycin, mol. wt 615.63	3	217.4	133.8[a]	1569.8	966.4[a]	344.4	212.0 (161.1–262.9)	698.1	429.8 (324.8–534.7)	336.7	207.3 (149.8–264.8)	792.4	487.8 (364.1–611.5)
	5	77.0	47.4[a]	415.3	255.7[a]	253.4	156.0 (98.0–241.1)	494.5	304.4 (211.1–397.7)	64.3	39.6[a]	240.7	148.2
	7	ND	ND	193.8	119.3[a]	233.6	143.8 (75.9–211.6)	438.7	270.1 (180.0–359.9)	ND	ND	ND	ND
Amphotericin B, mol. wt 923.49	3	0.08	0.07 (0.05–0.09)	0.16	0.15 (0.11–0.19)	0.04	0.04 (0.01–0.06)	0.12	0.11 (0.09–0.13)	0.04	0.04 (0.03–0.05)	0.10	0.09 (0.07–0.10)
	5	0.04	0.04[a]	0.21	0.19[a]	0.02	0.02[a]	0.04	0.04[a]	0.03	0.03 (0.02–0.04)	0.08	0.07 (0.05–0.08)
	7	0.16	0.15[a]	0.01	0.01[a]	0.06	0.06 (0.04–0.09)	0.13	0.12 (0.09–0.15)	0.01	0.01[a]	0.03	0.03[a]
Azithromycin, mol. wt 748.98	3	28.4	21.3 (14.3–28.3)	56.0	42.0 (29.1–54.9)	5.7	4.3 (1.3–9.9)	20.6	15.4 (11.2–19.6)	19.9	14.9 (12.0–17.8)	40.9	30.6 (25.7–35.5)
	5	21.7	16.3 (10.3–24.2)	45.0	33.7 (27.4–43.0)	ND	ND	4.3	3.2[a]	9.7	7.3 (2.7–11.9)	31.0	23.2 (17.7–28.7)
	7	ND	ND	14.7	11.0	ND	ND	18.3	13.7 (3.3–24.1)	ND	ND	10.1	7.6 (1.7–13.4)

ND, not determined; it was not possible to determine the IC_{50} values because the lowest concentrations used inhibited >50% of growing parasites.
CI values are in brackets when the IC_{50} was calculated by linear regression analysis. CI values are only given for μg/mL.
[a]IC determined by linear interpolation.
[b]Data already published.[12]

Table 2. Mean ∑FICs of interactions between paromomycin sulphate and partner drugs against intracellular amastigotes

Leishmania spp.	Paromomycin sulphate + drug	Days of incubation	Mean ∑FIC ± SD at the IC_{50} level	Mean ∑FIC ± SD at the IC_{90} level	Nature of interaction[a]
L. (*L.*) *amazonensis*	meglumine antimoniate	5	0.68 ± 0.13	0.88 ± 0.26	indifferent – indifferent
	miltefosine	3	0.34 ± 0.20	0.35 ± 0.13	synergistic – synergistic
	amphotericin B	5	1.39 ± 0.17	0.46 ± 0.15	indifferent – synergistic
	azithromycin	5	0.64 ± 0.05	0.65 ± 0.17	indifferent – indifferent
L. (*V.*) *braziliensis*	meglumine antimoniate	7	0.29 ± 0.21	0.49 ± 0.25	synergistic – synergistic
	miltefosine	7	1.36 ± 1.52	1.14 ± 0.81	indifferent – indifferent
	amphotericin B	3	0.10 ± 0.08	0.95 ± 0.12	synergistic – indifferent
	azithromycin	3	0.78 ± 0.26	1.28 ± 0.14	indifferent – indifferent
L. (*L.*) *infantum chagasi*	meglumine antimoniate	5	0.50 ± 0.33	0.67 ± 0.19	indifferent – indifferent
	miltefosine	5	ND	0.06 ± 0.04	synergistic
	amphotericin B	5	ND	0.46 ± 0.20	synergistic
	azithromycin	5	0.48 ± 0.26	0.88 ± 0.26	synergistic – indifferent

ND, not determined; it was not possible to determine the FIC values at the IC_{50} level because the lowest concentrations in the drug combinations used inhibited >50% of growing parasites.
[a]∑FIC ≤0.5, synergistic; ∑FIC >4.0, antagonistic; ∑FIC >0.5–4.0, indifferent.[17]

observed in the assessments of drug associations for *Leishmania donovani* and *Plasmodium falciparum*, in which the interactions were classified from indifferent at the IC_{50} level to synergistic at the IC_{90} level, and from indifferent at the IC_{50} level to synergistic at the IC_{90} and IC_{99} levels, respectively.[6,18]

These *in vitro* experimental data indicate synergistic associations between currently used antileishmanial drugs and paromomycin sulphate against the three tested *Leishmania* species. At least two associations were synergistic against the three species evaluated: against *L.* (*L.*) *amazonensis*, paromomycin plus amphotericin B and paromomycin plus miltefosine; against *L.* (*V.*) *braziliensis*, paromomycin plus meglumine antimoniate and paromomycin plus amphotericin B; and against *L.* (*L.*) *infantum chagasi*, paromomycin plus meglumine antimoniate, paromomycin plus amphotericin B and paromomycin plus azithromycin.

Drug interactions were previously studied for *Leishmania* (*Leishmania*) *donovani*. Seifert and Croft[6] observed a synergistic interaction between miltefosine and sodium stibogluconate and indifference when miltefosine was combined with amphotericin B, paromomycin or sitamaquine. In another study, the interaction between miltefosine and amphotericin B was also considered to be indifferent.[6,19] Seifert *et al.*[20] evaluated the interactions between sitamaquine and five other antileishmanial drugs and found synergism between sitamaquine and pentamidine.

Although our *in vitro* study has provided evidence for synergistic and indifferent (additive) antileishmanial combinations, these data do not necessarily imply *in vivo* effectiveness and indifference, respectively. Seifert and Croft,[6] for example, did not observe *in vitro* synergism for the combinations of miltefosine and paromomycin or miltefosine and amphotericin B against *L.* (*L.*) *donovani*, but *in vivo* tests indicated enhanced activity of these same combinations. Thus, *in vivo* tests of antileishmanial drugs in combination against these three *Leishmania* species are currently ongoing.

This work provides an *in vitro* preclinical dataset that supports future studies addressing multidrug treatment schedules against New World leishmaniasis.

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Transparency declarations

None to declare.

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