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Original Article

Effect of Butionin-Sulfaximine and Fluphenazine as Trypanothione Inhibitory Drugs on Promastigotes and Axenic Amastigotes of *Leishmania Peruviana* and *Leishmania Braziliensis*

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ABSTRACT

Background: Leishmaniasis is a disease caused by the *Leishmania* parasite, which is difficult to diagnose, causes disfigurement and is difficult to treat. **Objectives:** To determine the effect of Butionin-Sulfaximine (BSO) and Fluphenazine on trypomastigotes and axenic amastigotes of *Leishmania peruviana* and *Leishmania braziliensis*. **Methods:** A study was performed with Butionin-Sulfaximine (BSO), Fluphenazine, and Glucantime (positive control,) utilizing respective concentrations of 41.7 mg/ml, 4.17 mg/ml, and 50 mg/ml for twenty-four hours on axenic amastigotes. **Results:** A significant difference (*P < 0.05) was found between the negative control group, Fluphenazine, and BSO within both the axenic amastigotes of *L. peruviana* (5.5 X 10^5 / ml for the negative control, 0.15 X 10^5 / ml for Fluphenazine, and 0.7 X 10^5 / ml for BSO) and *L. braziliensis* (6.9 X 10^5/ml for the negative control, 0.18 X 10^5/ml for Fluphenazine, and 0.22 X 10^5/ml for BSO). Another significant difference (*P < 0.05) was found in the promastigotes of *L. peruviana* (5.9 X 10^5 / ml for the negative control, 0.66 X 10^5 / ml for Fluphenazine, and 3.1 X 10^5 / ml for BSO) and *L. braziliensis* (8.7 X 10^5/ml for the negative control and 5.68 X 10^5/ml for Fluphenazine). **Conclusions:** From this, we conclude Fluphenazine and BSO present promising antiparasitic effects against axenic amastigotes of *L. peruviana* and *L. braziliensis* in both pharmacological tests of the *in vivo* model and their potential future use.

Key words: Butionin-Sulfaximine, Fluphenazine, *Leishmania*, Axenic Amastigote.

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INTRODUCTION

Leishmaniasis is a chronic disease caused by certain parasites of the *Leishmania* genus, being difficult to treat due it not being easily diagnosable, the use of medication with considerable adverse effects, the development of stigmas against those carrying it, and the co-infections that present themselves in the progression of the disease.[1]

The evolutive (epidemiological) clinical classification of integumentary leishmaniasis in Peru is described as: cutaneous andean leishmaniasis (known colloquially as "Uta") cutaneous-mucosal andean leishmaniasis, cutaneous jungle leishmaniasis, and Cutaneous-mucosal jungle leishmaniasis (known colloquially as "Espundia.") Depending on the evolution time of the disease, it is either classified as early (less than a year) or late (over a year).[2]

The clinical form of the signs and symptoms caused by *Leishmania Peruviana* is known as "UTA".[3,4] In the case of *Leishmania braziliensis*, these cause destructive degeneration on both the cutaneous and mucosal level, with this species also being the most related to antimonial resistance.[3,4] The most common of these are Sodium Stibogluconate and Glucantime, acting as a frontline against the disease. These are highly nephrotoxic and hepatotoxic, a cause of aplastic anemia, and are not guaranteed to cure the patient *via* their usage. For these reasons, patient adherence to a plan is not simple.[3]

The use of drugs against *Leishmania* sp., usually *in vitro*, is carried out in intracellular amastigotes that are difficult to maintain or by using promastigotes, with the latter model being very different from the phase of the parasite in humans, therefore producing results that cannot be extrapolated to the actual situation that arises due to the disease.[5-7]

The enzymes Trypanothione Spermidine and Trypanothione Reductase are unique to kinetoplastid parasites, and could therefore be used as potential drug targets.[8,9] Trypanothione Reductase is homodimeric, composed of three domains dependent on the unique trypanothione in *Leishmania* and *Trypanosoma*, and is essential to the parasite as an antioxidant molecule. The metabolic process of the enzyme could serve as a specific therapeutic target against flagellate parasites.[10]

There are drugs, such as chlorpromazine, that have been tested with certain degrees of specificity in Trypanothione Reductase despite their considerable adverse effects.[8,11-14] In this sense, it is possible that Butionin-Sulfaximine (BS) could specifically inhibit the Trypanothione enzyme within the parasite.[11-14] According to molecular studies, both Fluphenazine and Perphenazine could also have specific effects against flagellate parasites, acting in the inhibition of Trypanothione Reductase.[12]

Although antimonials are the drugs of choice to treat the different clinical forms of leishmaniasis, various side effects can be observed from the usage of these medications in therapy, which leads to treatment abandonment by part of the patient in many cases.[5-7] As such, there is a need to seek new metabolites that are more effective in treating the

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disease. Previous studies have shown that, in order to extrapolate *in vitro* tests of antileishmanicidals, models in which the parasite is under conditions similar to which it is found in when infecting human beings (Amastigote).[6,7] In this sense, the use of "Promastigotes" of very different form and function from the parasite found in mammals may be used as a comparison group.

The use of intracellular amastigotes is highly costly and complex, which is why their use is limited.[7] With this in mind, *in vitro* axenic amastigotes have been developed. These are free-living parasites living in artificial culture mediums with similar form and function to the parasites found in human cells, making them very useful in identifying new drugs and/or antiparasitic substances.

Our study utilized parasite reductive enzyme-inhibiting drugs that have reported fewer side effects compared to on-market antimonials that have already had their bioavailability, bioequivalence, and adverse effects evaluated. The objective of our study was to figure out the antiparasitic effects of Butionin-Sulfaximine (BSO) and Fluphenazine on axenic amastigotes of *Leishmania peruviana* and *Leishmania braziliensis*.

MATERIAL AND METHODS

Obtaining axenic amastigotes and promastigotes

The promastigotes of reference strains HB86 (L. *peruviana*) and LC53 (L. *braziliensis*) were obtained through a donation from the Trypanosomatid laboratory of the Universidad Peruana Cayetano Heredia and were kept *in vitro* at 25 C° in an RPMI medium during transport Subsequently, they were transformed into axenic amastigotes by adjusting the pH to 4.7 and the temperature to 35 C°, producing the axenic amastigote model. The transformation conditions in Schneider's medium were based on previous studies,[7,15] properly adjusting pH and temperature conditions.

Use of Butionin-Sulfaximine (BSO) and Fluphenazine as Trypanothione inhibitory drugs on promastigotes and axenic amastigotes of *Leishmania peruviana* and *Leishmania braziliensis*

Axenic amastigotes in log phase growth on the third day were seeded in 96-well culture microplates: 120 µl for the control group and in a proportion of 20 µl / 100µl of drug with respect to the Schneider medium, with an initial inoculum of 2.93 X 10^5/ml for *L. peruviana* and 2.25 X 10^5/ml for *L. braziliensis*. Butionin-Sulfaximine (BSO), Fluphenazine, and Glucantime (Positive Control) were used at concentrations of 41.7 mg/ml, 4.17 mg/ml, and 50 mg/ml respectively for twenty-four hours, based on previously reported therapeutic doses.[11-14] Additionally, 120 µl of Schneider's medium was used. The cultures were kept at a temperature of 35° C at a pH of 4.7 Butionin-Sulfaximine (BSO) was chosen for its inhibitory effects on Trypanothione Reductase (TR) in *Leishmania*, while Fluphenazine was chosen for its weak/moderate sedative effect and its probable antiparasitic effect as a tricyclic compound that presumes a molecular mechanism on the TR enzyme of the parasite.[11,12] Each study group had five statistical repetitions.

Promastigotes in log phase growth on the third day were seeded in 96-well culture microplates: 120 µl for the control group and in a proportion of 20 µl / 100µl of drug with respect to the Schneider medium, with an initial inoculum of 1.74 X 10^5/ml for *L. peruviana* and 1.20 X 10^5/ml for *L. brazilienses*. Butionin-Sulfaximine (BSO), Fluphenazine, and Glucantime (Positive Control) were used at concentrations of 41.7 mg/ml, 4.17 mg/ml, and 50 mg/ml respectively for twenty-four hours, based on previously reported therapeutic doses. Additionally, 120 µl of Schneider's medium was used. The cultures were kept at a temperature of 35° C at a pH of 4.7. Butionin-Sulfaximine (BSO) was chosen for its inhibitory effects on Trypanothione Reductase (TR) in *Leishmania*, while Fluphenazine was chosen for its weak/moderate sedative effect and its probable antiparasitic effect as a tricyclic compound that presumes a molecular mechanism on the TR enzyme of the parasite.[11,12] Each study group had five statistical repetitions.

Statistical analysis

A one-way analysis of variance (ANOVA) with a significance level of 95%, $P < 0.05$, was performed for a comparison between the drugs used and the control groups for both axenic amastigotes and promastigotes.

RESULTS

A significant difference (*$P < 0.05$) was found between the negative control group, Fluphenazine, and BSO within both the axenic amastigotes of *L. peruviana* (5.5 X 10^5 / ml for the negative control, 0.15 X 10^5 / ml for Fluphenazine, and 0.7 X 10^5 / ml for BSO) and *L. braziliensis* (6.9 X 10^5/ml for the negative control, 0.18 X 10^5/ml for Fluphenazine, and 0.22 X 10^5/ml for BSO).

Another significant difference (*$P < 0.05$) was found in the promastigotes of L. *peruviana* (5.9 X 105 / ml for the negative control, 0.66 X 105 / ml for Fluphenazine, and 3.1 X 105 / ml for BSO) and L. *braziliensis* (8.7 X 105/ml for the negative control and 5.68 X 105/ml for Fluphenazine).

DISCUSSION

Our study on axenic amastigotes presented a significant difference between the control group, Fluphenazine, and Butionin-Sulfaximine

Table 1: Axenic amastigotes against evaluated drugs.

Leishmania peruviana				*Leishmania brasilienses*			
Control	Glucantime 50mg/ml	Fluphenazine 4,17mg/ml	Butionin-Sulfaximine 41,7mg/ml	Control	Glucantime 50mg/ml	Fluphenazine 4,17mg/ml	Butionin-Sulfaximine 41,7mg/ml
5,50 +/- 1.62 x 10^5/ml	*0.36 +/-0.18 x 10^5/ml	*0.15 +/-0.05 x 10^5/ml	*0.70 x 0.72 x 10^5/ml	6,9 +/- 1.42 x 10^5/ml	*0,18 +/- 0.05 x10^5/ml	*0,18 +/-0.00 x10^5/ml	*0,22 x 0.00 x10^5/ml

All values are expressed as mean (10^5/ml) ± SD (n = 5); Unimodal Analysis of Variance (ANOVA). *$P < 0.05$, significant when compared to control.

Table 2: Promastigotes against evaluated drugs.

Leishmania peruviana				*Leishmania brazilienses*			
Control	Glucantime 50mg/ml	Fluphenazine 4,17mg/ml	Butionin-Sulfaximine 41,7mg/ml	Control	Glucantime 50mg/ml	Fluphenazine 4,17mg/ml	Butionin-Sulfaximine 41,7mg/ml
5,9 +/-2.63 x10^5/ml	*0,76 +/- 0.25 x10^5/ml	*0,66 +/- 0.36 x10^5/ml	*3,1 +/- 0.55 x10^5/ml	8,7 +/- 1.47 x10^5/ml	*0,80 +/- 0.12x 10^5/ml	*5,68 +/-2.28x 10^5/ml	6,80 +/- 1.23 x10^5/ml

All values are expressed as mean (10^5/ml) ± SD (n = 5); Unimodal Analysis of Variance (ANOVA). *$P < 0.05$, significant when compared to control.

at concentrations of 4.17mg/ml and 41.7mg/ml respectively. Fluphenazine, known commercially as MODECATE, contains fluphenazine decanoate as an active ingredient, which is used to treat cases of schizophrenia and paranoid psychosis.[16] It is interesting to note that, despite using a dose that is six times less (4.17mg/ml) than that of the tolerable pharmacological dose in mental disorder cases (25mg/ml),[16] the drug showed a marked antiparasitic effect in axenic amastigotes on a magnitude of 36.37 x $x10^5$ for *L. peruviana* and 38.33 x105 for *L. braziliensis*, higher than that of the negative controls. When Fluphenazine was compared to Glucantime (positive control) the effect of fluphenazine was greater by 2.4 $x10^5$ in the case of L. *peruviana*, with *L. braziliensis* being similar *when compared with the positive control* despite the fact that Glucantime was used at 50mg/ml with a value 2500 times higher than what is used at a therapeutic level equivalent to 20mg/kg~0.02mg/ml.[17] An additional item of note is that a single dose of Fluphenazine at 25mg/ml could affect mental disorders for up to four weeks.[16] Given this, the results showed a drug with the potential to be tested at even higher doses presuming a considerable antiparasitic effect, with prolonged effects depending on the pharmacokinetics of the drug.[16] In the case of BSO, the antiparasitic effect was greater in magnitudes of 7.86 $x10^5$ for *L. peruviana* and 31.36 $x10^5$ for *L. braziliensis* when compared to the negative controls.

In the case of promastigotes, a statistically significant difference was shown between the negative control group and Fluphenazine and Butionin-Sulfaximine at concentrations of 4.17mg/ml and 41.7mg/ml respectively in the case of *L. peruviana* and only for Fluphenazine at 4.17 mg/ml for *L. braziliensis*. The drug Fluphenazine showed a marked antiparasitic effect in the promastigotes with a magnitude 8.94 x 10^5 greater for *L. peruviana* and a magnitude greater of 1.53 $x10^5$ for *L. braziliensis* when compared to the negative controls. As observed, Fluphenazine had a lesser antiparasitic effect in promastigotes than in axenic amastigotes. In the case of BSO, when compared to the negative controls, the antiparasitic effect was greater by a magnitude of 1.91 $x10^5$ for *L. peruviana* with no statistically significant difference for *L. braziliensis, observing* a lesser or no antiparasitic effect of BSO against promastigotes compared to axenic amastigotes. Even though BSO was used at a magnitude 189.55 times greater with respect to the antiparasitic effect that was used in a previous study with an anti-trypanosomatid effect used at 200mg/kg equivalent to ~ 0.22 mg/ml, the antiparasitic effects were less than the effects of Fluphenazine.[18] The differentiated effect of the drugs used against promastigotes and axenic amastigotes may be due to the differing metabolome of the two parasitic stages.[6,7] Therefore, it is better to use a model close to that which develops in humans in the amastigote stage. In addition, the drugs used in this study are market-approved and are specifically tailored to inhibit the Trypanothione Reductase of the parasite, which means that they are easy to use.

According to what was observed in the axenic amastigotes, the antiparasitic effect of fluphenazine was significant against those of L. *peruviana* and L. *braziliensis*. The antiparasitic effect was also significant, the case of BSO, although to a lesser extent. In regard to the antiparasitic effect against promastigotes of *L. peruviana* and *L. braziliensis,* Fluphenazine presented a significant effect, although lesser in value when compared to that which it showed in axenic amastigotes. In regard to BSO, it only presented a significant antiparasitic effect against *Leishmania peruviana*.

The limitations of our study were based on the use of models such as the axenic amastigote and promastigotes that are different from the infective phase in humans, which is the intracellular amastigote. Therefore, future studies on intracellular amastigotes are necessary.

CONCLUSION

Based on what has been stated, it is concluded that Fluphenazine at sub-therapeutic doses demonstrated significant antiparasitic effects on both axenic amastigotes and promastigotes of *L. peruviana* and *L. braziliensis*. In the case of BSO, the antiparasitic effect was significant in the axenic amastigotes of both *L. peruviana* and *L. braziliensis* and the promastigotes of L. *peruviana*. As such, the use of fluphenazine as a specific antiparasitic drug for use against L. *peruviana* and L. *braziliensis* in *in vivo* models is promising, and complementary studies should be carried out, such as those on mean doses and collateral effects.

AUTHOR CONTRIBUTIONS

Data curation, JRJ, MMG, AMZ; project administration, JRJ, MMG; supervision, JRJ; writing—original draft, JRJ, MMG, AMZ.; writing—review and editing, JRJ. All authors have read and agreed to the published version of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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