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# In vitro effects of purine and pyrimidine analogues on *Leishmania donovani* and *Leishmania infantum* promastigotes and intracellular amastigotes

Samira Azzouz<sup>1,2</sup> and Philippe Lawton<sup>1,2\*</sup>

<sup>1</sup>Université de Lyon, Université Claude-Bernard Lyon I, ISPB-Faculté de Pharmacie, Lyon, France;

<sup>2</sup>Institut de recherche pour le développement (IRD), UMR InterTryp IRD/CIRAD, campus international de Baillarguet, Montpellier, France

## Abstract

Inhibition of parasite metabolic pathways is a rationale for new chemotherapeutic strategies. The pyrimidine and purine salvage pathways are thus targets against *Leishmania donovani* and *L. infantum*, causative agents of visceral human leishmaniasis and canine leishmaniosis. The antiproliferative effect of the pyrimidine analogues Cytarabine and 5-fluorouracil and of the purine analogues Azathioprine and 6-mercaptopurine was evaluated in vitro on the promastigote and the intracellular amastigote stages of the parasite. Cytarabine and 5-fluorouracil were the best inhibitors against promastigotes, whereas 5-fluorouracil and azathioprine displayed the best efficacy against the amastigote stage. The ultrastructural study showed an important cytoplasmic vacuolization and with azathioprine and 5-fluorouracil, a mitochondrial swelling and appearance of autophagosome-like structures. Alterations of the kinetoplast were also observed with 5-fluorouracil, all these damages eventually resulting in an autolysis process that triggered the subsequent death of the intracellular parasites.

## Keywords

*L. donovani*, *L. infantum*, purine analogues, pyrimidine analogues, antiproliferative effect, ultrastructural modifications

## Introduction

Leishmaniasis is a disease caused by a number of species of protozoa of the genus *Leishmania*. Of the four major clinical types of this infection, visceral leishmaniasis or Kala-azar caused in humans by *L. donovani* and human visceral and canine leishmaniosis caused by *L. chagasi/infantum* are the most severe. The first-line therapies against visceral leishmaniasis are the two pentavalent antimonial compounds, sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), while pentamidine isethionate and amphotericin B desoxycholate are the two second-line agents (Monge-Maillo and López-Vélez 2013, Sundar and Chakravarty 2013). Among their numerous drawbacks, they require parenteral administration, they have significant toxic side effects and widespread resistance to them has been reported mainly in India, Kenya and Sudan (Croft and Olliaro 2011). Despite its effectiveness and its safety, liposomal amphotericin B is costly (Balasegaram *et al.* 2012). The development of the parasite's resistance to current drugs and with the exception of liposomal amphotericin B, their lack of efficacy against VL/HIV co-infections has emphasized the need for new

drugs. Miltefosine inhibits the phospholipid and sterol biosynthesis in Trypanosomatids and is effective in vivo against *Leishmania*, even by the oral route. However, its long half-life and its teratogenic potential are important drawbacks, as development of drug resistance may eventually lead to a premature inefficacy of this very important drug (Dorlo *et al.* 2012, Pachioni *et al.* 2013).

A rational discovery of novel antiparasitic drugs should be based on parasite-specific metabolisms (Vincent and Barrett 2015). Pyrimidine biosynthesis is a vital biological process, achieved both by *de novo* synthesis and by pyrimidine salvage pathways and enzymes of this pathway are considered as potential drug targets. Like most parasitic protozoa, *Leishmania* is also an obligate purine auxotroph and hence depends on its own purine salvage pathway that has also been considered as a target for novel treatments against leishmaniasis. The purine analogue allopurinol alone or in combination with other drugs has been proven to be effective against canine leishmaniosis (Farca *et al.* 2012), but its pharmacokinetic properties are a major limitation to its use for treatment of human leishmaniasis (Croft *et al.* 2006).

\*Corresponding author: philippe.lawton@univ-lyon1.fr

In the present work, we tested drugs used against other diseases in humans, two purine analogues – 6-mercaptopurine and azathioprine – and two pyrimidine derivatives – cytarabine and 5-fluorouracil – on the promastigote and amastigote stages of *L. donovani* and *L. infantum*, two agents of visceral diseases that exhibit a clinical polymorphism. As a purine analogue and a drug used against canine leishmaniasis, allopurinol was used as a control.

## Materials and Methods

### Drugs

The control drugs Glucantime and allopurinol, the pyrimidine analogues 5-fluorouracil, cytarabine (Cytosine  $\beta$ -D-arabinofuranoside) and the purine analogues azathioprine and 6-mercaptopurine were purchased from Sigma Aldrich (St. Quentin Fallavier, France). They were dissolved in DMSO and 1mM stock solutions were made extemporaneously by dilution with culture medium. Serial dilutions ranging from 0.1 to 100  $\mu$ M were performed in the relevant culture media.

### Cytotoxicity tests

Mouse peritoneal macrophages J774A.1 (ECACC, 91051511) were cultured in RPMI 1640 (Sigma Aldrich, St. Quentin Fallavier, France). When the cells had developed a monolayer, they were incubated in 96-well plates for 4 hours with 100  $\mu$ L of the drugs at concentrations ranging from 0.1 to 100  $\mu$ M with a maximum of 0.1% (v/v) DMSO. The cell viability was assessed after 24 hr, 48 hr and 72 hr using a methyl-tetrazolium salt (MTS)/phenazine methosulphate (PMS) solution (CellTiter 96<sup>®</sup>, Promega, Madison, WI, USA). The absorbance of the formazan product was measured at 490 nm with a Multiskan EX plate reader (Thermo Fisher Scientific, Villebon sur Yvette, France). The IC<sub>50</sub> values were calculated from the drug concentration-response curves.

### Promastigote culture and assays

The *Leishmania donovani* strain LCR-133 (*Leishmania* Reference Center, Jerusalem, Israel) used in this study was isolated in 1967 from a human case of Kala-azar in Behenber (Ethiopia) and the *L. infantum* strain was isolated from an infected dog from Spain. The promastigotes were grown in 75 cm<sup>2</sup> culture flasks at 22°C in TC-199 medium supplemented with 20% heat-inactivated foetal bovine serum and 2% penicillin-streptomycin (PAA, Vélizy Villacoublay, France). At the end of the exponential growth phase, the cultures were centrifuged at 1500 rpm for 15 min, the promastigotes were diluted at  $1 \times 10^6$  parasites/ml and 200  $\mu$ L of the suspension were distributed in duplicates into 96 multi-well plates with the analogues at final concentrations ranging from 0.1 to 100  $\mu$ M. The inhibition of promastigote growth was deter-

mined with a Leica DM4000B microscope at a  $\times 100$  magnification using a Malassez haemocytometer after 4 and 24 hr. The growth inhibition was calculated using the following formula:  $I = 100 - [T/C \times 100]$ , where I = percentage of inhibition, C = number of parasites in the control, T = number of parasites in the treated wells.

### Amastigote assay

To test the activity of the nucleoside analogues on the intracellular amastigote stage of the parasite, J774A.1 macrophages were cultured in triplicates on sterile glass coverslips in 24-well plates at a mean cell density of 75,000 cells/well. After adhesion of the cells, the medium was removed and replaced with fresh medium containing the *Leishmania* promastigotes at a 10 parasites per cell ratio. After 16 hrs at 37°C, the medium was removed, the infected cultures were washed 3 times with serum-free RPMI medium and incubated for 4 hrs with the various compounds at final concentrations ranging from 1 to 50  $\mu$ M. The cultures were then washed with fresh medium and subsequently allowed to grow for 24, 48 and 72 hrs. The coverslips were removed, fixed with methanol and stained with Giemsa. The intracellular amastigotes were counted by light microscopy and the percentage of inhibition was calculated as above. A minimum of 500 cells was counted in each replicate. Control cultures were infected as described above but without the drugs. All the experiments were repeated at least 3 times.

### Statistical analysis

The Prism<sup>®</sup> 6 software (Graph Pad Software, Berkeley, CA, USA) was used to calculate the half maximum inhibitory concentrations (IC<sub>50</sub>) by nonlinear regression and to evaluate the statistical significance of the growth inhibition.

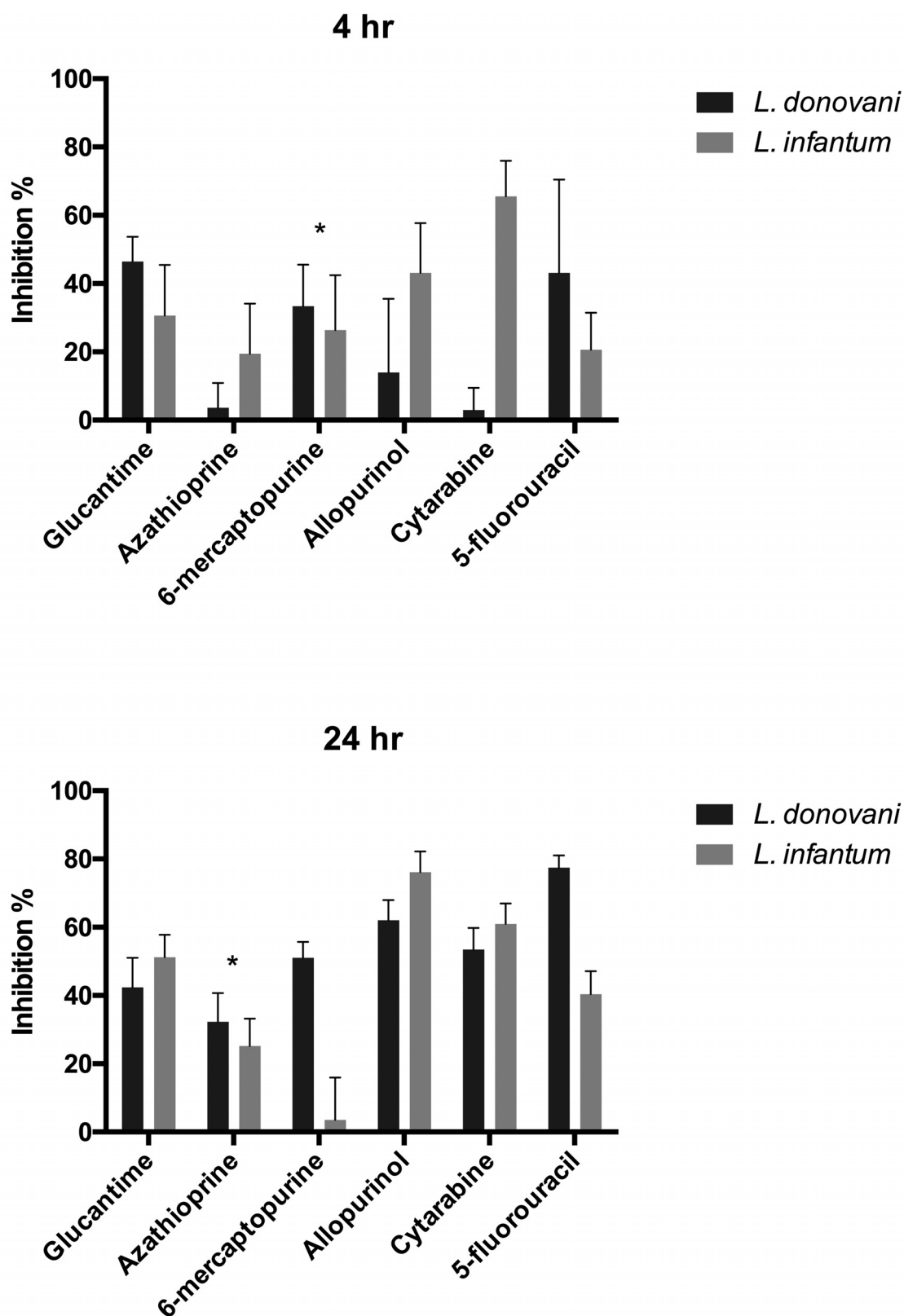
### Transmission electron microscopy (TEM) of infected macrophages

After 24 hr, control and treated cultures were fixed with 4% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, post-fixed with 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, dehydrated in a graded series of acetone and embedded in Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Jeol 1400 JEM transmission electron microscope (Tokyo, Japan) set at 80 kV.

## Results

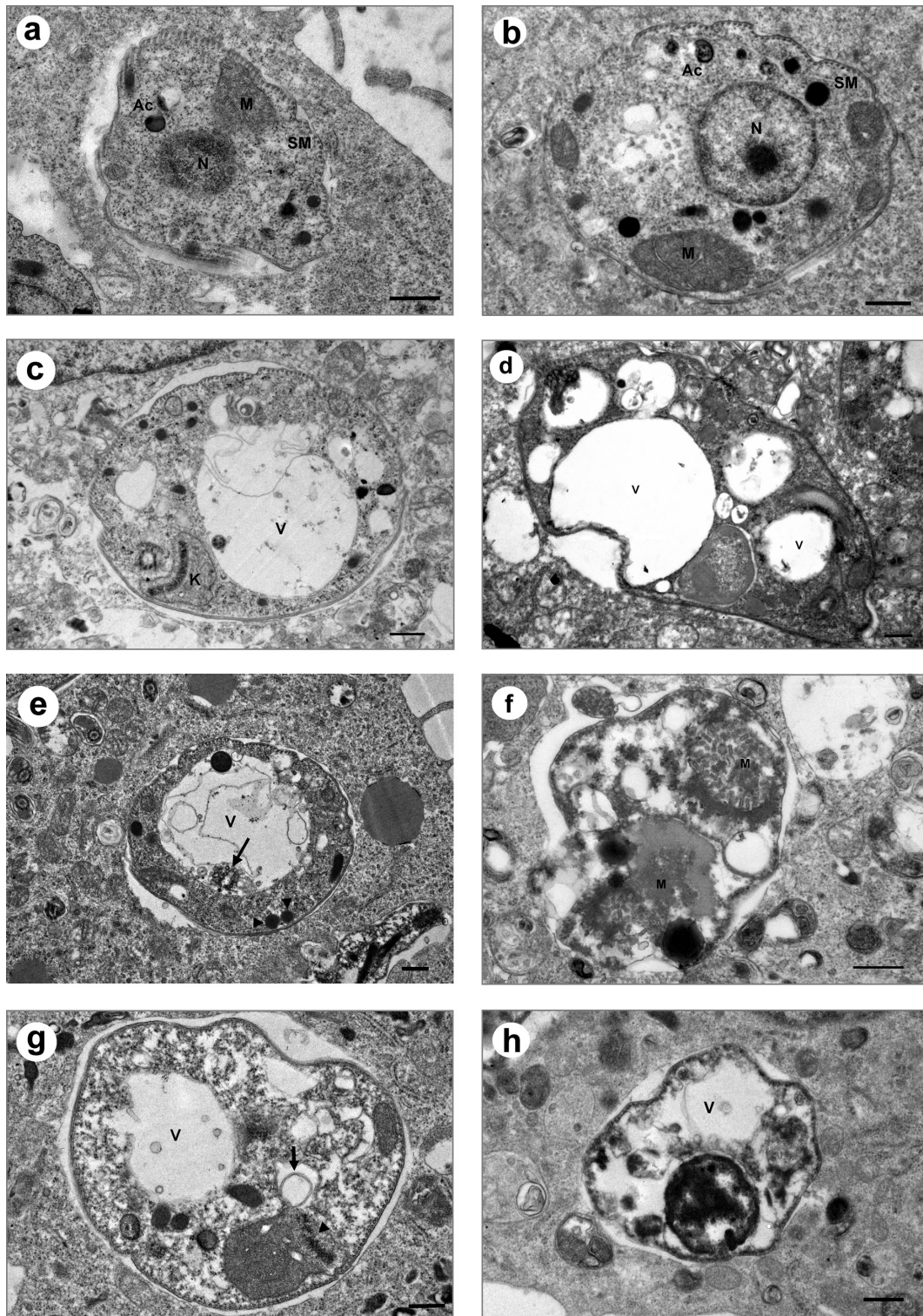
### Cytotoxicity

The cell viability decreased proportionally with the time of incubation with Amphotericin B, the only toxic control drug and



**Fig. 1.** Example of the differential inhibitory effects of the analogues (50  $\mu$ M) on *L. donovani* and *L. infantum* promastigotes. The parasites at a starting concentration of  $0.8 \times 10^6$ /ml were incubated for 4 and 24 hrs with the nucleoside analogues. The final parasite concentrations in the control wells were  $2.1 \pm 0.23 \times 10^6$ /ml for *L. donovani* and  $0.93 \pm 0.1 \times 10^6$ /ml for *L. infantum*. Results of three separate experiments. \*: no significant difference ( $p > 0.05$ ) between the *L. donovani* and *L. infantum* strains





**Fig. 2.** Ultrastructural alterations caused by a 4 hr-incubation of intracellular amastigotes with 10  $\mu$ M allopurinol, azathioprine and 5-fluorouracil after 24 hrs. (a, b) Controls: *L. donovani* (a) and *L. infantum* (b) intracellular amastigotes showing the nucleus (N), a layer of sub-pellicular microtubules (SM), the mitochondrion (M) and acidocalcisomes (AC). (c, d) Allopurinol: extensive vacuolization (V) with a normal kinetoplast (K) in *L. donovani* (c) and *L. infantum* (d) in the presence of the control drug. (e, f) Azathioprine: presence of lipid bodies (arrowheads), multivesicular material inside the flagellar pocket (arrows) in *L. donovani* (e) and disruption of the mitochondrion (M) in *L. infantum* (f) amastigotes. (g, h) 5-fluorouracil: autophagosome-like structures (arrow) and alteration of the kinetoplast (arrowhead) in *L. donovani* (g) and cell autolysis in *L. infantum* (h). Bar: 0.5  $\mu$ m

**Table I.** Cytotoxicity of the analogues for J744-A1 macrophages. The means  $\pm$  SD of the IC<sub>50</sub> values are given in  $\mu$ M

		24 hr	48 hr	72 hr
Controls	Amphotericin B	5.4 $\pm$ 1.1	3.6 $\pm$ 0.7	2.2 $\pm$ 0.4
	Glucantime	42.4 $\pm$ 0.4	>100	>100
	Allopurinol	>100	>100	>100
	6-mercaptopurine	>100	>100	>100
	Cytarabine	>100	>100	>100
	Azathioprine	>100	>100	>100
	5-fluorouracil	>100	>100	>100

the other compounds displayed IC<sub>50</sub> values higher than 100  $\mu$ M, glucantime being slightly more toxic (Table I).

### In vitro antiproliferative effects on *Leishmania donovani* and *L. infantum*

#### Effect on the promastigote stage

Direct observation of treated promastigotes by light microscopy showed that they lost their motility. Generally, the compounds' inhibitory effect began at the onset of incubation and after 4 hr, a difference in sensitivity could be seen between the strains tested, cytarabine being much more active on the canine *L. infantum* strain (Fig. 1). There was a significant difference between the two strains after 24 hrs, except for azathioprine. Among the control compounds, allopurinol displayed a far better inhibitory activity than glucantime.

#### Effect on the amastigote stage

The antiproliferative effect on amastigotes is shown in Table II. In the control cultures, the parasite load amounted approximately to 60% at 24 hrs and was still 40% at 72 h. Among the control drugs, allopurinol was the most potent. On both strains, only 5-fluorouracil and azathioprine were inhibitory, whereas the other compounds were ineffective.

**Table II.** Effect of the analogues against intracellular *L. donovani* and *L. infantum* amastigotes. The means  $\pm$  SD of the IC<sub>50</sub> values are given in  $\mu$ M. The infected macrophages were incubated for 4 hrs with the drugs and their inhibitory activity was evaluated after 72 hrs

IC <sub>50</sub> ( $\mu$ M)	<i>L. donovani</i>	<i>L. infantum</i>
Glucantime	26.7 $\pm$ 8.5	1.0 $\pm$ 0.1
Allopurinol	17.0 $\pm$ 0.7	0.6 $\pm$ 0.3
Azathioprine	1.9 $\pm$ 0.7	2.8 $\pm$ 1.4
6-mercaptopurine	>100	>100
Cytarabine	>100	>100
5-Fluorouracil	1.0 $\pm$ 0.2	3.0 $\pm$ 1.3

### Effect of azathioprine and 5-fluorouracil on the amastigote ultrastructure

Since 5-fluorouracil and azathioprine were the most effective against this stage, their effect on the morphology of the intracellular amastigotes was studied (Fig. 2). In the treated amastigote-infected cells, the intracellular parasites displayed severe structural damages. In the presence of allopurinol, an extensive vacuolization of the cytoplasm was observed and in addition, the presence of lipid bodies and mitochondrial swelling with loss of the matrix content was found in azathioprine-treated cultures. In 5-fluorouracil-treated amastigotes, autophagosome-like structures were observed together with alterations of the kinetoplast structure that led to autolysis of the parasites.

## Discussion

The combination of Glucantime and of the purine analogue allopurinol is considered so far to be the most effective therapy for canine leishmaniasis (Solano-Gallego *et al.* 2011), which is not the case in humans, due to pharmacokinetic properties (Croft and Olliaro 2011). Infection of the mammalian host depends on the metacyclic promastigote form and it has been reported recently that metacyclogenesis is highly influenced by nucleosides, especially adenosine (Serafim *et al.* 2012). In purine stress conditions, *Leishmania* parasites maximize salvage by up-regulating specific enzymes and transporters of the purine metabolic pathway (Carter *et al.* 2010, Martin *et al.* 2014). Although resistance mechanisms to purine starvation allow parasite survival, this pathway is a target of choice for rational drug design (De Koning *et al.* 2005, Vincent and Barrett 2015). For instance, the nucleoside analogues immucillins reduced in vitro the replication of *L. infantum* intramacrophagic amastigotes (Freitas *et al.* 2015). The pyrimidine nucleoside analogue 5-fluorouracil is converted in vivo into the active metabolite 5-fluoroxuridine monophosphate (F-UMP) that incorporates into RNA, thereby inhibiting cell growth. Despite the ability of *Leishmania* parasites to both biosynthesize and salvage pyrimidines from their host environment, *L. donovani* mutants deficient in the enzyme of pyrimidine salvage LdUPRT, were hypersensitive to high concentrations of uracil, 5-fluorouracil



and 4-thiouracil (Soysa *et al.* 2013). We thus chose to test some purine and pyrimidine analogues for their ability to inhibit in vitro the multiplication of both stages of *L. donovani* and *L. infantum*.

The tested compounds and the control drug allopurinol showed a low cytotoxicity on macrophages. The higher cytotoxicity was found with amphotericin B, which was not subsequently used, and to a much lesser extent, with glucantime.

The antiproliferative activity tests on promastigotes evidenced that cytarabine and the control drug allopurinol were the most effective after 24 hrs, compared to Glucantime. It is interesting to note that, except for cytarabine and allopurinol, the promastigotes of *L. donovani* were generally more sensitive than those of *L. infantum*. Azathioprine is a prodrug metabolized into 6-mercaptopurine and in humans, glutathione S-transferase (GST) is involved (Eklund *et al.* 2006). In promastigotes, involvement of the trypanothione-based redox metabolism could be possible (Leroux and Krauth-Siegel 2015). It is not surprising that its effect seemed delayed compared to the other analogues, while the effect of its active metabolite 6-mercaptopurine was observable at the beginning of incubation. The pyrimidine analogue 5-fluorouracil was generally more active on *L. donovani* than on the *L. infantum* canine strain, while it was the contrary for cytarabine.

When the analogues were incubated with intracellular amastigotes, cytarabine and 6-mercaptopurine were ineffective, whereas 5-fluorouracil and azathioprine displayed the highest activity, with IC<sub>50</sub> values lower than 10 µM. The possibility that the inefficient compounds could be metabolized before reaching the intracellular parasite might thus be raised. The inhibitory effect was observable after 72 hrs, but incubation for 4 hrs is closer to what happens in vivo and in vitro, where exogenous purines are metabolized within 6 hrs in the culture medium (Lawton *et al.* 2003). This emphasizes that in vitro inhibition tests should always be performed at first on the amastigote stage, the molecules displaying varying activities according to the insect or the mammalian stages and to their extra- or intracellular status and their respiratory metabolism (Mondal *et al.*, 2014).

Transmission electron microscopy evidenced global alterations of the intracellular amastigotes after treatment with the analogues. Allopurinol produced an intense cytoplasm vacuolization, but in addition, azathioprine and 5-fluorouracil triggered also an intense mitochondrial swelling and with the latter, alterations of the kinetoplast possibly due to degradation of the DNA structure. The presence of autophagosome-like structures could be related with a remodelling process of damaged organelles by autophagy, resulting in an autolysis process.

## Conclusion

The present work confirms the deleterious effect of nucleoside analogues against two *Leishmania* strains, human and ca-

nine. The pyrimidine analogue 5-fluorouracil was effective against both stages. Conversely, the deleterious effect of azathioprine was more visible in amastigotes, due to its probable rapid metabolism in the host cell. Our results confirm that in vitro tests of potential drug candidates should be made on intracellular amastigotes, since the observed effects can vary according to the culture conditions and the free or intracellular status of the parasites.

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