Effects of purine nucleosides on the in vitro growth of Cryptosporidium parvum

Philippe Lawton, Carine Hejl, Roselyne Mancassola, Muriel Naciri, Anne-Françoise Pétavy

EFFECTS OF PURINE NUCLEOSIDES ON THE *IN VITRO* GROWTH OF *CRYPTOSPORIDIUM PARVUM*

Philippe LAWTON, Carine HEJL, Roselyne MANCASSOLA*, Muriel NACIRI* and Anne-Françoise PETAVY.

Département de Parasitologie et Mycologie Médicale, Faculté de Pharmacie, Université Claude-Bernard, Lyon, France.

* INRA, Unité de Pathologie aviaire et de Parasitologie, Nouzilly, France.

FEMSLE-2003-0547

Running title: Purine nucleosides enhance the *in vitro* growth of *C. parvum*.

Correspondence to: Philippe Lawton, Département de Parasitologie et Mycologie Médicale, ISPB-Faculté de Pharmacie, 8 avenue Rockefeller, F-69373 Lyon Cedex 08, France.

Tel.: +33 4 78 77 72 77 – Fax: +33 4 78 77 70 58 – E-mail: lawton@univ-lyon1.fr

Abstract

The effect of purine nucleosides on the *in vitro* growth of *Cryptosporidium parvum* was studied. Culturing the parasite in THP-1 cells for 72 h in growth medium supplemented with adenosine or inosine improved the parasite yields especially on the first 48 h. Similar results were obtained with parasite cultured in MDBK cells and incubated for 24 h with inosine. The addition of inosine on 72 h cultures enhanced the growth of *C. parvum* in THP-1 cells, especially the trophic stages, whereas the analogue formycin B was toxic to the parasites and induced a marked decrease in the gamont stages. The monitoring of the added purine nucleosides by HPLC showed that at 37°C in the presence of THP-1 cells, a rapid uptake of inosine occurred with hypoxanthine being the main purine present after 2 h in the medium.

Index descriptors and abbreviations: *Cryptosporidium parvum*; Inosine; Adenosine; *In vitro* culture; THP-1 cells; ADA: Adenosine deaminase (E.C.3.5.4.4); HGXPRT: hypoxanthine, guanine, xanthine phosphoribosyltransferase (E.C.2.4.2.8); HPLC: high performance liquid chromatography; p.i.: post-infection.

1. Introduction

Parasitic protozoa are purine auxotrophs [1] and it is likely that their environment triggers the types of purine they use [2]. The salvaged purines are presumed to be in the nucleobase or nucleoside form, since phosphorylated nucleotides do not cross the membranes [3]. The nucleoside transport in parasites usually involves adenosine, and to a lesser extent, inosine and hypoxanthine [4]. Molecular biology techniques recently allowed the isolation and characterization of the transporter genes in Trypanosomatids [5, 6] and of the PfNT1 nucleoside transporter from *P. falciparum* [7-9]. These transport systems are equilibrative, except for the presence of a proton symporter in *Trypanosoma* [10]. The complexity of purine metabolism makes it difficult to study, especially in intracellular parasites. Metabolic studies on Trypanosomatids evidenced that purine stress induced adaptations in its metabolism [11, 12]. Apicomplexa have been less studied, essentially *Toxoplasma gondii* [13, 14], *Plasmodium falciparum* [15], *Babesia* [16] and recently, HGXPRT was evidenced in *Cryptosporidium parvum* [17]. Since the *in vitro* cultivation of this intestinal protozoan pathogen is still difficult despite recent improvements [18] and that the use of the non-adherent cell line THP-1 provides an additional mean for studying the biology of this parasite [19], this study was aimed to evaluate the ability of exogenous purine nucleosides to enhance the rate of infected cells *in vitro*, and thus to point out to this metabolism as essential to the parasite.

2. Materials and methods

2.1 Cells

The myelomonocytic cell line THP-1 (ECACC # 88081201, Sophia Antipolis, France) was continuously maintained in RPMI 1640 (Sigma, L'Isle d'Abeau, France) supplemented with 25 mM HEPES (Sigma H-3375), 200 U ml^{-1} of penicillin, 200 µg ml^{-1} of streptomycin (Sigma P-0781) and 10% fetal calf serum (Dutscher, Brumath,

France) at 37°C in a 5% CO_2 moist atmosphere. The cell suspension was diluted once a week when the cell density reached about 1 x 10^6 ml^{-1}. Madin Darby Bovine Kidney cells (MDBK; ATCC 6071, Rockville, MD) were grown as monolayers in the same conditions and routinely passaged using trypsin-EDTA (Sigma, L'Isle d'Abeau, France).

2.2 Parasites

Cryptosporidium parvum oocysts were passaged in neonatal calves (INRA, Nouzilly, France) and purified as previously described [20] except that the oocyst suspension was stored at 4°C in sterile water with 400 U ml^{-1} of penicillin, 400 μg ml^{-1} of streptomycin until use.

2.3 Infection of cells with C. parvum

Prior to infection, the cells were collected and pelleted for 4 min at 160 x g. They were diluted in complete medium and kept in the incubator until use. The MDBK cells were periodically resuspended to minimize their adherence. Infection of the cells was carried out as previously described [21]. Briefly, the surface-sterilized oocysts were mixed with the cell suspension at an oocyst to cell ratio of 1:1, carefully layered on top of a 60% Percoll solution and centrifuged 10 min at 1500 x *g*. Excystation was allowed to proceed at 37°C for 1.5 h in the parasites-cells layer at the interface between the Percoll solution and the medium. This layer was then retrieved with a Pasteur pipette and washed once in RPMI 1640 for 9 min at 600 x *g*. Most of the cell debris and oocysts shells stayed on top of a 30% Percoll solution after centrifugation 10 min at 1500 x *g*, while the parasitized cells pellet was subsequently washed once at 600 x *g* and 2 x at 160 x *g* as above and distributed in a 24-well Falcon microplate (Becton-Dickinson, Meylan, France) at an approximate density of 4 x 10^6 cells ml^{-1} under reduced oxygen in a candle jar. The medium was replaced twice a day until use for the experiments. For the experiments with MDBK cells, the 30% Percoll step was omitted

and the infected cells were distributed in 6-wells microplates fitted with 22 x 22-mm glass coverslips.

2.4 Effect of purine nucleosides

Adenosine (Sigma A-9251), inosine (Sigma I-4125) and formycin B (Sigma F-7254) were dissolved in RPMI 1640 as 40 mM stock solutions (10.68 mg ml^{-1},10.73 mg ml^{-1} and 10.77 mg ml^{-1}, respectively) and stored at -20°C when not used immediately.

For growth enhancement experiments, initial concentrations of 200 µM adenosine and inosine were tested for 72 h on 24 h-old parasite cultures in THP-1 cells. Prior to use, the stock solutions were diluted 1:100 in growth medium to give concentrations of 400 µM and distributed under 100 µl in a 96-well Nunclon microplate (Nunc, Denmark). Control wells received 100 µl of medium alone. In each well, 100 µl of the washed parasitized cell suspension (final volume: 200 µl; mean cell concentration: 4 x 10^6 ml^{-1}) were added, mixed and incubated in a candle jar at 37°C. Each nucleoside was tested in triplicate. Every day, 100 µl of medium were carefully removed and replaced with 100 µl of fresh medium containing 200 µM of the relevant purine nucleosides.

In experiments with MDBK cells, only 24 h-old *C. parvum* confluent cultures were used to minimize problems due to the sloughing of cells. The growth medium was aspirated, the cell monolayer was washed with RPMI 1640 and medium supplemented with 100 µM inosine was added. The parasites were cultured for an additional 24 h, the coverslips were then removed and processed as described below.

Inosine (100 µM) and formycin B (20 µM) were tested on 72 h-old *C. parvum* cultures in THP-1 cells. The parasitized cells were washed 3 x for 5 min at 160 x *g* RPMI 1640 before incubation in triplicate with the drugs for 24 h in a 96-well microplate.

When needed, the aspect and the concentration of the cells in the cultures were controlled with a Malassez counting cell after a 1:10 dilution in saline.

2.5 Evaluation of parasite growth

The content of the wells was thoroughly mixed and centrifuged for 1 min in microtubes at 15,000 revs/min in a Beckman Avanti centrifuge. Thin smears were made, fixed in 100% methanol and stained with 20% Giemsa and 1% Alcian blue as previously described [20]. In experiments with MDBK cells, the coverslips were removed, the cells were fixed and stained likewise. At least 1000 cells were counted with an oil immersion x100 objective. The percentage of cells infected with the various development stages of the parasite was assessed, as well as the number of multiparasitized cells. The growth enhancement or inhibition percentages were calculated using the following formula: 100 x [Percent of infected cells in the treated wells / Percent of infected cells in the control wells] for the relevant parasite stages. The Student's *t*-test was used to assess the statistical significance of the results. Graphs and statistical tests were performed using GraphPad Prism 3.0c and InStat 3 0cx for Macintosh (GraphPad Software, San Diego, Ca, USA).

2.6 Determination of the purines

To evaluate the fate of the added purine nucleosides at 37°C, 100 µL of complete medium containing 200 µM of nucleosides were mixed with the same volume of either (1) a 24 h-old THP-1 cell culture suspension, (2) the supernatant after removing the cells by centrifugation or (3) growth medium alone, and incubated in triplicates at 37°C for 6 h in each well of a 96-well microplate. Samples were taken every 2 h, centrifuged if needed and frozen until processing.

The purine nucleosides were assessed by reverse-phase HPLC on a Kontron 422 system (Kontron Instruments, France). The samples were injected into a Hypersil 5 ODS column (250 x 4.6 mm) according to Suchail *et al*. [22] modified from Alexiou *et al*. [23], except that the 25 mM $NH_4H_2PO_4$ mobile phase was supplemented with 10% (v/v) methanol. The assay was performed at a flow rate of 0.5 ml min^{-1} and the detector was set at 254 nm.

3. Results and discussion

Adenosine and inosine at 200 µM as long-term growth additives induced an overall enhancement in the number of parasitized THP-1 cells, especially for the first 48 h, when asexual stages are predominant, evidencing a metabolic effect. Although both nucleosides were effective on trophozoites, meronts, gamonts and total number of parasites, inosine usually gave better results (Fig. 1). Dose-response experiments evidenced that above 100 µM inosine, there was no statistical difference (not shown) and therefore this concentration was used in subsequent experiments. Inosine was then tested for 24 h on 24 h-old confluent *C. parvum*-infected MDBK cells and displayed a similar effect (Fig.2).

Since our culture system allows sequential testing of compounds on the same parasitized cells batch [21], inosine was added to 72 h p.i. parasite cultures for 24 h, at a moment when the number of parasites begins to decrease and the sexual stages appear [20]. The culture yields increased about 30% and there were again about 20% more trophic stages, with more than twice the number of gamont-infected cells (Fig. 3). By contrast, the inosine analog formycin B was inhibitory, especially on the trophozoites and gamont stages (Fig. 4). A similar effect had been previously reported with the antiviral drug 2', 3'-dideoxyinosine but at a higher concentration [19], possibly due to the fact that the sugar moiety cannot be enzymatically removed in formycin B.

Without any purine supplied with RPMI 1640, the pool available for the parasite originates from the fetal calf serum and the host cells. Mammalian cells are able to synthesize the purine ring *de novo* and also have a variety of nucleoside transporters, essentially equilibrative [24]. The concentrative nucleoside transporters are mainly found on certain cell types such as intestinal cells and cells of the immune system [25], such as the THP-1 cells of monocytic origin. The regulation of these transport systems is important in macrophage activation and proliferation [26]. The inhibitory activity of formycin B suggests that a system involving transport from the extracellular medium and transfer through parasite transporters is likely to occur. This system is not

exclusive, since there was no 100% inhibition and the parasite may use the *de novo*-synthesized purines from its host cell.

The fate of the added purines was monitored on growth medium with and without THP-1 cells and at various temperatures. Adenosine was quickly converted to inosine by ADA present in the calf serum (not shown). Thus, the use of adenosine as additive is not suitable if the medium is not used immediately. Conversely, only about half of the initial inosine concentration was eventually hydrolyzed to hypoxanthine after one week at 4°C. At 37°C however, this process took 6 h, even with supernatants from 24 h THP-1 cultures. In the presence of THP-1 cells, hypoxanthine was the main purine found in the culture medium after 2 h (Fig. 5). No difference was noticed using parasitized cells (not shown). The rapid decrease in inosine concentration in culture is probably due to its uptake by the cells rather than caused by a release of cellular enzymes, since THP-1 culture supernatants gave the same decay rates than growth medium alone. However, hypoxanthine being the only purine found in culture conditions, it is likely to be the nucleobase transported into the host cells after 2 hours. It has been reported to facilitate *P. falciparum* growth in culture [27, 28] and to allow reduced serum concentrations for culturing *B. bovis* [29]. It is also noteworthy that *in vivo* at the parasite dwelling site, extensive uptake of nucleosides occurs, since the highest levels of transporters are on the proximal intestine of rabbits [30], the human jejunum cells poor in *de novo* metabolism, express the CNTs at their apical ends [31] and *C. parvum* alters the permeability of intestinal host cells [32].

This study shows the importance of the purine metabolism in *C. parvum* and its use to enhance culture yields *in vitro*. Improvements in the cultivation of this parasite will allow deeper studies on this important metabolism.

4. References

[1] Marr, J.J. and Ullman, B. 1995. In "Biochemistry and molecular biology of parasites" (Marr, J.J. and Müller, M., eds), pp 323-336, Academic Press.

[2] Ghosh, M. and Mukherjee, T. 2000. Stage-specific development of a novel adenosine transporter in *Leishmania donovani* amastigotes. Mol. Biochem. Parasitol. 108, 93-99.

[3] Carter, N.S., Landfear, S.M. and Ullman, B. 2001. Nucleoside transporters of parasitic protozoa. Trends Parasitol. 17, 142-145.

[4] Ogbunude, P.O.J. and Baer, H.P. 1993. Nucleoside transport in parasites – current status and methodological aspects. Int. J. Biochem. 25, 471-477.

[5] Landfear, S.M. 2001. Molecular genetics of nucleoside transporters in *Leishmania* and African trypanosomes. Biochem. Pharmacol. 62, 149-155.

[6] Vasudevan, G., Ullman, B. and Landfear, S.M. 2001. Point mutations in a nucleoside transporter gene from *Leishmania donovani* confer drug resistance and alter substrate specificity. Proc. Natl. acad. Sci. USA 98, 6092-6097.

[7] Carter, N.S., Ben Mamoun, C., Liu, W., Silva, E.O., Landfear, S.M., Goldberg, D.E. and Ullman, B. (2000) Isolation and functional characterization of the PfNT1 nucleoside transporter gene from *Plasmodium falciparum*. J. Biol. Chem. 275, 10683-10691.

[8] Parker, M.D., Hyde, R.J., Yao, S.Y.M., McRobert, L., Cass, C.E., Young, J.D., McConkey, G.A. and Baldwin, S.A. 2000. Identification of a nucleoside/nucleobase transporter from *Plasmodium falciparum*, a novel target for anti-malarial chemotherapy. Biochem. J. 349, 67-75.

[9] Rager, N., Ben Mamoud, C., Carter, N.S. and Ullman, B. 2001. Localization of the *Plasmodium falciparum* PfNT1 nucleoside transporter to the parasite plasma membrane. J. Biol. Chem. 276, 41095-41099.

[10] De Koning, H.P., Watson, C.J. and Jarvis, S.M. 1998. Characterization of a nucleoside/proton symporter in procyclic *Trypanosoma brucei brucei*. J. Biol. Chem. 273, 9486-9494.

[11] Gero, A.M. 1998. Purine stress in *Crithidia*: adaptation of a parasite to environmental stress. Parasitol. Today 14, 277-281.

[12] De Koning, H.P., Watson, C.J., Sutcliffe, L. and Jarvis, S.M. 2000. Differential regulation of nucleoside and nucleobase transporters in *Crithidia fasciculata* and *Trypanosoma brucei brucei*. Mol. Biochem. Parasitol. 106, 93-107.

[13] Schwab, J.C., Afifi Afifi, M., Pizzorno, G., Handschumacher, R.E. and Joiner, K.A. 1995. *Toxoplasma gondii* tachyzoites possess an unusual plasma membrane adenosine transporter. Mol. Biochem. Parasitol. 70, 59-69.

[14] Ghérardi, A., Sarciron, M.E., Pétavy, A.F. and Peyron, F. 1999. Purine pathway enzymes in a cyst-forming strain of *Toxoplasma gondii*. Life Sci. 65, 1733-1738.

[15] Upston, J.M. and Gero, A.M. 1995. Parasite-induced permeation of nucleosides in *Plasmodium falciparum* malaria. Biochim. Biophys. Acta 1236, 249-258.

[16] Matias, C., Nott, S.E., Bagnara, A.S., O'Sullivan, W.J. and Gero, A.M. 1990. Purine salvage and metabolism in *Babesia bovis*. Parasitol. Res. 76, 207-213.

[17] Doyle, P.S., Kanaani, J. and Wang, C.C. 1998. Hypoxanthine, guanine, xanthine phosphoribosyltransferase activity in *Cryptosporidium parvum.* Exp. Parasitol. 89, 9-15.

[18] Hijjawi, N.S., Meloni, B.P., Morgan, U.M. and Thompson, R.C.A. 2001. Complete development and long-term maintenance of *Cryptosporidium parvum* human and cattle genotypes in cell culture. Int. J. Parasitol. 31, 1048-1055.

[19] Lawton, P., Hejl C., Sarciron M.E., Mancassola R., Naciri M. and Pétavy A.F. 1999. Use of a non-adherent cell culture system for testing the effect of 2', 3' - dideoxyinosine against *Cryptosporidium parvum*. FEMS Microbiol. Lett. 176, 235-240.

[20] Lawton, P., Naciri, M., Mancassola, R. and Pétavy, A.-F. 1996. Cultivation of *Cryptosporidium parvum* in a non-adherent human monocytic cell line. J. Microbiol. Meth. 27, 165-173.

[21] Lawton, P., Mancassola R., Naciri M. and Pétavy A.F. 2001. Use of Percoll for the infection of cells *in vitro* with *Cryptosporidium parvum* oocysts. J. Microb. Meth. 46, 81-84.

[22] Suchail S., Sarciron M.E. and Pétavy A.F. 1998. Purine metabolism in *Echinococcus multilocularis*. Comp. Biochem. Physiol. 120B, 633-637.

[23] Alexiou, M. and Leese, H. (1994) Enzymes of purine salvage and catabolism in the mouse preimplantation embryo measured by high performance liquid chromatography. J. Reprod. Fertil. 101, 151-158.

[24] Cass, C.E., Young J.D. and Baldwin, S.A. 1998. Recent advances in the molecular biology of nucleoside transporters of mammalian cells. Biochem. Cell. Biol. 76, 761-770.

[25] Baer, H.P., Moorji, A., Ogbunude, P.O. and Serignese, V. 1992. Sodium-dependent nucleoside transport in mouse lymphocytes, human monocytes, and hamster macrophages and peritoneal exsudate cells. Can. J. Physiol. Pharmacol. 70, 29-35.

[26] Soler, C., Garcia-Manteiga, J., Valdes, R., Xaus, J., Comalada, M., Casado, F.J., Casado, M., Pastor-Anglada, A., Celada, A. and Felipe, A. 2001. Macrophages require different nucleoside transport systems for proliferation and activation. FASEB J. 15, 1979-1988.

[27] Zolg, J.W., MacLeod, A.J., Dickson, I.H. and Scaife, J.G. 1982. *Plasmodium falciparum*: modifications of the *in vitro* culture conditions improving parasite yields. J. Parasitol. 68, 1072-1080.

[28] Asahi, H., Kanazawa, T., Kajihara, Y., Takahashi, K. and Takahashi T. 1996. Hypoxanthine: a low molecular weight factor essential for growth of erythrocytic *Plasmodium falciparum* in a serum-free medium. Parasitology 113, 19-23.

[29] Neves, L., Cross, H.F., Loureiro, L., Akça, A., Hommel, M. and Trees, A.J. 2001. Addition of hypoxanthine to culture media allows in vitro cultivation of *Babesia*

bovis and B. *bigemina* at reduced serum concentrations. Parasitology, 123, 357-363.

[30] Roden, M., Paterson, A.R. and Turnheim, K. 1991. Sodium-dependent nucleoside transport in rabbit intestinal epithelium. Gastroenterology 100, 1553-1562.

[31] Patil, S.D. and Unadkat, J.S. 1997. Sodium-dependent nucleoside transport in the human intestinal brush-border membrane. Am. J. Physiol. 272, G1314-G1320.

[32] Capet, C., Kapel, N., Huneau, J.F., Magne, D., Laikuen, R., Tricottet, V., Benhamou, Y., Tomé, D. and Gobert, G.G. 1999. *Cryptosporidium parvum* infection in suckling rats: impairment of mucosal permeability and Na^+-glucose cotransport. Exp. Parasitol. 91, 119-125.

Legends to figures

Fig. 1: Effect of medium supplemented with 200 µM inosine or adenosine on the growth of *C. parvum* in THP-1 cells. The purine nucleosides were added 24 h after infection of the cells and the various parasite stages were monitored after a 24 h (A), 48 h (B) and a 72 h (C) additional incubation, with a daily medium change, as described in the Materials and Methods section. Controls account for 100 %. Results (mean ± S.D.) from 3 separate experiments.✶: statistically not significant ($p > 0.05$).

Fig. 2: Effect of 100 µM inosine on 24 h-old *C. parvum* cultures in MDBK cells. The parasite stages were counted after 24 h. Controls account for 100 %. Results (mean ± S.D.) from 3 separate experiments.

Fig. 3: Effect of 100 µM inosine on 72 h-old *C. parvum* cultures in THP-1 cells. The parasite stages were counted after 24 h. Controls account for 100 %. Results (mean ± S.D.) from 3 separate experiments.

Fig. 4: Inhibitory effect of 20 µM formycin B on 72 h-old *C. parvum* cultures. The parasite stages were counted after 24 h. Results (mean ± S.D.) from 3 separate experiments.

Fig. 5: HPLC monitoring of the composition of the inosine-supplemented growth medium at 37°C. A: decay of inosine. B: appearance of hypoxanthine. Growth medium (▲), supernatant from a 24 h THP-1 cell culture (●), incubation in the presence of THP-1 cells (□). Results (mean ± S.D.) from 3 separate experiments.

Figure 1

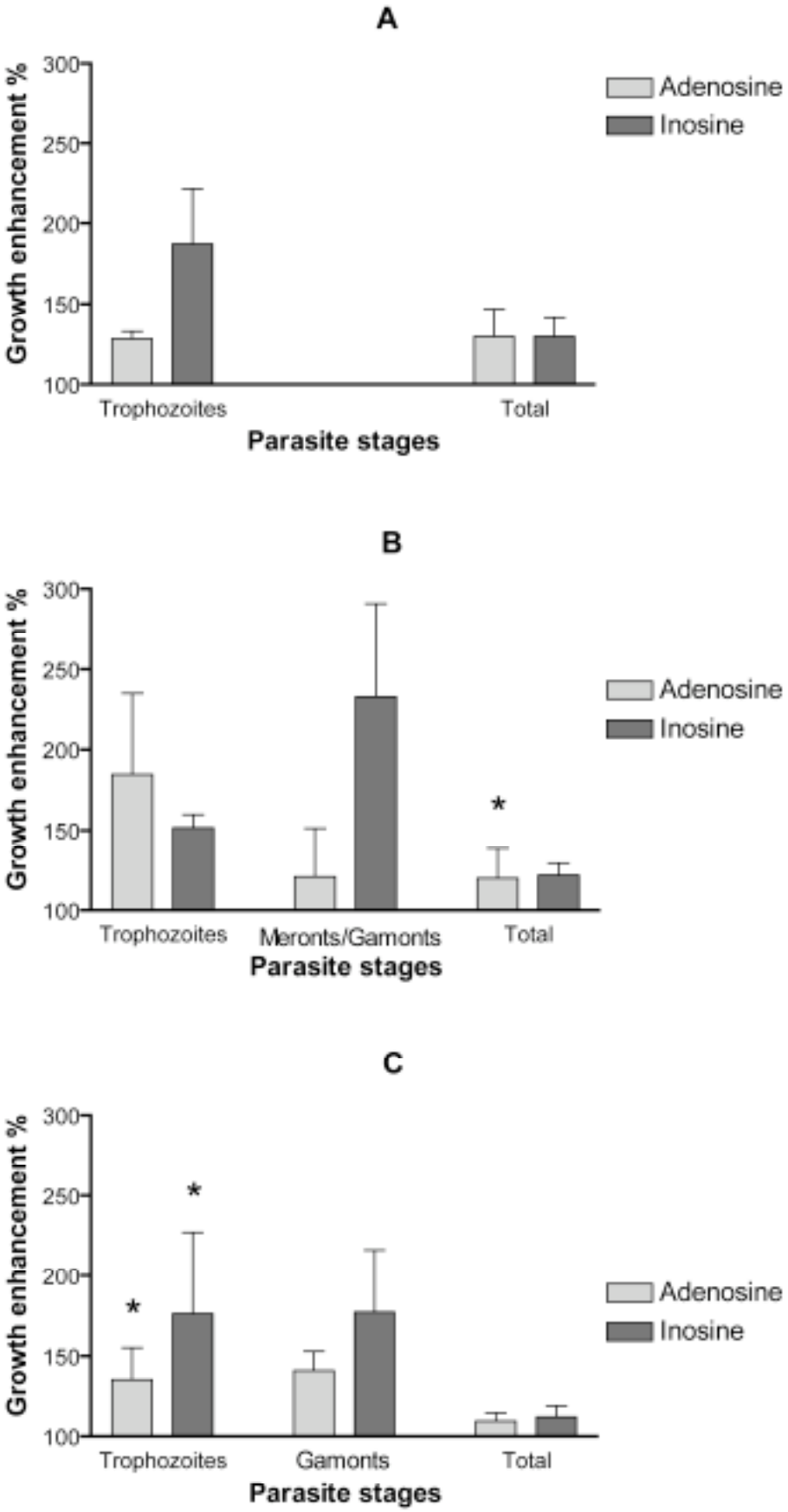
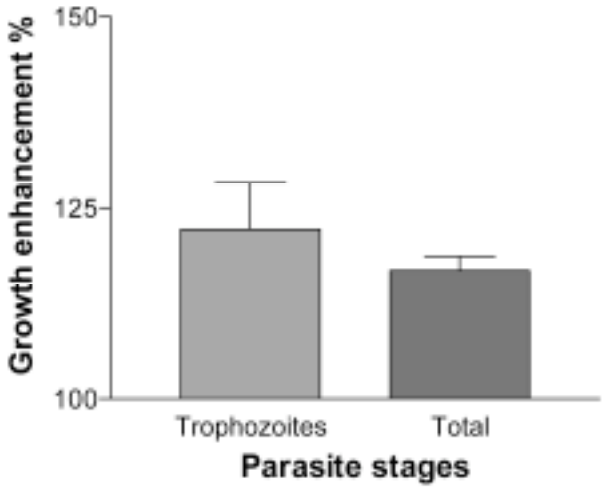

A
Growth enhancement %
300
250
200
150
100
Adenosine
Inosine
Trophozoites
Total
Parasite stages
B
Growth enhancement %
300
250
200
150
100
Adenosine
Inosine
*
Trophozoites
Meronts/Gamonts
Total
Parasite stages
C
Growth enhancement %
300
250
200
150
100
*
*
Adenosine
Inosine
Trophozoites
Gamonts
Total
Parasite stages

Figure 2



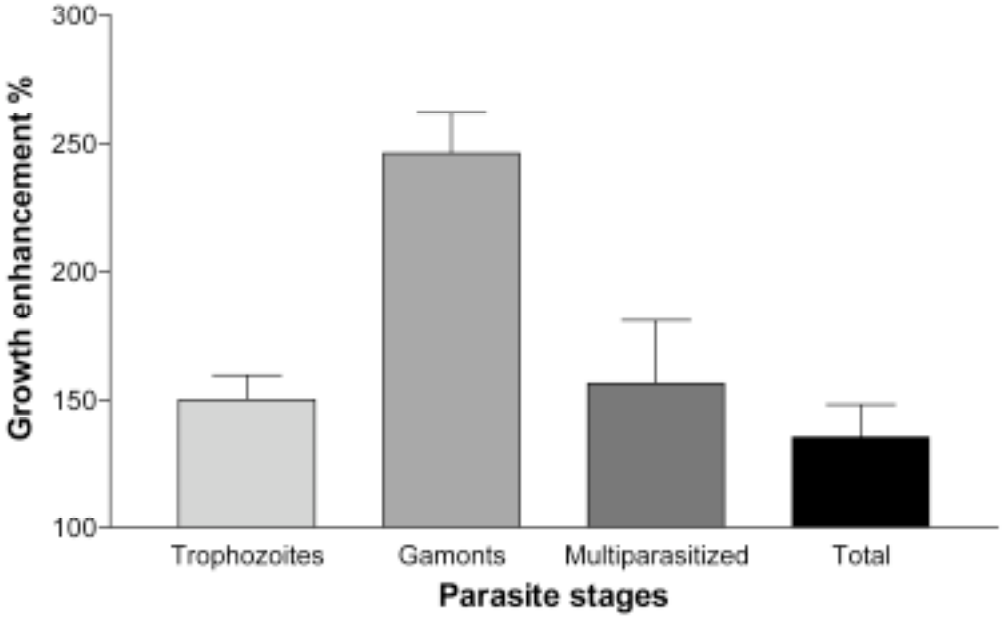


Figure 3

Figure 4

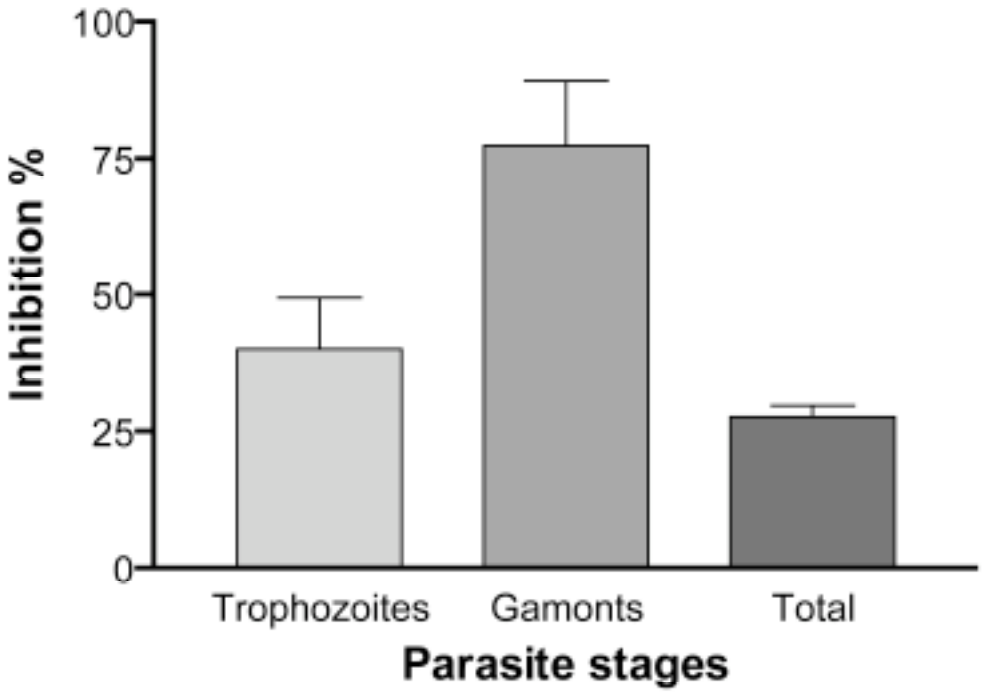
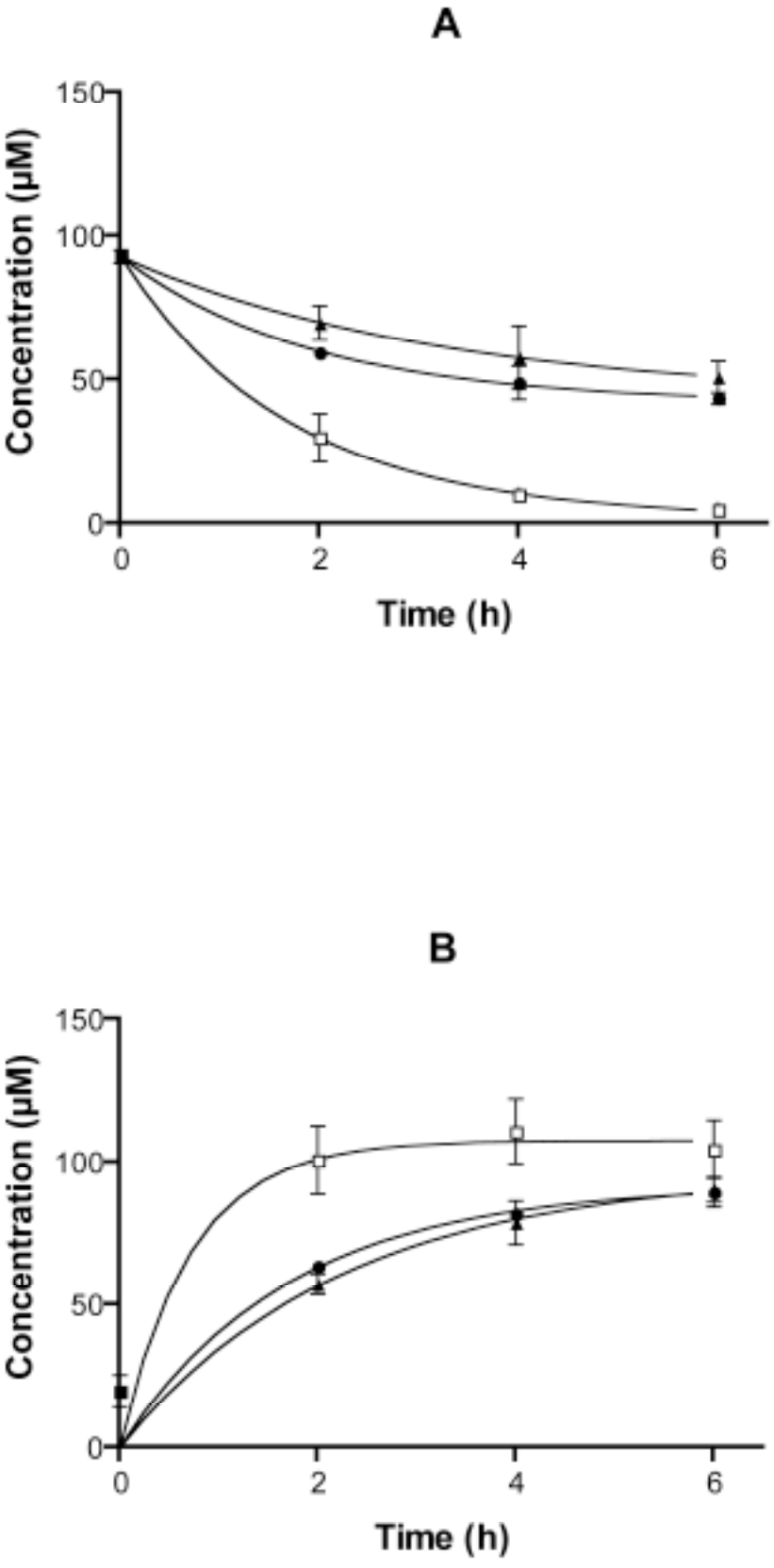

100
75
50
25
0
Inhibition %
Trophozoites
Gamonts
Total
Parasite stages

Figure 5


A
150
100
50
0
Concentration (µM)
0
2
4
6
Time (h)
B
150
100
50
0
Concentration (µM)
0
2
4
6
Time (h)