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MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Arrest of *Trypanosoma brucei rhodesiense* and *T. brucei brucei* in the S-phase of the cell cycle by (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((S)-HPMPA)

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Abstract

African trypanosomes are incapable of purine de novo synthesis. They use salvage pathways to meet their purine requirements. Therefore, purine analogues appear as potential candidates to interfere in trypanosome metabolism. The acyclic adenosine analogue (S)-9-(-3-hydroxy-2-phosphonylmethoxypropyl)adenine ((S)-HPMPA) expressed antitrypanosomal activity in vitro and vivo. When exposed to 20 μM (S)-HPMPA, trypanosomes were arrested in the S-phase of the cell cycle and were unable to enter G2-phase. Thymidine uptake and incorporation was inhibited almost completely. Only nuclear DNA replication was inhibited, while mitochondrial DNA replication and kinetoplast division was not inhibited. The antitrypanosomal effect was reversible when cells were exposed for 12 h. As a control, aphidicolin arrested trypanosomes in the G1-phase of the cell cycle at a concentration of 30 μM. At 20 μM (S)-HPMPA, glycolysis was not effected, while leucine and adenine uptake were reduced with prolonged exposure.

Keywords: *Trypanosoma brucei* spp.; (S)-HPMPA; Aphidicolin; Adenosine analogues; Cell cycle

1. Introduction

The rationale to employ purine analogues as potential antitrypanosomal compounds is the fact that all investigated kinetoplastids rely on preformed purine bases and nucleosides present in the circulatory system of their host since they are

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; FACS, fluorescence activated cell sorter; IC_{50}, concentration which inhibits metabolic activity of trypanosomes by 50%; MIC, minimum inhibitory concentration; (S)-HPMPA, (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine.

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unable to synthesize them de novo [1–3]. Bases, such as hypoxanthine and xanthine, are produced continuously in man by catabolism of purine nucleotides. Therefore, bloodstream form trypanosomes have access to sufficient resources to meet their purine requirements by salvage.

The unique features of the purine salvage systems of trypanosomes represent an adaptation to the environment in the mammalian host. However, the need for exogenous purines cause them to be selectively susceptible to the cytotoxic effects of several purine analogues such as formycin B, 9-deazainosine, or methylthioadenosine analogues [4–6]. We have shown that some phosphonylmethoxyalkylpurines express antitrypanosomal activity [7]. These acyclic nucleoside phosphonates are unique in that the phosphorus atom is attached to the alkyl side chain of the purine base via an O–C–P bond which is resistant to enzymatic degradation when compared with the usual C–O–P bond. Initially, phosphonylmethoxyalkylpurines were synthesized as antiviral agents [8–10]. Subsequently, the antiparasitic activity of some of these purine analogues has been observed for *Plasmodium falciparum* [11] and *Trypanosoma brucei brucei* [7].

Among the acyclic nucleoside phosphonates tested, (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((S)-HPMPA) (Fig. 1) expressed activity against human pathogenic *T. b. rhodesiense* and *T. b. gambiense*, and against animal pathogenic species such as *T. b. brucei*, *T. congolense* and *T. evansi* [12]. Trypanosomes were eliminated in vitro at concentrations which were 15000-fold lower than those which were toxic for mammalian cells [13]. More importantly, mice infected with *T. b. rhodesiense* were cured by (S)-HPMPA [12].

Consequently, we were interested in the mode of action of this potent purine analogue. (S)-HPMPA is an inhibitor of viral DNA synthesis, most likely by inhibition of DNA polymerase [14]. Furthermore, de Vries et al. [15] showed the inhibition of purified Plasmodium α- and γ-like DNA polymerase by the diphosphorylated (S)-HPMPApp. The aim of this study was to investigate the mode of action of (S)-HPMPA against human pathogenic *T. b. rhodesiense*.

2. Materials and methods

2.1. Parasites

T. b. rhodesiense STIB (Swiss Tropical Institute) 900 is a derivative of STIB 704 which was isolated in 1982 at St Francis Hospital, Ifakara, in Tanzania from a human patient. After several passages in rodents and a cyclic passage in *Glossina morsitans morsitans*, a cloned population was adapted to axenical growth in vitro. *T. b. brucei* STIB 920 is a derivative of STIB 348 which was isolated in 1971 in the Serengeti National Park (Tanzania) from a hartebeest (*Alcelaphus buselaphus cokii*). Following several passages in rodents and a cyclic passage in *G. m. morsitans*, a cloned population was adapted to axenic growth.

2.2. Cultivation of parasites

T. b. rhodesiense and *T. b. brucei* were propagated in vitro in a liquid medium consisting of Minimal Essential Medium (MEM, Gibco-RBL No. 072-1100 powder) with Earle's salts supplemented with 1 mg ml^{-1} glucose, 10 μl ml^{-1} MEM non-essential amino acids (100 $\times$), 2.2 mg ml^{-1} $NaHCO_3$ and 10 mM HEPES. The medium was further supplemented with 2 mM sodium pyruvate, 0.2 mM 2-mercaptoethanol, 0.016 mM thymidine [16], 0.1 mM hypoxanthine and 15% heat inactivated horse serum (self prepared from horse blood obtained from a local slaughterhouse). All cultures were kept in 24-multiwell-plates (Costar, Cambridge, MA) at 37°C in a humidified atmosphere in 5% CO_2. Cultures were subpassaged to a density of 10^3–10^5 trypanosomes ml^{-1} every 2nd or 3rd day. Try-

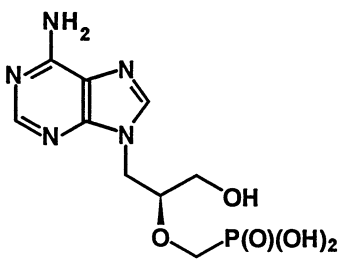

Fig. 1. Chemical structure of (S)-HPMPA.

panosomes in logarithmic growth phase were used for determination of drug sensitivites.

2.3. Drugs

(S)-HPMPA sodium salt was synthesized as described by Holý et al. [17]. (S)-HPMPA was dissolved in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) as a 33 mM stock solution, sterile filtered and stored at −20°C. Aphidicolin was purchased from Merck (ABS, Zürich, Switzerland), dissolved as a 15 mM stock solution in 50% methanol and stored at −20°C. The final methanol concentration in culture was below 0.1% (v/v) which did not affect growth of trypanosomes (data not shown).

2.4. In vitro assays

Drug sensitivities were assessed in vitro as previously described [18,19]. Briefly, trypanosomes were seeded at appropriate densities such that after the 72 h incubation period trypanosomes were at the end of the logarithmic growth phase. Cells were propagated in the appropriate medium for 72 h in the presence of various drug concentrations. All assays were carried out in 96-multiwell-plates (Costar) at 37°C. The minimum inhibitory concentration (MIC, defined as the concentration at which no trypanosomes or cells with normal motility or morphology were detected) was determined microscopically by viewing cultures with an inverted microscope (100 × magnification). IC_{50} values were obtained using a fluorescence assay and plotting of the relative growth of trypanosomes against the drug concentration and by linear interpolation of selected values above and below the 50% inhibition mark [19].

2.5. Staining with 4′,6-diamidino-2-phenylindol (DAPI)

Trypanosomes were fixed in 70% ethanol, centrifuged at 1500 × *g* for 5 min and resuspended in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 8.0) containing 1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (Sigma). After 30 min of incubation at room temperature, cells were transferred on slides and fluorescence was evaluated with a fluorescence microscope.

2.6. Measurement of DNA content

Trypanosome samples were fixed and stained according to Garside et al. [20]. Trypanosomes were washed and suspended at 10^6 ml^{-1} in ice cold Earle's Balanced Salts Solution (EBSS, 54 mM KCl, 116 mM NaCl, 26 mM $NaHCO_3$, 1 mM $NaH_2PO_4H_2O$, 5.5 mM D(+)-glucose) with 0.5 mM EDTA. Cells were fixed by adding 95% ethanol dropwise with constant mixing to 70% final concentration. Fixed samples were stored at 4°C until further use. For staining, cells were resuspended in PBS pH 8.0 containing 50 μg ml^{-1} propidium iodide and 40 μg ml^{-1} RNAse for at least 30 min. Intensity of red fluorescence was measured using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Europe) collecting linear red fluorescence (emission peak 590 nm) and forward light scatter over 1024 channels. Debris and doublet organisms have been gated out using the Doublet Discrimination Module, where fluorescence area (FL2-A) is measured against fluorescence width (FL2-W). A total of 10000 cells was counted from each sample. Relative percentages of organisms in the three cell cycle stages were calculated by CellFIT software (Becton Dickinson).

2.7. Adenine and leucine uptake

Trypanosomes were harvested in the logarithmic phase and suspended to 10^7 ml^{-1} in complete medium lacking adenine or leucine. At the starting point of incubation ($t = 0$) [^{3}H]adenine (24 Ci mmol^{-1}) or [^{3}H]leucine (126 Ci mmol^{-1}, Amersham, Rahn AG, Switzerland) was added in a concentration of 2 or 5 μCi ml^{-1} respectively. Cells were incubated at 37°C and aliquots of 2×10^6 cells were taken at different time intervals. Trypanosomes were subsequently centrifuged in Spin-X tubes with a 0.2 μM membrane (Costar, Tecnomara, Switzerland) at 1200 × *g* for 20 s and

filters were washed 2 × with ice cold PBS pH 8.0 and dried at room temperature. Radioactivity was counted in Ecoscint A scintillation cocktail (National Diagnostics, Atlanta, Georgia).

2.8. Thymidine uptake and incorporation

For thymidine uptake and incorporation, cells were treated as for leucine and adenine uptake, but were suspended at 2×10^5 ml^{-1} in complete medium without thymidine, supplemented with 10% heat inactivated horse serum. [^{3}H]Thymidine (10 μCi ml^{-1}, Amersham, Rahn AG, Switzerland) was added and after an incubation for 24 h at 37°C aliquots of 2×10^6 trypanosomes were washed 2 × with ice cold PBS pH 8.0 and transferred to glass fiber GF/C filters (Whatman, ABS, Zürich, Switzerland). Filters were dried only for uptake measurement. For incorporation experiments, acid insoluble material was collected by precipitation with 10% trichloroacetic acid. Filters were washed 3 × with ice cold 5% trichloroacetic acid supplemented with 20 mM sodium pyrophosphate [21], and 1 × with 70% ethanol. Radioactivity was counted in Ecoscint A scintillation cocktail (National Diagnostics, Atlanta, Georgia).

2.9. Pyruvate production

Pyruvate production was measured by the oxidation of NADH to NAD$^+$ in the presence of lactate dehydrogenase. A dilution row from 2–20 mM sodium pyruvate was made in 100 mM Tris–HCl buffer, pH 7.2 to get a standard curve. Supernatants of the first centrifugation step of the adenine and leucine uptake assay were also diluted in 100 mM Tris–HCl, pH 7.2, and 0.25 mM NADH was added. Extinction (E1) was determined firstly at 340 nm. Extinction (E2) was measured again 5 min after addition of lactate dehydrogenase. The sodium pyruvate concentration can be determined from the difference between E1 and E2 (ΔE), the dilution factor of the supernatants (V), and the gradient of the standard curve (S) as follows: sodium pyruvate concentration [mM] = ΔE × V × S.

Table 1
Sensitivity of *T. b. brucei* STIB 920 and *T. b. rhodesiense* STIB 900 to (S)-HPMPA and aphidicolin

Inhibitor	MIC [nM]	IC_{50} [nM]
T. b. brucei STIB 920		
(S)-HPMPA	600	34
Aphidicolin	532	118
T. b. rhodesiense STIB 900		
(S)-HPMPA	600	49
Aphidicolin	532	120

Values are the mean of two experiments each carried out in duplicate.

2.10. Cell volume determination

The average cell volume of trypanosomes was measured in a CASY-cell analysing system (Schärfe System, Reutlingen, Germany) and determined in femtoliter (fL), using a 60 μm capillary. The principal of measurement is based on the change of axial electrical resistance which is proportional to the cell volume.

3. Results

The in vitro effects of (S)-HPMPA and aphidicolin on *T. b. brucei* and *T. b. rhodesiense* are summarized in Table 1. (S)-HPMPA was more active than aphidicolin but both compounds had IC_{50} values in the nM-range. There was no difference in the efficacy of each compound against the two trypanosome subspecies.

When *T. b. brucei* were exposed to 20 μM (S)-HPMPA, a change in the distribution of different cell cycle stages within a population was observed by FACS analysis. The number of cells in G1- and G2-phase diminished (Fig. 2, panel A) while the number of cells in the S-phase increased as indicated by the shift of the peak of cells in G1-phase (Fig. 2, 0 h) towards the S-phase. After 24 h exposure to (S)-HPMPA, the majority of cells was in the S-phase of the cell cycle (Fig. 2, 24 h). Continuous exposure to the drug arrested the population in the S-phase (Fig. 2, 36 h) while the amount of cells in G1- and G2-phase remained negligible. A similar result was obtained with *T. b.*

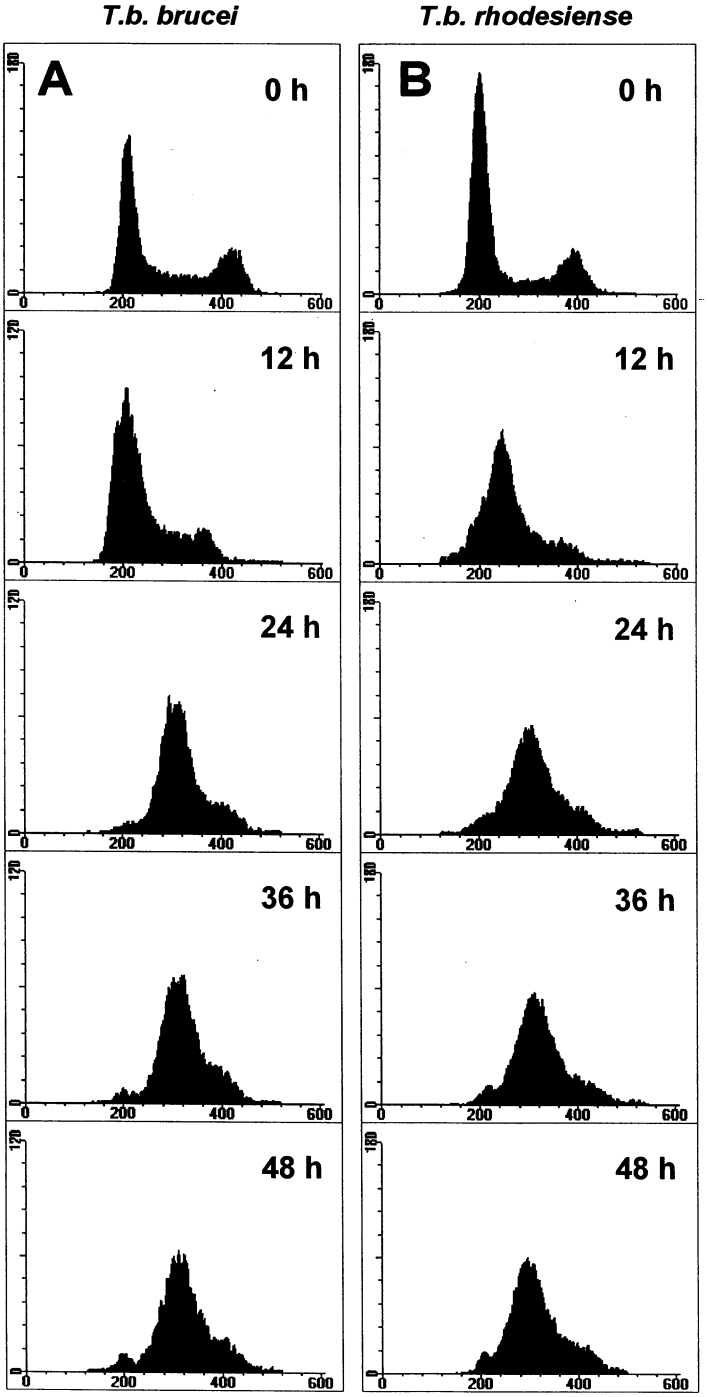

Fig. 2. FACS analysis of DNA content of (S)-HPMPA-arrested *T. b. brucei* (panel A) and *T. b. rhodesiense* (panel B). Histograms show the distribution of DNA content of trypanosomes at indicated time points after exposure to 20 μM (S)-HPMPA. Cells were fixed, stained with propidium iodide and analysed with a FACScan flow cytometer (Material and methods). The x-axis shows the channel number which is proportional to fluorescence signals and indicates DNA content. The y-axis shows the number of events in each channel which is proportional to cell numbers.

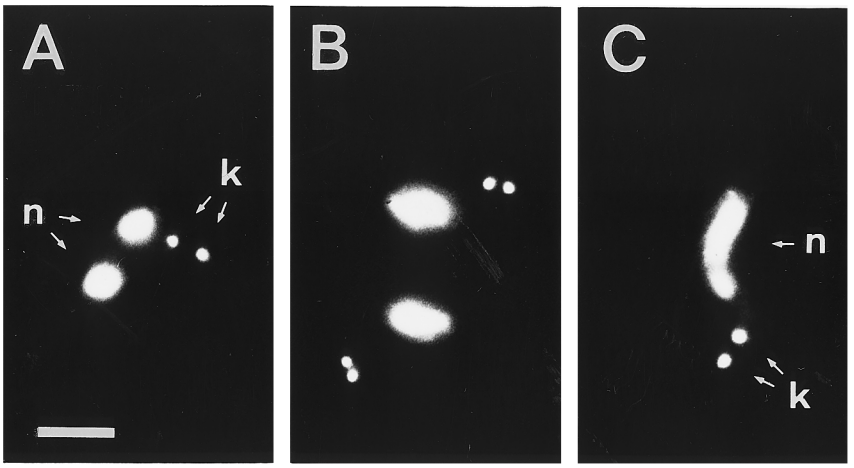

Fig. 3. DAPI-stained *T. b. brucei* (STIB 920) after exposure to 20 μM (S)-HPMPA. (A) A dividing trypanosome in control cultures containing two nuclei (n) and two kinetoplasts (k). (B) Two trypanosomes after 12 h incubation with 20 μM (S)-HPMPA containing one enlarged nucleus and two kinetoplasts each. (C) A trypanosome representing all individuals of a population after 36 h incubation with 20 μM (S)-HPMPA containing one enlarged nucleus and two kinetoplast. The bar represents 5 μm.

rhodesiense. The majority of trypanosomes was arrested in the S-phase of the cell cycle during 48 h incubation with 20 μM (S)-HPMPA (Fig. 2, panel B).

Inhibition of DNA-synthesis was also estimated by determination of thymidine uptake and incorporation. When cells were exposed to 20 μM (S)-HPMPA for 24 h uptake of [^{3}H]thymidine was reduced by $86 \pm 3\%$ compared with control cultures (5640 cpm $[2 \times 10^6$ cells$]^{-1}$). Similarly, thymidine incorporation was inhibited by $89 \pm 2\%$ compared with control cultures (4030 cpm $[2 \times 10^6$ cells$]^{-1}$).

The block of trypanosomes in the S-phase of the cell cycle was confirmed by DAPI stained *T. b. rhodesiense* after exposure to 20 μM (S)-HPMPA (Fig. 3). After 12 h exposure, the number of cells containing two kinetoplasts and one nucleus increased while cells with one kinetoplast and one nucleus or cells with two kinetoplasts and two nuclei diminished. After 36 h exposure, 98% of the cells had two or more kinetoplasts but one nucleus only (Table 2).

After incubation with 20 μM (S)-HPMPA for 12 h, the effect of the inhibitor was reversible. *T. b. rhodesiense* stopped dividing, the majority of trypanosomes died and cultures were reduced to a density below 10^5 ml^{-1}. However, on the 6th day after exposure, dividing forms reappeared and the population-doubling time returned to control values. The effect of (S)-HPMPA was irreversible after incubation for 36 h and all trypanosomes died.

The eukaryotic DNA polymerase inhibitor aphidicolin was chosen as a control compound. The effect of aphidicolin on the distribution of different cell cycle stages within a population of *T. b. rhodesiense* and *T. b. brucei* is shown in Fig. 4. After 3 h of incubation with 30 μM aphidicolin cultures contained predominantly cells in G1-phase and hardly any cells in G2-phase.

The cytotoxic effect of (S)-HPMPA on other essential cellular functions of trypanosomes is illustrated in Fig. 5. Exposure of *T. b. rhodesiense* to 20 μM (S)-HPMPA for 24 h initially slowed down adenine uptake but after 120 min uptake in (S)-HPMPA-treated trypanosomes reached the same level (5 pmol $[2 \times 10^6$ cells$]^{-1}$) as in control cultures (Fig. 5(A)). Leucine uptake was slightly elevated in trypanosomes incubated for 12 h with (S)-HPMPA but reduced in cells after exposure for 24 h (Fig. 5(B)). The average cell volume increased from 60 to 82 fL when cells were exposed for 12 h to 20 μM (S)-HPMPA. A further increase was observed up to 96 fL after 18 h drug exposure. Further incubation with (S)-HPMPA

Table 2
Distribution of DNA containing cell organells in untreated control and (S)-HPMPA treated (20 μM) *T. b. rhodesiense* cultures

Organelles	Control (%)	12 h (S)-HPMPA (%)	36 h (S)-HPMPA (%)
One kinetoplast/one nucleus	53	12	0
Two kinetoplasts/one nucleus	35	82+3[a]	98+2[a]
Two kinetoplasts/two nuclei	12	3	0

[a] More than two kinetoplasts.

did not result in a further increase of cell volume. Based on pyruvate production no difference was observed for glycolysis in (S)-HPMPA-treated cells compared with untreated trypanosomes (Fig. 5(C)).

4. Discussion

The results obtained show that both *T. b. rhodesiense* and *T. b. brucei* are arrested by the nucleoside analogue (S)-HPMPA in the S-phase of the cell cycle. This effect was observed after an incubation time which is slightly longer than the population doubling time of these species ($\approx$ 9 h). Cells which were in the G2-phase prior to (S)-HPMPA exposure entered a new cycle, but almost no cells previously in G1- or S-phase entered G2-phase indicating that DNA synthesis is inhibited during replication of trypanosomes. Inhibition of DNA synthesis was also followed by incorporation experiments with radiolabelled thymidine. Assuming that thymdine is taken up by an equilibrative transport system, (S)-HPMPA inhibited the incorporation of thymidine almost completely at concentrations which did not affect other cell functions. It must be noted that incorporation of thymidine was minimal even in untreated cells which is in agreement with earlier observations by Brun [22] and Mutomba and Wang [23], because trypanosomes contain only relatively low levels of thymidine kinase [24].

The FACS analysis was confirmed by observations of DAPI-stained trypanosomes which clearly showed that the effect of (S)-HPMPA is restricted to the nucleus and does not affect the kinetoplast. This is opposite to the effect of most other trypanocides such as isometamidium, quinapyramine and diminazene, which interfere with division of the kinetoplast by inhibition of topoisomerase type II while the nucleus appears not to be affected [25]. Mul et al. [14] provided evidence that diphosphorylated (S)-HPMPA [(S)-HPMPApp] is inhibiting adenovirus DNA synthesis by inhibition of the viral DNA polymerase suggesting that the DNA polymerase is the prime target for (S)-HPMPApp. This hypothesis was substantiated by Lurain et al. [26] who showed that point mutations in the DNA polymerase gene of human cytomegalovirus resulted in resistance to (S)-HPMPA. Detailed analysis demonstrated that (S)-HPMPApp inhibits cellular DNA polymerases α, δ and ε with a pronounced activity against DNA polymerase ε [27]. Furthermore, DNA polymerase α-like enzymes of *Plasmodium falciparum* and *P. berghei* and a γ-like DNA polymerase of *P. falciparum* were inhibited by (S)-HPMPApp [15]. Unfortunately, it was not possible to investigate the effect of (S)-HPMPApp on extracts containing trypanosomal DNA polymerase activity in this study because diphosphorylated (S)-HPMPApp was not available.

The effect of aphidicolin, an inhibitor of eukaryotic DNA polymerase, on trypanosomes was determined as a positive control of inhibition of DNA synthesis. *T. b. rhodesiense* and *T. b. brucei* were arrested by aphidicolin in the G1-phase of the cell cycle when incubated with 30 μM aphidicolin. A similar result was obtained by Mutomba and Wang [23] who observed this effect at 10 μg ml^{-1} (29 μM) aphidicolin. The targets of aphidicolin are the nuclear replicative DNA polymerases α and δ [28] while polymerases β and γ are not affected [28,29]. As nuclear polymerases α and δ are inhibited, neither DNA synthesis on the leading nor on the lagging strand of the replication

fork is possible. Thus, the DNA replication in S-phase is completely inhibited by interaction of aphidicolin with the replicative DNA polymerases.

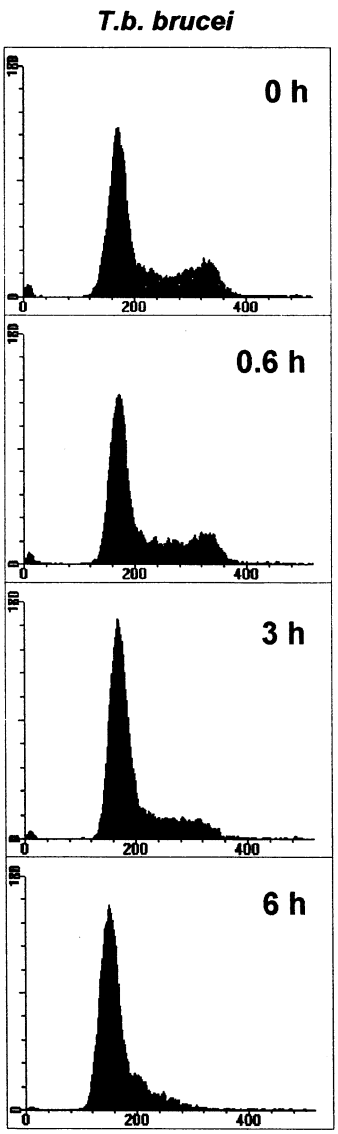

Fig. 4. FACS analysis of DNA content of aphidicolin-arrested *T. b. brucei* (STIB 920). Histograms show the distribution of DNA content of trypanosomes at indicated time points after exposure to 30 μM aphidicolin. Cells were fixed, stained with propidium iodide and analysed with a FACScan flow cytometer (Material and methods). The *x*-axis shows the channel number which is proportional to fluorescence signals and indicates DNA content. The *y*-axis shows the number of events in each channel which is proportional to cell numbers.

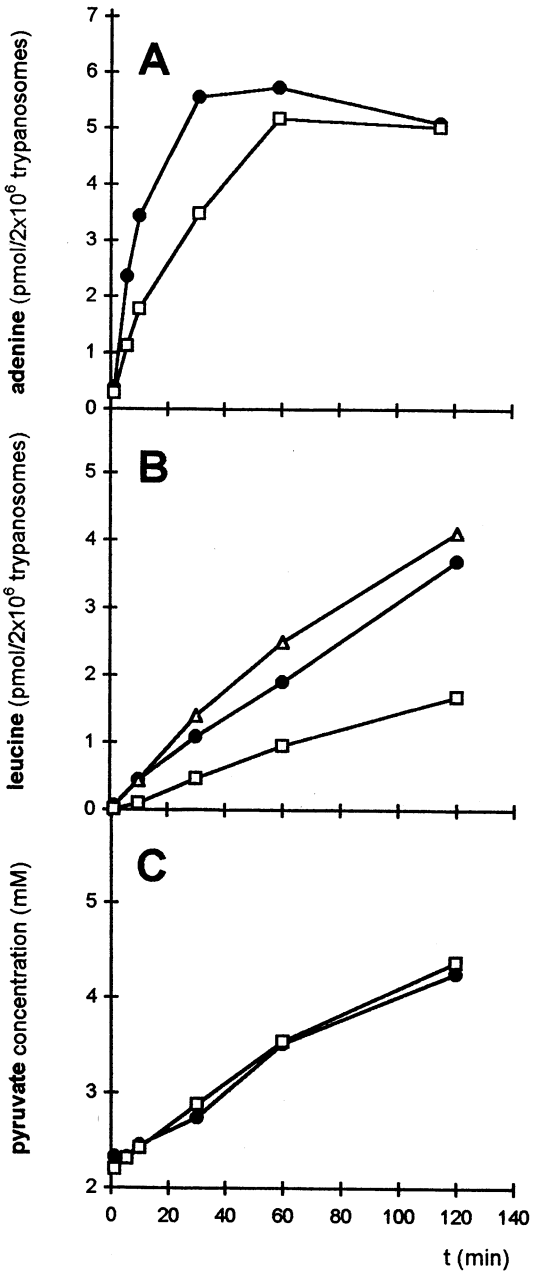

Fig. 5. The effect of (S)-HPMPA on adenine and leucine uptake, and on pyruvate production of *T. b. rhodesiense* STIB 900. Trypanosomes were exposed to 20 μM (S)-HPMPA for 12 h (Δ) and 24 h (□) prior to the measurements (Materials and methods), and metabolic parameters were compared with those of trypanosomes in control cultures (●).

Synchronisation of trypanosome cultures using (S)-HPMPA was not possible although the effect of (S)-HPMPA was reversible after incubation for 12 h at 20 μM. While all cells were arrested in the S-phase of the cycle, the lag phase to recovery was too long and when trypanosomes restarted growth and dividing forms were detected, cultures were already desynchronized. Similarly, Mutomba and Wang [23] were unable to synchronize trypanosome populations with aphidicolin due to the long lag period to recovery after drug incubation.

The potential of purine analogues as antiprotozoal agents has been demonstrated previously. An example is allopurinol, an inhibitor of hypoxanthine-guanine phosphoribosyltransferase [30]. However, results of clinical trials against leishmaniasis have been equivocal [31]. (S)-HPMPA inhibited selectively extracellular trypanosomes in vitro at concentrations (IC_{50} about 0.1 μM) which did not affect mammalian cells nor intracellular haemoflagellates [12]. Intraerythrocytic forms of *P. falciparum* were inhibited at higher concentrations (IC_{50} of 40 μM) [11]. *P. berghei* infected mice were cured with dosages (total of 28 mg kg^{-1}) at which signs of toxicity could be detected [32]. However, *T. b. rhodesiense* infected mice could be cured with less than the above dosages (two applications with 10 mg kg^{-1}) [12]. It should also be noted that (S)-HPMPA reduced mortality in mice infected intracerebrally with herpes virus [9] suggesting that the drug is able to cross the blood brain barrier, a prerequisite for a compound against late stage sleeping sickness. The selective activity of the nucleoside analogue (S)-HPMPA supports the approach of surveying inhibitors of protozoan DNA polymerases for new antiparasitic leads.

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