

MECHANISM OF ACTION OF A NITROIMIDAZOLE-THIADIAZOLE DERIVATE UPON *TRYPANOSOMA CRUZI* TISSUE CULTURE AMASTIGOTES

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Megazol (CL 64,855) a very effective drug in experimental infections by Trypanosoma cruzi, and also in in vitro assays with vertebrate forms of the parasite, had its activity upon macromolecule biosynthesis tested using tissue culture-derived amastigote forms. Megazol presented a drastic inhibition of [3H]-leucine incorporation, and only a partial inhibition of [3H]-thymidine and [3H]-uridine incorporation, suggesting a selective activity upon protein synthesis. Comparing the three drugs, megazol was more potent than nifurtimox and benznidazole in inhibiting protein and DNA synthesis. Megazol showed a 91% of inhibition of [3H]-leucine incorporation whereas nifurtimox and benznidazole, 0% and 2%, respectively. These latter two drugs inhibited the incorporation of all the precursors tested at similar levels, but the concentration of benznidazole was always three times higher, suggesting different mechanisms of action or, more probably, a greater efficiency of the 5-nitrofuranyl derivative in relation to the 2-nitroimidazole. So, we conclude that the mode of action of megazol is different from the ones of nifurtimox and benznidazole and that its primary effect is associated with an impairment of protein synthesis.

Key words: *Trypanosoma cruzi* – chemotherapeutic agents – amastigotes – macromolecule biosynthesis – radioactive precursors – action of thiadiazole derivative – megazol

The chemotherapy of Chagas' disease is still inadequate, and the two clinically used drugs – nifurtimox and benznidazole – present a high incidence of important side effects, with a controversial efficacy at the chronic stage of the disease (Brener, 1979; Avila, 1983; Gutteridge, 1985). In this context, efforts are in progress to find new therapeutic agents active against *Trypanosoma cruzi* which would not be harmful to the host.

A promising drug is megazol (2-amino-5-(1-methyl-5-nitro-2-imidazolyl)-1,3,4-thiadiazole-CL 64855), synthesized by Berkelhammer & Asato (1968), that showed suppressive activity against trypanosomatids in experimental rodent infections (Berkelhammer & Asato, 1968; Burden & Racette, 1968). Brener and co-workers studied intensively this drug, observing a marked curative effect on experimental rodent infections, with significantly higher levels of cure than similar treatments with nifurtimox and

benznidazole (Filardi & Brener, 1982). Megazol was considered one of the most active compounds against *T. cruzi* being also effective against parasite strains which are resistant to the two clinically used drugs (Filardi & Brener, 1984, 1985). Electron microscopic analysis of the heart of the megazol-treated animals showed progressive alterations in the parasite, culminating after 36 h of treatment, with a partial or total lysis (Maria et al., 1984).

Megazol was tested in our *in vitro* system of trial of drugs based on the direct effect upon the parasite and its proliferation and on the interaction with heart muscle cell (De Castro & Meirelles, 1987). This thiadiazole derivative proved to be a very potent drug at levels similar to nifurtimox and benznidazole, leading to inhibition of proliferation of amastigote forms in axenic medium or intracellularly in heart muscle cells. Total lysis of trypomastigote forms was observed after treatment for 24 h with 50 μ M megazol. Ultrastructurally this drug led to several alterations on *T. cruzi*, being very characteristic a vacuolization, a loss of structures in the cytoplasm, and a reduction of the number of ribosomes and swelling of the kinetoplast (De Castro & Meirelles, 1987).

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The investigation of the action of currently used drugs and of newly synthesized compounds is a fundamental step to identify the mechanisms of their selective toxicity toward the parasite. As studies of the incorporation of macromolecules precursors can shed some light on the mechanism of action of drugs, we propose to use this approach to further investigate the action of megalol on amastigote forms of *T. cruzi*.

MATERIALS AND METHODS

Parasites — We used the Y strain of *T. cruzi* (Silva & Nussenzweig, 1953). The amastigote forms were obtained from the supernatant of a J774G-8 macrophage cell line (Carvalho & de Souza, 1983; De Castro et al., 1987) as follows: macrophages in Dulbecco's modified Eagle medium plus 5% fetal bovine serum (DMES) were infected with bloodstream trypomastigotes in the ratio 1:10 cell per parasite. After 24 h, the medium was changed and trypanosomes that did not penetrate the cells were discarded. Thereafter the medium was changed every 2 days. After 6-8 days of infection, the culture supernatants consisted of amastigotes, very few trypomastigotes and cell debris. The amastigote forms were obtained by differential centrifugation and when their percentage was less than 93-95%, further purification was carried out by metrizamide gradient until about 99% purity was obtained.

Macromolecule precursors incorporation — Amastigote forms were collected and washed once with KRT buffer (25 mM Tris-HCl, pH 7.2, 1.2 mM Mg₂SO₄, 2.6 mM CaCl₂, 120 mM NaCl, and 100 mM glucose). A quantity of 5.10⁶ cells/ml was incubated in the buffer solution containing [3H]-thymidine (2 µCi/ml), [3H]-leucine (1 µCi/ml) or [3H]-uridine (2 µCi/ml), in the presence or absence of the drugs. At the chosen intervals, aliquots were taken and the cells were lysed with sodium dodecyl sulphate (0.01%) (De Castro et al., 1987). Following the addition of TCA (10% w/v), the samples were filtered through glass fiber filters (Whatman GF/A), and the filters washed three times with 5 ml cold phosphate buffered saline (PBS), dried and counted in a scintillation counter (Beckman Model LS-700).

As stock solutions of megalol, nifurtimox and benznidazole were prepared in DMSO, we tested also the effect of this solvent on the

incorporation of the precursors at concentrations three times higher (about 0.5%) than the ones used in the experiments, detecting no alterations of the incorporation as compared to the controls. During the assays, the cells were monitored by phase microscopy, with no apparent alteration on the cell morphology.

Chemicals — Dulbecco's modified Eagle medium and fetal calf serum were obtained from Microbiologica and [methyl-3H]thymidine, [L-4, 5-3H(N)]-leucine and [3H(G)]-uridine from New England Nuclear. All other reagents were of analytical grade. Megazol (CL 64,855) and the other drugs were kindly provided by Dr Zigman Brener (Centro de Pesquisas René Rachou — FIOCRUZ).

RESULTS

[3H]leucine incorporation — Megazol (50 and 100 µM) caused a drastic inhibition of [3H]leucine incorporation as compared even to higher concentrations of nifurtimox (100 and 200 µM) and benznidazole (300 and 400 µM) (Table I). In concentrations four times higher, in the case of nifurtimox, and eight times higher for benznidazole than the ones used with megalol, no effect on protein synthesis was observed, whereas inhibition of 77% was detected with this drug (Table I). The effect of the thiazol derivate was dose-dependent, showing a significant decrease of the incorporation in the range of (12 to 50 µM), whereas 300 µM benznidazole showed results similar to the control (Fig. 1).

[3H]-uridine incorporation — After 120 min of incubation, megalol (100 µM) caused only a partial inhibition (23%) of the RNA precursor incorporation, at somewhat lower levels as compared to nifurtimox and benznidazole, 38 and 33% respectively (Table II). These two drugs showed similar percent of inhibition of RNA synthesis, but as benznidazole was assayed at a concentration three times higher, these results indicate that it is less effective than nifurtimox.

[3H]-thymidine incorporation — Megazol (100 µM) showed a more potent effect on the inhibition of the DNA precursor incorporation than 100 µM nifurtimox and 300 µM benznidazole, being the values of percentual inhibition after 120 min respectively, 70, 48 and 50% (Fig. 2). As pointed out in the assays of [3H]

TABLE I

Inhibition of [3H]-leucine incorporation by amastigotes of *Trypanosoma cruzi* caused by drugs

Incub. time	Benznidazole		Nifurtimox		Megazol	
	300 uM	400 uM	100 uM	200 uM	50 uM	100 uM
30	0.99 + 0.02 ^a	0.91 + 0.04	0.90 + 0.05	0.89 + 0.03	0.29 + 0.02	0.29 + 0.01
60	0.94 + 0.02	0.98 + 0.05	0.89 + 0.02	0.82 + 0.04	0.22 + 0.05	0.15 + 0.00
90	0.90 + 0.01	0.78 + 0.03	0.93 + 0.03	0.72 + 0.03	0.18 + 0.02	0.12 + 0.01
120	0.98 + 0.02	1.02 + 0.05	1.02 + 0.04	0.86 + 0.04	0.23 + 0.02	0.09 + 0.01

a. treated/control.

TABLE II

Inhibition of [3H]-uridine incorporation by amastigotes of *Trypanosoma cruzi* caused by drugs

Incub. time	Control		Benz. 300 uM		Nif. 100 uM		Meg. 100 uM	
	fmol/10 ⁶	cell T/C ^a	fmol/10 ⁶	cell T/C	fmol/10 ⁶	cell T/C	fmol/10 ⁶	cell T/C
30	25.4 + 0.5	1.00	24.6 + 0.5	0.97	31.6 + 1.2	1.24	28.5 + 0.4	1.12
60	31.7 + 1.3	1.00	24.7 + 1.2	0.78	30.0 + 3.8	0.95	27.7 + 1.8	0.87
90	38.4 + 1.3	1.00	24.8 + 2.3	0.65	29.4 + 2.6	0.77	23.2 + 0.6	0.60
120	38.7 + 1.0	1.00	26.0 + 2.7	0.67	24.1 + 0.9	0.62	29.8 + 2.1	0.77

a: T/C = treated/control.

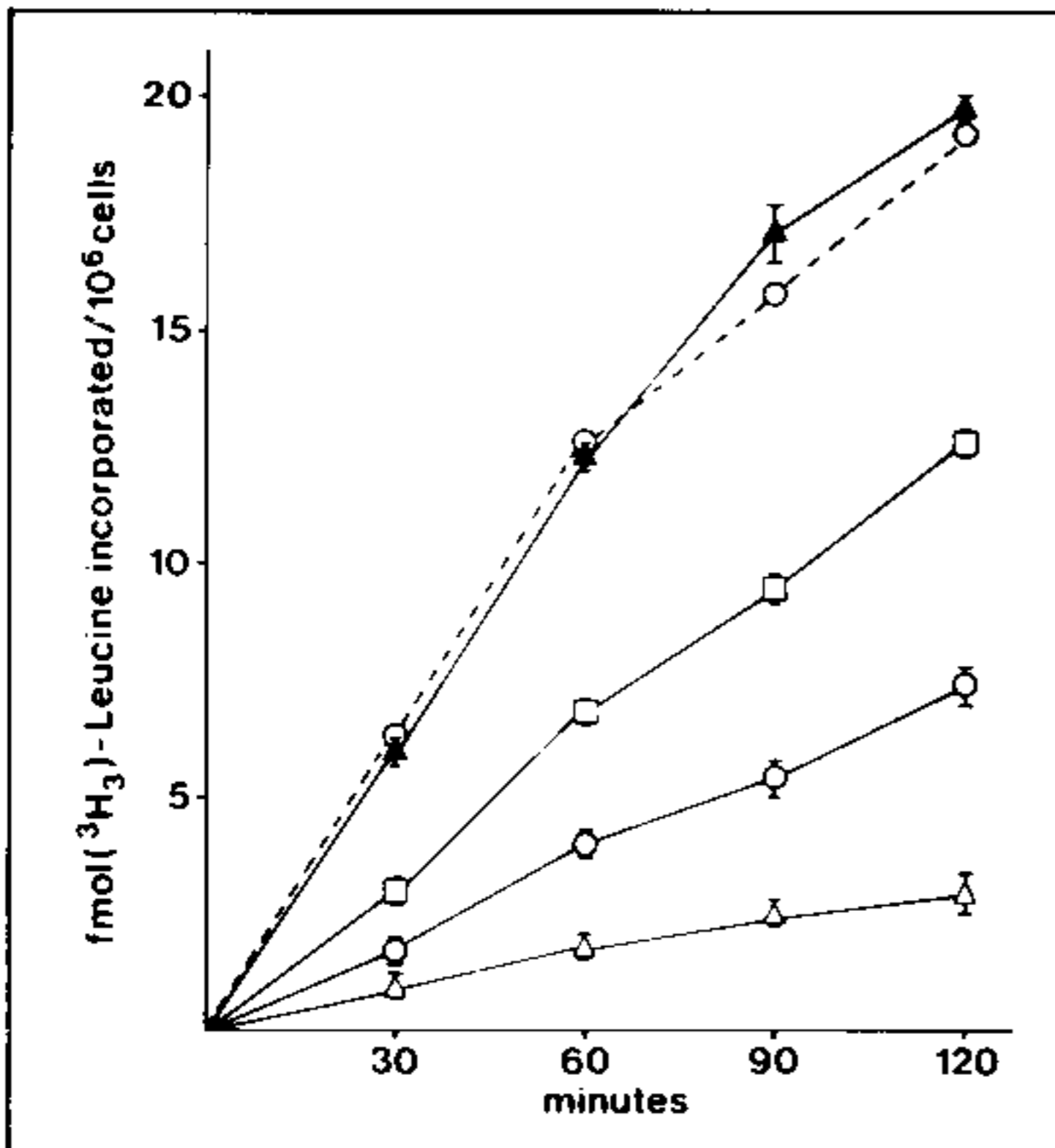


Fig. 1: dose-dependent inhibition of [3H]-leucine incorporation by amastigote forms of *Trypanosoma cruzi*. (▲) control, (●) 300 μM benznidazol, (□) 50 μM megazol, (○) 25 μM megazol and (△) 12.5 μM megazol. Each value shown is the mean of triplicate incubations.

uridine incorporation, comparing benznidazole and nifurtimox, the latter drug revealed more efficacy in inhibition DNA synthesis.

DISCUSSION

In this work, the important feature is the finding that the mode of action of megazol upon amastigote forms of *T. cruzi* involves selective inhibition of protein synthesis, measured by [3H]-leucine incorporation, whereas nifurtimox and benznidazole, even at higher concentrations, caused no alteration on the incorporation pattern. In the case of nucleic acid synthesis, the three drugs tested showed similar values of percent of inhibition being the effect on [3H]-thymidine incorporation stronger than that on [3H]-uridine. As benznidazole was assayed at a concentration three times higher than nifurtimox (Table II), these results indicate the latter drug is more effective and that probably different mechanisms are involved. While oxidative stress has been implicated in the mechanism of nifurtimox

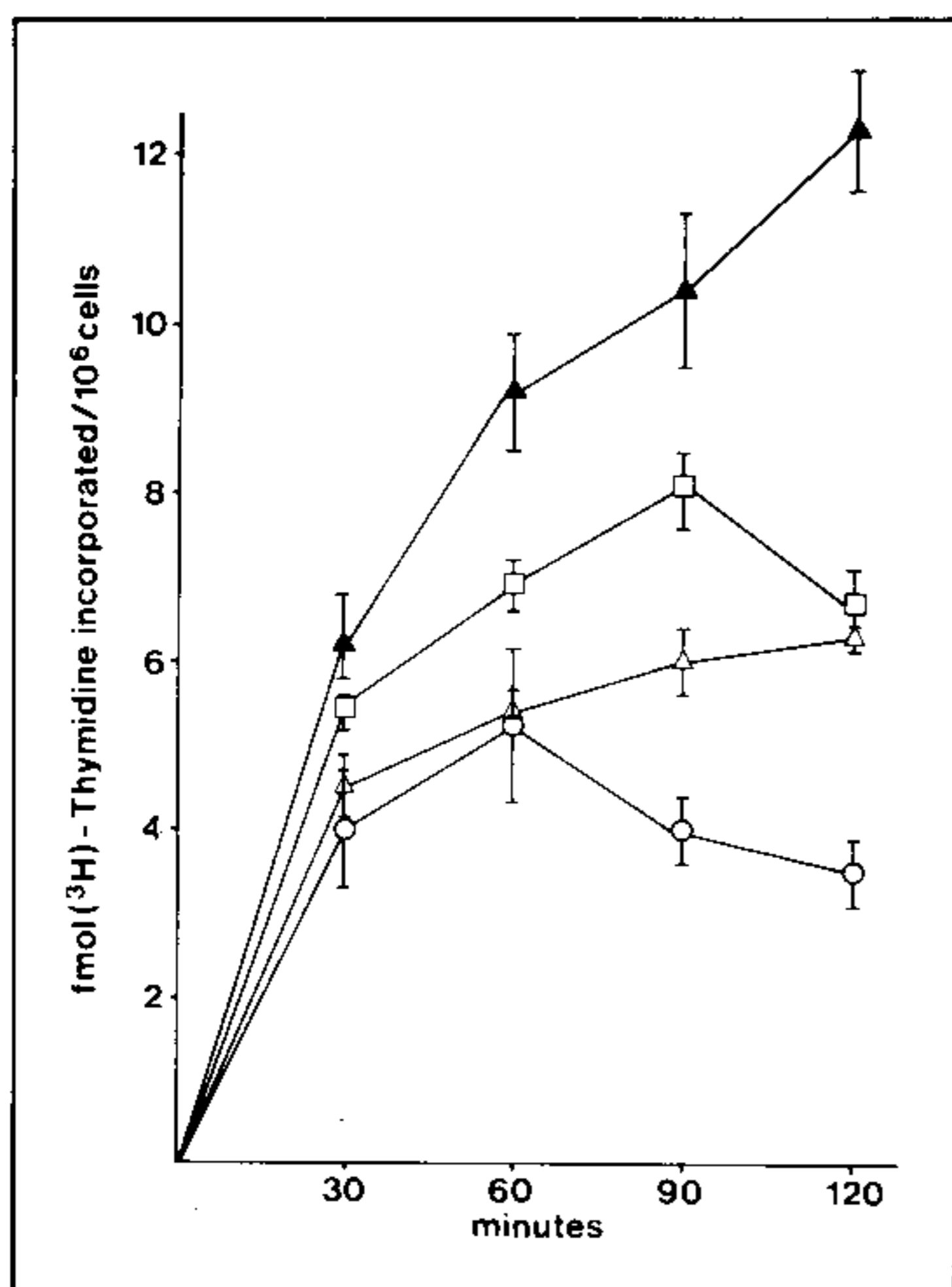


Fig. 2: effect of drugs on [3H]-thymidine incorporation by amastigote forms of *Trypanosoma cruzi*. (▲) control, (□) 300 μM benznidazol, (△) 100 μM nifurtimox and (○) 100 μM megalol. Each value shown is the mean of triplicate incubations.

toxicity on *T. cruzi*, the process has been ruled out as a toxic mechanism of benznidazole (Moreno et al., 1982; Do Campo & Moreno, 1985).

Several groups, using epimastigote forms (Sims & Gutteridge, 1979; Gugliotta et al., 1980; Goijman et al., 1984; Goijman & Stoppani, 1985), have used the assay of macromolecule precursor incorporation sometimes with different results. Analysing the nitrofurans SQ-18.506, Sims & Gutteridge (1979) considered that its mode of action upon epimastigotes involves inhibition of nucleic acid synthesis, whereas Gugliotta et al. (1980) suggested that the primary effect of this drug is on protein synthesis.

Goijman & Stoppani (1985) observed an inhibition of macromolecule synthesis of epimastigotes in the presence of benznidazole and nifurtimox, being the latter also more active. The levels of inhibition of incorporation were similar for nucleic acids for both forms of *T. cruzi*, however a direct correlation between

these results can not be established, since different strains were used — Tulahuen for epimastigotes and Y strain for amastigotes. Analyzing the effect of benznidazole on epimastigote forms of the Y strain, Polak & Richle (1978) showed that DNA synthesis is much less susceptible to the inhibitory effect of the drug than RNA and protein synthesis.

Comparing the effect of benznidazole upon amastigote (present paper) and epimastigote forms (Polak & Richle, 1978) of the same *T. cruzi* strain (Y), after 120 min of incubation in the presence of 300-380 μM benznidazole we observed that: (a) epimastigotes were more susceptible to the inhibitory action of the drug upon protein synthesis, whereas amastigotes were not susceptible at all; (b) in relation to DNA synthesis benznidazole impaired the amastigotes incorporation of the precursor but showed no effect upon epimastigote forms; (c) both forms were susceptible at the level of RNA synthesis. These experimental data once more point out to discrepancies in the results obtained when comparing the effect of drugs on epimastigotes and vertebrate forms of *T. cruzi* (Schlemper et al., 1977, Avila et al., 1981, McCabe et al., 1985).

Comparison between megalol and the used drugs — nifurtimox and benznidazole —, led to the suggestion that different mechanisms are involved in the mode of action of these compounds. Augusto et al. (1986) have already pointed out that the mechanism of action of megalol probably does not involve nitro anion radicals as in the case of nitrofurans (Do Campo & Moreno, 1984), since the thiadiazol derivate radical could not be detected in conditions in which it was evident such an effect for nifurtimox. Besides in microsomal fractions of *T. cruzi*, the generation of hydroxyl radicals was two times smaller with megalol than using nifurtimox.

Megalol has a definitive effect on protein synthesis of amastigotes of *T. cruzi* at concentrations at which nifurtimox and benznidazole presented no effect. Macromolecule synthesis proved to be a suitable parameter for the assay of therapeutic agents. We conclude that the mode of action of the thiadiazol derivate is different from those of these two latter drugs, being its primary effect associated with the impairment of protein synthesis by amastigote forms.

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REFERENCES

- AUGUSTO, O.; ALVES, M. J. M.; COLII, W.; FILARDI, L. S. & BRENER, Z., 1986. Megazol reductive metabolism. *Mem. Inst. Oswaldo Cruz*, 81 (suppl.): QT 16.
- AVILA, J. L., 1983. New rational approaches to Chagas' disease chemotherapy. *Interciência*, 8: 405-417.
- AVILA, J. L.; AVILA, A. & MUNOZ, E., 1981. Effect of allopurinol on different strains of *T. cruzi*. *Mol. Biochem. Parasitol.*, 11: 51-54.
- BERKELHAMMER, G. & ASATO, G., 1968. 2-amino-5-(1-methyl-5-nitro-2-imidazolyl) -1, 3, 4-thiadiazole: A new antimicrobial agent. *Science*, 162: 1146.
- BRENER, Z., 1979. Present status of chemotherapy and chemoprophylaxis of human trypanosomiasis in the western hemisphere. *Pharmac. Therap.*, 7: 71-90.
- BURDEN, E. J. & RACETTE, E., 1968. 2-amino-5-(1-methyl-5-nitro-2-imidazolyl) -1, 3, 4-thiadiazole, a new microbial agent. IX- Action against hemoflagellate infections in laboratory animals. *Antim. Agents Chemother.*, 7: 545-547.
- CARVALHO, T. U. & DE SOUZA, W., 1983. Separation of amastigotes and trypomastigotes of *T. cruzi* from cultured cells. *Z. Parasitok.*, 69: 571-575.
- DE CASTRO, S. L. & MEIRELLES, M. N. L., 1987. Effect of drugs on *T. cruzi* and its interaction with heart muscle cell "in vitro". *Mem. Inst. Oswaldo Cruz*, 82: 209-218.
- DE CASTRO, S. L.; MEIRELLES, M. N. L. & OLIVEIRA, M. M., 1987. *T. cruzi*: Adrenergic modulation of cAMP role in proliferation and differentiation "in vitro". *Exp. Parasitol.*, 64: 368-375.
- DO CAMPO, R. & MORENO, S. J., 1984. Free radical metabolites in the mode of action of chemotherapeutic agents and phagocytic cells on *T. cruzi*. *Rev. Infect. Dis.*, 6: 233-238.
- DO CAMPO, R. & MORENO, S. N. J., 1985. Biochemical toxicology of antiparasitic compounds used in the chemotherapy and chemoprophylaxis of american trypanosomes. *Rev. Biochem. Toxicol.*, 7: 159-204.
- FILARDI, L. S. & BRENER, Z., 1982. A nitroimidazole-thiadiazole derivative with curative action in experimental *T. cruzi* infection. *Ann. Trop. Med. Parasitol.*, 76: 293-297.
- FILARDI, L. S. & BRENER, Z., 1984. A rapid method for testing "in vivo" the susceptibility of different strains of *T. cruzi* to active chemotherapeutic agents. *Mem. Inst. Oswaldo Cruz*, 79: 221-225.
- FILARDI, L. S. & BRENER, Z., 1985. Efeito curativo do 2-amino-5-(1-metil-5-nitro-2-imidazolil)-1, 3, 4-tiadiazol (megazol) em animais inoculados com cepas de *T. cruzi* resistentes ao nifurtimox e benznidazol. *XXII Reun. Pesq. Bás. Doença de Chagas*, Brasil, p. 135.
- GOIJMAN, S. G.; FRASCH, A. C. C. & STOPPANI, A. O. M., 1984. Efectos diferentes del nifurtimox y el benznidazol sobre la biosíntesis de DNA, RNA y proteínas en *T. cruzi*. *Medicina*, 44: 261-270.
- GOIJMAN, S. G. & STOPPANI, A. O. M., 1985. Effects of nitroheterocyclic drugs on macromolecule synthesis and degradation in *T. cruzi*. *Biochem. Pharmacol.*, 34: 1331-1336.
- GUGLIOTTA, J. L.; TANOWITZ, H. B.; WITTNER, M. & SOEIRO, R., 1980. *T. cruzi*: Inhibition of protein synthesis by nitrofurans SQ 18.506. *Exp. Parasitol.*, 49: 216-224.
- GUTTERIDGE, W. E., 1985. Existing chemotherapy and its limitations. *Br. Med. Bull.*, 41: 162-168.
- MARIA, T. A.; FILARDI, L. S. & BRENER, Z., 1984. Ultrastructural alterations of intracellular stages and effect on blood forms of *T. cruzi* induced "in vivo" by 2-amino-5-(methyl-5-nitro-2-imidazolyl)-1, 3, 4-thiadiazole. *Rev. Soc. Bras. Med. Trop.*, 17: 89-93.
- MCCABE, R. E.; ARAUJO, F. G. & REMINGTON, J. S., 1985. *In vivo* and *in vitro* effects of cyclosporin in A on *T. cruzi*. *Am. J. Trop. Med. Hyg.*, 32: 960-962.
- MORENO, S. N. J.; DO CAMPO, R.; MASON, R. R.; LEON, W. & STOPPANI, A. O. M., 1982. Different behaviour of benznidazole as free radical generator with mammalian and *T. cruzi* microsomal preparations. *Arch. Biochem. Biophys.*, 218: 585-591.
- POLAK, A. & RICHLÉ, R., 1978. Mode of action of the 2-nitroimidazole derivative benznidazole. *Ann. Trop. Med. Paras.*, 72: 45-51.
- SCHLEMPER, B. R. Jr; CHIARI, E. & BRENER, Z., 1977. Growth inhibition drug test with *T. cruzi* culture forms. *J. Protozool.*, 24: 544-547.
- SILVA, L. H. P. & NUSSENZWEIG, V., 1953. Sobre uma cepa de *T. cruzi* virulenta para o camundongo branco. *Folia Clin. Biol.*, 20: 191-207.
- SIMS, P. & GUTTERIDGE, W. E., 1979. Mode of action of a 5-nitrofurans drug (SQ 18.506) against *T. cruzi*. *Int. Soc. Parasitol.*, 9: 61-67.