In Vitro and in Vivo Assays of 3,5-Disubstituted-Tetrahydro-2*H*-1,3,5-Thiadiazin-2-Thione Derivatives against *Trypanosoma cruzi*

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Cytotoxicity assays of 24 new 3,5-disubstituted-tetrahydro-2H-1,3,5-thiadiazin-2-thione derivatives were performed. The 17 compounds with higher anti-epimastigote activity and lower cytotoxicity were, thereafter, screened against amastigote of Trypanosoma cruzi. *Out of these 17 derivatives S-2d was selected to be assayed in vivo, because of its remarkable trypanocidal properties. To determine toxicity against J774 macrophages, a method based on quantification of cell damage, after 24 h, was used. Cell respiration, an indicator of cell viability, was assessed by the reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan. Anti-amastigote activity was estimated after 48 h by microscopic counts of May Grünwald-Giemsa-stained monolayers. Nifurtimox and benznidazole were used as reference drugs. For the in vivo experiences, mice were infected with 10^4 blood trypomastigotes and then treated during 15 days with S-2d or nifurtimox by oral route. All of the compounds were highly toxic at 100 µg/ml for macrophages and a few of them maintained this cytotoxicity even at 10 µg/ml. Of the derivatives assayed against amastigotes 3k and S-2d showed an interesting activity, that was held even at 1µg/ml. It is demonstrated that the high anti-epimastigote activity previously reported is mainly due to the non-specific toxicity of these compounds. In vivo assays assessed a reduction of parasitemia after administration of S-2d to infected mice.*

Key words: antichagasic drugs - amastigotes - J774 macrophages - cytotoxicity assays - antitrypanosomal activity assays - thiadiazine thione derivatives

Trypanosoma cruzi is the etiological agent of Chagas disease. It is estimated that 16-18 million people are chronically infected (WHO 1993). The current treatment is dependent on two nitroheterocyclic drugs, the nitrofuran nifurtimox (Lampit ®), whose production has now been discontinued, and the 2-nitroimidazole benznidazole (Rochagan ®) (Croft et al. 1997). Both drugs are effective in reducing the severity of acute and congenital Chagas disease, but have no role in the therapy of chronic infections. Both must be administrated for extended periods, often cause severe side effects and achieve parasitological cure in only about 50% of treated patients (Kirshhoff 1994). There is a considerable need for the development of new compounds to approach the chemotherapy for this disease.

In this context new 3,5-disubstituted-tetrahydro-2*H*-1,3,5-thiadiazin-2-thione derivatives have been synthesized. In a previous work it was reported the antibacterial, antifungal, antiviral, anthelmintic, and tuberculostatic activities of tetrahydro-2H-1,3,5-thiadiazin-2-thione as prodrugs. The isothiocyanates formed by hydrolysis of the thiadiazine ring interact with and inactivate cysteine proteinases (Ertan et al.1992).

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The anti-epimastigote activity of these new thiadizines was previously evaluated (Ochoa et al. 1999). All of them were effective at 100 µg/ml, and some of them even at 1 µg/ml (1m, S-1n, RS-2b, 2c, S-2d, RS-2f, S-2g and S-2j). Once these first in vitro studies were accomplished, new biological assays have been performed in the present work, to study the non-specific toxicity and anti-amastigote activity of these compounds.

MATERIALS AND METHODS

Cell culture - Murine J774 macrophages is a cell line that was kindly provided by the National Centre for Sanitary Microbiology, Virology and Immunology of Instituto Carlos III (Spain). It was grown in plastic 25 ml flasks in RPMI 1640 medium (Sigma) supplemented with 20% heat inactivated (30 min 56ºC) foetal calf serum (FCS) and 100 IU penicillin/ml + 100 µg/ml streptomycin, in a humidified 5% CO_2/95% air atmosphere at 37ºC and subpassaged once a week.

Parasites - *T. cruzi* (Y strain) was grown at 28ºC in liver infusion tryptose (LIT) supplemented with 10% FCS and antibiotics. Epimastigote forms were harvested on day 14 of culture (stationary phase) and washed three times in Grace medium. To induce metacyclogenesis, parasites were then cultured into fresh Grace medium supplemented with 10% FCS and haemin (25 µg/ml). Nine days after cultivation at 28ºC, metacyclic forms were counted in order to infect macrophages. The proportion of metacyclic forms was around 30% at this stage.

Cell infection - J774 macrophages were detached by EDTA-PBS (ethylendiamine tetraacetic acid- phosphate-

buffered saline) treatment and counted by a haemocytometer. Cells were seeded at a density of 50,000 cells/well in 24-wells microplates (NUNC) with rounded coverslips on the bottom. Then 500,000 trypomastigotes and fresh medium were added, giving a final volume of 2 ml. Attachment and invasion of host cells were allowed for 24 h.

Cytotoxicity to macrophages - J774 macrophages were seeded (70,000 cells/well) in 96-well flat bottom microplates (NUNC) with 200 µl of medium. The cells were allowed to attach for 24 h at 37ºC and then exposed to the compounds (100, 10 and 1 µg/ml) for 24 h. Afterwards, the cells were washed with PBS and incubated (37ºC) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 0.4 mg/ml for 60 min. MTT solution was removed and the cells solubilized in DMSO (100 µl). The extend of reduction of MTT to formazan within cells was quantified by measurement of optical density at 595 nm (OD_{595}) in a plate reader EL_x800 from Bio-Tek instruments Inc. (Hattori & Nakanishi 1995). Each concentration was assayed three times and six cell growth controls were used in each test. The assays were twice performed. Cytotoxicity percentages (%C) were determined as:

$$\%C = [1-(ODd-ODdm)/(ODc-ODm)] \times 100$$

were ODd: mean of OD_{595} of wells with macrophages and different concentrations of derivative; ODdm: mean of OD_{595} of wells with different derivative concentrations in medium; ODc: mean of OD_{595} of wells with macrophages and no compound (growth controls); and ODm: mean of OD_{595} of wells with only medium. The cytotoxic dose 50 (CD_{50}) was defined as the concentration of drug, which decreases OD_{595} in 50% in relation to control cultures.

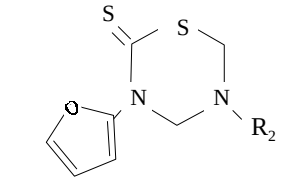
Anti-amastigote activity - After cell infection, culture medium was removed, and suspensions of compounds in fresh medium were added to final concentrations non-toxic for macrophages (i.e. concentrations < CD_{50}). After 48 h, the coverslips were fixed and stained with May Grünwald Giemsa and the number of amastigotes/100 macrophages (No. A/100 Mø) were estimated. Anti-amastigote activity (%AA) was expressed as:

$$\%AA = [1-(\text{No. A/100 Mø})p/(\text{No. A/100 Mø})c] \times 100$$

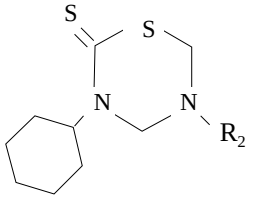
All experiments were run at least in triplicate and the results are given as mean ± standard deviation (Mendez et al. 1999).

Anti-Trypanosoma in vivo test - Three groups of 10 NMRI female mice of 45 days (at the beginning of test), weighting 20-22 g, were prepared. One was an infection control, the second was treated with nifurtimox 50 mg/kg/d given by gavage for 15 days and the third was administrated the new compound, that was dissolved in 2% carboxymethyl cellulose. The animals were infected by intraperitoneal injection of 10^4 blood trypomastigotes. The treatment was initiated three days after infection and maintained during 15 days. The level of parasitemia was checked by counting in a Neubauer Chamber the number of parasites in 5 µl of blood drawn from the tail of the mice and diluted 1:10 in ammonium chloride (Barr et al. 1995).

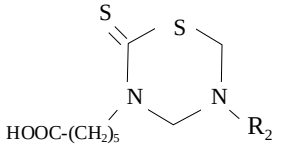
Source of compounds - The synthesis of the 24 new 3,5-disubstituted-tetrahydro-2*H*-1,3,5-thiadiazin-2-thione derivatives was described elsewhere (Ochoa et al. 1999).



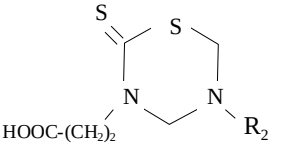
RS-1d: R_2=CH(CH_2Ph)-COOH;
1m: R_2= $(CH_2)_2$-COOH;
n: R_2= CH[CH_2-$CH(CH_3)_2$]-COOH



2a: R_2= $(CH_2)_5$-COOH; RS-2b: R_2= CH(COOH)-CH_2-COOH;
2c: R_2= CH_2-COOH; S-2d: R_2=CH(CH_2Ph)-COOH;
RS-2d: R_2=CH(CH_2Ph)-COOH;
S-2e: R_2= CH[$CH(CH_3)_2$]-COOH;
RS-2f: R_2= CH[$(CH_2)_2$COOH]-COOH;
S-2g: R_2= $CH(CH_3)$-COOH;
2h: R_2= CH_2-CONH-CH_2-COOH;
2i: R_2 = CH[$(CH_2)_2SCH_3$]-COOH;
S-2j: R_2= CH(CH_2CONH$_2$)-COOH;
2k: R_2= C[$(CH_3)_2$]-COOH; 2m: R_2= $(CH_2)_2$-COOH;
S-2n: R_2= CH[CH_2-$CH(CH_3)_2$]-COOH; 2o: R_2= cyclohexyl



3a: R_2= $(CH_2)_5$-COOH; 3c: R_2= CH_2-COOH;
3k: R_2= C[$(CH_3)_2$]-COOH; 3m: R_2= $(CH_2)_2$-COOH;
S-3n: R_2= CH[CH_2-$CH(CH_3)_2$]-COOH



4a: R_2= $(CH_2)_5$-COOH

Fig. 1: chemical structure of 3,5-disubstituted-tetrahydro-2H-1,3,5-thiadiazin-2-thione derivatives assayed

TABLE I

Cytotoxicity (%) of thiadiazine derivatives on J774 macrophages

Compound	1 µg/ml	10 µg/ml	100 µg/ml
Nifurtimox	2.7±3.4	3±3.6	17.8±11.9
Benznidazole	7.5±5.2	5.4±4.2	3.4±4.6
RS-1d	19.1±10.5	87.8±11.1	96.8±5.2
1m	11.6±9.1	71.2±5.8	95.9±2.3
1n	6.8±7.9	15.5±11	98.4±1.5
2a	12±6.6	15.7±6.5	92.9±4.8
RS-2b	5.1±6.7	13.1±7.7	95.2±5.4
2c	6±5.8	86.5±5.1	97.3±2.6
S-2d	2.6±3.5	4.8±5.9	92.5±4.5
RS-2d	2.8±3.3	19.3±3.7	86.7±7.6
S-2e	4.6±5.5	13.1±4.7	95.1±6.3
RS-2f	7.9±7.7	12.7±7.2	92.3±2.2
S-2g	11.3±9.6	29.3±10.1	96.5±2.8
2h	4.2±3.3	7±5.4	75±7.6
2i	6.8±5.6	21±8	80.7±2.6
S-2j	5±3.7	14.9±4.2	95.2±5
2k	3±3.5	13.8±9.1	87.1±7.1
2m	11.4±9	67.6±17.7	93.6±5.1
S-2n	3.8±2.7	19.1±10.8	86±5.1
2o	7.2±8.5	16.4±8.4	91.5±4.2
3a	3.9±5	2.3±2.6	98±2.2
3c	12.6±10.6	33.4±7	98.4±1.3
3k	3.7±4.5	6.4±6.7	91.1±7.4
3m	5.3±5.3	21±10.2	95.3±2.4
S-3n	1.9±2.8	6.1±8.7	98.7±1.5
4a	19±8.1	76.1±8.9	97.1±3

TABLE II

Activity of thiadiazine derivatives against amastigote forms of *Trypanosoma cruzi*

Compond	1 µg/ml	10 µg/ml	100 µg/ml
Nifurtimox	67.5±6.3	84.9±3.6	95±1.5
Benznidazole	40.3±3.1	82.1±1.5	92.4±3.8
1n	42±3	42±3	a
2a	18±9.6	18.2±7	a
RS-2b	0±7.4	0±14.4	a
S-2d	31.7±6.2	58.3±5.5	a
RS-2d	0±2.1	12.1±5.4	a
RS-2f	0.6±6.7	13.2±9.1	a
S-2g	24.1±7.5	33.3±4.9	a
2i	0±18.9	67.7±7.1	a
S-2j	10.7±14.2	54.8±3.6	a
2k	12±5.6	56.3±3.8	a
S-2n	7.1±3	64±8.9	a
2o	0±16.2	60.3±3.1	a
3a	24.2±11.1	25.6±17.9	a
3c	27±8.2	58±3.8	a
3k	39.8±2.9	58.9±1.9	a
3m	10.8±8.9	41±7	a
S-3n	9.9±4	20.1±5.9	a

a: toxic doses to macrophages J774

Their structures are shown in the Fig. 1. Nifurtimox (Lampit; Bayer, Buenos Aires, Argentina) and benznidazole (Rochagan; Roche, Rio de Janeiro, Brazil) were used as reference drugs in every assay.

RESULTS

Cytotoxicity of these new compounds is shown in Table I. As it can be observed every compound is toxic for macrophages at 100 µg/ml, thus they could not be assayed at this concentration against amastigotes. Compounds RS-1d, 1m, 2c, 2m and 4a were highly toxic also at 10 µg/ml for macrophages, for this reason they were not assayed against amastigotes. Compounds 2h and S-2e were only active against epimastigotes at 100 µg/ml (Ochoa et al. 1999), due to the high value of cytotoxicity observed for these compounds at this concentration, we suggest that the previously described anti-*T. cruzi* activity was the result of non-specific toxicity.

There are several products active against amastigotes (%AA > 50%) at 10 µg/ml: S-2d, S-2j, 2k, 2i, 2n, 2o, 3c and 3k. At 1µg/ml most of them lose their effectiveness. The %AA of compounds 1n, S-2d and 3k are close to 40% at 1 µg/ml, which is similar to that of benznidazole (Table II).

Compound S-2d was assayed in vivo to examine its ability to provide a cure for *T. cruzi* infected mice. Initially a dosage of 50 mg/kg/day was used and a reduction of parasitemia was observed (Fig. 2a), but this reduction was

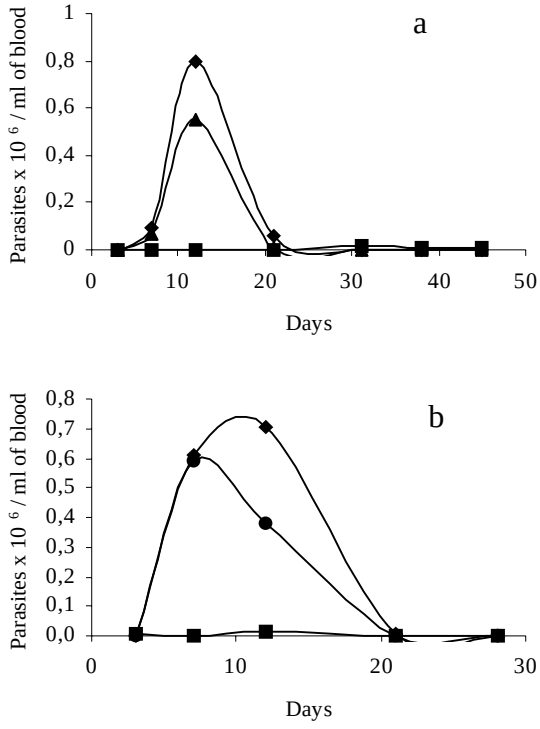


Fig. 2: parasitemias in infected NMRI mice after treatment of with S-2d. Control group (◆); nifurtimox treated group (■); group treated with 50 mg/kg/day of compound S-2d (▲); group treated with 100 mg/kg/day of compound S-2d (●).

not as remarkable as the one produced by nifurtimox. Thus, a higher dosage was administrated (100 mg/kg/day). An improvement of results was not observed. Mortality cases were not recorded for any of the groups (Fig. 2b).

DISCUSSION

Once the first screening against epimastigotes were performed (Ochoa et al. 1999), cytotoxicity assays were required to determine whether the high activity of this series of new compounds was due to a specific or non-specific toxicity. All the compounds in this series are highly toxic for mammalian cells in spite of carboxyalkyl substituents at 5th position that were supposed to decrease the toxicity (Ertan et al. 1992). None of the products with a $(CH_2)_5$-COOH substituent at 3rd position were toxic at 10 µg/ml but, in general, they were also less effective against epimastigotes than the others (Ochoa et al. 1999).

New potential anti-chagasic drugs must be screened in vitro against both extracellular epimastigotes and amastigote infected cells. Epimastigotes have the advantage that are not so expensive to maintain and the method employed is easy to perform, but screening against amastigote forms seems to be a more selective method. All compounds active against amastigotes were also active against epimastigotes, but the other way around is not always true (Martinez Díaz et al. 2000). From the derivatives with a furfuryl substituent at 3rd position only 1n was assayed against amastigotes, the rest of them had a toxicity far to high, which means that anti-epimastigote activity was mainly due to their cytotoxicity properties. Tetrahydro-2*H*-1,3,5-thiadiazine-2-thione derivatives had shown activity against other microorganisms and parasites (Ochoa et al. 1999) and with this paper we have also demonstrated their anti-trypanosome activity, though no structure-activity relationship could be found. Several compounds were effective against amastigotes at 10 µg/ml, but only S-2d and 3k are worth to continue with in vivo experiments because of their trypanocidal activity at 10 µg/ml and similar activity to benznidazole at 1 µg/ml, showing almost no cytotoxicity at these concentrations.

Derivative 3k was not further investigated because it is a mixture of stereoisomeres in an undetermined proportion, impossible to separate. Compound S-2d produces at 50 and 100 mg/kg/day a reduction of parasitemia in infected mice, but no dosage-activity relation was observed and no parasitological cure was achieved. The fact that no greater decrease in parasitemia values was recorded after administration of a dosage of S-2d 100 mg/kg/day, induces us to believe that some kind of problem regarding the intestinal absorption is the cause for the failure of this derivative to eradicate the infection in treated mice.

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