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New thiadiazine derivatives with activity against *Trypanosoma cruzi* amastigotes

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Abstract. The cytotoxicity of 18 new 1,2,6-thiadiazin-3,5-dione 1,1-dioxides was evaluated. This group of products was previously assayed against epimastigotes of *Trypanosoma cruzi* and some of them showed a high antiprotozoal activity. Thereafter 13 compounds with a high anti-epimastigote activity and low cytotoxicity were selected to be assayed against amastigotes. Some of the products showed the same or even lower cytotoxicity than nifurtimox and benznidazole, but most of them were very toxic for macrophages at 100 µg/ml. Only one of the compounds had an anti-amastigote activity similar to that of reference drugs at 10 µg/ml, but unfortunately this disappeared at lower concentrations.

Chagas' disease is a major health problem in South and Central America, affecting 16-18 million people (WHO 1991). In spite of such high prevalence, only two synthetic compounds, nitroheterocycles nifurtimox (Lampit®) and benznidazole (Rochagan®), are in use (Croft et al. 1997). Both are effective in the early stages of trypanosomiasis, but are practically useless in the chronic disease. Only 50% of patients are parasitologically healed after treatment (Kirchhoff 1994). The limited efficacy as well as their toxic side effects justify the continued research for trypanocidal substances.

In this way, new 1,2,6-thiadiazine-3,5-dione 1,1-dioxides have been synthesised (Di Maio et al. 1999). In previous works were reported the anti-*Trypanosoma cruzi* properties of 3,5-diamino-4-(5'-nitro-2-furfuryl-idene) 4*H*-thiadiazine 1,1-dioxide and those of some 4-heteroarylidene-1,2,6-thiadiazine-3,5-dione 1,1-dioxide (Atienza et al. 1992, Herrero et al. 1992). The presence of nitro substituents in the pentaheterocyclic moiety, as a source of free radicals, seems to be a factor that increases anti-*T. cruzi* activity (Di Maio et al. 1999).

Previous anti-*T. cruzi* activity of these new thiadiazines was evaluated on epimastigotes. Almost all of them were effective at 100 µg/ml. Some of them remained active at 10 µg/ml (3a, 3b, 3c, 3d, 4a, 4b, 4c and 4d), but none at 1 µg/ml (Di Maio et al. 1999). After the first screening, new *in vitro* studies have been performed to analyse the nonspecific toxicity and anti-amastigote activity of the compounds.

MATERIALS AND METHODS

Cell culture. Murine J774 macrophages were grown in plastic 25ml 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. *Trypanosoma cruzi* Chagas, 1909 (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 in 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-well 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 another 24 h. Afterwards, the cells were washed with PBS and incubated (37ºC) with 3-(4,5-

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dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 0.4 mg/ml for 60 min. MTT solution was removed and the cells solubilised in dimethyl sulphoxide (100 µl). The extent of reduction of MTT to formazan within cells was quantified by measurement of OD_{595} (Hattori and 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 follows:

$$\%C=[1-(ODp-ODpm)/(ODc-ODm)]*100$$

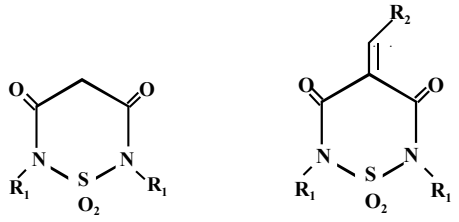
where ODp represents the mean OD_{595} value recorded for wells with macrophages containing different doses of product; ODpm represents the mean OD_{595} value recorded for different concentrations of product in medium; ODc represents the mean OD_{595} value recorded for wells with macrophages and no product (growth controls), and ODm represents the mean OD_{595} value recorded for medium/control wells. The cytotoxic dose 50 (CD_{50}) was defined as the concentration of drug that decreases OD_{595} up to 50% of that in 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:

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

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

Source of compounds. The synthesis of the 18 new 1,2,6-thiadiazine-3,5-dione 1,1-dioxides was described elsewhere (Di Maio et al. 1999). Their structures are shown in Fig. 1. Nifurtimox (Lampit; Bayer, Buenos Aires, Argentina) and benznidazole (Rochagan; Roche, Rio de Janeiro, Brazil) were used as reference drugs in every assay.



2 R_2 absent
3 R_2=5-nitro-2-thienyl
4 R_2=5-nitro-2-furyl
5 R_2=*p*-nitrophenyl

a R_1=cyclohexyl
b R_1=n-butyl
c R_1=n-hexyl
d R_1=benzyl
e R_1=phenethyl

Fig. 1. Chemical structure of 1,2,6-thiadiazine-3,5-dione 1,1-dioxides assayed. Structure of derivatives 2 (R_2 absent) is shown on the left, that of derivatives 3, 4 and 5 (R_2 nitro-substituted) is shown on the right.

RESULTS

Table 1 shows the cytotoxicity of these new compounds. As it can be seen compounds non-substituted in 4th position were not toxic for macrophages at 100 µg/ml, while the rest of compounds (3, 4 and 5 derivatives) with the exception of 5a, 5d and 4e had a %C > CD_{50}, thus they could not be assayed afterwards against amastigotes at this concentration. At lower concentrations there was no toxicity.

Activity against amastigotes is shown in Table 2. None of the compounds had activity greater than the reference drugs. Compound 3c had good trypanocidal effect at 10 µg/ml, but this disappeared at 1 µg/ml. Compound 3a had high activity at 100 µg/ml but also a relatively high toxicity (close to CD_{50}). At 10 µg/ml and 1 µg/ml it maintained some effect against amastigotes.

DISCUSSION

The complexity of the clinical entity caused by *Trypanosoma cruzi* makes the pharmacological screening of new compounds a hard and tedious job. Sensitivity of extracellular stages to drugs is usually higher than that of intracellular amastigotes (Martínez Díaz et al. 2000), so that the first screening on epimastigotes may produce a number of false positives. However, screening against epimastigotes maintained in liquid medium is easier and more economic than test on cellular cultures of amastigotes. In our opinion potential antichagasic drugs must be first selected on extracellular epimastigotes and thereafter on amastigote-infected cells.

Other 1,2,6-thiadiazine-3,5-dione 1,1-dioxides have been previously synthesised and assayed against *T. cruzi* (Atienza et al. 1992, Herrero et al. 1992), and 5-nitro-2-furyl derivatives had important activity, both against epimastigotes and amastigotes. The presence of nitro substituents, resembling in this way the structure of nifurtimox and benznidazole, seems to enhance the activity of these compounds. That is the reason why new 1,2,6-thiadiazines with 5-nitro-2-thienyl, 5-nitro-2-furyl and *p*-nitrophenyl substituents were synthesised along with their analogues without this heterocyclic moiety. The nitro substituents do not only enhance the anti-trypanosome activity but also the nonspecific toxicity. Thus analogues without the heterocyclic moiety were not toxic at 100 µg/ml, but only 2c had some anti-amastigote activity and at the same time, was the most toxic (%C ≈ 37%). In general, compounds with the heterocyclic moiety were considered toxic at 100 µg/ml (%C ≥ 50%), but these values hardly ever reached %C close to 90-100%, that the real toxic products have. As for the anti-amastigote activity, compound 3a had an important one at 100 µg/ml that was maintained at lower concentrations; at 1 µg/ml

Table 1. Cytotoxicity (%) of thiadiazine derivatives to J774 macrophages at different drug concentrations. Means of three tests ± standard deviation.

Product	100 µg/ml		10 µg/ml		1 µg/ml	
	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2
nifurtimox	11.7 ± 3.7	23.9 ± 11.1	0.6 ± 3.9	0 ± 7.3	0.3 ± 3.5	0.7 ± 6.3
benznidazole	0 ± 3.8	2.9 ± 6.8	1.9 ± 5.4	7.1 ± 2.7	6.9 ± 6.1	8.1 ± 1.6
2b	17.9 ± 8	21.9 ± 5.6	0 ± 6.8	0 ± 7.9	3.6 ± 9.0	0 ± 4.2
2c	0 ± 1.3	0 ± 8.2	0 ± 1.9	0 ± 5.8	0 ± 4.0	0 ± 5.3
2e	39.8 ± 8.1	34.7 ± 2.1	6.5 ± 3.9	0 ± 8.2	0 ± 6.6	0 ± 5.6
3a	48.3 ± 8.6	48.1 ± 10.6	16.2 ± 3.8	7.0 ± 7.0	0.4 ± 4.9	0 ± 8.2
3b	61.2 ± 6.7	58.6 ± 6.2	4.0 ± 2.2	0 ± 2.6	0 ± 3.6	0 ± 4.2
3c	94.6 ± 3.7	94.6 ± 3.7	4.1 ± 6.4	1.9 ± 0.8	0 ± 1.9	0 ± 1.1
3d	50.9 ± 2.1	58.2 ± 7.5	0 ± 8.8	0 ± 13.5	0 ± 3.2	0 ± 0.2
3e	74.3 ± 1.2	72.8 ± 2.8	0 ± 4.6	0 ± 3.2	0 ± 12.0	0 ± 5.9
4a	75.0 ± 1.2	80.1 ± 4.1	7.7 ± 4.7	4.6 ± 5.5	0 ± 8.9	0 ± 2.9
4b	58.3 ± 0.9	55.6 ± 7.3	0 ± 3.6	0 ± 5.5	0 ± 1.7	0 ± 4.4
4c	97.0 ± 1.0	91.0 ± 1.2	2.9 ± 9.0	1.9 ± 1.2	1.0 ± 4.2	0 ± 1.1
4d	55.8 ± 2.8	52.0 ± 2.4	0 ± 4.2	1.0 ± 5.6	4.4 ± 2.1	10.1 ± 5.9
4e	16.3 ± 9.3	14.1 ± 13.7	0 ± 12.6	0 ± 18.3	0 ± 3.7	0 ± 14.1
5a	2.1 ± 6.7	0 ± 10.6	8.0 ± 3.3	0 ± 4.8	3.1 ± 2.9	0 ± 3.3
5b	96.6 ± 4.7	93.5 ± 2.6	0 ± 3.6	0 ± 9.6	0 ± 6.0	0 ± 3.1
5c	100 ± 1.3	98.8 ± 1.6	8.7 ± 9.0	8.9 ± 6.3	0 ± 1.7	4.7 ± 4.2
5d	13.2 ± 8.0	17.9 ± 6.8	0 ± 5.1	0 ± 3.7	0.2 ± 4.2	0 ± 11.6
5e	100 ± 1.3	98.8 ± 2.8	0 ± 10.0	0.2 ± 2189	0 ± 3.1	7.9 ± 1.6

Table 2. Trypanocidal effects of several thiadiazine derivatives on amastigote forms at different drug concentration. Means of at least three tests ± standard deviation.

Product	100 µg/ml	10 µg/ml	1 µg/ml
nifurtimox	95.0 ± 1.5	84.9 ± 3.6	67.5 ± 6.3
benznidazole	92.4 ± 3.8	82.1 ± 1.5	40.3 ± 3.1
2b	6.6 ± 5.1	6.6 ± 18.8	0 ± 23.9
2c	0 ± 11.5	2.3 ± 4.1	0 ± 25.0
2e	75.1 ± 15.7	0 ± 24.9	0 ± 14.7
3a	75.8 ± 6.4	42.7 ± 7.1	9.9 ± 4.5
3b	*	65.8 ± 5.1	0 ± 6.3
3c	*	82.2 ± 2.5	0 ± 5.6
3d	*	0 ± 3.8	0 ± 12.0
4c	*	0 ± 15.5	0 ± 23.2
4d	*	0 ± 4.4	4.4 ± 7.6
4e	38.2 ± 7.8	12.0 ± 10.8	2.5 ± 13.6
5a	59.9 ± 4.2	0 ± 5.7	0 ± 11.2
5b	*	4.4 ± 23.7	32.4 ± 7.3
5d	26.2 ± 15.7	14.2 ± 6.2	7.4 ± 4.6

* toxic doses to macrophages J774

some activity was left. Although a complete clearance of amastigotes was not observed in any case, the new 1,2,6- thiadiazines assayed are more active that the ones described in previous works.

Based on the trypanocidal activities and the cytotoxicity experiments, the most interesting compound for animal studies of trypanosomal infection is 3a. Further work designed to examine the ability of compound 3a to provide a cure for *T. cruzi*-infected mice is currently in progress.

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