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Short communication

Sulphur-containing derivatives structurally related to fenoxycarb are potent growth inhibitors against the intracellular form of *Trypanosoma cruzi*

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Abstract

Sulphur-containing derivatives structurally related to the insect growth regulator fenoxycarb were shown to be extremely active antiproliferative agents against the amastigote form of *Trypanosoma cruzi* in in vitro assays. All of these drugs had previously been proved to be remarkably potent growth inhibitors against the epimastigote form of the parasite.

Keywords: *Trypanosoma cruzi*; Amastigotes; Growth inhibitors; Antiparasitic agents

1. Introduction

Chagas' disease or American trypanosomiasis is an important health problem that affects around twenty million people in Central and South America [1]. Around 2–3 million individuals develop the typical symptoms of this disease that results in 50 000 yearly deaths [2]. The causative agent of this disease is the haemoflagellate protozoan *Trypanosoma cruzi*, which is transmitted in rural areas to humans and other mammals by reduviid bugs such as *Rhodnius prolixus* and *Triatoma infestans* [3,4]. The main route of transmission is the result of blood-sucking activity of Chagas' disease vectors on mammals when feeding in a cyclic process. The parasite presents three main morphological forms in a complex life cycle. It replicates within the crop and midgut of Chagas' disease vectors as the epimastigote form, it is released with the insect excrements as the nondividing highly infective metacyclic trypomastigotes that invade mammalian tissues via wounds provoked by blood sucking action. The parasite multiplies intracellularly as amastigotes, the clinically more relevant form of the parasite, which is released as the nondividing bloodstream trypomastigote form that invades other tissues [3,4].

Despite the progress made in the study of *T. cruzi* biochemistry and physiology [5], in which several crucial enzymes for parasite survival (not present in the host) have been identified as potential targets for the design of new drugs [6–11], the chemotherapy to control this parasitic infection remains undeveloped. It is based on old and quite unspecific drugs associated with long term treatments that give rise to severe side effects. In fact, although nifurtimox (4-([5-nitrofurfurylidene]-amino)-3-methylthiomorpholine-1,1-dioxide) and benznidazole (*N*-benzyl-2-nitro-1-imidazoleacetamide), the only two drugs currently in use for clinical treatment of this disease [12,13], are able to wipe out parasitaemia and reduce serological titres, they are not specific enough to all *T. cruzi* strains to guarantee complete

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cure [14–17]. The chronic stage of this disease leads to irreversible cardiac and digestive disorders. In addition, as this parasite may be transmitted by transfusion of contaminated blood, it is very important to have an efficient agent to eradicate the bloodstream trypomastigotes from blood banks. The only drug clinically in use for blood sterilization is crystal violet (*N*-{4-bis[[4-(dimethylamino)-phenyl]methylene]-2,5-cyclohexadien-1-ylidene}*N*-methyl-methanaminium chloride) [18], which in turn suffers from some disadvantages regarding its safety [19]. In the last few years, this illness has been encountered even where it is not endemic such as in Southern California, in the US, as a consequence of transfusion of contaminated blood from immigrants [20–23]. This awful illness, associated to poverty and bad housing quality, does not attract the pharmaceutical industry as a result of the lack of commercial motives; therefore, efforts to develop new and safer drugs have to be carried out mainly by academic institutions.

Ergosterol biosynthesis inhibitors promised to be interesting chemotherapeutic agents not only for fungi but also for different parasites. Depletion of endogenous sterols produces impaired growth of the parasite and this sterol biosynthetic pathway differs significantly compared with the mammalian host [24]. The blockage of this metabolic pathway has been extensively studied and some drugs show potential as chemotherapeutic agents [25–27].

2. Materials and methods

Experiments on the intracellular form of the parasite were carried out on *T. cruzi*-infected L_6E_9 myoblasts (Y strain) as previously described [28]. Confluent myoblast cell monolayers were prepared on 0.9×0.9 cm coverslips in tissue culture chambers (three coverslips per treatment). Myoblasts were trypsinized and counted in a Neubauer haemocytometer. The same number of cells were inoculated into each chamber. The monolayers were washed three times with phosphate buffered saline (PBS) at 37°C after 4 h. Some monolayers were exposed to a suspension of tissue culture-derived trypomastigotes in DMEM-10% FBS (1 ml/chamber). The final concentration of trypomastigotes was adjusted to a ratio of 4:1 parasites to L_6E_9 cells. The parasites were allowed to internalize within the myoblast for 24 h. At this time, a set of *T. cruzi*-infected cultures was fixed and stained with Giemsa and was designated the 24-h control culture. The media from the remaining slides was removed and fresh DMEM-10% FBS alone (control) or containing compounds 3–9 (see Results section) was added to the cultures. Compound 2 and nifurtimox were used as positive controls. After a further 24 h of incubation at 37°C, a set of *T. cruzi*-infected cultures (untreated control and drug-treated) was fixed and stained with Giemsa. Media were removed from other cultures and again, fresh DMEM-10% FBS alone (control) or containing compounds 3–9 (see Fig. 1 for structures) was added to the cultures. Cultures were incubated (37°C) for a further 24 h after which they were fixed and stained with Giemsa. Infection was assessed by the percentage of myoblasts with intracellular parasites and by the number of parasites present in 100 myoblasts. A minimum of 200 cells was screened in each culture.

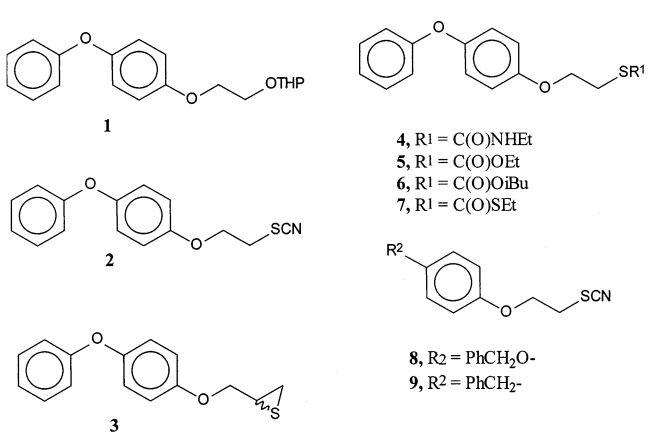

Fig. 1. Chemical structures of potent growth inhibitors against intracellular *T. cruzi* structurally related to fenoxycarb.

3. Results and discussion

In the present study, we report the biological activity of sulphur-containing derivatives structurally related to fenoxycarb (*N*-ethyl 2[4-phenoxyphenoxyethyl] carbamate) [29], against the intracellular form of the parasite. 4-Phenoxyphenoxyethyl tetrahydropyranyl ether (compound 1) that had previously exhibited ability to control proliferation of the epimastigote forms of *T. cruzi* [30,31] was also a very active inhibitor of amastigotes [28]. It is worth noting that these drugs, originally designed as juvenile hormone analogues for Chagas' disease vectors, *T. infestans* and *R. prolixus* [32,33], became cell growth inhibitors bearing in mind that *T. infestans* treated with juvenile hormones were less susceptible to natural infection with *T. cruzi* [34]. There is good reason to believe that the mode of action of these compounds is the blockage of the sterol biosynthetic pathway [35,36] at an early stage [37]. According to the mode of action of these drugs, compound 1 is devoid of biological activity against the nondividing trypomastigotes [38]. We have demonstrated that the presence of the 4-phenoxyphenoxy as a nonpolar skeleton is very important in maintaining a high growth inhibitory action [37,39,40]. As this pathway differs in *T. cruzi* and mammalian cells [19], it would not be expected to have toxic side effects in the host cells.

Table 1
Growth inhibition of intracellular *T. cruzi* by compounds 3–9

	% Myoblast with parasites	Parasites No. per 100 myoblasts (%I)[a]
Compound 3 (μg/ml)		
None (24 h)	28.3 ± 4.8	60.5 ± 17.5
None (72 h)	18.0 ± 4.0	667.3 ± 77.3
1.0	14.3 ± 2.3	531.8 ± 28.3
2.5	20.5 ± 2.0	503.8 ± 17.3
5.0	19.0 ± 2.5	577.0 ± 10.5
Compound 4 (μg/ml)		
None (24 h)	41.3 ± 3.8	113.0 ± 8.0
None (72 h)	19.3 ± 2.8	570.0 ± 17.8
1.0	20.8 ± 1.3	180.8 ± 2.8 (68)
2.5	12.3 ± 0.8	59.0 ± 6.5 (90)
5.0	11.5 ± 4.5	28.5 ± 14.5 (95)
Compound 5 (μg/ml)		
None (24 h)	41.3 ± 3.8	113.0 ± 8.0
None (72 h)	19.3 ± 2.8	570.0 ± 17.8
1.0	16.0 ± 1.5	188.3 ± 10.5 (67)
2.5	35.0 ± 5.0	282.5 ± 72.3 (51)
5.0	11.3 ± 1.3	131.3 ± 38.8 (77)
Compound 6 (μg/ml)		
None (24 h)	41.3 ± 3.8	113.0 ± 8.0
None (72 h)	19.3 ± 2.8	570.0 ± 17.8
1.0	19.8 ± 2.3	571.0 ± 65.0
2.5	23.3 ± 3.8	323.8 ± 104 (43)
5.0	27.0 ± 0.0	167.8 ± 23.3 (71)
Compound 7 (μg/ml)		
None (24 h)	41.3 ± 3.8	113.0 ± 8.0
None (72 h)	19.3 ± 2.8	570.0 ± 17.8
1.0	23.0 ± 1.5	554.8 ± 18.3
2.5	26.5 ± 1.5	462.0 ± 37.0 (19)
5.0	19.3 ± 6.8	179.5 ± 63.5 (69)
Compound 8 (μg/ml)		
None (24 h)	28.3 ± 4.8	60.5 ± 17.5
None (72 h)	18.0 ± 4.0	667.3 ± 77.3
1.0	18.5 ± 1.0	340.3 ± 32.8 (49)
2.5	19.8 ± 0.8	378.3 ± 193 (43)
5.0	14.3 ± 1.8	104.5 ± 3.5 (84)
Compound 9 (μg/ml)		
None (24 h)	30.0 ± 2.0	99.0 ± 4.5
None (72 h)	20.0 ± 1.0	515.0 ± 24.0
1.0	18.3 ± 1.3	135.8 ± 4.8 (73)
2.5	12.5 ± 4.5	56.0 ± 13.5 (89)
5.0	12.0 ± 1.0	41.3 ± 3.8 (92)

[a] I, percent inhibition of growth compared to control.

Experiments were set up to determine the activities of compounds 3–9 against *T. cruzi*-infected L_6E_9 myoblasts. Sulphur-containing derivatives structurally related to fenoxycarb proved to be extremely potent inhibitors of *T. cruzi* proliferation [37]. The inhibitory action previously observed for 4-phenoxyphenoxyethyl thiocyanate (compound 2) against epimastigotes with an IC_{50} close to 2 μM was present against amastigotes at the low nanomolar range [37]. This form was more sensitive to these drugs in all cases with the exemption of drug 3, which was toxic for myoblasts. The rest of the tested drugs had similar activity to that formerly observed against the epimastigote form of the parasite [37] (see Table 1). The amastigotes were more sensitive to this family of drugs in all cases as has been noticed for several sterol biosynthesis inhibitors. Thus, when compound 4 was used, there was a significant dose-dependent reduction in the percentage of myoblasts containing amastigotes and in the number of parasites per 100 myoblasts, this effect being more noticeable after 48 h. Thiolcarbamate 4 ($IC_{90} = 2.5$ μg/ml), at 5.0 μg/ml, substantially eradicated all parasites, and basically reduced the percentage of infected cells to a half. Drug 4, at a concentration as low as 1.0 μg/ml exhibited a significant growth impairing action with inhibition values close to 70%. The replacement of the nitrogen atom by an oxygen atom which gives rise to the thiolcarbonate derivative 5 slightly lessened the biological activity compared with compound 4. Thiolcarbonates 6 and 7 (IC_{50} values of 3.1 and 4.1 μg/ml, respectively) were less active than 4 and 5. Both drugs were ineffective at concentrations of 1.0 μg/ml against the intracellular amastigotes. Drugs 8 and 9 that have the thiocyanate moiety at the polar end, proved to be extremely potent growth inhibitors of the intracellular form of the parasite. At a concentration of 1.0 μg/ml, compound 8 was able to reduce growth by 50% ($IC_{50} = 1.0$ μg/ml), while at 5.0 μg/ml 85% of growth was inhibited. Similar results were observed for drug 9 ($IC_{90} = 2.5$ μg/ml). At a concentration of 1.0 μg/ml thiocyanate derivative 9 inhibited growth by 73% while at a concentration of 5.0 μg/ml the parasite numbers were reduced to less than 10% of the controls and the percentage of infected myoblasts by nearly half. Drug 2 and nifurtimox were used as positive controls with IC_{90} values of 1.0 and 2.1 μg/ml, respectively. At the concentrations used, with the exemption of drug 3, the compounds were not toxic to the host cells as judged by their normal morphology and no direct effects of the drugs were detected on the parasites incubated alone for as long as 1 h under similar conditions. In conclusion, the presence of a sulphur atom in the aliphatic side chain plays a critical role in molecular recognition and the activity previously observed against the epimastigote form of the parasite correlates very well with these results.

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