Trypanosoma cruzi: Inhibition of Parasite Growth and Respiration by Oxazolo(thiazolo)pyridine Derivatives and Its Relationship to Redox Potential and Lipophilicity

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**Program of Clinical and Molecular Pharmacology, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, P.O. Box 70086 Santiago 7, Chile; †Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Salamanca, Spain; and ‡Laboratory of Bioelectrochemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, P.O. Box 233, Santiago, Chile*

Maya, J. D., Morello, A., Repetto, Y., Rodríguez, A., Puebla, P., Caballero, E., Medarde, M., Núñez-Vergara, L. J., Squella, J. A., Ortiz, M. E., Fuentealba, J., and San Feliciano, A. 2001. *Trypanosoma cruzi:* Inhibition of parasite growth and respiration by oxazolo(thiazolo)pyridine derivatives and its relationship to redox potential and lipophilicity. *Experimental Parasitology* **99,** 1–6. Chagas' disease constitutes a therapeutic challenge because presently available drugs have wide toxicity to the host and are generally ineffective in the chronic stages of the disease. A series of oxazolo(thiazolo)pyridene derivatives were studied on *Trypanosoma cruzi* epimastigote growth and oxygen consumption and their electrochemical (redox) potentials and lipophilicity. The derivatives produced different degrees of parasite growth and respiration inhibition on CL Brener, LQ, and Tulahuen strains of *T. cruzi* epimastigotes. Respiratory chain inhibition appears to be a determinant of the trypanosomicidal activity of these compounds, since a significant correlation between respiration and culture growth inhibition was found. A similar correlation was found, within the different structural subfamilies, between toxic effects and the ability of the compounds to be oxidized in aqueous media. The inhibition of respiration and of parasite growth in culture increases with the lipophilicity of the substituents on the oxazolopyridine nucleus. No difference in the action of these derivatives was found among the different parasite strains. It is concluded that these compounds may have a potential usefulness in the treatment of Chagas' disease.

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Index Descriptors and Abbreviations: oxazolopyridines; thiazolopyridine; *Trypanosoma cruzi*; epimastigotes; oxygen consumption; apparent partition coefficient; oxidation peak potential; respiratory chain.

INTRODUCTION

There are over 20 million people infected with *Trypanosoma cruzi* in Latin America, and mortality indices range from 8 to 12%, depending on patient's age and physiological state (WHO, 1998). This makes Chagas' disease (American trypanosomiasis) a serious health problem. The available antichagasic therapies are inadequate; in the first place, nifurtimox and benznidazole, classical nitroheterocyclic antichagasic agents, have serious side effects that force half of the patients to stop treatment. Because of nifurtimox's toxicity and its ineffectiveness in chronic stages of Chagas' disease, it is no longer used in some countries. Additionally, the many different parasite strains so far isolated show important differences in susceptibility (Filardi and Brener 1987; Gustafsson *et al.* 1987; Maya *et al.* 1997; Morello *et al.* 1994; WHO 1998). Due to these problems, hundreds of chemical compounds, both natural and synthetic, have been tested as

antichagasic agents, but the potential of actual toxicity and the low water solubility of many of these have curtailed their use (Chiari *et al.* 1991; Rivas *et al.* 1999; Sepulveda-Bosa and Cassels 1996).

Among the tested compounds, a series of nitroaryl-1,4-dihydropyridine and 3-chlorophenyl-1,4-dihydropyridine derivatives proved to be active against several strains of *T. cruzi* epimastigotes (Maya *et al.* 2000; Nuñez-Vergara *et al.* 1997). The results demonstrated that all compounds have an inhibitory effect on the growth and respiration of parasites. Due to the positive results previously found and with the aim of predicting the antichagasic activity of new compounds structurally related to the dihydropyridines, we have now extended the studies to a series of fused heterocyclic analogues, with the basic skeleton of ozaxolo(thiazolo)pyridines.

MATERIALS AND METHODS

Chemicals and drugs. Tryptose, fetal calf serum, yeast extract, and tryptone were obtained from Difco. Hemin and all other chemicals were purchased from Sigma Chemical Co. For oxazolo(thiazolo)pyridines, synthesis of compounds 1 to 8 (Fig. 1) was reported previously (Caballero *et al.* 1996; San Feliciano *et al.* 1991).

Apparent partition coefficient determination (Papp). Studies were carried out in a system containing octanol and 50 mM phosphate buffer, pH 7.4. Both phases were mutually saturated at 23°C prior to use. Drugs were dissolved in octanol to obtain final concentrations about 0.5 mM. After the equilibrium was achieved, absorbance in both phases were determined by UV-Vis spectroscopy (Unicam UV 160 spectrophotometer) between 345 and 360 nm, depending on the derivative. Final drug concentrations were calculated from the corresponding calibration curves. All the experiments were performed in triplicate.

Retention time (Rt) determination of oxazolo(thiazolo) derivatives by HPLC. HPLC measurements were carried out by using a Waters assembly equipped with a Model 600 Controller pump and a Model 996 Photodiode Array Detector. The acquisition and treatment of data were made by means of Millenium version 2.1 software. As a chromatographic column, a Bondapak/Porasil C-18 column of 3.9 × 150 mm was used. As a column guard, a C18 Bondapak (30 × 4.6 mm) was employed. The injector was a 20-μl Rheodine valve.

Chromatograms were obtained through the photodiode array detector at 340 nm. An isocratic method was employed, using a mobile phase composed of acetonitrile/water (60/40) at 1 ml/min and applying helium spurting (30 ml/min) to remove dissolved gases. The temperature was kept constant at 35°C.

Electrochemical measurements. All the studies were carried out in aprotic media (dimethylformamide containing 0.1 M tetrabutylammonium perchlorate) or in aqueous media (ethanol/Britton Robinson buffer, pH 7.4, KCl) using an electrochemical BAS equipment Model 50W. A glassy carbon electrode, an Ag/AgCl-electrode, and a platinum wire electrode were used as the working electrode, reference electrode, and auxiliary electrode, respectively. Consequently, oxidation peak potential values were measured against an Ag/AgCl reference electrode (Nuñez-Vergara *et al.* 1997, 1999). Drugs were used at a 0.5 mM concentration.

Parasites. *T. cruzi* epimastigotes (CL Brener, Tulahuén, and LQ strains), from our collection, were grown at 28°C in Diamond's monophasic medium as reported earlier (Aldunate *et al.* 1986), with blood replaced by 4 μM hemin. Fetal calf serum was added to a final concentration of 4%. For parasites, 80 × 10^6 cells correspond to 1 mg of protein or 12 mg of fresh weight.

Oxygen uptake. The parasites were harvested at the fourth or fifth day of growth by centrifugation at 500*g* for 10 min and then washed and resuspended with 0.05 M potassium phosphate buffer, pH 7.4, containing 0.107 M sodium chloride. Respiration measurements were carried out polarographically with a Clark No. 5331 electrode (Yellow Spring Instruments) in a Gilson 5/6 oxygraph (Letelier *et al.* 1990). The chamber volume was 2 ml and the temperature was 28°C. The number of parasites used for the assays was equivalent to 2 mg of protein. Drugs were added at a 100 μM final concentration in DMSO. Control respiration was 35 ± 5 n-at. oxygen/min/mg of protein. Values are expressed as the mean ±SD of three or more independent experiments.

Epimastigote growth inhibition. Four to five different concentrations of each drug dissolved in dimethylsulfoxide were added to a suspension of 3 × 10^6 *T. cruzi* epimastigotes/ml (Tulahuen and LQ strains and CL Brener). Final concentrations in the culture growth were between 10 and 250 μM for each drug. Parasite growth was followed by nephelometry for 10 days (Ferreira *et al.* 1988; Aldunate *et al.* 1992). No toxic effect of dimethylsulfoxide (DMSO) alone was observed.

The growth constant (K_c) for each drug concentration employed and the control was calculated using the epimastigote exponential growth curve (regression coefficient >0.97, $P < 0.05$). The slope resulting from plotting the Ln of nephelometry lecture versus time corresponds to the K_c (hours^{-1}). The IC_{kc50} is defined as the drug concentration needed to diminish the control growth K_c in 50%, calculated by linear regression analysis from the K_c values at the employed concentrations.

Drug toxicity. To perform toxicity determinations, parasite suspensions (3 × 10^6 cells/ml of CL Brener epimastigotes suspended in Diamond media) were incubated for 2 and 24 h at 28°C, with drugs at a 100 μM concentration in DMSO. Changes in parasite motility and shape were microscopically (40× magnification) observed (Letelier *et al.* 1990).

Toxicity grades expressed as 0, 1, 2, 3, and 4 represent the sequential changes in motility, shape, and lysis of parasites (Letelier *et al.* 1990).

No effect on cell growth, oxygen consumption, or drug toxicity attributable to DMSO was observed at the maximum concentration used of 2%.

Statistical analysis. Pearson's correlation and linear regression analysis were performed using Prism Graphpad software from Graphpad Software Inc.

RESULTS AND DISCUSSION

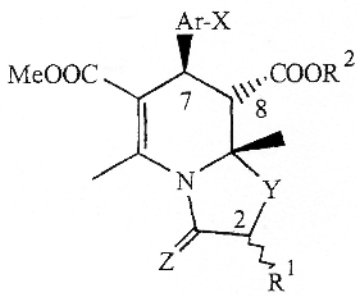
Nifurtimox, benznidazole, and many 1,4-dihydropyridines, such as nicardipine and felodipine, have inhibitory effects upon epimastigote growth and oxygen uptake (Maya

et al. 2000). The oxazolo(thiazolo)pyridine derivatives evaluated in this work share some structural similarity with dihydropyridines (Fig. 1). To determine the inhibitory ability of these compounds, we investigated their effect on epimastigote growth in culture and respiration.

Table I shows oxazolo(thiazolo)pyridines effect on the growth of *T. cruzi*, CL Brener, epimastigotes. The inhibition is expressed as the IC_{Kc50} (see culture growth inhibition under Materials and Methods for experiments and calculations). A global dose–response dependence trend was observed. The 3-chlorophenyl oxazolopyridines **2, 3**, and **4** and the thio analogue **8** are more potent inhibitors than the nitro **5** and **6** and the unsubstituted furyl **7** derivatives. These potency values are comparable to that of nifurtimox and benznidazole, which have IC_{Kc50} of 10 and 20 μM, respectively, under the same experimental conditions.

With these results, some general structure–activity relationships observations can be made. The presence of the chlorophenyl moiety seems to be important for antitrypanosomal activity. Nevertheless, if the comparison is restricted to the 3-chlorophenyl-oxazolopyridines **1** to **4**, an influence on activity is observed for the size (lipophilicity) of the R^2 substituent, attached to the ester group at position C-8. The presence of a carbonyl function at position C-3 also induces enhancement of the antiparasitic potency (compound **4** vs compound **1**).

Values for the inhibition of parasite respiration by the same derivatives, at 100 μM concentration, are also included in Table I. When parasite growth and respiration inhibitions are studied together for all the compounds, a reasonably good positive correlation is found (Pearson's correlation coefficient, 0.8883, $P < 0.005$), indicating that the respiratory chain is an important target for this type of compound (Fig. 2A, Table I). When the lipophilicity of the substituents is considered (Table I, Fig. 2B), it becomes clear that an increase in lipophilicity increases the inhibitory action of these



Compound	Ar	X	Y	Z	R^1	R^2	Type
1	phenyl	3-Cl	O	H_2	Methyl	Methyl	chlorophenyl-oxazolopyridines
2	phenyl	3-Cl	O	H_2	H	Allyl	
3	phenyl	3-Cl	O	H_2	H	Benzyl	
4	phenyl	3-Cl	O	O	Methyl	Methyl	
5	phenyl	3-NO_2	O	H_2	H	Methyl	nitrophenyl-oxazolopyridines
6	phenyl	3-NO_2	O	H_2	H	Ethyl	
7	2-furyl	H	O	H_2	H	Methyl	furyl-oxazolopyridine
8	phenyl	3-Cl	S	H_2	H	Methyl	chlorophenyl-thiazolopyridine

FIG. 1. Chemical structures of oxazolo(thiazolo)pyridines.

TABLE I

Effect of Oxazolo(thiazolo)pyridine Derivatives on Culture Growth, Oxygen Uptake, and Toxicity upon *Trypanosoma cruzi* Epimastigotes (CL Brener) and Oxidation Potentials and the Lipophylicity of Drugs

Drugs[a]	Inhibition of epimastigote growth[b] [IC_{Kc50} (μM)]	Inhibition of respiration[c] (%)	Toxicity (h)[d]		Peak potential[e] E_p (mV)		Lipophilicity[f]	
			2	24	aqueous	DMF	Apparent K_p	Retention time (min)
3	8.3 ± 1.0	70.6 ± 6	0	3	938	1118	10.9 ± 0.9	5.6 ± 0.1
8	9.6 ± 0.6	90.0 ± 4	3	4	928	1126	8.7 ± 1.1	4.9 ± 0.1
4	14.6 ± 2.5	68.5 ± 7	2	3	—	—	20.0 ± 1.3	8.8 ± 0.1
2	14.0 ± 1.2	24.5 ± 4	1	4	948	1084	5.2 ± 0.9	4.4 ± 0.3
6	287.4 ± 65.5	10.0 ± 3	1	1	950	1126	17.0 ± 2.3	6.8 ± 0.1
5	214.6 ± 45.7	10.0 ± 3	1	1	978	1158	14.3 ± 1.5	6.6 ± 1.1
1	147.8 ± 10.7	8.0 ± 3	1	2	952	1068	12.5 ± 1.8	6.0 ± 0.2
7	147.8 ± 13.1	5.0 ± 2	1	2	974	1138	2.3 ± 0.5	3.0 ± 0.2

[a] See Fig. 1 for chemical structures.

[b] The IC_{Kc50} corresponds to the concentration of drug needed to inhibit 50% of the control culture growth.

[c] Calculated with respect to control (35 ± 5 n-at, oxygen/min/mg protein) at a 100 μM drug concentration at 28°C.

[d] Toxicity grades expressed as 0 (Control); 1, 2, 3, and 4 represent the sequential changes in motility, shape, and lysis of parasites at a 100 μM drug concentration.

[e] E_p is the anodic peak potential value measured in aqueous media or in dimethylformide expressed in millivolts.

[f] K_p is the apparent partition coefficient octanol/buffer, pH 7.4.

Note. All values are expressed as the mean ±SD of three or more independent experiments. For further details see Materials and Methods.

compounds. Thus, the chlorophenyl group increases inhibition compared with the nitrophenyl substituent. It also should be noted that the values of respiratory inhibition induced by compounds **3** and **8** are inverted with respect to those found for growth inhibition. The greater potency of 8 with respect to its parental compound 3 can be explained by: (i) the higher lipophilicity of the sulfur atom of the thiazolo derivative **8**, with respect to that of the oxygen atom of the oxazolo derivative **3**, (ii) the possible oxidation of **8**, withdrawing some oxygen of the medium during the experiment, or (iii) the incipient formation of the sulfoxide of **8**, which could display a higher potency than the parent compound as a respiratory inhibitor.

Table I also shows the results of toxicity measured as

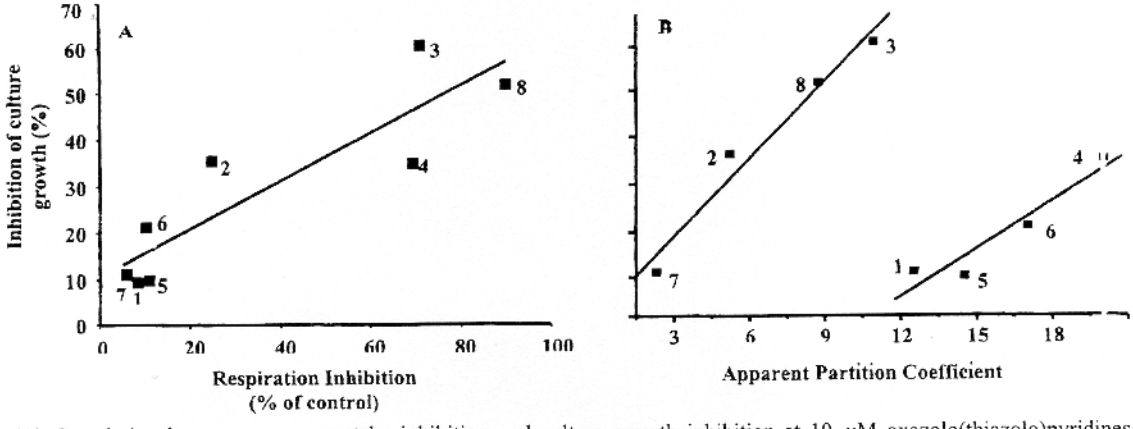


FIG. 2. (A) Correlation between oxygen uptake inhibition and culture growth inhibition at 10 μM oxazolo(thiazolo)pyridines on *T. cruzi* epimastigotes. Pearson's correlation and regression coefficients are 0.8883 and 0.7891, respectively ($P = 0.0032$). (B) Relationship between apparent partition coefficient and percentage of inhibition of cultures at 10 μM concentration of oxazolo(thiazolo)pyridines. Oxygen uptake inhibition and apparent partition coefficient obtained from Table I.

changes in motility, shape, and lysis induced by the oxazolo(thiazolo)pyridine derivatives at 2 and 24 h. The most toxic compounds at 24 h were compound **3, 8, 4**, and **2**. These results agree reasonably well with those for growth inhibition, indicating that the changes observed are mainly due to the inhibition of the respiratory chain of the parasites and their energy production.

To compare strain susceptibilities, studies with Tulahuen and LQ strains of *T. cruzi* were conducted. Results similar to those reported in Table I were obtained (data not shown). These studies are consistent with previous work where drugs showing inhibition of the respiratory chain also showed no important differences among different strains of *T. cruzi* (Aldunate *et al.* 1986, 1992; Ferreira *et al.* 1988).

In order to substantiate the biological results we have conducted some additional studies. Thus, the apparent partition coefficients (Papp = octanol/phosphate buffer, pH 7.4) and retention times of all the derivatives were determined (Table I). A good correlation between the above-mentioned parameters was found. These results are consistent with the fact that there is a close parallel between the retention of drugs on reverse-phase high-pressure liquid chromatography columns and the octanol–water partition coefficients. All compounds exhibited Papps higher than unity, evidencing the lipophilic character of the studied derivatives.

In Fig. 2B, the correlation between the percentage of inhibition cultures and the apparent partition coefficients of octanol-phosphate buffer, pH 7.4, is shown. Two types of linear correlation were found: a first group of derivatives represented by the chlorophenyloxazolopyridines **2** and **3**, furyloxazolopyridine **7**, and chlorophenylthiazolopyridine **8**, with $r = 0.98$; a second group ($r = 0.95$) including the nitrophenyloxazolopyridines **5** and **6**; and the other chlorophenyloxazolopyridines **1** and **4**, compound **4** being the most lipophillic derivative, with an apparent coefficient of 20 (Table I). At present, we have no explanation for the two types of linear correlations. Nevertheless, lipophilicity, which indicates penetration of the drugs into the parasite, is important for respiration inhibition, but other factors, such as calcium homeostasis, might also be involved. However, two well-known channel calcium antagonists, verapamil and diltiazem, which can inhibit epimastigote calcium uptake, do not have trypanocidal activity. Other dihidropyridine derivatives with trypanocidal activity, such as nifedipine and nicardipine, do not have inhibitory activity on parasite calcium homeostasis, supporting the idea that inhibition of respiration or other factors, but not calcium homeostasis, is involved (Nuñez-Vergara *et al.* 1997).

Also, we have correlated the pharmacological effects and the ease of the derivatives to becoming oxidized. As can be seen from Table I, when all the compounds are globally considered, they showed no strict correlation ($r^2 = 0.62$) between oxidation peak potential and corresponding growth inhibitory effects on *T. cruzi* epimastigotes. Nevertheless, if only aqueous E_p values are taken into account and the comparisons are made excluding those with high lipophilicity (Table I, compounds **1, 4, 5**, and **6**), a good correlation between parasite growth or respiration inhibition ($r^2 = 0.87$) and the corresponding oxidation ease for each compound can be observed. This could support the recent results that we reported for several 3-chlorophenyl-1,4-dihydropyridines, which showed a good correlation between toxic effects upon *T. cruzi* and ease of oxidation (Maya *et al.* 2000).

Much extensive chemical and pharmacological work must be done in order to design, synthesize, and evaluate new molecules, to explain thoroughly the structure–activity relationships and the mechanistic aspects mentioned in this paper. Nevertheless, it can be concluded that within the family of oxazolo(thiazolo)pyridines related to those evaluated in this work, compounds can be found displaying significant inhibitory effects on culture growth and oxygen uptake, as well as toxic effects inducing changes in shape and motility or lysis on the parasites. They constitute a new structural category of potentially useful anti-Chagas agents.

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