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In vitro anti-parasitic activity of Cyclosporin A analogs on *Trypanosoma cruzi*

Jacqueline Búa,* Andrés M. Ruiz, Mariana Potenza and Laura E. Fichera

Instituto Nacional de Parasitología 'Dr. Mario Fatala Chabén' ANLIS Carlos G. Malbrán; Consejo Nacional de Investigaciones, Científicas y Técnicas. Av. Paseo Colón 568 (1063) Buenos Aires, Argentina

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Abstract—Cyclosporin A (CsA) nonimmunosuppressive analogs were evaluated against *Trypanosoma cruzi* and on *Tc*CyP19, a cyclophilin of 19 kDa. Two out of eight CsA analogs, H-7-94 and F-7-62 showed the best anti-parasitic effects on all in vitro assays. Their IC_{50} values were 0.82 and 3.41 μM, respectively, compared to CsA IC_{50} value 5.39 μM on epimastigote proliferation; and on trypomastigote lysis their IC_{50} values were 0.97 and 2.66 μM compared to CsA IC_{50} value 7.19 μM. H-7-94 and F-7-62 were also more effective than CsA in inhibiting trypomastigote infection. The enzymatic activity of *Tc*CyP19 was inhibited by all CsA derivatives, suggesting this target is involved in the trypanocidal effects observed.
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1. Introduction

Chagas' disease, caused by the protozoon *Trypanosoma cruzi*, is considered one of the most important tropical parasitic diseases by the World Health Organization.[1]

The chemotherapy to control Chagas' disease is based on nonspecific drugs such as benznidazole, associated with a high incidence of serious side effects.[2,3]

The study of potential new drug targets has recently increased due to the *T. cruzi* genomic DNA sequences that are becoming available. In a previous study, we have described a *T. cruzi* cyclophilin of 19 kDa (*Tc*CyP19) isolated from CL Brener clone.[4] Cyclophilins are a family of proteins that mediate folding events catalyzing the interconversion of the *cis* and *trans* isomers of peptidyl prolyl bonds in peptides or proteins (peptidyl–prolyl *cis–trans* isomerase activity, PPIase). These proteins are known to be the major cellular target for the immunosuppressant drug Cyclosporin A (CsA),[5] which demonstrated to exhibit anti-parasitic effects on some protozoa and helminths.[6]

CsA has an inhibitory effect on CyP enzymatic isomerization,[7] and we showed that *Tc*CyP19 had a CsA-sensitive PPIase activity.[4]

The CyP–CsA complex binds to calcineurin and blocks its serine-threonin protein phosphatase activity. This binding inhibits the expression of interleukin-2 in T cells, which leads to immunosuppression.[8] However the mechanisms of action of the anti-parasitic activity of CsA remain unclear.

The immunosuppressive activity of CsA in vivo, interferes with its anti-parasitic effect, exacerbating the *T. cruzi* and other parasitic infections,[9] with the exception of malaria and some worms.[10] Therefore, synthetic CsA derivatives with an in vitro and in vivo significant less immunosuppressive activity,[11] were considered of interest for the evaluation of their trypanocidal activity.

We have therefore undertaken the study of anti-*T. cruzi* effects in vitro by CsA analogs with decreased immunosuppressive activity. We were also interested in the assignment of the *T. cruzi* CyP19 as the cellular target for CsA derivatives.

Keywords: Trypanocidal; Cyclophilin; Cyclosporin A; Peptidyl–prolyl *cis–trans* isomerase.
* Corresponding author. Tel.: +54-11-4331-4010; fax: +54-11-4331-7142; e-mail: jacbua@yahoo.com

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2. Chemistry

A-4-16 (8-azido-6,7-dihydro-MeBmt)-1-cyclosporin (cyclosporin = Cyclosporin A); B-5-49 (8-methoxy-6,7-dihydro-MeBmt)-1-cyclosporin; C-5-34 (6,7-dihydro-MeBmt)-1-(Thr[$OCH_2CH_2CH_2$-OH])-2-cyclosporin (dihydro-cyclosporin C derivative); D-6-45 (7-carboisopropyloxy)(7-desmethyl)MeBmt-1-cyclosporin (COO-$CH(CH_3)_2$); E-6-44 D-Thr-8-cyclosporin (replacement of D-Ala by D-Thr in position 8); F-7-62 (7-phenyl-6,7-dihydro-(7-desmethyl)MeBmt)-1-cyclosporin; H-7-94 (7-phenyl)(7-desmethyl) MeBmt-1-cyclosporin (6,7 *trans* double bond). These CsA analogs were kindly given by Dr. Horst Zahner, of University of Giessen, Germany, originally donated by J. F. Borel, from Novartis, Basel, Switzerland.

SDZ 211-810 (4-hydroxy)MeLeu 4-cyclosporin. Cyclosporin A and SDZ 211-810 derivative were a gift of Novartis Pharma Ltd, Basel, Switzerland.

Me is methyl and Bmt1 is 4-butenyl-4-methyl threonine.

The CsA derivatives were synthesized starting from CsA molecule.[12] The two most potent derivatives H-7-94 and F-7-62, (Fig. 1) were synthesized following three standard organical procedures. Briefly, the first step is the ozonolysis of CsA and formation of MeBmt-6-aldehyde, which is then reacted with a Wittig reagent made from benzylbromide, which give two isomers that are separated to give pure H-7-94. The other isomer can be reduced with hydrogen and a palladium carbon catalyst in ethanol as solvent, to get F-7-62 derivative.

3. Results and discussion

The inhibition of the epimastigote growth, the lysis of the bloodstream trypomastigote form, the interference of development as intracellular amastigotes and the inhibition of the 19 kDa *T. cruzi* cyclophilin enzymatic activity were analyzed in this report.

The effect of CsA and the best CsA derivatives on *Trypanosoma cruzi* epimastigotes growth[13] is shown in Figure 2. IC_{50} were estimated at 72 h from nonlinear regression analysis of parasite growth rate versus drug concentration and are summarized in Table 1. H-7-94 and F-7-62 had an IC_{50} of 0.82 and 3.41 μM, respectively, while CsA showed an IC_{50} of 5.39 μM. E-6-44 and D-6-45 had a similar effect as CsA, and the other derivatives were from two to sixfold less effective. H-7-94 and F-7-62 CsA analogs, showed the best cytotoxic effects on epimastigotes in concentrations comparable to the IC_{50} reported for other protozoan parasites as *Entamoeba histolytica*,[14] and *Plasmodium vivax*.[15]

It is known that bloodstream forms are targets for *T. cruzi* screening in blood banks and we have also confirmed *Tc*CyP19 cyclophilin expression in trypomastigotes. Then we considered necessary to analyze the lytic action of CsA analogs on bloodstream trypomastig-

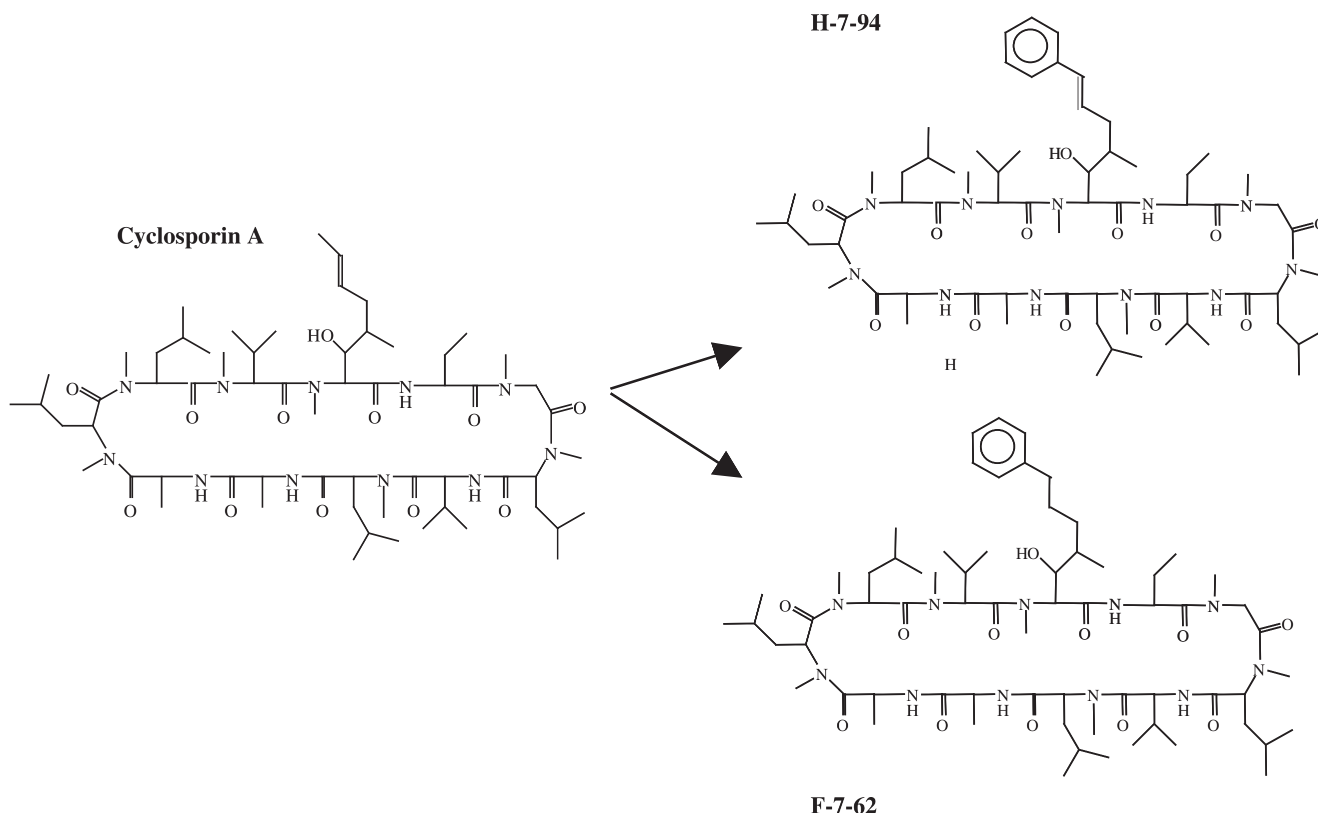

Figure 1. Structures of Cyclosporin A, H-7-94, and F-7-62 CsA derivatives.

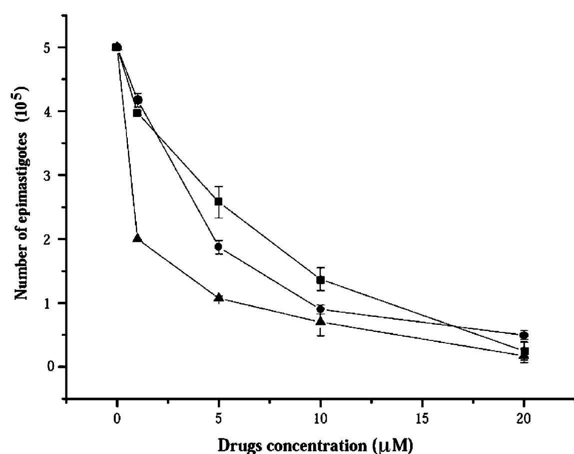

Figure 2. The figure shows the inhibition of epimastigotes proliferation at 72 h of treatment with CsA—squares, H-7-94—triangles, and F-7-62—circles CsA derivatives.

otes[16] as a way of searching new drugs for blood sterilization. CsA analogs H-7-94 and F-7-62 exhibited at a micromolar level, a clear lytic effect on trypomastigotes, with 2.7 and 7-fold lower drug concentration than CsA (Table 1).

We have analyzed the toxic effects of CsA and CsA analogs on Vero cell cultures, before studying the parasite infection in vitro. Cultures treated with different drug concentrations were checked by microscopy at 24 and 48 h, to observe cell morphology and confluent growth. H-7-94 and F-7-62 CsA derivatives did not modify the proliferation rate and morphological appearance of treated cells, at 50 μM concentration, compared with nontreated control cultures. In addition, the trypan blue exclusion test showed 95% cell viability for H-7-94, F-7-62, and nontreated controls. Cultures treated with CsA, A-4-16, and B-5-49 began to show rounding up of the cells with detachment from the substrate at 24 h. Some granularity around the nucleus was observed at 48 h after treatment. Around 50% necrotic cells for this group of drugs, was observed at 48 h after treatment. CsA analogs C-5-34, D-6-45, E-6-44, and SDZ 211-810 showed signs of cellular damage, such as morphological deterioration and cytoplasmic vacuolation at 24 h after treatment, and after 48 h cultures showed more than 90% detached and necrotic cells.

When evaluation of CsA toxicity on mammalian cells was performed at 5 and 25 μM drug concentration, cells showed more than 90% viability then, we analyzed the effects of the nontoxic CsA analogs, at these concentrations on the *T. cruzi* infection in vitro.[17] The number of infected cells in cultures treated with CsA, H-7-97, and F-7-62 was significantly lower than in nontreated cultures ($p < 0.01$, $p < 0.005$, $p < 0.004$, respectively) at 25 μM drug concentration, while at 5 μM drug concentration only H-7-97, F-7-62 CsA analogs were significant ($p < 0.01$ and $p < 0.03$, respectively) (Fig. 3a). The percentage of infection inhibition achieved by CsA, H-7-97 and F-7-62 respect to the control was 56%, 75.8%, and 80.4%, respectively, at 25 μM drug concentration and is shown in Figure 3b. The infection rates were determined as the number of Vero cells containing amastigotes in the cytoplasm. A total of 300 cells were evaluated for each drug treatment in randomly selected fields. The percentage of infection inhibition was calculated as [(experimental infected cells − control infected cells)/control infected cells] × 100.

In Vero cell cultures, H-7-97 and F-7-62 had a marked inhibitory effect on the infection and on the capability of amastigote development in a dose-dependent manner.

Although CsA is known to elicit free radicals in vivo, we think that the trypanocidal effects observed in these in vitro results, are not based on free radical CsA induction since there is evidence about a low physiological concentration of free radicals in vitro, induced by CsA.[18] Moreover, in *Leishmania major*, the intracellular CsA leishmanicidal effect by murine macrophages is independent from reactive nitrogen, since no influence was observed when L-*N*-(1-iminoethyl) lysine, a potent and selective inhibitor of mouse inducible nitric oxide synthase, was added.[19]

CsA was used as a positive control and the effects of CsA and derivatives on the parasite was at the micromolar level. Benznidazole and Nifurtimox, the usual drugs for chemotherapy of Chagas' disease have shown an anti-*T. cruzi* activity at micromolar concentrations[20–22]

Table 1. CsA and CsA analogs inhibition of *T. cruzi* epimastigotes growth, lytic effect on *T. cruzi* trypomastigotes and inhibition on *Tc*CyP19 PPIase activity

Compounds	Epimastigote inhibition growth IC_{50} in μM	Trypomastigote lysis EC_{50} in μM	Peptidyl–prolyl *cis–trans* isomerase inhibition IC_{50} in nM
H-7-94	0.82	0.97	12.54
F-7-62	3.41	2.66	13.30
Cyclosporin A	5.39	7.19	14.42
E-6-44	6.93	13.39	16.94
D-6-45	7.64	58.59	36.28
A-4-16	10.55	7.98	23.16
SDZ 211-810	11.39	153.27	19.33
C-5-34	18.39	36.76	22.11
B-5-49	33.75	76.07	25.50

IC_{50} values for inhibition of epimastigote proliferation were estimated at 72 h after treatment. The EC_{50} values for trypomastigotes lysis was determined after 24 h drug treatment. Three to four independent experiments, in duplicate were done for each drug concentration.

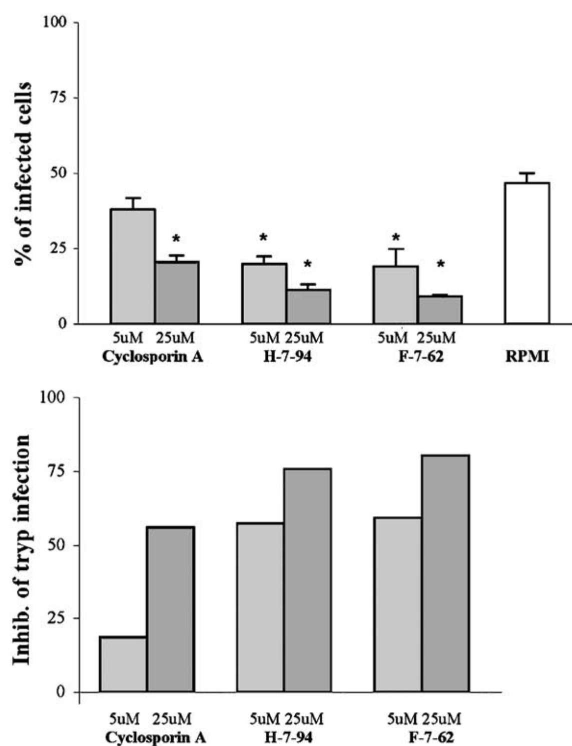

Figure 3. CsA and CsA analogs inhibition of *T. cruzi* trypomastigotes infection on mammalian cells. (a) The number of infected cells in cultures treated with CsA, H-7-97, F-7-62 was significantly lower than in nontreated cultures at 25 μM drug concentration. (b) Shows the inhibition of parasite penetration achieved by CsA, drugs respect to medium control. The percentage of parasite inhibition was 56, 75.8, and 80.4 for CsA, H-7-97, and F-7-62, respectively, at 25 μM drug concentration.

and we did not consider their inclusion as control in this study.

Since the anti-*T. cruzi* activity exerted by CsA, could be assigned to the interference of the main role of *T. cruzi* cyclophilins, we studied the inhibition of CsA derivatives on *Tc*CyP19 PPIase activity.

For this purpose we expressed a recombinant *Tc*CyP19 cyclophilin that had been previously cloned in pQE30 plasmid vector (Qiagen, Hilden, Germany) and expressed in *E. coli*. XL1 Blue strain.[4] The recombinant protein was purified by affinity chromatography on a Ni_2-nitriloacetate agarose (Qiagen, Hilden, Germany) following manufacturer's protocols.

We have previously shown that the purified recombinant His-*Tc*CyP19 clearly accelerated the rate of *cis–trans* isomerization of the peptide substrate comparing with the control without the recombinant protein and demonstrated is an active PPIase.[4]

In this report we performed PPIase activity at 5 °C,[23] with improved technique modifications, in which the percentage of the peptide substrate in the *cis* conformation was increased.[24] The susceptibility of *Tc*CyP19 enzymatic activity to CsA analogs was performed using 20 nM of recombinant protein preincubated with several concentrations of CsA and CsA analogs,[25] and all compounds inhibited the PPIase assay. The inhibition of the enzymatic activity by H-7-94 and F-7-62 (IC_{50} = 12.54 and 13.30 nM, respectively) was significantly lower than Cyclosporin A IC_{50} (IC_{50} = 14.42 nM) ($p < 0.05$) (Table 1).

The depletion of *Tc*CyP19 PPIase activity by CsA derivatives suggest that the anti-parasitic effect of CsA analogs is probably exerted by the binding of the CyP–CsA analog complex.

However, in *Toxoplasma gondii*, the CsA analog antiparasitic activity was performed through the inhibition of a P-glycoprotein but did not bind to cyclophilins.[26] In our study, H-7-94 and F-7-62 CsA derivatives, have strongly bound to *Tc*CyP19 but both compounds proved to exhibit a negligible immunosuppressive activity,[11] unlike CsA and other CsA analogs, which showed a physical and functional interaction with calcineurin, suppressing IL-2 gene expression.

In *Cryptococcus neoformans*, it was reported that SDZ 211-810, (4-OH) Me Leu[4]-CsA, also used in this study, formed a complex with cyclophilin A, which bound the fungal calcineurin with more efficiency than the CsA itself, but the 4-hydroxy substitution on the leucine 4 residue prevented the binding to mammalian calcineurin.[27] Another example in *Saccaromices cerevisiae* showed that a CsA analog when bound to CyP18 did not interact with calcineurin.[28]

Taking into account the biological evaluation against *T. cruzi* parasites by H-7-94 and F-7-62 CsA analogs and relating their inhibitory effects with its chemical structure, we could remark that both compounds contained a phenyl group in the MeBm1 residue (Fig. 1), and both showed a better trypanocidal activity than the other analogs tested. Whether this substitution is responsible for a more efficient binding with the parasite cyclophilin proteins remains to be further studied in a tridimensional molecular system.

Molecular functions of *T. cruzi* cyclophilins need further experimental evidence, as other genes codifying *T. cruzi* cyclophilins have been found (data not shown). *Tc*CyP19 cyclophilin could be a useful tool to study protein–protein interactions by proteomic techniques, to better elucidate the mechanisms of action of the antiparasitic effects of CsA and derivatives, in addition to in vivo studies of these drugs in the *T. cruzi* experimental infection.

We consider H-7-94 and F-7-62 CsA analogs very interesting compounds in the search for new anti-trypanosomal drugs as we have demonstrated their anti-*T. cruzi* activity, and their inhibitory effect on *Tc*CyP19 cyclophilin, proving to be one molecular target.

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17. Effect of CsA and CsA analogs on the in vitro trypomastigote infection: cultures with 10^4 Vero cells per well were treated with CsA, H-7-97, and F-7-62CsA analogs and infected with trypomastigotes at a 10:1 parasite-to-cell ratio. Parasites were incubated with drugs for 2 h at 37 °C in 5% CO_2 and then, thoroughly washed with serum-free medium and incubated with complete medium without drugs for 36 h. Infected and drug nontreated cell cultures were used as controls with 0.3% ethanol. Cell cultures were set up in triplicate for each drug concentration. Covers with cells were removed from the culture plates after 36 h of treatment, gently rinsed with phosphate buffer saline, air dried, fixed in ethanol, and stained with Giemsa. Statistical analysis: experiments were performed in duplicate or in triplicate, as indicated, in two to four independent experiments. The dose response curves were obtained by linear regression analysis using computer programs. Statistical analysis of the in vitro parasite experiments and enzyme kinetics was performed using Student's *t* test. $p<0.05$ was regarded as statistically significant.
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