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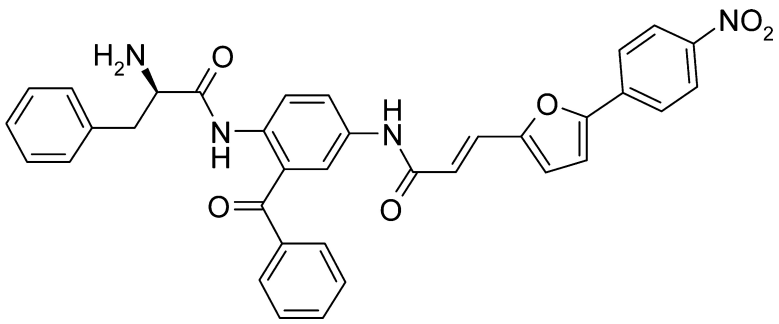

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Benzophenone-Based Farnesyltransferase Inhibitors with High Activity against *Trypanosoma cruzi*

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Less toxic drugs are needed to combat the human parasite *Trypanosoma cruzi* (Chagas's disease). One novel target for antitrypanosomal drug design is farnesyltransferase. Several farnesyltransferase inhibitors based on the benzophenone scaffold were assayed in vitro and in vivo with the parasite. The common structural feature of all inhibitors is an amino function which can be protonated. Best in vitro activity (LC_{50} values 1 and 10 nM, respectively) was recorded for the *R*-phenylalanine derivative **4a** and for the *N*-propylpiperazinyl derivative **2f**. These inhibitors showed no cytotoxicity to cells. When tested in vivo, the survival rates of infected animals receiving the inhibitors at 7 mg/kg body weight/day were 80 and 60% at day 115 postinfection, respectively.

Introduction

The global burden of Chagas's disease is 649 000 DALYs (disability adjusted life years).[1] *Trypanosoma cruzi*, the causal agent, is found throughout much of Mexico and Central and South America. It is transmitted to humans primarily by insects (subfamily *Triatominae*), which carry trypomastigotes, the infective stage of the parasite, in their feces. Infected blood is also a route of Chagas's disease transmission, especially since blood control is not implemented in all health services in affected countries. The probability of getting an infection can be as high as 2 per 100 transfusions.[2] Organs can also function as vehicles, and the parasite has been transmitted through transplantation.[1] For these reasons novel antitrypanosomal therapeutics are urgently needed. In this context, farnesyltransferase inhibitors are of growing importance. Farnesyltransferase (FTase) is a heterodimeric enzyme which catalyzes the posttranslational modification of several proteins involved in intracellular signal transduction. Thereby, a farnesyl residue is transferred from farnesylpyrophosphate to the thiol of a cysteine residue in the C-terminal CAAX consensus sequence (C: cysteine; A: amino acid with aliphatic side chain; X: variable amino acid) of farnesylated proteins.[3,4] FTases of different trypanosomes have been identified and characterized.[5−7] Furthermore, it has been shown that different FTase inhibitors affect the growth of trypanosomatids.[8−11] In this context, it is important to note that the investigations of the antitrypanosomal efficacy of FTase inhibitors concentrated on *T. brucei* while only few inhibitors were tested against the causative agent of Chagas's disease, *T. cruzi*. We therefore focused our efforts on *T. cruzi* and joined the active search for better inhibitors through synthesis and experimental testing of different series of our previously described[12−17] benzophenone-based FTase inhibitors.

Synthesis. The synthesis of most inhibitors employed in this study has been described.[12−16] The preparation of the heterocyle-substituted derivatives will be outlined in detail in a different publication.[17] The complete sets of analytical data are provided in these publications. Briefly, commercially available 2-amino-5-nitrobenzophenone was acylated by α-chlorophenylacetic acid chloride. Then, the α-chloro was substituted by different heterocyclic amines. Reduction of the nitro group and subsequent acylation with appropriate phenylfurylacrylic acid chlorides yielded the target compounds (Scheme 1). For the preparation of the *p*-chlorophenylacetic acid-substituted compound, α-chlorophenylacetic acid was replaced by α-bromo-*p*-chlorophenylacetic acid, prepared from *p*-chlorophenylacetic acid.

Homology Modeling. The sequences of the alpha and beta subunits of *Trypanosoma cruzi* FTase (Q8WR04 and Q8WR02) were retrieved from SWISS-Prot[18] and aligned to rat FTase (Q04631 and Q02293) using T-COFFEE.[19] Based on the rat crystal structure (pdb code 1qbq), a homology model was computed using MOBILE[20] and MODELLER.[21] Significant variation is observed in the amino acids constituting the far aryl binding pocket due to a high uncertainty in the sequence alignment of this region. The procedure for docking of ligand **2l** into the active site of the rat enzyme with AUTODOCK has been described previously.[22,17]

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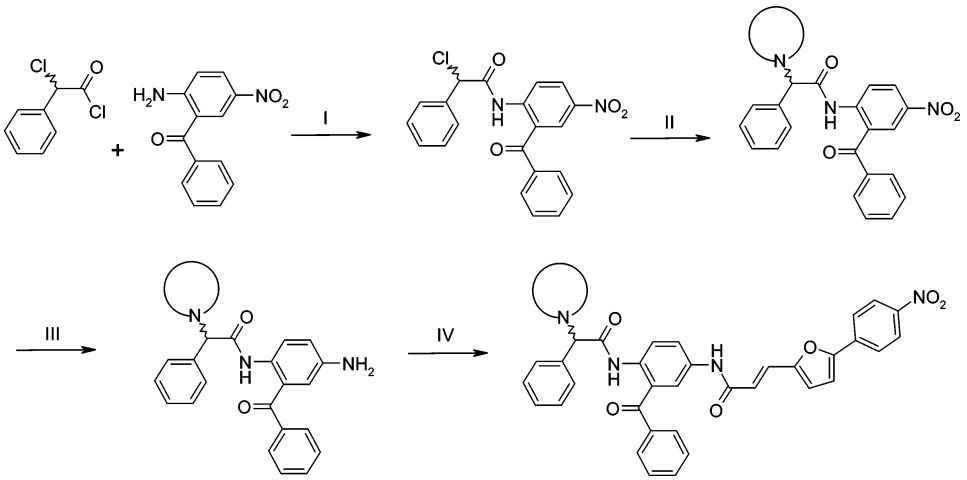
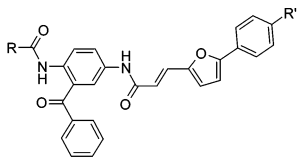
Scheme 1[a]



[a] (i) Toluene/dioxane, reflux, 2 h; (ii) (4-oxo)piperidine or morpholine or N-substituted piperazine, acetonitrile, reflux, 24 h; (iii) $SnCl_2 \times 2H_2O$, ethyl acetate, reflux, 2 h; (iv) 3-[5-(4-nitrophenyl)-2-furyl]acrylic acid chloride, toluene/dioxane, reflux, 2 h.

Table 1. Benzophenone-Based FTase Inhibitors without Basic Functions in the Side Chain



	R	R'	IC_{50} (nM) *Sc*FTase	LC_{50} (μM) *T. cruzi*	CC_{50} (μM)
1a[13]		$-NO_2$	35	> 100	> 85
1b[12]		$-NO_2$	26	50	> 57
1c[14]		$-CH_2-CH_3$	90	50	nd
1d[14]		$-SO_2-CH_3$	30	50	nd

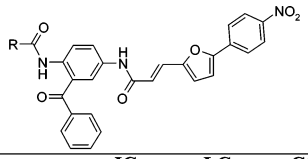
Results and Discussion

In the first round of the in vitro lysis assays inhibitors **1a**−**d** (Table 1) and **2a** and **2l** (Table 2) were employed. The more potent compounds were inhibitors **2a** and **2l** with LC_{50} values in the low micromolar range. Although only six inhibitors were employed in the first round, an important structure activity relationship can be delineated from this sample. Notable activity was only displayed by the two methylpiperazine substituted inhibitors (LC_{50} values: 10 μM). This is most probably attributable to the higher solubility of this type of compounds in comparison to those benzophenone-based inhibitors lacking an amino group which can be protonated. To exemplify this, compounds of type **1** display a solubility <0.06 mM in water, whereas the solubility of inhibitor **2a** is >3.33 mM.[22] Therefore, only compounds possessing protonable amino groups were included in the second round. Inhibitors of this second round can be structurally divided into the amino acid derivatives **3** and **4** (Tables 3 and 4) and the heterocyle bearing compounds **2** (Table 2).

In the series of heterocycle substituted FTase inhibitors the methylpiperazine derivative **2a** served as a lead structure. Most variations of the heterocycle led to a significant reduction in activity with the LC_{50} values of most compounds being at 50 μM or higher. However, also in this series notably more active inhibitors were found. The 4-oxopiperidyl compound **2d** and the cyclohexylpiperazinyl derivative **2i** were found to be 10-fold more active ($LC_{50} = 1.0$ μM) than the methylpiperazinyl derivative **2a**. The propylpiperazinyl derivative **2f** with an LC_{50} value of 10 nM was 3 orders of magnitude more active than **2a** and again among the most active antiprotozoal FTase inhibitors described so far. A striking feature is the inactivity of the structurally closely related ethylpiperazinyl derivative **2e** in comparison to the propylpiperazinyl derivative **2f** and also in comparison to the methylpiperazinyl derivative **2a** in trypanosome lysis assay. There is no readily available explanation even on the basis of flexible docking (see infra), but it should be noted that this compound is also virtually inactive in the farnesyltransferase inhibition assay (IC_{50} > 10 μM), so that the data seem consistent.

To gain insight into the structure−activity relationships of this type of benzophenone-based FTase inhibitors, we previously performed some docking studies with the inhibitors **2a** and **2l**[17,22] using the crystal structure of rat FTase.[23] In this study, we additionally prepared a homology model for the *T. cruzi* enzyme (*Tc*FTase) (Figure 1). *Tc*FTase shows low sequence homology to the human or rat enzymes with an identity of 22%. *Tc*FTase is significantly larger than rat FTase with 628 vs 377 residues in the α-subunit, and 588 vs 437 residues in the β-subunit. Amino acid side chains contributing to substrate binding, thus forming the active site, are well-known from crystal structures.[23] The active site residues are well conserved with 72% sequence identity (39 of 54 residues). Amino acid substitutions are located on opposite sides of the inhibitor binding site affecting the binding of the nitrophenylfurylacryloyl and the 2-(methylpiperazinyl)-2-phenylacetyl moieties, respectively. As stated above, in the region binding the nitrophenylfurylacryloyl moiety there

Table 2. FTase Inhibitors with an α-Heterocycle-Substituted Residue



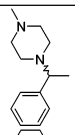
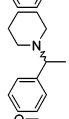
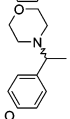
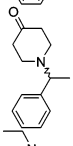
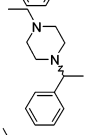
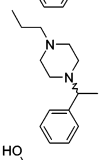
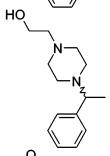
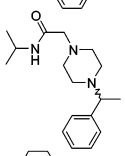
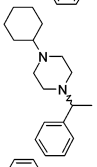
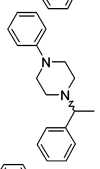
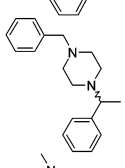
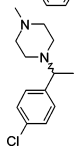
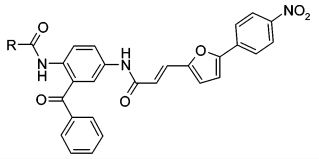
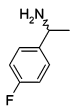
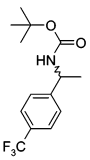
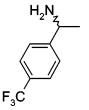
	R	IC_{50} (nM) *Sc*FTase	LC_{50} (μM) *T. cruzi*	CC_{50} (μM)
2a[17,22]		10	10	37
2b[17]		455	50	nd
2c[17]		660	50	> 76
2d[17]		1100	1.0	nd
2e[17]		> 10000	> 50	nd
2f[17]		125	**0.01**	68
2g[17]		430	10	71
2h[17]		340	> 50	nd
2i[17]		340	1.0	42
2j[17]		300	> 50	nd
2k[17]		660	50	nd
2l[17,22]		125	10	38.6

Table 3. FTase Inhibitors with Phenylglycine Substructure

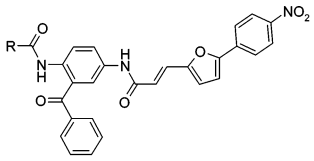


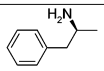
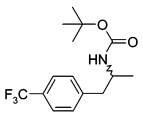
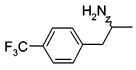
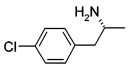
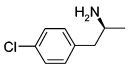
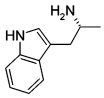
	R	IC_{50} (nM) *Sc*FTase	LC_{50} (μM) *T. cruzi*	CC_{50} (μM)
3a[15]		46	> 50	> 80
3b[15]		8	> 50	> 80
3c[15]		25	50	> 78
3d[15]		8	50	nd
3e[15]		17	10	nd

is a high uncertainty in the sequence alignment. However, the replacements of Lys 356β by Cys, Gln 48β by Met, Glu 47β by Pro and Thr 44β by Asp are likely not to have any significant effect on inhibitor **2l** binding because they are too distant from the inhibitor. The majority of amino acid substitutions occurs in the pocket where the 2-(methylpiperazinyl)-2-phenylacetyl moiety is bound. While most substitutions probably do not affect inhibitor binding significantly, the replacement of Ala 129α by Gly and Gln 162α by Asn creates more space in the vicinity of the N-4 of the piperazinyl residue. While this could be a possible explanation of the high activity of the *N*-propylpiperazinyl-substituted inhibitor **2f**, the inactivity of inhibitor **2e** remains unexplained.

With the phenylglycine derivatives **3**, notable activity was only recorded for the *p*-trifluoromethyl derivative **3e** with an LC_{50} value of 10 μM while other phenylglycine derivatives were at least 5-fold less active. For comparison, the boc-protected derivative of **3e** (**3d**) was also tested. Not surprisingly, it displayed a significantly lower activity than its parent compound, again, most probably attributable to its lower solubility. When the homologue of the phenylglycine derivative **3b**, the *R*-phenylalanine derivative **4a**, was assayed, a striking enhancement in antitrypanosomal activity was observed. The phenylalanine derivative **4a** displayed the highest activity of the second test series with an LC_{50} value of 1 nM. Furthermore, this compound is among the FTase inhibitors with the highest in vitro activity against trypanosome parasites recorded. Because of the high activity of the phenylalanine derivative **4a**, several more amino acid derivatives were prepared (Table 4). The most important compound of these additional amino acid derivatives is the enantiomer of **4a**, the *S*-phenyl-

Table 4. FTase Inhibitors with Different α-Aminoacyl Residues



	R	IC_{50} (nM) *Sc*FTase	LC_{50} (μM) *T. cruzi*	CC_{50} (μM)
4a[16]		116	**0.001**	> 78
4b[16]		3	> 10	> 79
4c[16]		285	> 50	nd
4d[16]		95	50	nd
4e[16]		60	1.0	> 74.5
4f[16]		70	> 10	> 74.5
4g[16]		360	1.0	> 83
4h[16]		500	0.1	47.8
4i[16]		150	0.1	> 83
4j[16]		1770	> 10	> 81
4k[16]		500	> 10	> 81
4l[16]		580	10	nd

alanine derivative **4b**. For these two compounds a striking stereodifferentiation is visible. While the *R*-enantiomer **4a** is the most active compound of the whole study (LC_{50} = 1 nM) the *S*-enantiomer **4b** is more than 4 orders of magnitude less active with an LC_{50} value > 10 μM. Apart from the methionine derivatives **4j** and **4k** which both display only weak activity, stereodifferentiation is also observed for other amino acid derivatives although this effect is not as pronounced as in the case of the phenylalanine enantiomers. No general preference for the *R*- or *S*-enantiomer is visible in this series. The activity of most inhibitors in this series was in the micromolar range with LC_{50} values between 1 and 10 μM. However, the *R*-leucine derivative **4h** and the *S*-isoleucine derivative **4i** displayed submicromolar activity with LC_{50} values of 100 nM. As in the series of the heterocylic substituted inhibitors **2**, also in this series there is no obvious explanation for the structure−activity relationships. Because of the large number of rotatable bonds in the amino acid derivatives, no reliable docking solution could be obtained possibly helping in the interpretation of the SAR.

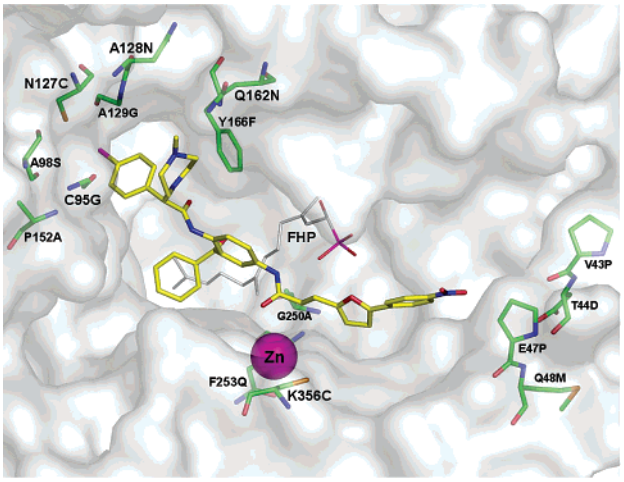


Figure 1. Homology model of farnesyl transferase from *Trypanosoma cruzi* based on the crystal structure of the rat enzyme (pdb entry 1qbq). The docked inhibitor **2l** (yellow) lies on top of the farnesylpyrophosphate analogue FHP (white) and the Zn^{2+} ion (magenta). The majority of amino acid substitutions are located in the specificity pocket.

Since in this second round two compounds were active in the nanomolar range these two inhibitors (**4a** and **2f**) were selected for further in vivo evaluation. No toxicity in terms of cell morphology or confluent growth was detected on Vero cells treated with drug diluent (DMSO 1/1000) or with compounds **4a** and **2f** at maximal concentrations of 100 μM. Confirming these results, an independent assessment established the CC_{50} values against HeLa cell of selected inhibitors of the first two test series being in the range of 37 to >78 μM with the best inhibitors being at 68 μM (**2f**) and > 78 μM (**4a**).

When performing the in vivo assays, no alteration in behavior or aspect was recorded in the uninfected/treated toxicity control group, receiving 39 doses. Parasitemia levels in blood were assessed twice a week by microscopic examination. Infected groups (five animals per group) reached the parasitemia peaks ($0.8-1.2 \times 10^6$ parasites/mL) at around 24−27 days pi. Groups treated daily with inhibitors at ≈3.5 mg/kg body weight/day presented higher parasitemia than the control group, while those receiving at ≈7 mg/kg/day behaved like the control (not shown). On day 50 pi parasites were no more detectable in surviving mice with our method of optical microscopic examination (detection limit ≥10^4 parasites/mL).[24]

Animal mortality stopped at day 34 pi (Figure 2). Animals treated at ≈3.5 mg/kg/day performed as the controls. Unexpectedly, group **4a** at 3.2 mg/kg/day displayed 0% survival. Although it is in accordance to the higher parasitemia registered, we have no explanation for this result. The survival in the control group (infected, no drug) was 16% (1/6). Survival rates of 60% (3/5) and 80% (4/5) were observed in mice treated with inhibitors at 7 and 6.4 mg/kg/day (**2f** and **4a**, respectively). On day 115 pi the animals were still alive. These results, and the fact that toxicity seems to be low or absent, single out compounds **2f** and **4a** as the most promising inhibitors.

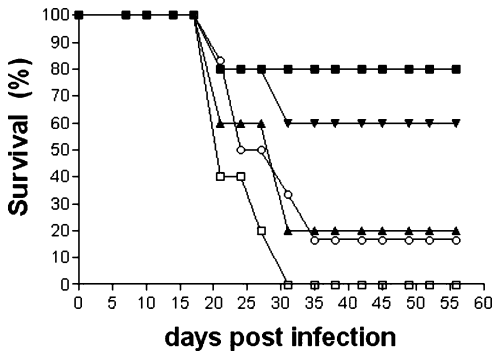


Figure 2. Survival of infected and treated animals. Mice groups received the following inhibitors: **2f** 3.5 mg/kg/day (▲), **2f** 7 mg/kg/day (▼), **4a** 3.2 mg/kg/day (□), **4a** 6.4 mg/kg/day (■). The control group (○) received no drug.

We compared these results with those obtained previously with other molecules. We reported that some Green tea (*Camelia sinensis*) catechins were very active in vitro against *T. cruzi* trypomastigotes (MBC_{50} at the picomol level),[25] but we cannot compare their in vivo effect since is currently under study. We also communicated that the in vitro effect of methoprene (a juvenile hormone analogue) on *T. cruzi* trypomastigotes was excellent, but only 60% of the treated infected mice survived.[26] Our present survival result obtained administering **4a** is similar to the obtained by Garzoni et al.[27] using Risedronate, an inhibitor of the farnesyl pyrophosphate synthase. Both results are not comparable due to differences in the treatment protocols. However, it is remarkable that our administration route (oral vs iv) is more demanding, speaking in favor of the stability and efficacy of our compounds. Interestingly, both chemotherapeutic targets contribute to the same metabolic products.

Summary and Conclusion

From several benzophenone-based FTase inhibitors carrying a protonable nitrogen in the side chain two inhibitors (**4a** and **2f**) displayed promising in vitro activity against *T. cruzi* with LC_{50} values of 1 nM and 10 nM, respectively. When further evaluated in vivo, the phenylalanine derivative **4a**, which was more active in vitro, proved to be slightly superiour to the propylpiperazinyl-substituted inhibitor **2f**. However, further structural variation of the amino acid portion of **4a** did not yield better inhibitors. Therefore, the propylpiperazinyl derivative **2f** seems to be the most promising lead structure for further development of antitrypanosomal FTase inhibitors.

Experimental Section

Farnesyltransferase Inhibition. Yeast farnesyltransferase inhibition of all compounds employed in this study has been published.[12−17] The assay is described and referenced there.

Parasites. Mice blood containing *T. cruzi* bloodstream trypomastigotes (Tulahuen strain, Tul 2 stock) was obtained by cardiac puncture from infected BALB/c mice.

In Vitro Assays. Test substances were dissolved in DMSO (10 mM) before being diluted in the culture medium BHI (brain heart infussion 0.37%, DIFCO). Five microliters of each compound was added to 95 μL of blood containing 5×10^4 trypomastigotes. Controls received just DMSO (final dilution 1/1000). Incubations were performed in polystyrene 96-well U-plates at 4 °C during 24 h. Five microliters of blood from each well was then microscopically examined under a 18 mm-diameter coverslip at 400× magnification.[24] Each dilution condition was done in triplicate. Each complete experiment (samples and control) was repeated 3−4 times for confirmation. The percentage of lysis was calculated considering the control as 100%. The LC_{50} (lythic concentration 50) was defined as the minimal concentration of compound required to lyse 50% of the trypomastigotes.

In Vivo Assays. For the in vivo assays, 60 days old BALB/c males were used. The uninfected/treated toxicity control group received the same drug concentration as the treated groups, and the general aspect of the animals was recorded. The infected/treated groups consisted of five animals, while the infected/non treated control group had six. All of them were injected by intraperitoneal route with 500 *T. cruzi*-bloodstream trypomastigotes. From 30 min after infection, doses of the most promising drugs (**4a** and **2f**) or physiological solution were administered orally (25 μL) five times a week. The total number of doses was 39, except group **4a** 3.2 mg/kg body weight/day, that received 21 doses (see survival in Figure 2). The final drug doses were ≈3.5 or ≈7 mg/kg/day. Parasitemia levels were estimated by microscopic examination of blood samples taken from the tail twice a week. Parasitemia and mortality were recorded.

Cytotoxicity Assays. The test substances were dissolved in DMSO (10 mM) before being diluted in the cell culture medium (1:200). HeLa (DSM ACC 57) cells were grown in RPMI 1640 culture medium (GIBCO BRL 21875−034) supplemented with 25 μg/mL gentamicin sulfate (BioWhittaker 17−528Z) and 10% heat inactivated fetal bovine serum (GIBCO BRL 10500−064) at 37 °C in high density polyethylene flasks (NUNC 156340). The adherent HeLa cells were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS-containing 0.02% EDTA (Biochrom KG L2163). For each experiment approximately 10 000 cells were seeded with 0.1 mL of RPMI 1640 containing 25 μg/mL gentamicin sulfate (BioWhittaker 17−528Z), but without HEPES, per well of the 96-well microplates (NUNC 167008). For the cytotoxic assay, HeLa cells were preincubated for 48 h without the test substances. The dilutions of the test substances were carried out carefully on the monolayers of HeLa cells after the preincubation time. The HeLa cells were further incubated for 72 h at 37 °C in a humidified atmosphere and 5% CO_2. The adherent HeLa cells were fixed with 25% glutaraldehyde and stained with a 0.05% solution of methylene blue for 15 min. After gently washing, the stain was eluted with 0.2 mL of 0.33 N HCl per well. The optical densities were measured at 660 nm in SUNRISE microplate reader (TECAN). For data analysis, the Magellan software (TECAN) was used.

Vero cells were grown in RPMI 1640 culture medium (DIFCO) and 5% heat inactivated fetal bovine serum (BIOSER, Buenos Aires). The toxicity control was performed incubating cell cultures (10^4 cells/well, in triplicate for each drug concentration) with different concentrations of drugs at 37 °C in 5% CO_2. DMSO was used as drug diluent, reaching a final concentration of 1/1000 in the assays. Drugs (10 mM stock solutions) were used at final concentrations of 1 nM, 10 nM, 100 nM, and 1 μM. Cell morphology and confluent growth were checked at 24, 48, and 72 h.

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Supporting Information Available: Sequence alignment for *T. cruzi* and rat farnesyltransferase. This material is available free of charge via the Internet at http://pubs.acs.org.

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