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Pyrazol(in)e derivatives of curcumin analogs as a new class of anti-*Trypanosoma cruzi* agents

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Abstract

Aim: In this work, we report the synthesis and biological evaluation of a small library of fifteen functionalized 3-styryl-2-pyrazolines and pyrazoles, derived from monocarbonyl and dicarbonyl curcuminoids, respectively, as antitrypanosomal agents. **Methods & results:** The products were prepared *via* the cyclization reaction between the corresponding curcuminoids and the appropriate hydrazines. All of the derivatives synthesized were investigated for their antitrypanosomal activities. Compounds **4a** and **4e** showed significant activity against epimastigotes of *Trypanosoma cruzi* with IC₅₀ values of 5.0 and 4.2 µM respectively accompanied by no toxicity to mammalian non-cancerous cells, whereas compound **6a** was found to effectively inhibit *T. cruzi* triosephosphate isomerase. **Conclusion:** The 16-fold (for **4a**) and 6-fold (for **4e**) higher potencies of these derivatives in comparison to their curcuminoid precursors, make them an attractive and promising new family of *T. cruzi* inhibitors.

Keywords

pyrazolines; 4,5-dihydropyrazoles; antitrypanosomal agents; Chagas disease; *Trypanosoma cruzi*

Introduction

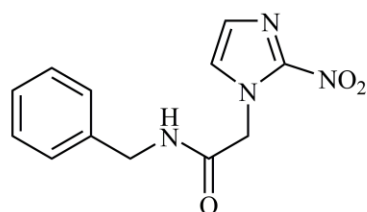
Chagas disease, also known as American trypanosomiasis, is a potentially life-threatening illness caused by the parasite *Trypanosoma cruzi*. More than six million people, mostly in Latin America are estimated to be infected by *T. cruzi* [1]. The only available drugs for its treatment are benznidazole and nifurtimox (Fig. 1). However, both medicines are far from ideal. In the benznidazole case, adverse reactions occur in up to 40% of treated adult patients, while the treatment is only for a short duration and carried out once [2]. The other medication, nifurtimox, is the only one available in some countries, including the United States of America. It produces, in general, frequent, but mild side effects, however, special attention should be paid to potentially severe symptoms like depression, rash, and anxiety [3]. It is also contraindicated for people with a background of neurological or psychiatric disorders [1]. Given these facts, there is an urgent need for new efficient, and safer drugs [4].

Curcumin or diferoylmethane, the most abundant bioactive natural product extracted from roots of *Curcuma longa* has been reported as an effective agent for the treatment of *T. cruzi* infections [5]. More specifically, curcumin was the most active typanocidal agent among the major constituents of *C. longa*, with IC₅₀ value of 10.13 μ M on epimastigotes compared to demethoxycurcumin and bisdemethoxycurcumin which exhibited IC₅₀ values of 11.07 and 45.33 μ M, respectively. Moreover, being a natural product low systemic toxicity was observed. Finally, *in vivo* studies on *T. cruzi*-infected mice revealed a significant reduction in parasitemia and tissue parasite load, increased survival rate and improved pathological conditions [6]. Therefore, curcumin can serve as a promising lead compound for the treatment of Chagas disease. However, it suffers from poor bioavailability, chemical instability and fast systemic elimination [7].

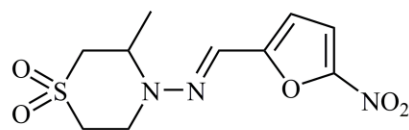
Monocarbonyl analogs of curcumin (MACs) represent a highly attractive and in some cases a highly promising category of curcumin synthetic analogs. In many cases, they demonstrate higher potency than the diketo-curcuminoid counterparts, being at the same time easier to produce and more stable [8]. Alvarez *et al.* have reported the synthesis and antitrypanosomal evaluation of cyclohexanone, cyclopentanone, cycloheptanone

curcuminoids bearing thiophene, furan and unsubstituted phenyl rings with IC₅₀ values against epimastigotes of *T. cruzi* lower than 5 µM. Those compounds were potent and selective inhibitors of triosephosphate isomerase of *T. cruzi*. This enzyme is a promising drug target in Chagas disease [9,10]. More recently, Din *et al.* synthesized thirty-four symmetrical and unsymmetrical cyclohexanone-based curcuminoids bearing substituted phenyl rings as antiparasitic compounds. They found that two unsymmetrical molecules with methoxy substitutions on the phenyl rings were the most potent compounds against epimastigotes of *T. cruzi* (IC₅₀ values of 5.2 and 3.0 µM, respectively) displaying at the same time low cytotoxicity against Vero cells [11].

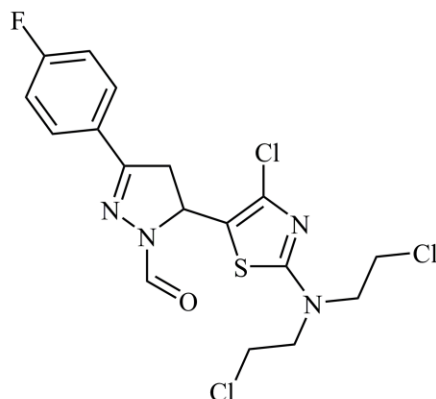
On the other hand, 2-pyrazolines, five-membered heterocycles usually derived from chalcones or monocarbonyl curcuminoids, are versatile molecules with prominent pharmacological profile [12-14], which includes anticancer [15-17], anti-inflammatory [18,19], antiviral [20] and antidepressant activities [21]. The reports on antitrypanosomal activities of pyrazoline derivatives are scarce, and not all of them demonstrate active products [22,23]. Cohen *et al.* have published the synthesis and biological evaluation of more than one hundred thiosemicarbazone derivatives as inhibitors of the trypanosomal cysteine protease cruzipain. Among them, a few thiocarboxamide pyrazolines (Fig. 1) were identified as highly potent with IC₅₀ values in the nanomolar range [24]. In two recent publications, Insuasty *et al.* reported the synthesis and antitrypanosomal activity of novel caffeine-based pyrazolines [25] and thiazolyl-pyrazoline derivatives bearing nitrogen mustard (Fig. 1) [26].



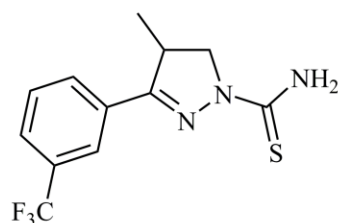
Benznidazole



Nifurtimox



N-Formyl thiazolyl-pyrazoline



Thiocarboxamide pyrazoline

Fig 1. Heterocyclic antitrypanosomal agents.

All the aforementioned evidence prompted us to design and synthesize substituted pyrazole/2-pyrazolines generated from curcuminoid precursors aiming to combine these two bioactive structures into one molecule, thus further improving the promising activities of curcuminoids. Herein, we report the synthesis of a small library of this class of pyrazole/pyrazolines to perform: a) an initial *in vitro* screening against the epimastigote form of *T. cruzi*, b) investigation of triosephosphate isomerase (*TcTIM*) inhibition and c) evaluation of toxicity in mammalian cells.

Results and Discussion

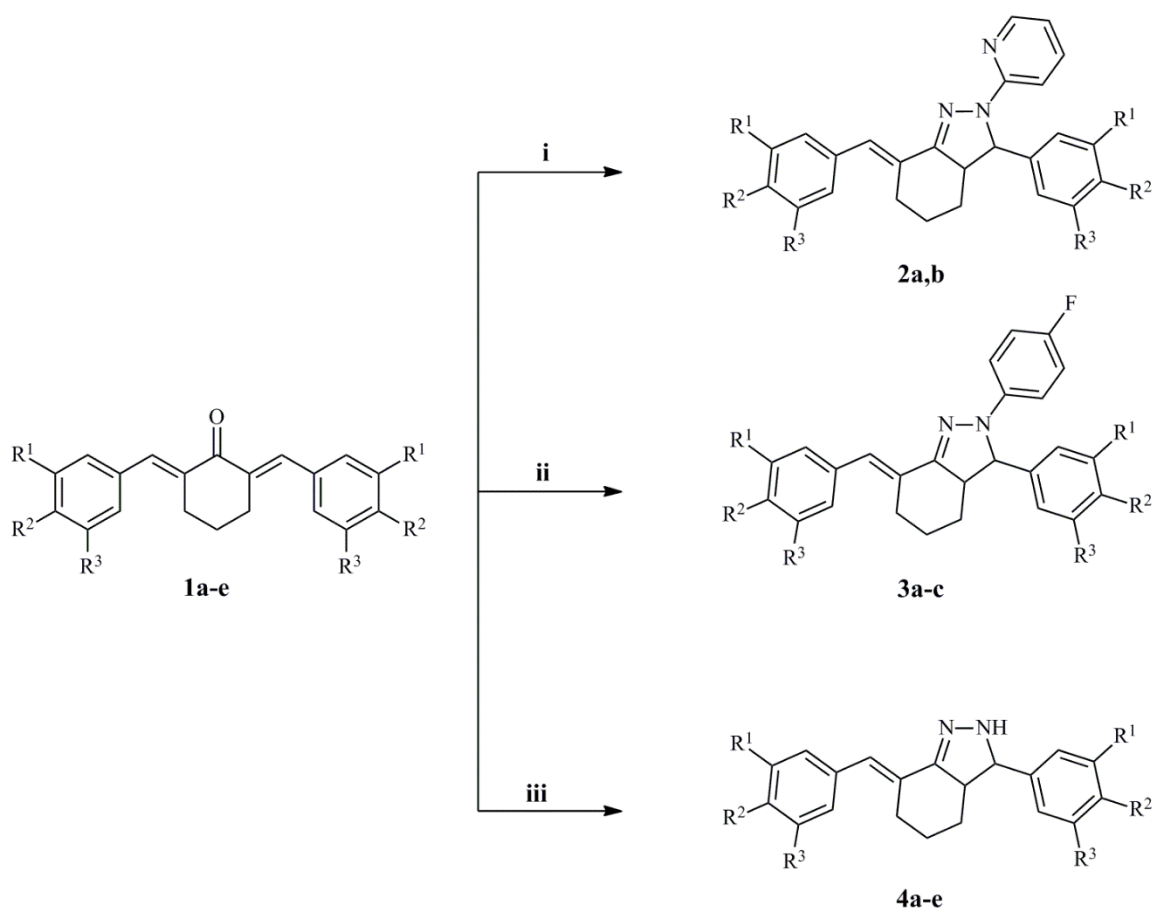
Chemistry

The thirteen pyrazoline derivatives (**1** - **5**) were prepared from the corresponding cyclohexanone-based (**1a-e**) or acetone-based (**1f,g**) monocarbonyl precursors. The compounds can be grouped as *N*-pyridine pyrazolines **2a,b**, *N*-*p*-fluorophenyl pyrazolines **3a-c**, unsubstituted (NH) pyrazolines **4a-e** and *N*-benzoic or *N*-benzoate pyrazolines **5a-c**. All products were prepared from inexpensive starting materials, in moderate to high yields,

in high purity as evidenced by elemental analysis and NMR, without the need of any chromatographic method for purification.

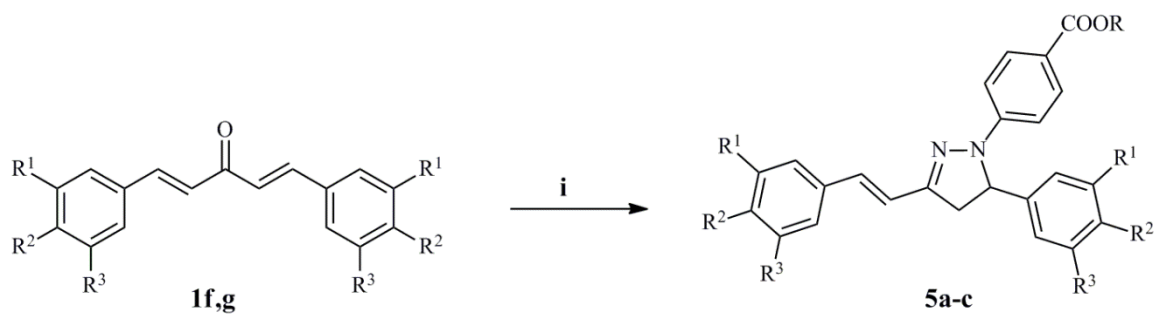
Pyrazolines **2a,b** were synthesized using 2-hydrazinopyridine in neat ethanol under reflux for three days. In particular, the synthesis and characterization of **2a** has been reported previously [27]. Fluorophenyl derivatives **3a-c** were prepared using hydrochloric 4-fluorophenyl hydrazine in the presence of sodium ethoxide in ethanol.

The synthesis of unsubstituted pyrazolines **4a-e** proved a challenging task, due to instability and decomposition of the products when dissolved in most organic solvents. This behavior was not unexpected, since it has been documented in the literature [28-31]. The two main obstacles we had to overcome in order to obtain a high purity product were: a) the autoxidation of the products upon exposure to the atmosphere [28,31] and b) the fact that they cannot be purified by chromatographic methods [29,30]. Therefore, the reaction, handling of the mixture and any work-up were done strictly under nitrogen atmosphere to avoid the exposure to oxygen. Moreover, when precipitate was formed at the end of the reaction period, as in the cases of **4a,b,e** this had to be collected only within a few hours after the end of the reaction. Failing to do so, resulted in the formation of a mixture of at least two inseparable products. In the case of products that did not precipitate spontaneously, as in compounds **4c, d**, a few drops of water were added in the solution after completion of the reaction.



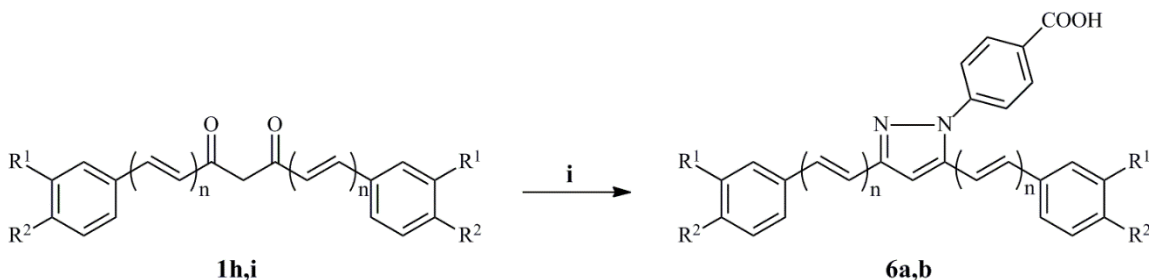
Scheme 1. Synthesis of pyrazolines **2a,b**, **3a-c** and **4a-e** from cyclohexanone monocarbonyl curcuminoids **1a-e**. Reagents and conditions: i) **1a,b**, 2-hydrazinopyridine, ethanol, 3 days, reflux; ii) **1a-c**, 4-fluorophenyl hydrazine hydrochloride, NaOEt, EtOH, r.t. to reflux, 24 h, iii) **1a-e**, hydrazine hydrate, EtOH, reflux, 1 h

Compounds **5a-c** were synthesized in glacial acetic acid under heating and their preparations have been recently reported along with full characterization and X-ray crystallographic analysis of **5b** [32,33].



Scheme 2. Synthesis of pyrazolines **5a-c** from monocarbonyl curcuminoids **1f,g**. Reagents and conditions: i) **1f,g**, 4-hydrazinobenzoic acid (for **5a,b**) or ethyl 4-hydrazinobenzoate (for **5c**), glacial acetic acid, 100 °C, 5 h.

Pyrazoles **6a,b** were synthesized according to literature procedures [34,35], slightly modified. These compounds were anticipated to provide an insight as to whether the activity might be affected by the presence of the pyrazole rather than the pyrazoline moiety in the molecule.



Scheme 3. Synthesis of pyrazoles **6a,b** from dicarbonyl curcuminoids **1i,j**. Reagents and conditions: i) **1h,i**, 4-hydrazinobenzoic acid, glacial acetic acid, Et₃N, reflux, 12 h.

The novel compounds **2b**, **3a-c** and **4c** were fully characterized by means of ¹H and ¹³C NMR and elemental analysis. The formation of the pyrazoline ring is mainly characterized by the appearance of a doublet signal (4.90 to 4.56 ppm) for the compounds **2a,b** and **3a-c** and a doublet of doublets (4.29 to 4.32 ppm) for the unsubstituted (NH) pyrazolines **4a-e** or the pyrazolines **5a-c** not bearing the cyclohexyl ring (5.49 to 5.61 ppm) corresponding to the adjacent to N-1 methine. In addition to the other expected signals, the ¹H NMR of the unsubstituted derivatives **4a-e** showed a doublet (*J* = 5.0 Hz) at δ 7.26 to 7.34 ppm assigned to the proton of the secondary nitrogen (N-1) of the ring.

Biological evaluation

Trypanosomicidal activity

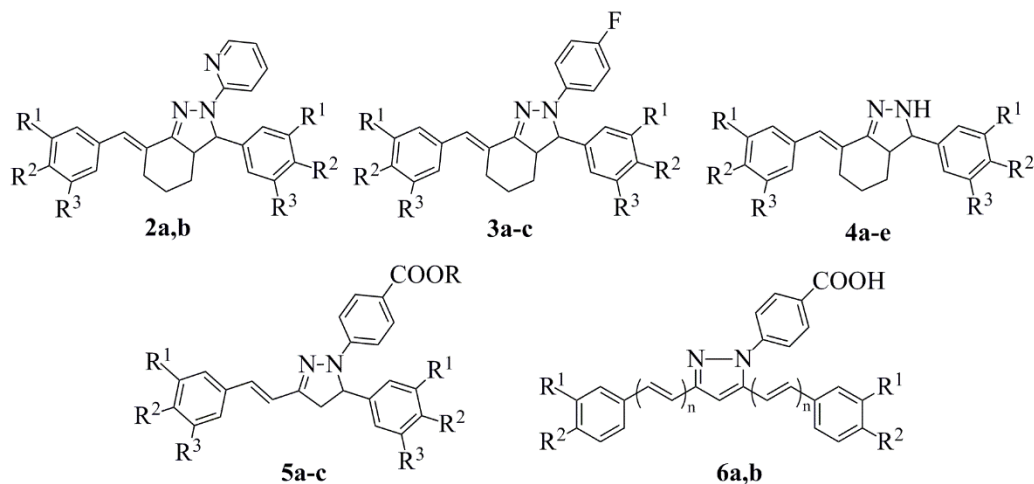
It was hypothesized that a TIM dimer interface inhibitor would be a successful strategy to identify molecules with selective antiparasitic activity. Therefore, curcumin analogs have been previously described as a potent and selective inhibitor of *Tc*TIM, but these analogs have metabolic stability problems and they are also considered as PAINS compounds as Michael acceptors [ref.]. The new derivatives described in the present work address these

issues by the reaction of this Michael acceptor motif towards the formation of nitrogen heterocyclic analogues. Table 1 summarises the results on the biological evaluation of the fifteen heterocyclic compounds synthesized. Firstly, as a consequence of our initial hypothesis the inhibition potential of *Tc*TIM was evaluated. Compound **6b** was the only molecule which exhibited interesting *Tc*TIM inhibitory activity with an IC₅₀ value of 15 µM. It is also important to note that **6b** has no inhibition effect at 100 µM on the human enzyme (HsTIM), demonstrating a selectivity of this molecule for *Tc*TIM.. However, this IC₅₀ value is almost twice the one obtained for curcumin. Moreover, **6b** did not exhibit any significant activity against *T. cruzi* epimastigotes which indicates that the *Tc*TIM inhibition potential alone was not enough to evoke antitrypanosomal activity.

Regarding the Trypanosomicidal activity against epimastigotes of *T. cruzi* three of the tested pyrazol(in)es exhibited moderate to good anti-*T. cruzi* activity. More specifically, **4a** and **4e** were the most active of all, exhibiting IC₅₀ values of 5 and 4.2 µM, respectively. It is worth noting that these values are smaller than the IC₅₀ value of the reference drug, Nifurtimox, which was at the range of 7 µM, whereas curcumin exhibited an IC₅₀ value of 10 µM. Therefore, compounds **4a** and **4e** are highly promising Trypanosomicidal scaffolds for further exploitation. They share the common feature of *para*-substitution of the phenyl ring and 2-NH-pyrazoline derivatisation of the bis-arylidene skeleton. It seems that the nature of this substitution is not critical for this activity since **4a** possesses a –OMe group whereas **4e** is *p*-fluoro derivative. In the case of **4b** reduced activity was observed resulting in a three times higher IC₅₀ value. This activity drop might be related to the di-substituted motif of the aromatic moiety. Interestingly, the *para*-substituted active derivatives **4a** and **4e** were found to be much less active against murine macrophages. This additional feature of selectivity is highly desirable for a compound to be further exploited as a potential Trypanosomicidal agent.

Table 1. Inhibition of *Tc*TIM, Trypanosomicidal activity against epimastigotes of *Trypanosoma cruzi*, cytotoxicity against murine cells, and selective index of compounds **2a,b**, **3a-c**, **4a-e**, **5a-c** and **6a,b**.

Groups of compounds

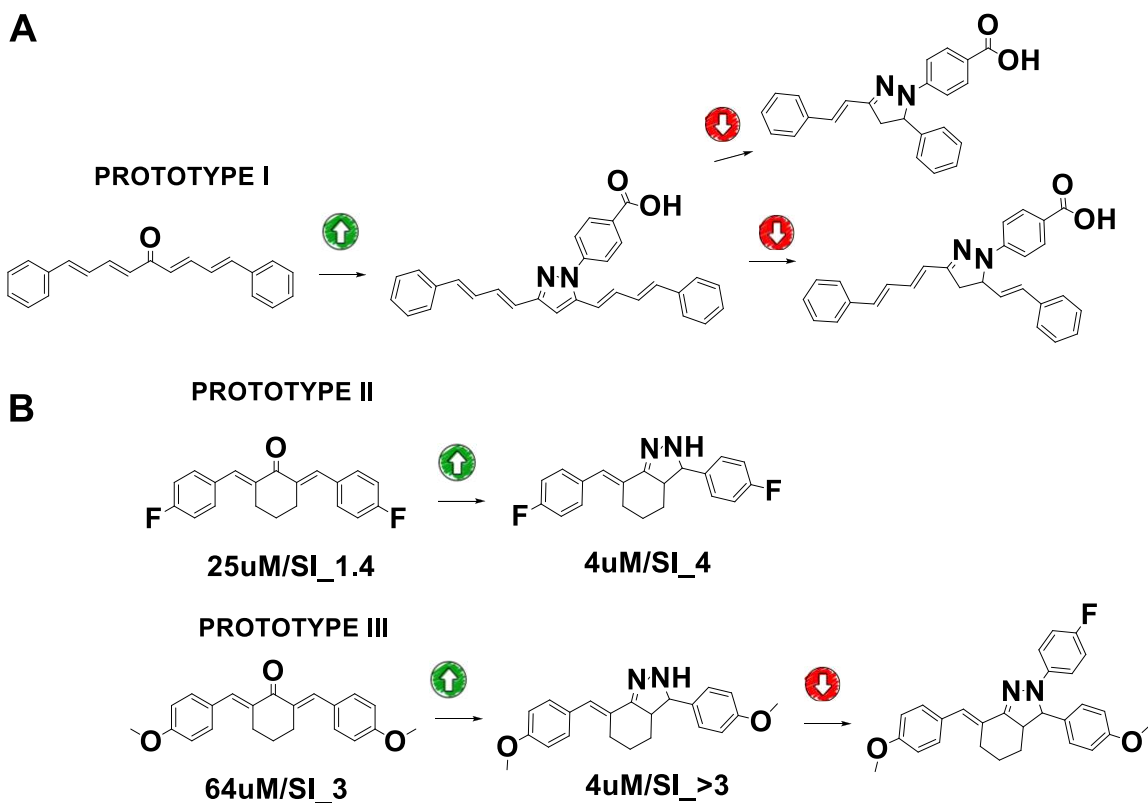


Compound	R ¹	R ²	R ³	IC ₅₀ TIM from <i>T.</i> <i>cruzi</i> (μM)	IC ₅₀ epimastigotes <i>T. cruzi</i> (μM)	IC ₅₀ Murine macrophages	Selectivity Index
2a	H	OMe	H	>100	>25	nd	nd
2b	OMe	OMe	H	>100	>25	nd	nd
3a	H	OMe	H	100	>25	nd	nd
3b	OMe	OMe	H	>100	>25	nd	nd
3c	OMe	OMe	OMe	>100	>25	nd	nd
4a	H	OMe	H	>100	5	>15	>3
4b	OMe	OMe	H	>100	15	11	<1 (toxic)
4c	OMe	OMe	OMe	>100	>25	nd	nd
4d	H	OH	H	>100	>25	nd	nd
4e	H	F	H	>100	4.2	15	4
5a (R = H)	H	H	H	>100	>25	nd	nd
5b (R = H)	OMe	OMe	H	>100	>25	nd	nd
5c (R = Et)	OMe	OMe	H	>100	>25	nd	nd

6a (n=1)	OMe	OMe	H	>100	>25	nd	nd
6b (n=2)	H	H	H	15	>25	nd	nd
Curcumin				7	10	5	<1 (toxic)
Nifurtimox				>100	7	>100	>15

Scheme 4A provides a rationalization of the results of this study compared to previous data on the parental arylideneketones [9]. The new analogs demonstrate better *Tc*TIM activity and trypanosomicidal activity than the parental prototypes. More specifically, in the case of **6b** resulting from the prototype I, the carboxyl moiety may potential interact with the protein which is essential for this enzymatic inhibition activity [9]. It is also worth mentioning that the pyrazole scaffold of **6a** presents the highest degree of symmetry out of the fifteen molecules tested being in accordance with the notion that symmetry is essential for inhibition activity [10]. The inactivity against *T. cruzi* epimastigotes could be attributed to the presence of the ionizable carboxylic group, which may increase the difficulty of cell permeability, as it has been previously observed and reposted [36].

In the case of the new trypanosomicidal compounds **4a** and **4e**, compound **4e** was 6 times more potent than the prototype II (Scheme 4B). Compound **4a** was 16 times more potent than the prototype III and both exhibited improved toxicology profile since they were much less toxic against murine macrophages. The incorporation of a fluorophenyl or pyridine in the pyrazoline ring results in loss of activity.



Scheme 4. A) Structure relationship in *TcTIM* activity and B) Structure relationship in epimastigote activity.

The predicted pharmacokinetic parameters and oral toxicity in rats are according to the drug-likeness. Also, the LD₅₀ was higher than the reference drug Benznidazole and curcumin (Table 2).

Table 2. Predicted pharmacokinetic parameters and oral toxicity *in vivo*.

Compound	Solubility (mg/mL)	Gastrointestinal Absorption	Brain Permeability	CYP inhibitor	P-gp* substrate	Lipophilicity	Oral Rat **LD50 mg/kg
4a	3.3 x 10 ⁻³	High	Yes	Yes	No	4.0	1630
4e	2.1 x 10 ⁻³	High	Yes	Yes	No	4.6	1200

Benznidazole	2.3×10^{-3}	High	No	No	No	0.5	517
Curcumin	4.2×10^{-3}	High	No	No	No	3.0	1015

*P-glycoprotein substrate, **Calculated by Toxicity Estimation Software Tool (TEST)

Computational study

The docking studies showed that the predicted interaction site is similar to the curcumin. However, the conformation for curcumin is better than in the **6b** analogue because of a π - π interaction and hydrogen bond in the hydroxyl substitution in the aromatic ring. This interaction was not favorable in the **6b** analog, but the conformation of **6b** interact closer to an essential loop for the dimer stability (the total binding data in the supporting information). Also, compound **6b** has no inhibition effect at 100uM in the human enzyme (*HsTIM*), demonstrating the selectivity of this molecule for *TcTIM*.

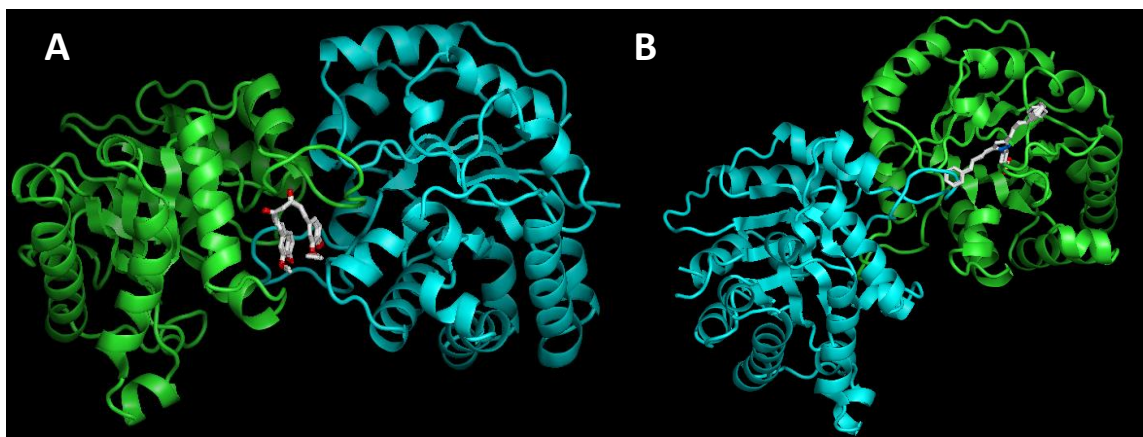


Fig. 2. Binding site interaction predicted by Docking, full energy calculations are in supporting information. **A)** is the binding site of curcumin and **B)** binding site of **6b**.

Experimental part

General

All reagents were purchased from Sigma-Aldrich, Alfa Aesar, and TCI and used without further purification. Melting points were determined with a Gallenkamp MFB-595 melting point apparatus. NMR spectra were recorded with a Bruker Avance 500 MHz spectrometer

operating at 500 MHz (^1H) and 125 MHz (^{13}C) or with a Bruker Avance 250 MHz spectrometer operating at 250 MHz (^1H) and 62.5 MHz (^{13}C). Chemical shifts are reported in ppm relative to DMSO- d_6 (^1H : $\delta = 2.50$ ppm, ^{13}C : $\delta = 39.52 \pm 0.06$ ppm). Elemental analyses were performed... Starting materials **1a-c**, **1e-g** and **1i,j** were prepared following our published procedures [32,34]. Pyrazolines **2a**, **5a-c** and pyrazoles **6a,b** were prepared according to our published protocols without modifications [27,32-35].

Synthesis of 2,6-bis((E)-4-hydroxybenzylidene)cyclohexan-1-one 1d

A mixture of cyclohexanone (0.98 g, 10 mmol) and 4-hydroxybenzaldehyde (2.44 g, 20 mmol) in ethanol (2 mL) was stirred at 40 °C. 0.2 mL of concentrated hydrochloric acid (37%) was added dropwise. The resulting clear solution was stirred at 40 °C for 24 h. After cooling to room temperature, a small volume of ethanol (6-8 mL) was added and the precipitated solid was filtrated under vacuum. The crude product was washed with cold ethanol (2 \times 6-8 mL) and then recrystallized from ethanol to give the pure product as olive green solid.

Yield: 1.68 mg (55%), mp, ^1H NMR (250 MHz, DMSO- d_6): 1.71 (2H, quin, $J = 5.4$ Hz, $\text{CH}_2\text{CH}_2\text{CH}_2$), 2.85 (4H, t, $J = 5.4$ Hz, $\text{CH}_2\text{CH}_2\text{CH}_2$), 6.84 (4H, $J = 8.6$ Hz, Ph), 7.41 (4H, $J = 8.6$ Hz, Ph), 7.54 (2H, s, $\text{CH}=\text{C}$), 9.93 (2H, brs, OH).

General synthetic procedure for compounds 2a-b

Pyrazoline **2b** was prepared following the published synthetic protocol for **2a** [27].

(E)-7-(3,4-dimethoxybenzylidene)-3-(3,4-dimethoxyphenyl)-2-pyridyl-3,3a,4,5,6,7-hexahydro-2H-indazole 2b

Yield: 138 mg (57%), mp, ^1H NMR (500 MHz, DMSO- d_6): 1.46 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.67 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.86 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.10 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.50 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.92 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.72 (6H, s, OCH_3), 3.77 (6H, s, OCH_3), 4.90 (1H, d, $J = 10.6$ Hz, $\text{CH}-\text{N}$), 6.73 (1H, br, Pyr), 6.83 (1H, d, $J = 7.5$, Ph), 6.89 (2H, d, $J = 6.5$ Hz, Ph), 6.98 (2H, br, Ph), 7.02 (1H, br, Ph), 7.12 (1H, s, $\text{CH}=\text{C}$), 7.39 (1H, d, $J = 7.5$ Hz, Pyr), 7.59 (1H, br t, $J = 6.8$ Hz, Pyr), 7.94 (1H, br d, $J = 3.6$ Hz, Pyr), ^{13}C NMR (62.5 MHz, DMSO- d_6): 23.8 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 28.4 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 28.9 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 55.48 (2 \times MeO), 55.5 (2 \times MeO), 56.1 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 70.1 ($\text{CH}-\text{N}$), 109.5 (Ph), 110.7 (Pyr), 111.6 (Ph), 111.9 (Ph), 113.4 (Ph), 115.3 (Pyr), 117.7 (Ph), 122.2 (Ph), 125.9 ($\text{CH}=\text{C}$), 128.9 (Ph), 129.0 (Ph), 136.0 ($\text{CH}=\text{C}$), 136.9 (Pyr), 147.1 (Pyr), 147.6 (Ph), 148.3 (Ph), 148.4 (Ph), 148.8 (Ph), 155.4 ($\text{C}=\text{N}$), 157.2 (Pyr).

General synthetic procedure for compounds 3a-c

Compound **1a-c** (0.50 mmol) and 4-fluorophenylhydrazine hydrochloride (244 mg, 1.50 mmol) were added in anhydrous ethanol (10 mL) containing sodium ethoxide (3.00 mmol) prepared in situ by addition of Na^o (69 mg, 3.00 mmol) in anhydrous ethanol (10 mL). The reaction mixture was stirred under reflux for 24 h.

(E)-7-(4-methoxybenzylidene)-3-(4-methoxyphenyl)-2-(4-fluorophenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **3a**

Yield: 138 mg (62%), mp, ¹H NMR (500 MHz, DMSO-*d*₆): 1.36-1.43 (1H, m, CH₂CH₂CH₂CH), 1.61-1.68 (1H, m, CH₂CH₂CH₂CH), 1.85-1.87 (1H, m, CH₂CH₂CH₂CH), 1.99-2.00 (1H, m, CH₂CH₂CH₂CH), 2.40-2.46 (1H, m, CH₂CH₂CH₂CH), 2.85-2.91 (2H, m, CH₂CH₂CH₂CH and CH₂CH₂CH₂CH), 3.75 (3H, s, OCH₃), 3.78 (3H, s, OCH₃), 4.56 (1H, d, *J* = 12.6 Hz, CH-N), 6.93-6.99 (8H, m, Ph), 7.11 (1H, s, CH=C), 7.33-7.38 (4H, m, Ph), ¹³C NMR (62.5 MHz, DMSO-*d*₆): 23.6 (CH₂CH₂CH₂CH), 28.1 (CH₂CH₂CH₂CH), 28.3 (CH₂CH₂CH₂CH), 55.07 (MeO), 55.13 (MeO), 56.6 (CH₂CH₂CH₂CH), 73.0 (CH-N), 113.8 (Ph), 114.5 (Ph), 115.0 (d, ²*J*_{C-F} = 22.0 Hz, Ph), 115.9 (d, ³*J*_{C-F} = 7.5 Hz, Ph), 125.2 (CH=C), 127.2 (Ph), 128.6 (Ph), 128.7 (CH=C), 130.9 (Ph), 133.5 (Ph), 143.5 (d, ⁴*J*_{C-F} = 2.0 Hz, Ph), 153.6 (C=N), 156.4 (d, ¹*J*_{C-F} = 236.0 Hz), 158.5 (Ph), 158.7 (Ph).

(E)-7-(3,4-dimethoxybenzylidene)-3-(3,4-dimethoxyphenyl)-2-(4-fluorophenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **3b**

Yield: 156 mg (62%), mp, ¹H NMR (500 MHz, DMSO-*d*₆): 1.37-1.45 (1H, m, CH₂CH₂CH₂CH), 1.61-1.69 (1H, m, CH₂CH₂CH₂CH), 1.86-1.89 (1H, m, CH₂CH₂CH₂CH), 2.01-2.02 (1H, m, CH₂CH₂CH₂CH), 2.50 (1H, m, CH₂CH₂CH₂CH), 2.91-2.97 (2H, m, CH₂CH₂CH₂CH and CH₂CH₂CH₂CH), 3.74 (3H, s, OCH₃), 3.75 (6H, s, OCH₃), 3.78 (6H, s, OCH₃), 4.53 (1H, d, *J* = 12.6 Hz, CH-N), 6.95-7.01 (10H, m, Ph), 7.13 (1H, s, CH=C), ¹³C NMR (62.5 MHz, DMSO-*d*₆): 23.6 (CH₂CH₂CH₂CH), 28.19 (CH₂CH₂CH₂CH), 28.24 (CH₂CH₂CH₂CH), 55.5 (MeO), 56.5 (CH₂CH₂CH₂CH), 73.5 (CH-N), 109.4 (Ph), 111.6 (Ph), 112.2 (Ph), 113.3 (Ph), 115.0 (d, ²*J*_{C-F} = 22.0 Hz, Ph), 115.9 (d, ³*J*_{C-F} = 7.5 Hz, Ph), 118.1 (Ph), 122.2 (Ph), 125.6 (CH=C), 128.8 (CH=C), 128.9 (Ph), 134.0 (Ph), 143.8 (d, ⁴*J*_{C-F} = 2.0 Hz, Ph), 148.21 (Ph), 148.24 (Ph), 148.4 (Ph), 149.2 (Ph), 153.7 (C=N), 156.4 (d, ¹*J*_{C-F} = 236.0 Hz).

(E)-7-(3,4,5-trimethoxybenzylidene)-3-(3,4,5-trimethoxyphenyl)-2-(4-fluorophenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **3c**

Yield: 167 mg (59%), mp, ^1H NMR (500 MHz, $\text{DMSO-}d_6$): 1.39-1.46 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.62-1.70 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.87-1.89 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.06-2.08 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.50 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.93-2.99 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.66 (3H, s, OCH_3), 3.68 (3H, s, OCH_3), 3.76 (6H, s, OCH_3), 3.79 (6H, s, OCH_3), 4.54 (1H, d, $J = 12.6$ Hz, CH-N), 6.71 (2H, s, Ph), 6.73 (2H, s, Ph), 6.99-7.04 (4H, m, Ph), 7.14 (1H, s, CH=C), ^{13}C NMR (62.5 MHz, $\text{DMSO-}d_6$): 23.6 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 28.2 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 28.3 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 55.9 ($4 \times \text{MeO}$), 56.4 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 59.9 ($2 \times \text{MeO}$), 60.1 ($2 \times \text{MeO}$), 73.9 (CH-N), 102.9 (Ph), 107.1 (Ph), 112.2 (Ph), 113.3 (Ph), 115.2 (d, $^2J_{\text{C-F}} = 22.0$ Hz, Ph), 116.0 (d, $^3J_{\text{C-F}} = 7.5$ Hz, Ph), 125.9 (CH=C), 130.1 (Ph), 131.7 (Ph), 136.6 (Ph), 137.0 (CH=C), 137.5 (Ph), 143.8 (d, $^4J_{\text{C-F}} = 2.0$ Hz, Ph), 152.6 (Ph), 153.4 (Ph), 153.6 (Ph), 149.2 (Ph), 153.7 (C=N), 156.4 (d, $^1J_{\text{C-F}} = 236.0$ Hz).

General synthetic procedure for compounds 4a-e

(E)-7-(4-methoxybenzylidene)-3-(4-methoxyphenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **4a** [37,38]

Yield: 134 mg (77%), mp, ^1H NMR (500 MHz, $\text{DMSO-}d_6$): 1.30-1.38 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.43-1.50 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.83-1.89 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.34-2.39 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.55-2.61 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.81-2.84 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.74 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 4.29 (1H, dd, $J = 14.2$ Hz/5.0 Hz, CH-N), 6.91 (2H, d, $J = 8.6$ Hz, Ph), 6.94 (6.91 (2H, d, $J = 8.6$ Hz, Ph), 6.95 (1H, s, CH=C), 7.26 (1H, d, $J = 5.0$ Hz, NH), 7.31 (2H, d, $J = 8.6$ Hz, Ph), 7.35 (2H, d, $J = 8.6$ Hz, Ph)

(E)-7-(3,4-dimethoxybenzylidene)-3-(3,4-dimethoxyphenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **4b** [39]

Yield: 136 mg (67%), mp, ^1H NMR (500 MHz, $\text{DMSO-}d_6$): 1.31-1.39 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.44-1.51 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.84-1.86 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.90-1.91 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.36-2.42 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.59-2.64 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.86-2.89 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.74 (3H, s, OCH_3), 3.76 (9H, s, OCH_3), 4.29 (1H, dd, $J = 14.2$ Hz/5.0 Hz, CH-N), 6.91-6.96 (6H, m, Ph), 7.04 (1H, s, CH=C), 7.27 (1H, d, $J = 5.0$ Hz, NH).

(E)-7-(3,4,5-trimethoxybenzylidene)-3-(3,4,5-trimethoxyphenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **4c**

Yield: 138 mg (59%), mp, ^1H NMR (500 MHz, $\text{DMSO-}d_6$): 1.32-1.40 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.47-1.54 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.85-1.87 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.95-1.97 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.41-2.46 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.60-2.67 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.89-2.92 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 3.64 (3H, s, OCH_3), 3.67 (3H, s, OCH_3), 3.78 (12H, s, OCH_3), 4.32 (1H, dd, $J = 14.2$ Hz/5.0 Hz, CH-N), 6.65 (2H, s, Ph), 6.73 (2H, s, Ph), 6.96 (1H, s, CH=C), 7.34 (1H, d, $J = 5.0$ Hz, NH). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$): 23.9 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 28.1 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 52.8 ($\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 55.8 ($2 \times \text{MeO}$), 55.9 ($2 \times \text{MeO}$), 59.9 (MeO), 60.0 (MeO), 72.4 (CH-N), 104.0 (Ph), 106.9 (Ph), 124.2 (CH=C), 131.1 (Ph), 132.0 (Ph), 136.6 (Ph), 136.8 (Ph), 137.0 (CH=C), 152.6 (Ph), 152.8 (Ph), 153.6 (C=N).

(E)-7-(4-hydroxybenzylidene)-3-(4-hydroxyphenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **4d** [39,40]

Yield: 138 mg (59%), mp, ^1H NMR (500 MHz, $\text{DMSO-}d_6$): 1.29-1.37 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.40-1.47 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.82-1.88 (2H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$ and $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.31-2.36 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.52-2.57 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.81-2.84 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 4.23 (1H, dd, $J = 14.2$ Hz/5.0 Hz, CH-N), 6.73 (2H, d, $J = 8.6$ Hz, Ph), 6.76 (6.91 (2H, d, $J = 8.6$ Hz, Ph), 6.90 (1H, s, CH=C), 7.17 (1H, d, $J = 5.0$ Hz, NH), 7.19-7.23 (4H, two overlapped d, $J = 8.6$ Hz, Ph), 9.31 (1H, br, OH), 9.53 (1H, br, OH).

(E)-7-(4-fluorobenzylidene)-3-(4-fluorophenyl)-3,3a,4,5,6,7-hexahydro-2H-indazole **4e** [39]

Yield: 139 mg (86%), mp, ^1H NMR (500 MHz, $\text{DMSO-}d_6$): 1.32-1.40 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.47-1.52 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.83-1.86 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 1.90-1.92 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.36-2.42 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.59-2.65 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 2.79-2.82 (1H, m, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}$), 4.38 (1H, dd, $J = 14.2$ Hz/5.0 Hz, CH-N), 7.00 (1H, s, CH=C), 7.17 (2H, d, $J = 9.0$ Hz, Ph), 7.21 (2H, d, $J = 9.0$ Hz, Ph), 7.40-7.43 (3H, m, NH and Ph), 7.46-7.49 (2H, m, Ph).

Inhibition of Triosephosphate Isomerase [9].

Expression and purification of proteins: *Tc*TIM and *Hs*TIM were expressed in *Escherichia coli* and purified as described in the literature. After purification, the enzyme, dissolved in 100 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol (pH 8), was precipitated

with ammonium sulfate (75% saturation) and stored at 4°C. Before use, extensive dialysis against 100 mM triethanolamine/10 mM EDTA (pH 7.4) was performed. Protein concentration was determined by absorbance at 280 nm for *Tc*TIM ($\epsilon=33460 \text{ M}^{-1}\text{cm}^{-1}$) and *Hs*TIM ($\epsilon= 33460 \text{ M}^{-1}\text{cm}^{-1}$). Enzymatic activity was determined following the conversion of glyceraldehydes-3-phosphate into dihydroxyacetone phosphate in a coupled enzyme assay. The decrease in absorbance at 340 nm was followed in a multiplate reader Varioskan™ Flash Multimode Reader (Thermo Scientific™, Waltham, MA, USA) at 38°C. The reaction mixture (1 mL, pH 7.4) contained 100 mM triethanolamine, 10 mM EDTA, 0.2 mM NADH, 1 mM glyceraldehydes-3-phosphate and 0.9 units of α -glycerol phosphate dehydrogenase. The reaction was initiated by the addition of 5 ng/mL of the TIM of interest. For the inhibition studies, TIM was incubated at a concentration of 5 mg/mL in a buffer containing 100 mM triethanolamine, 10 mM EDTA, pH 7.4, and 10% of DMSO at 37°C for 1h. The mixture also contained the compounds, dissolved in DMSO, at the indicated concentrations. After 1 h, 10 μ L was withdrawn and added to a final volume of 100 μ L of the reaction mixture for the activity assay. The inhibition assay was performed in a 96-well microplate. None of the molecules tested here affected the activity of α -glycerol phosphate dehydrogenase, the enzyme used in the coupled assay. The IC₅₀ value was taken as the concentration of drug needed to reduce the enzymatic activity to 50% by analysis using OriginLab8.5® sigmoidal regression (% of enzymatic activity vs. the logarithm of the compound concentration). The experiments were performed in triplicate in two independent experiments.

Docking [41].

The crystal structure PDB ID 1TCD of *Tc*TIM was used as a target in the subsequent docking studies. Compounds were modeled using their SMILES codes from the Chemoffice software. Then, both target protein and ligands were prepared using AutoDockTools v1.5.6: the polar hydrogen atoms were added, the non-polar hydrogens were merged, and the Gasteiger partial atomic charges were calculated. Finally, all the possible rotatable bonds were assigned for each compound. Four distinct docking experiments were then carried out with the program AutoDock Vina v1.1.2: a search on the entire surface of *Tc*TIM in its dimeric and monomeric forms as well as a targeted docking in the active site only, with and without the flexibility of the side chains of the residues defining the binding pocket. Compounds were treated as fully flexible in each experiment. The search grid was defined accordingly to encompass the considered areas.

A visual examination of the resulting poses was performed using PyMOL (Schrödinger, Delano Scientific, LLC, New York, NY, USA).

Anti-Parasitic Test *In Vitro* [9].

For the *in vitro* anti-*T. cruzi* activity, we used epimastigotes of the Tulahuen 2 strain (genotype TcVI) grown in an axenic medium (BHI-Tryptose). Cells from a 5–7-days-old culture were inoculated in fresh culture medium to give an initial concentration of 1×10^6 cells/mL. The absorbance at 600 nm of the cells in culture was measured every day. At day five, the medium was inoculated with different doses of the compounds (25, 12.5, 6.25, 3.13, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05) μM from a freshly prepared stock solution in DMSO (DMSO concentration in the culture milieu never exceeded 0.4%). Control parasites were cultivated in 0.4% DMSO. Each concentration of the compound was evaluated in duplicate. At five days, the absorbance of the culture was measured and compared to the control and the IC₅₀ values calculated for each compound using OriginLab8.5® sigmoidal regression. Each experiment was done in duplicate, and each concentration was tested in triplicate.

Nonspecific *In Vitro* Cytotoxicity of Mammalian Cells [9].

J774.1 murine macrophages (ATCC, USA) were grown in a DMEM culture medium containing 4 mM L-glutamine and supplemented with 10% FCS. Cells were seeded in a 96-well plate (5×10^4 cells in 200 μL culture medium) and incubated at 37 °C in a 5% CO₂ atmosphere for 48 h, to allow cell adhesion before drug testing. Afterward, cells were exposed for 48 h to the compounds (25–400 μM) or the vehicle for control (0.4% DMSO), and additional controls (cells in medium) were used in each test. Cell viability was then assessed by measuring the mitochondria-dependent reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan. For this purpose, MTT in sterile PBS (0.2% glucose), pH 7.4, was added to the macrophages to achieve a final concentration of 0.1 mg/mL, and the cells were incubated at 37 °C for 3 h. After removing the medium, formazan crystals were dissolved in 180 μL of DMSO and 20 μL of MTT buffer (0.1 M glycine, 0.1 M NaCl, 0.5 mM EDTA, pH 10.5), and the absorbance at 560 nm was measured. The IC₅₀ was defined as the drug concentration at which 50% of the cells were viable, relative to the control (no drug added), and was determined by analysis using OriginLab8.5® sigmoidal regression (% of viable cells compared to the logarithm of the compound concentration). Tests were performed in triplicate.

Calculation of the Pharmacokinetic Parameters [42,43].

The predictions were made with the open-access SwissADME software (<http://www.swissadme.ch>), a tool that allows the prediction of different pharmacokinetic parameters such as water solubility, gastrointestinal absorption, skin penetrability, lipophilicity, bioavailability, etc (Figure 2S). The oral Lethal Doses 50 in Rat was estimated using the Toxicity Estimation Software Tool (TEST). For both of them were used the SMILES codes generated with the ChemBioOffice 2010 program.

Conclusions

Fifteen new compounds were synthesized and chemical and biological characterized against *TcTIM* and epimastigotes of *T. cruzi*. One compound was a novel and selective *TcTIM* inhibitor, and two compounds were effective trypanosomicidal agents. The 16-fold (for **4a**) and 6-fold (for **4e**) higher potencies of these derivatives in comparison to their curcuminoids precursors make them an attractive and promising new family of trypanosomicidal compound. Also, compounds **4a** and **4e** can proceed to the next stage in the drug development process to an *in vivo* and metabolic stability studies.

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