Design and synthesis of new (E)-cinnamic N-acylhydrazones as potent antitrypanosomal agents

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · JUNE 2012

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2012.05.041 · Source: PubMed

15 AUTHORS, INCLUDING:

Samir A. Carvalho
Fundação Oswaldo Cruz
23 PUBLICATIONS **199** CITATIONS
SEE PROFILE

James L Wardell
Fundação Oswaldo Cruz
860 PUBLICATIONS **4,212** CITATIONS
SEE PROFILE

Gustavo Trossini
University of São Paulo
27 PUBLICATIONS **178** CITATIONS
SEE PROFILE

Carlos Alberto Manssour Fraga
Federal University of Rio de Janeiro
257 PUBLICATIONS **2,823** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 54 (2012) 512–521

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design and synthesis of new (*E*)-cinnamic *N*-acylhydrazones as potent antitrypanosomal agents

Samir A. Carvalho [a,b], Larisse O. Feitosa [a], Márcio Soares [a], Thadeu E.M.M. Costa [a], Maria G. Henriques [a], Kelly Salomão [c], Solange L. de Castro [c], Marcel Kaiser [d], Reto Brun [d], James L. Wardell [e], Solange M.S.V. Wardell [f], Gustavo H.G. Trossini [g], Adriano D. Andricopulo [h], Edson F. da Silva [a], Carlos A.M. Fraga [b,i,*]

[a] *Instituto de Tecnologia em Fármacos – Farmanguinhos, Fundação Oswaldo Cruz, 21041-250 Rio de Janeiro, RJ, Brazil*
[b] *Programa de Pós-Graduação em Química, Instituto de Química, Universidade Federal do Rio de Janeiro, 21949-900 Rio de Janeiro, RJ, Brazil*
[c] *Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21040-900, Rio de Janeiro, RJ, Brazil*
[d] *Medical Parasitology and Infection Biology, Swiss Tropical Institute, CH-4002 Basel, Switzerland*
[e] *Centro de Desenvolvimento Tecnológico em Saúde (CDTS), Fundação Oswaldo Cruz, 21040-900 Rio de Janeiro, RJ, Brazil*
[f] *CHEMSOL, 1 Harcourt Road, Aberdeen AB15 5NY, Scotland, UK*
[g] *Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, 05508-000 São Paulo, Brazil*
[h] *Laboratório de Química Medicinal e Computacional, Centro de Biotecnologia Molecular Estrutural, Instituto de Física de São Carlos, Universidade de São Paulo, 13560-590 São Carlos, Brazil*
[i] *Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, PO Box 68023, 21941-902 Rio de Janeiro, RJ, Brazil*

ARTICLE INFO

Article history:
Received 23 February 2012
Received in revised form
18 May 2012
Accepted 30 May 2012
Available online 7 June 2012

Keywords:
N-Acylhydrazone
Cinnamic acid
Chagas' disease
Trypanosoma cruzi
Cruzain inhibitors

ABSTRACT

We report herein the synthesis and trypanocidal profile of new (*E*)-cinnamic *N*-acylhydrazones (NAHs) designed by exploiting molecular hybridization between the potent cruzain inhibitors (*E*)-1-(benzo[*d*][1,3]dioxol-5-yl)-3-(4-bromophenyl)prop-2-en-1-one and (*E*)-3-hydroxy-*N*′-((2-hydroxynaphthalen-1-yl)methylene)-7-methoxy-2-naphthohydrazide. These derivatives were evaluated against both amastigote and trypomastigote forms of *Trypanosoma cruzi* and lead us to identify two compounds that were approximately two times more active than the reference drug, benznidazole, and with good cytotoxic index. Although designed as cruzain inhibitors, the weak potency displayed by the best cinnamyl NAH derivatives indicated that another mechanism of action was likely responsible for their trypanocide action.

1. Introduction

Chagas' disease (CD) is caused by the intracellular obligatory parasite *Trypanosoma cruzi*. CD is the major cause of infectious cardiopathy and represents an important public health problem. It is broadly dispersed in 18 developing countries in South and Central America [1] and affects approximately eight million people in Latin America, of whom 30–40% either have or will develop cardiomyopathy, digestive megasyndromes, or both [2]. Recently, CD has become a major concern due to globalization, which results in immigration of infected individuals to non-endemic regions, thus spreading the disease [3]. Nifurtimox and benznidazole (Bzn) were empirically introduced into the clinical therapy regime for CD over four decades ago.

Abbreviations: AMC, 7-amino-4-methylcoumarin; Bzn, benznidazole; CD, Chagas' disease; DMES, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; ESI, electrospray ionization; FBS, fetal bovine serum; HOBt, *N*-hydroxybenzotriazole; HPLC, high pressure liquid chromatography; IR, infrared; MS, mass spectra; MTT(4,5-dimethylthiazol-2yl)-2,5-dimethyl tetrazolium bromide, 3-; NAH, *N*-acylhydrazone; NMR, nuclear magnetic resonance; ppm, parts per million; SI, selectivity index; TLC, thin-layer chromatography.

* Corresponding author. Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, PO Box 68023, 21941-902 Rio de Janeiro, RJ, Brazil. Tel.: +55 21 2562 6503; fax: +55 21 2562 6478.
E-mail address: cmfraga@ccsdecania.ufrj.br (C.A.M. Fraga).
URL: http://www.farmacia.ufrj.br/lassbio

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2012.05.041

Neither drug is ideal because they present variable results depending on the phase of the disease (they are only effective in the acute and recent chronic phases of the infection), the dose and duration of the treatment, and patient age and endemic region, as well as showing undesirable secondary side effects [4].

T. cruzi contains a cathepsin L-like cysteine protease termed cruzain (cruzipain), responsible for the major proteolytic activity of all life-cycle stages of this parasite [5]. Selective inhibitors of this protease cure *T. cruzi* infection in animal models using both mice [6] and dogs [7], making this enzyme a prime target for potential trypanocidal drugs [8].

The privileged status of the *N*-acylhydrazone framework [9] has been widely exploited in the design of new bioactive compounds with different pharmacological profiles [10], and the pharmacophoric character of this group has been identified during the development of some cysteinyl protease inhibitors [11–13]. Moreover, chalcones [(2*E*)-1,3-diphenylprop-2-en-1-one] share some similarity with the four-atom acylhydrazone linker and modeling studies suggest that they could occupy similar positions and adopt similar binding modes in the active site of the malarial falcipain-2 [14], a related cysteine protease from *Plasmodium falciparum* [15] that has more than 90% homology with cruzain at the binding site residues [16,17]. In addition, the unsaturated aryl ketone subunit of chalcones can act as good Michael acceptors, which would result in the irreversible inhibition of cruzain through the formation of a covalent adduct with the nucleophilic amino acid cysteine in the active site. Although the anti-*T. cruzi* activity of chalcones is well described [18], there are few studies associated with cruzain inhibition assays [11,18].

In our effort to develop potent trypanocidal compounds, we constructed a new class of cinnamic *N*-acylhydrazone (NAH) derivatives designed by molecular hybridization between two classes of cruzain inhibitors represented by the chalcone derivative (**1**) (IC_{50} = 20 μM) [19] and the naphthyl NAH derivative (**2**) (IC_{50} = 0.6 μM) [11]. This class aimed to enhance the inhibitory profile against the targeted enzyme by offering two electrophilic sites (A and B) capable of binding with the nucleophilic cysteine residue of cruzain (Fig. 1).

Both intracellular amastigotes and circulating trypomastigotes forms of *T. cruzi* are relevant in drug development assays, since they are present in the vertebrate host during the acute and chronic phases of Chagas' disease. For this reason, we first synthesized and evaluated the activity of the cinnamyl *N*-acylhydrazones (**3**–**17**), presenting substituents with different stereoelectronic properties only in the phenyl subunit D (Fig. 1), against amastigote and trypomastigote forms of *T. cruzi*. Next, aiming to enhance the trypanocidal activity observed in the screening of NAH derivatives **3**–**17**, we proposed structural changes in the phenyl ring C of two potent compounds (Fig. 1). For each new substituent on the phenyl subunit C, two new cinnamic NAH derivatives were synthesized (**18**–**25**) (Table 1). In addition, NAH derivative **26**, the retroisostere of **17**, was planned to investigate how the exchange of substituents between the two phenyl subunits (C) and (D) influenced the trypanocidal activity (Table 1). Unsaturated derivatives (**27**–**28**) and the naphthyl analogs (**29**–**30**) were structurally designed to investigate how the degree of conformational freedom and the loss of the pharmacophoric vinyl amide subunit would influence the trypanocidal activity (Table 1). Then, new cinnamic NAH derivatives (**18**–**30**) were evaluated *in vitro* against the bloodstream trypomastigote form of the parasite. The six most active compounds and Bzn were evaluated for toxicity to mammalian cells.

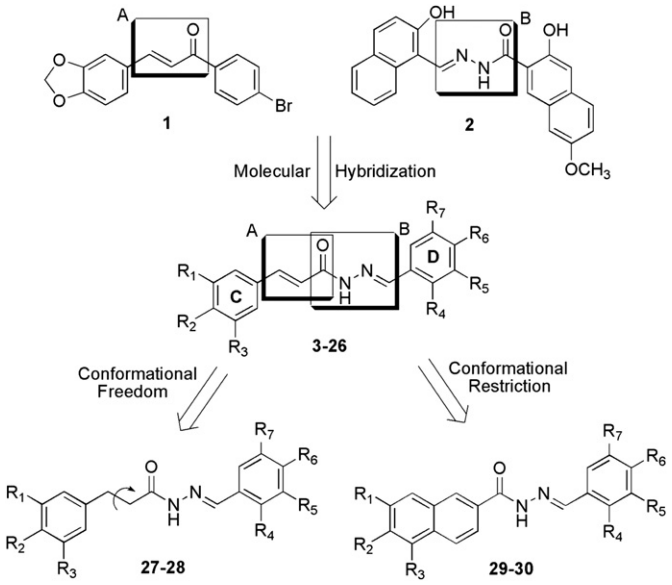


Fig. 1. Design of cinnamic *N*-acylhydrazone derivatives (**3**–**26**) and their conformationally-modified analogs (**27**–**30**). See Table 1 for the nature of substituents R_1–R_7.

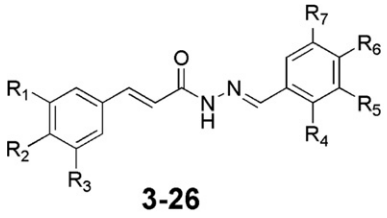
2. Results and discussion

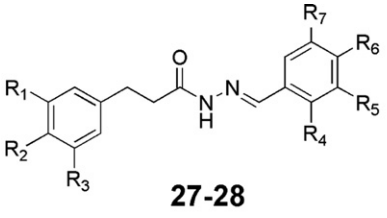
2.1. Chemistry

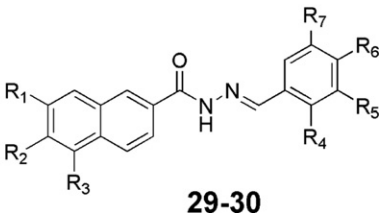
The planned synthetic route exploited to achieve the new NAH derivatives (**3**–**30**) are depicted in Schemes 1 and 2. (*E*)-Cinnamic acids (**31a**–**d**) were employed as starting materials. Non-commercially available (*E*)-cinnamic acids (**31e** and **31f**) were obtained in 75% and 70% yield, respectively, through the Knoevenagel–Doebner reaction of the corresponding aromatic aldehyde (**32a** and **32b**) with malonic acid in basic media [20,21]. The preparation of the cinnamic *N*-acylhydrazide derivatives involved the use of general methodology developed by Zhang and coworkers [22]. This methodology involved the pre-formation of esters and/or activated amides, followed by nucleophilic substitution of these intermediates with hydrazine hydrate. Through the use of HOBt/EDC as coupling reagent, *N*-acylhydrazides (**33a**–**33f**) were obtained in yields ranging from 83 to 92% (Scheme 1). Acylhydrazide derivatives **33g** and **33h** were respectively prepared from 3-phenylpropionic acid (**34**) and 2-naphtoic acid (**35**) by Fischer esterification and hydrazinolysis of the respective ester intermediates (Scheme 2) [23]. The target NAH derivatives (**3**–**30**) were obtained in good yields (Table 1) by acid catalyzed condensation of the hydrazines (**33a**–**33h**) with the corresponding aromatic aldehydes (ArCHO) in ethanol [23], as described in Schemes 1 and 2.

^{1}H NMR spectra and HPLC chromatograms of the synthesized NAH derivatives (**3**–**30**) were consistent with the presence of only one geometric isomer at the imine bond level, which X-ray diffraction data indicated as having the relative configuration (*E*). The molecular structure of (2*E*)-*N*-[(*E*)-4-chlorobenzylidene]-3-phenylprop-2-enohydrazide (**5**) in its mono-hydrate [24] was very similar to that of the bromo analog (**6**) reported here (Fig. 2). As described previously for other NAH derivatives [25], cinnamyl *N*-acylhydrazones (**3**–**30**) exist in equilibrium between the two stable conformers *sin-periplanar* (*sp*) and *antiperiplanar* (*ap*) in solution. These conformers are able to interconvert through rotation of the amide bond. Regarding the chemical shifts of these conformers, it is believed that the signal from the imine hydrogen being more downshield matches the *ap* conformer, as described by Palla and colleagues [26].

Table 1
Physical and spectral properties, *in vitro* trypanocidal activity, cytotoxicity and inhibition of cruzain of the cinnamic and structurally-related *N*-acylhydrazone derivatives (**3**–**30**).

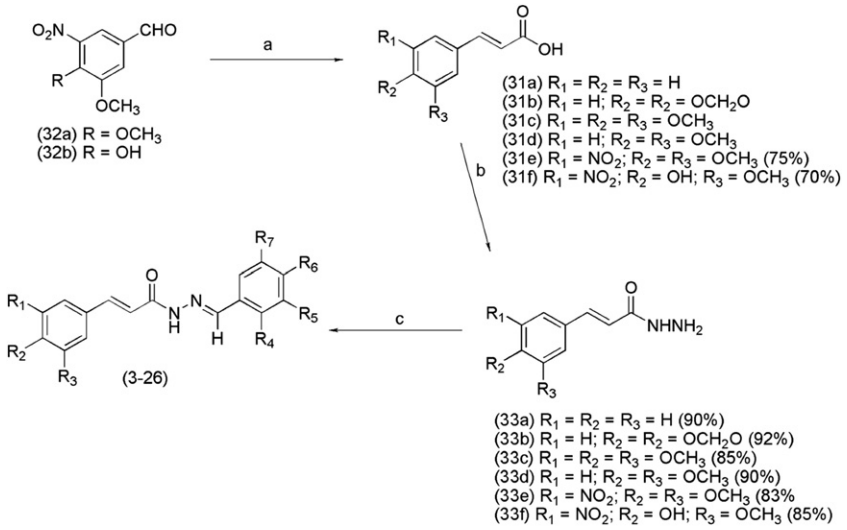




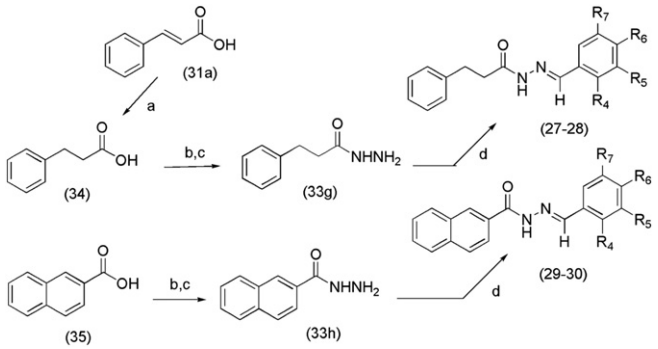


	R_1	R_2	R_3	R_4	R_5	R_6	R_7	Yield (%)	Mp (°C)	Conformational ratio (*ap*/*sp*′)[a]	IC_{50}[b] amastigote	IC_{50}[b] tripomastigote	IC_{50}[b] cytotoxicity	IC_{50}[b] cruzain	SI[c]
3	H	H	H	H	H	H	H	93	229	1:0.9	>100	>1100	ND		
4	H	H	H	H	H	F	H	83	190	1:0.6	>100	>1100	ND		
5	H	H	H	H	H	Cl	H	81	211	1:0.9	>100	>1100	ND		
6	H	H	H	H	H	Br	H	80	220	1:0.9	ND	>1100	ND		
7	H	H	H	H	H	NO_2	H	94	233	1:1	81	>1100	ND		
8	H	H	H	H	H	OCH_3	H	76	219	1:0.8	>100	>1100	ND		
9	H	H	H	H	H	OH	H	80	290	1:0.9	>100	>1100	ND		
10	H	H	H	OH	H	H	H	85	240	1:0.4	6.6	5.9 ± 1.2	420.2 ± 20.1	52.2 ± 4.0	71
11	H	H	H	H	OH	OH	H	80	229	1:0.3	>100	107.0 ± 21.0	ND		
12	H	H	H	H	O–CH_2–O		H	90	180	1:0.9	61	>1100	ND		
13	H	H	H	H	OCH_3	OCH_3	H	89	187	1:0.6	>100	>1100	ND		
14	H	H	H	H	OH	OCH_3	H	87	193	1:0.4	>100	>1100	ND		
15	H	H	H	H	OCH_3	OH	H	88	219	1:0.4	73	127.0 ± 10.0	ND		
16	H	H	H	H	OCH_3	OCH_3	OCH_3	91	185	1:0.6	70	71.0 ± 14.0	ND		
17	H	H	H	H	OCH_3	OH	NO_2	90	240	1:0.6	40	>1100	ND		
18	O–CH_2–O		H	OH	H	H	H	85	221	1:0.4	ND	5.6 ± 1.4	281.3 ± 14.8	74.8 ± 11.0	50
19	O–CH_2–O		H	H	OCH_3	OH	NO_2	83	255	1:0.7	ND	>1100	ND		
20	OCH_3	OCH_3	OCH_3	OH	H	H	H	82	222	ND	ND	78.8 ± 13.0	ND		
21	OCH_3	OCH_3	OCH_3	H	OCH_3	OH	NO_2	83	228	1:0.8	ND	18.0 ± 4.0	2413.0 ± 100	45.9 ± 6.0	134
22	NO_2	OCH_3	OCH_3	OH	H	H	H	87	206	1:0.3	ND	17.0 ± 0.4	550.2 ± 17.8	83.9 ± 6.0	32
23	NO_2	OCH_3	OCH_3	H	OCH_3	OH	NO_2	87	253	1:0.8	ND	16.0 ± 2.0	1865.0 ± 80.1	44.7 ± 3.0	116
24	OCH_3	OCH_3	H	OH	H	H	H	90	193	1:0.3	ND	65.0 ± 15.0	ND		
25	OCH_3	OCH_3	H	H	OCH_3	OH	NO_2	85	265	1:0.8	ND	>1100	ND		
26	NO_2	OH	OCH_3	H	H	H	H	81	216	1:0.8	ND	79.0 ± 13.0	ND		
27	H	H	H	OH	H	H	H	85	151	1:0.6	ND	18.0 ± 1.4	1630 ± 77.0	>100	90
28	H	H	H	H	OCH_3	OH	NO_2	88	197	1:1	ND	>1100	ND		
29	H	H	H	OH	H	H	H	89	209	1:1	ND	93.0 ± 12.0	ND		
30	H	H	H	H	OCH_3	OH	NO_2	88	197	1:0.9	ND	93.0 ± 20.0	ND		
Bzn	–	–	–	–	–	–	–	–	–	–	0.122	10.8 ± 0.4	3840.7 ± 110.2		356

[a] Relative ratio of amide bond conformers identified from the relative integration of signals related to the N–NH subunit of the NAH group.
[b] IC_{50} (μM).
[c] SI = Cytotoxicity IC_{50}/Trypanocide IC_{50} (trypomastigote forms of *T. cruzi*); ND = Not determined.



Scheme 1. Reagents and conditions: a) malonic acid, pyridine, $piperidine_{cat.}$, reflux, 1 h; b) (1) HOBt, EDC, CH_3CN, rt, 2 h; (3) inverse addition to hydrazine, CH_3CN, cyclohexane, 0–10 °C; c) ArCHO, EtOH, $HCl_{cat.}$, reflux.



Scheme 2. Reagents and conditions: a) $PdCl_2$, HCO_2H, H_2O, NaOH, 65 °C, 90%; b) EtOH, H_2SO_4, reflux, 2 h; c) NH_2NH_2 (aq. 80%), reflux, 3 h (**33g**, 90% yield, 2 steps)/(**33h**, 85% yield, 2 steps); d) ArCHO, EtOH, $HCl_{cat.}$, reflux.

2.2. Trypanocide activity

The *in vitro* screening of the cinnamyl NAH derivatives (**3**–**17**) against intracellular amastigote forms of *T. cruzi* showed that compound **10**, with an *o*-hydroxyphenyl group attached to the imine unit, was the most active (IC_{50} = 6.6 μM). This superior trypanocidal profile in comparison to the other derivatives could be explained by the ability of the *o*-hydroxybenzylidene *N*-acylhydrazone framework to form an electrophilic quinone methide intermediate that could interact with nucleophilic sites in target enzymes of *T. cruzi*, *e.g.* cysteine proteases [13].

The trypanocidal activity of the other derivatives varied in ring C was not as pronounced as for compound **10**. The comparison between the potencies of 5-NO_2–vanillinyl (**17**) and vanillinyl (**15**) NAH derivatives (IC_{50} = 40 and 73 μM, respectively) (Table 1) revealed that the presence of the NO_2 group significantly increased the trypanocidal activity, suggesting that this group may interfere with the redox system of the parasite. Piperonyl (**12**) and 3,4,5-trimethoxyphenyl (**16**) derivatives had only modest trypanocidal activity, with IC_{50} values of 61 μM and 70 μM, respectively (Table 1).

From these initial results, we proposed novel cinnamyl NAH derivatives (**18**–**25**) with structural changes in the phenyl subunit (C) (Fig. 1). For all of the new substituents introduced on the aromatic ring (C), two novel derivatives led to the highest trypanocidal activity in the first series: 2-hydroxyphenyl and 5-NO_2–vanillinyl.

Next, all NAH derivatives, including **3**–**17**, had their trypanocidal activity evaluated on bloodstream trypomastigotes (Table 1). In general, the presence of *o*-hydroxyphenyl provided better activity, corroborating the results obtained in the first series of assays on amastigotes. The derivatives that presented the most pronounced trypanocidal activity were **10** and **18**, with IC_{50} values of 5.6 and 5.9 μM, respectively (Table 1). The structural modifications introduced in the phenyl subunit C confirmed the pharmacophoric sketch of the introduced substituents [13,19], since most of the derivatives **18**–**25** were equally or more active than their unsubstituted counterparts (Table 1).

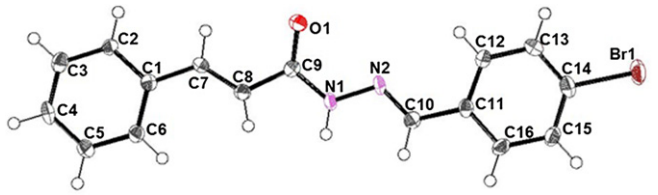


Fig. 2. Atom arrangements for (*E*)-*N*′-(4-bromobenzylidene)cinnamohydrazide (**6**).

Comparing the trypanocidal activity of the *o*-hydroxyphenyl and 5-NO_2–vanillinyl containing derivatives, it was observed that three of the five most active (**10**, **18** and **22**) contained an *o*-hydroxy substituted phenyl group. Moreover, when comparing the activity of each pair alone (**10** and **17**, **18** and **19**, **20** and **21**, **22** and **23** and **24** and **25**), only for compound **20** did the presence of a 5-NO_2–vanillinyl unit result in superior trypanocidal activity. For the pair of NAH derivatives **22** and **23**, the trypanocidal activity was equal (Table 1). Furthermore, the retro-isosteric replacement between the substituents attached to the phenyl subunits C and D improved activity against trypomastigotes for **26** (IC_{50} = 78.8 μM) more than the corresponding analog **17** (IC_{50} > 150 μM) (Table 1).

The reduction of the cinnamic double bond of compound **10** (IC_{50} = 5.9 μM) resulted in a remarkable decrease in the trypanocidal activity, as evidenced by the corresponding saturated analog **27** (IC_{50} = 18.0 μM). This profile could be related to the augmented conformational freedom acquired by **27**, allowing it to adopt different conformations from those of the most active NAH derivative **10** and decreasing the antiprotozoal activity. On the other hand, a more pronounced decline was observed in comparison of the trypanocidal activity of **10** with a more restricted conformational analog **29**. It is possible that reducing the degrees of conformational freedom of the double bond linked to the aromatic ring (C) prevented **29** from adopting the bioactive conformation of **10**.

Next, we evaluated the inhibitory effects of the six most active compounds against the enzyme cruzain from *T. cruzi* (Table 1) by monitoring the liberation of the fluorescent leaving group 7-amino-4-methylcoumarin from the peptide substrate Z–Phe–Arg–AMC [14,19]. The results demonstrated that these compounds (Table 1) did not substantially inhibit this enzyme, indicating that another mechanism of action must be involved in their trypanocidal activity. Derivatives **10**, **21** and **23** had the best inhibitory profile with IC_{50} values ranging from 44.7 to 52.2 μM. On the other hand, the cruzain inhibitory activity of unsaturated derivative **27** could not be determined accurately due to solubility limitations that decreased the sensitivity of the assay.

The six most active derivatives ($IC_{50} \leq 20$ μM) and Bzn had their activity against mammalian cells measured using the macrophage lineage J774 and the selectivity index (SI) (LC_{50} for cytotoxicity divided by IC_{50} for trypanocidal activity) was calculated. In this context, the five compounds with SI ≥50 (*i.e.*, **10**, **18**, **21**, **23** and **27**) were considered as good candidates for subsequent studies on trypanocidal activity in a murine model [27,28].

3. Conclusions

We have discovered five new potent *trans*-cinnamic *N*-acylhydrazones compounds with low cytotoxicity and excellent trypanocidal activity. Two of them (**10** and **18**) were two-fold more potent than the reference drug Bzn against trypomastigotes of *T. cruzi*. Although designed as cruzain inhibitors, none of the most active NAH derivatives were potent inhibitors of this enzyme, suggesting that a more complex mechanism of action is related to the displayed trypanocidal activity.

4. Experimental section

4.1. General procedures

Melting points were determined on a Buchi apparatus and are uncorrected. Infrared spectra were recorded on a Thermo Nicolet Nexus 670 spectrometer in potassium bromide pellets and frequencies are expressed in cm^{-1}. ^1H NMR and ^{13}C NMR spectra were recorded, at room temperature, on a Bruker Avance 500, Bruker Avance 400, Bruker DRX-300 and Bruker DRX-200

spectrometers operating at 500, 400, 300 and 200 MHz (^{1}H)/125, 100, 75, 50 MHz (^{13}C), respectively. Chemical shifts are reported in ppm (δ) downfield from tetramethylsilane used as internal standard. Low resolution mass spectra (MS) were obtained by electron-spray ionization in an LC/MS micromass ZQ 4000. Microanalysis data were obtained using a Perkin–Elmer 240 analyzer, using a Perkin–Elmer AD-4 balance.

The progress of all reactions was monitored by TLC, which was performed on 2.0 × 6.0 cm aluminum sheets precoated with silica gel 60 (HF-254, Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under ultraviolet light (254–265 nm).

4.2. General procedure for the synthesis of (E)-cinnamic acids (**31e** and **31f**)

The corresponding benzaldehyde derivative **32a** or **32b** (33 mmol) and malonic acid (72 mmol) were dissolved in a mixture of 15 mL of pyridine and 0.25 mL of piperidine and heated under reflux until TLC analysis indicated the end of the reaction. The reaction medium was cooled, neutralized with HCl (10% aq.), filtered in Buchner funnel, washed with water and dried, providing the appropriate (*E*)-cinnamic acids **31e** and **31f** as described next.

4.2.1. (2E)-3-(3,4-Dimethoxy-5-nitrophenyl)acrylic acid (**31e**)

87% yield; orange solid, mp 235 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.88 (s, 3H, Ar(C$_3$)OC$\underline{H}_3$), 3.94 (s, 3H, Ar(C$_4$)OC$\underline{H}_3$), 6.61 (d, 1H, J = 16.0 Hz, CH=C$\underline{H}$–COOH), 7.45 (d, 1H, J = 16.0 Hz, C$\underline{H}$=CH–COOH), 7.57 (d, 1H, J = 2.0 Hz, Ar(C$_2$)$\underline{H}$) and 7.62 (d, 1H, J = 2.0 Hz, Ar(C$_6$)H) ppm; ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.83 (Ar(C$_3$)O$\underline{C}$H$_3$), 61.71 (Ar(C$_4$)O$\underline{C}$H$_3$), 115.37 (Ar($\underline{C}$$_2$)H), 115.56 (Ar($\underline{C}$$_6$)H), 121.21 (CH=$\underline{C}$H–COOH), 130.76 (Ar($\underline{C}$$_1$)), 141.57 ($\underline{C}$H=CH–COOH), 142.35 (Ar($\underline{C}$$_5$)NO$_2$), 144.74 (Ar($\underline{C}$$_4$)OCH$_3$), 153.60 (Ar($\underline{C}$$_3$)OCH$_3$) and 167.32 (CH=CH–$\underline{C}$OOH) ppm; IR (KBr) ν_{max} cm^{-1}: 3383–3049 (ν O–H), 1654 (ν C=O), 1608 (ν N=O), 1276 (ν C–O–C); MS (ESI) *m*/*z*: 253.1, 252.1 (100%). Anal. Calcd for $C_{11}H_{11}NO_6$: C: 52.18; H: 4.79; N: 5.53. Found: C: 51.99; H: 4.78; N: 5.54.

4.2.2. (2E)-3-(4-Hydroxy-3-methoxy-5-nitrophenyl)acrylic acid (**31f**)

90% yield; yellow solid, mp 249 °C (Lit. [29] mp 249 °C); ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.95 (s, 3H, Ar(C$_3$)OC$\underline{H}_3$), 6.61 (d, 1H, J = 16.0 Hz, C$\underline{H}$=CH–COOH), 7.56 (d, 1H, J = 16.0 Hz, CH=C$\underline{H}$–COOH), 7.64 (s, 1H, Ar(C$_2$)$\underline{H}$), 7.77 (s, 1H, Ar(C$_6$)$\underline{H}$) ppm; ^{13}C NMR (125 MHz, DMSO-d_6) δ 56.86 (Ar(C$_3$)O$\underline{C}$H$_3$), 113.88 (Ar($\underline{C}$$_2$)H), 117.19 (Ar($\underline{C}$$_6$)H), 119.07 (CH=$\underline{C}$H–COOH), 125.15 (Ar($\underline{C}$$_1$)), 137.33 (Ar($\underline{C}$$_5$)NO$_2$), 142.32 ($\underline{C}$H=CH–COOH), 143.83 (Ar($\underline{C}$$_4$)O–H), 149.69 (Ar($\underline{C}$$_3$)OCH$_3$), 167.58 (CH=CH–$\underline{C}$OOH) ppm; IR (KBr) ν_{max} cm^{-1}: 3050 and 3100 (ν O–H), 1664 (ν C=O); MS (ESI) *m*/*z*: 241.9 (100%). Anal. Calcd for $C_{10}H_9NO_6$: C: 50.22; H: 3.79; N: 5.86. Found: C: 50.29; H: 3.78; N: 5.84.

4.3. General procedure for the synthesis of cinnamic acid hydrazides (**33a**–**33f**)

To a suspension of the corresponding cinnamic acid (**31a**–**31f**) (6.67 mmol, 1.0 equiv) in 15 mL CH_3CN at room temperature, was added HOBt (8 mmol, 1.2 equiv) in a single portion, followed by EDC (8 mmol, 1.2 equiv). The reaction was stirred at room temperature for 2 h, the time required for the complete consumption of cinnamic acid. The resulting mixture was then slowly added to a solution of hydrazine hydrate (13.34 mmol, 2.0 equiv) in 15 mL of CH_3CN kept between 0 and 10 °C. The reaction was usually completed upon the end of addition. Then, 15 mL of water was added. The reaction medium was extracted with ethyl acetate (3 × 30 mL) and aqueous sodium bicarbonate (1 × 50 mL). The organic phases were joined and the solvent was evaporated under reduced pressure to furnish the respective *N*-acylhydrazines (**33a**–**33f**), as described next.

4.3.1. (2E)-3-Phenylacrylohydrazide (**33a**)

90% yield; beige solid, mp 116–117 °C (Lit. [30] mp 116–117 °C); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.48 (s, 2H, –CONH–N$\underline{H}_2$), 6.55 (d, J = 16.0 Hz, 1H, CH=C$\underline{H}$–COOH), 7.33–7.46 (m, 4H, C$\underline{H}$=CH–COOH, Ar(C$_3$)$\underline{H}$, Ar(C$_4$)$\underline{H}$ and Ar(C$_5$)$\underline{H}$), 7.53–7.55 (d, 2H, Ar(C$_2$)$\underline{H}$ and Ar(C$_6$)$\underline{H}$), 9.37 (s, 1H, –CON$\underline{H}$–NH$_2$) ppm: ^{13}C NMR (100 MHz, DMSO-d_6) δ 120.28 (CH=$\underline{C}$H–COOH), 127.43 (Ar($\underline{C}$$_2$)H and Ar($\underline{C}$$_6$)H), 128.91 (Ar($\underline{C}$$_3$)H and Ar($\underline{C}$$_5$)H), 129.38 (Ar($\underline{C}$$_4$)H), 134.92 (Ar($\underline{C}$$_1$)), 138.16 ($\underline{C}$H=CH–COOH) and 164.47 (CH=CH–$\underline{C}$OOH) ppm; IR (KBr) ν_{max} cm^{-1}: 3292 and 3026 (ν N–H); 1664 (ν C=O); MS (ESI) *m*/*z*: 185.2 ($M^{+\cdot}$[+Na]) (100%). Anal. Calcd for $C_9H_{10}N_2O$: C: 66.65; H: 6.21; N: 17.27. Found: C: 66.58; H: 6.20; N: 17.24.

4.3.2. (2E)-3-(1,3-Benzodioxol-5-yl)acrylohydrazide (**33b**)

92% yield; beige solid, mp 151–152 °C (Lit. [31] mp 151–152 °C); ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.05 (s, 2H, O–C$\underline{H}_2$–O), 6.39 (d, J = 16.0 Hz, 1H, CH=C$\underline{H}$–COOH), 6.93 (d, J = 8.0 Hz, 1H, Ar(C$_5$)$\underline{H}$), 7.12 (dd, J = 8.0 Hz, J = 1.4 Hz, 1H, Ar(C$_6$)$\underline{H}$), 7.33 (d, J = 1.4 Hz, 1H, Ar(C$_2$)$\underline{H}$), 7.45 (d, J = 16.0 Hz, 1H, C$\underline{H}$=CH–COOH) ppm: ^{13}C NMR (100 MHz, $CDCl_3$) δ 101.24 (O–$\underline{C}$H$_2$–O), 106.03 (Ar($\underline{C}$$_2$)H), 108.29 (Ar($\underline{C}$$_5$)H), 115.36 (Ar($\underline{C}$$_6$)H), 123.85 (CH=$\underline{C}$H–COOH), 128.63 (Ar($\underline{C}$$_1$)), 141.37 ($\underline{C}$H=CH–COOH), 148.00 (Ar($\underline{C}$$_4$)), 149.03 (Ar($\underline{C}$$_3$)) and 167.00 (CH=CH–$\underline{C}$OOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3050 and 3100 (ν N–H), 1664 (ν C=O); MS (ESI) *m*/*z*: 229.0 ($M^{+\cdot}$[+Na]) (100%). Anal. Calcd for $C_{10}H_{10}N_2O_3$: C: 58.25; H: 4.89; N: 13.59. Found: C: 58.14; H: 4.88; N: 13.54.

4.3.3. (2E)-3-(3,4,5-Trimethoxyphenyl)acrylohydrazide (**33c**)

85% yield; white solid, mp 153–154 °C (Lit. [30] mp 153–154 °C); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.67 (s, 3H, Ar(C$_4$)OC$\underline{H}_3$), 3.80 (s, 6H, Ar(C$_3$)OC$\underline{H}_3$ and Ar(C$_5$)OC$\underline{H}_3$), 4.41 (s, 2H, –CONH–N$\underline{H}_2$), 6.48 (d, J = 16.0 Hz, 1H, CH=C$\underline{H}$–COOH), 6.87 (s, 2H, Ar(C$_2$)$\underline{H}$ and Ar(C$_6$)$\underline{H}$), 7.38 (d, J = 16.0 Hz, 1H, C$\underline{H}$=CH–COOH), 9.22 (s, 1H, –CONH–NH$_2$) ppm: ^{13}C NMR (100 MHz, DMSO-d_6) δ 55.87 (Ar(C$_3$)O$\underline{C}$H$_3$ and Ar(C$_5$)O$\underline{C}$H$_3$), 60.07 (Ar(C$_4$)O$\underline{C}$H$_3$), 104.88 (Ar($\underline{C}$$_2$)H and Ar($\underline{C}$$_6$)H), 119.53 (CH=$\underline{C}$H–COOH), 130.53 (Ar($\underline{C}$$_1$)), 138.34 ($\underline{C}$H=CH–COOH), 138.60 (Ar($\underline{C}$$_4$)), 153.06 (Ar($\underline{C}$$_3$) and Ar($\underline{C}$$_5$)), 164.58 (CH=CH–$\underline{C}$OOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3317 and 3240 (ν N–H), 1579 (ν C=O), 1247 (ν C–O–C): MS (ESI) *m*/*z*: 251.2 (100%). Anal. Calcd for $C_{12}H_{16}N_2O_4$: C: 57.13; H: 6.39; N: 11.10. Found: C: 56.99; H: 6.40; N: 11.13.

4.3.4. (2E)-3-(3,4-Dimethoxyphenyl)acrylohydrazide (**33d**)

90% yield; white solid, mp 217–218 °C (Lit. [31] mp 217–217.5 °C); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.76 and 3.78 (s, 6H, Ar(C$_3$)OC$\underline{H}_3$ and Ar(C$_4$)OC$\underline{H}_3$), 6.42 (d, J = 16.0 Hz, 1H, CH=C$\underline{H}$–COOH), 6.96 (d, J = 8.00 Hz, 1H, Ar(C$_5$)$\underline{H}$), 7.10 (dd, J = 8.00, 1H, Ar(C$_6$)$\underline{H}$), 7.13 (d, 1H, Ar(C$_2$)$\underline{H}$), 7.37 (d, J = 8.0 Hz, 1H, C$\underline{H}$=CH–COOH), 9.23 (s, 1H, –CONH–NH$_2$) ppm: ^{13}C NMR (100 MHz, DMSO-d_6) δ 55.43 (Ar(C$_4$)O$\underline{C}$H$_3$), 55.55 (Ar(C$_3$)O$\underline{C}$H$_3$), 110.11 (Ar($\underline{C}$$_5$)H), 111.76 (Ar($\underline{C}$$_2$)H), 117.88 (Ar($\underline{C}$$_1$)), 121.17 (CH=$\underline{C}$H–COOH), 127.70 (Ar($\underline{C}$$_6$)H), 138.34 ($\underline{C}$H=CH–COOH), 148.88 (Ar($\underline{C}$$_3$)OCH$_3$), 150.06 (Ar($\underline{C}$$_4$)OCH$_3$), 164.92 (CH=CH–$\underline{C}$OOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3321 and 3236 (ν N–H), 1510 (ν C=O) and 1263 (ν C–O–C): MS (ESI) *m*/*z*: 223.2 (100%). Anal. Calcd for $C_{11}H_{14}N_2O_3$: C: 59.45; H: 6.35; N: 12.60. Found: C: 59.41; H: 6.36; N: 12.57.

4.3.5. (2E)-3-(3,4-Dimethoxy-5-nitrophenyl)acrylohydrazide (**33e**)

83% yield; orange solid, mp 164–165 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.87 (s, 3H, Ar(C$_3$)OC$\underline{H}_3$), 3.93 (s, 3H, Ar(C$_4$)OC$\underline{H}_3$), 6.60

(d, J = 16.0 Hz, 1H, CH=CH–COOH), 7.45 (d, J = 16.0 Hz, 1H, CH=CH–COOH), 7.57 (d, J = 2.0 Hz, 1H, Ar(C_2)H), 7.62 (d, J = 2.0 Hz, 1H, Ar(C_6)H), 9.34 (–CONH–NH_2) ppm: ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.83 (Ar(C_3)OCH_3), 61.71 (Ar(C_4)OCH_3), 115.37 (Ar(C_2)H), 115.56 (Ar(C_6)H), 121.21 (CH=CH–COOH), 130.76 (Ar(C_1)), 141.57 (CH=CH–COOH), 142.35 (Ar(C_5)NO_2), 144.74 (Ar(C_4)OCH_3), 153.60 (Ar(C_3)OCH_3) and 167.32 (CH=CH–COOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3383–3049 (ν N–H), 1654 (ν C=O), 1608 (ν N=O) and 1276 (ν C–O–C): MS (ESI) m/z: 267.2 (100%). Anal. Calcd for $C_{11}H_{13}N_3O_5$: C: 49.44; H: 4.90; N: 15.72. Found: C: 49.35; H: 4.89; N: 15.68.

4.3.6. (2E)-3-(4-Hydroxy-3-methoxy-5-nitrophenyl)acrylohydrazide (**33f**)

85% yield; yellow solid, mp 160–161 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.92 (s, 3H, Ar(C_3)OCH_3), 6.58 (d, 1H, J = 16.0 Hz, CH=CH–COOH), 7.54 (d, 1H, J = 16.0 Hz, CH=CH–COOH), 7.62 (s, 1H, Ar(C_2)H), 7.74 (s, 1H, Ar(C_6)H) ppm; ^{13}C NMR (125 MHz, DMSO-d_6) δ 56.86 (Ar(C_3)OCH_3), 113.88 (Ar(C_2)H), 117.19 (Ar(C_6)H), 119.07 (CH=CH–COOH), 125.15 (Ar(C_1)), 137.33 (Ar(C_5)NO_2), 142.32 (CH=CH–COOH), 143.83 (Ar(C_4)O–H), 149.69 (Ar(C_3)OCH_3), 167.58 (CH=CH–COOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3383–3049 (ν N–H), 1654 (ν C=O), 1608 (ν N=O) and 1276 (ν C–O–C): MS (ESI) m/z: 252.1 (100%). Anal. Calcd for $C_{10}H_{11}N_3O_5$: C: 47.43; H: 4.38; N: 16.59. Found: C: 47.28; H: 4.38; N: 16.66.

4.4. General procedure for the synthesis of 2-naphthoic and 3-phenylpropanoic acid hydrazides (**33g** and **33h**)

To a solution of the corresponding carboxylic acid derivative (**34**) or (**35**) (3 mmol) in 10 mL of ethanol was added a catalytic amount of concentrated H_2SO_4 and the mixture was refluxed for 2 h, when analysis by TLC indicated the end of the reaction. Then, 3 mL of 80% hydrazine monohydrate was added. The reaction mixture was maintained under reflux for 3 h, when TLC indicated the total consumption of the ester intermediate. The media was poured onto ice and the resulting precipitate was filtered out, affording the corresponding acylhydrazide derivatives (**33g**) and (**33h**) as described next.

4.4.1. 3-Phenylpropanohydrazide (**33g**)

90% yield; beige solid, mp 160–161 °C (Lit. [32] mp 160–161 °C); ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.30 (t, J = 8.0 Hz, 2H, CH_2–CH_2–COOH), 2.79 (t, J = 8.0 Hz, 2H, CH_2–CH_2–COOH), 4.19 (s, 2H, –CONH–NH_2), 7.21 (m, 5H, Ar(C_2)H, Ar(C_3)H, Ar(C_4)H, Ar(C_5)H and Ar(C_6)H), 8.95 (s, 1H, –CONH–NH_2): ^{13}C NMR (100 MHz, DMSO-d_6) δ 30.36 (CH_2–CH_2–COOH), 35.24 (CH_2–CH_2–COOH), 125.96 (Ar(C_4)H), 128.18 (Ar(C_3)H and Ar(C_5)H), 128.27 (Ar(C_2)H and Ar(C_6)H), 140.90 (Ar(C_1)) and 173.76 (CH_2–CH_2–COOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3311 and 3180 (ν N–H), 1618 (ν C=O): MS (ESI) m/z: 187.1 (M^+[+Na]) (100%). Anal. Calcd for $C_9H_{12}N_2O$: C: 65.83; H: 7.37; N: 17.06. Found: C: 65.77; H: 7.35; N: 16.98.

4.4.2. 2-Naphthohydrazide (**33h**)

85% yield; white solid, mp 153 °C (Lit. [33] mp 152 °C); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.56 (s, 2H, –CONH–NH_2), 7.58 (m, 2H, Ar(C_5)H and Ar(C_7)H), 7.95 (m, 4H, Ar(C_6)H, Ar(C_8)H, Ar(C_3)H and Ar(C_4)H), 8.42 (s, 1H, Ar(C_1)H), 9.91 (s, 1H, –CONH–NH_2): ^{13}C NMR (100 MHz, DMSO-d_6) δ 123.82 (Ar(C_3)H), 126.64 (Ar(C_7)H), 127.24 (Ar(C_5)H), 127.47 (Ar(C_2)CONH–NH_2), 127.56 (Ar(C_4)H), 127.83 (Ar(C_6)H), 128.78 (Ar(C_8)H), 130.63 (Ar(C_1)H), 132.14 (Ar(C_9)), 134.03 (Ar(C_{10})) and 165.88 (Ar(C_2)CONH–NH_2) ppm: IR (KBr) ν_{max} cm^{-1}: 3311 and 3180 (ν N–H), 1618 (ν C=O): MS (ESI) m/z: 209.0 (M^+[+Na]) (100%). Anal. Calcd for $C_{11}H_{10}N_2O$: C: 70.95; H: 5.41; N: 15.04. Found: C: 70.74; H: 5.40; N: 15.00.

4.5. General procedure for the synthesis of NAH derivatives (**3**–**30**)

To a solution of the corresponding hydrazide derivative (**33a**–**33h**) (0.829 mmol) in absolute ethanol (90 mL) containing a catalytic amount of 37% aq. hydrochloric acid was added 0.87 mmol of desired aromatic aldehyde derivative. The mixture was stirred at reflux for 60 min, when extensive precipitation was observed. The reaction mixture was then poured into cold water, neutralized with 10% aqueous sodium bicarbonate solution. The precipitate formed was filtered out and dried, furnishing the target compounds in the yields as described next.

4.5.1. (E)-N′-Benzylidenecinnamohydrazide (**3**)

93% yield; white solid, mp 229 °C (Lit. [34] mp 225.5–227.5); ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.71 (d, J = 16.0 Hz, 1H, CH=CH–COOH), 7.40–7.47 (m, 6H, Ar(C_3)H, Ar(C_4)H, Ar(C_5)H, Ar($C_{3'}$)H, Ar($C_{4'}$)H and Ar($C_{5'}$)H), 7.57–7.66 (m, 2H, Ar($C_{2'}$)H and Ar($C_{6'}$)H), 7.70–7.76 (m, 3H, CH=CH–COOH, Ar(C_2)H and Ar(C_6)H), 8.06 and 8.24 (s, 1H, –N=CH–), 11.52 and 11.68 (s, 1H, –CONH–): ^{13}C NMR (100 MHz, DMSO-d_6) δ 117.08 and 120.21 (CH=CH–COOH), 126.89 and 127.09 (Ar($C_{3'}$)H) and (Ar($C_{5'}$)H), 127.73 and 128.20 (Ar($C_{2'}$)H and Ar($C_{6'}$)H), 128.79, 128.95 and 129.00 (Ar(C_2)H, Ar(C_3)H, Ar(C_5)H and Ar(C_6)H), 129.78 and 129.86 (Ar($C_{4'}$)H or Ar(C_4)H), 130.02 (Ar(C_4)H or Ar($C_{4'}$)H), 134.15 and 134.26 (Ar($C_{1'}$)), 134.63 and 134.77 (Ar(C_1)), 140.56 and 142.11 (CH=CH–COOH), 143.17 and 146.69 (–N=CH–), 161.41 and 165.99 (CH=CH–COOH) ppm: IR (KBr) ν_{max} cm^{-1}: 2989 and 2893 (ν N–H), 1647 (ν C=O), 1570 (ν C=C): MS (ESI) m/z: 273.1 (M^+[+Na]) (100%). Anal. Calcd for $C_{16}H_{14}N_2O$: C: 76.78; H: 5.64; N: 11.19. Found: C: 76.65; H: 5.63; N: 11.21.

4.5.2. (E)-N′-(4-Fluorobenzylidene)cinnamohydrazide (**4**)

83% yield; white solid, mp 190 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{16}H_{13}N_2FO$: C: 71.63; H: 4.88; N: 10.44. Found: C: 71.76; H: 4.89; N: 10.41.

4.5.3. (E)-N′-(4-Chlorobenzylidene)cinnamohydrazide (**5**)

81% yield; white solid, mp 211 °C (Lit. [35] mp 211.3 °C); Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{16}H_{13}ClN_2O$: C: 67.49; H: 4.60; N: 12.45. Found: C: 67.64; H: 4.61; N: 12.50.

4.5.4. (E)-N′-(4-Bromobenzylidene)cinnamohydrazide (**6**)

80% yield; white solid, mp 220 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{16}H_{13}BrN_2O$: C: 58.38; H: 3.98; N: 8.51. Found: C: 58.22; H: 3.97; N: 8.50.

4.5.5. (E)-N′-(4-Nitrobenzylidene)cinnamohydrazide (**7**)

94% yield; yellow solid, mp 233 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{16}H_{13}N_3O_3$: C: 65.08; H: 4.44; N: 14.23. Found: C: 65.23; H: 4.43; N: 14.19.

4.5.6. (E)-N′-(4-Methoxybenzylidene)cinnamohydrazide (**8**)

76% yield; beige solid, mp 219 °C (Lit. [35] mp 218–219 °C); Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{17}H_{16}N_2O_2$: C: 72.84; H: 5.75; N: 9.99. Found: C: 72.99; H: 5.76; N: 9.95.

4.5.7. (E)-N′-(4-Hydroxybenzylidene)cinnamohydrazide (**9**)

80% yield; yellow solid, mp 290 °C (Lit. [35] mp 290–291 °C); Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{16}H_{14}N_2O_2$: C: 72.16; H: 5.30; N: 10.52. Found: C: 72.08; H: 5.31; N: 10.49.

4.5.8. (E)-N′-(2-Hydroxybenzylidene)cinnamohydrazide (**10**)

85% yield; yellow solid, mp 240 °C (Lit. [35] mp 240 °C); ^{1}H NMR (500 MHz, DMSO-d_6) δ 6.71 (d, J = 16.0 Hz, 1H, CH=CH–COOH),

6.88–6.95 (m, 2H, Ar($C_{3'}$)H and Ar($C_{5'}$)H), 7.31 (m, 1H, Ar($C_{4'}$)H), 7.41–7.48 (m, 3H, Ar(C_3)H, Ar(C_4)H and Ar(C_5)H), 7.55–7.58 (m, 1H, Ar($C_{6'}$)H), 7.65–7.70 (m, 3H, CH=CH–COOH, Ar(C_2)H and Ar(C_6)H), 8.39 and 8.46 (s, 1H, –N=CH–), 10.10 and 11.18 (s, 1H, Ar($C_{2'}$)O–H), 11.50 and 11.95 (s, 1H, –CONH–): ^{13}C NMR (125 MHz, DMSO-d_6) δ 116.10 and 116.39 (Ar($C_{3'}$)H), 117.22 and 118.77 (CH=CH–COOH), 119.37, 119.49, 119.70 and 120.30 (Ar($C_{1'}$) and Ar($C_{5'}$)H), 127.86 and 128.17 (Ar(C_2)H and Ar(C_6)H), 129.01 and 129.07 (Ar(C_3)H and Ar(C_5)H), 129.33 (Ar(C_4)H or Ar($C_{4'}$)H), 130.03 (Ar(C_4)H or Ar($C_{4'}$)H), 131.06 and 131.42 (Ar($C_{6'}$)H), 134.56 and 134.83 (Ar(C_1)), 140.56 and 140.96 (CH=CH–COOH), 142.00 and 146.99 (–N=CH–), 156.38 and 157.36 (Ar($C_{2'}$)), 161.23 and 165.80 (CH=CH–COOH). IR (KBr) ν_{max} cm^{-1}: 3251 (ν O–H), 3059 (ν N–H), 1651 (ν C=O), 1546 (ν C=C), 1268 (ν C–O): MS (ESI) *m/z*: 289.2 ($M^{+\cdot}$[+Na]) (100%). Anal. Calcd for $C_{16}H_{14}N_2O_2$: C: 72.16; H: 5.30; N: 10.52. Found: C: 72.01; H: 5.31; N: 10.49.

4.5.9. (E)-N′-(3,4-Dihydroxybenzylidene)cinnamohydrazide (**11**)

80% yield; yellow solid, mp 229 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{16}H_{14}N_2O_3$: C: 68.07; H: 5.00; N: 9.92. Found: C: 68.28; H: 4.99; N: 9.89.

4.5.10. (E)-N′-(3,4-Methylenedioxybenzylidene)cinnamohydrazide (**12**)

90% yield; yellow solid, mp 180 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{17}H_{14}N_2O_3$: C: 69.38; H: 4.79; N: 9.52. Found: C: 69.50; H: 4.78; N: 9.54.

4.5.11. (E)-N′-(3,4-Dimethoxybenzylidene)cinnamohydrazide (**13**)

89% yield; yellow solid, mp 187 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{18}H_{18}N_2O_3$: C: 69.66; H: 5.85; N: 9.03. Found: C: 69.43; H: 5.86; N: 9.07.

4.5.12. (E)-N′-(3-Hydroxy-4-methoxybenzylidene)cinnamohydrazide (**14**)

87% yield; yellow solid, mp 193 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{17}H_{16}N_2O_3$: C: 68.91; H: 5.44; N: 9.45. Found: C: 69.13; H: 5.45; N: 9.42.

4.5.13. (E)-N′-(4-Hydroxy-3-methoxybenzylidene) cinnamohydrazide (**15**)

88% yield; yellow solid, mp 219 °C (Lit. [35] mp 218–219 °C); Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{17}H_{16}N_2O_3$: C: 68.91; H: 5.44; N: 9.45. Found: C: 69.11; H: 5.43; N: 9.47.

4.5.14. (E)-N′-(3,4,5-Trimethoxybenzylidene)cinnamohydrazide (**16**)

91% yield; white solid, mp 185 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{19}H_{20}N_2O_4$: C: 67.05; H: 5.92; N: 8.23. Found: C: 66.93; H: 5.91; N: 8.19.

4.5.15. (E)-N′-(4-Hydroxy-3-methoxy-5-nitrobenzylidene) cinnamohydrazide (**17**)

90% yield; yellow solid, mp 240 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{17}H_{15}N_3O_5$: C: 59.82; H: 4.43; N: 12.31. Found: C: 60.02; H: 4.44; N: 12.27.

4.5.16. (2E)-3-(1,3-Benzodioxol-5-yl)-N′-[(1E)-2-hydroxybenzylidene] acrylohydrazide (**18**)

95% yield; beige solid, mp 221 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.08 (s, 2H, O–CH_2–O), 6.51 (d, J = 16.0 Hz, 1H, CH=CH–COOH), 6.88–6.92 (m, 2H, Ar($C_{3'}$)H and Ar($C_{5'}$)H), 6.97 (d, J = 8.0 Hz, 1H, Ar(C_5)H), 7.15 (d, J = 8.0 Hz, 1H, Ar(C_6)H), 7.22 (s, 1H, Ar(C_2)H), 7.28 (t, 1H, Ar($C_{4'}$)H), 7.52–7.59 (m, 2H, CH=CH–COOH and Ar($C_{6'}$)H), 8.34 and 8.42 (s, 1H, –N=CH–), 11.20, 11.36 and 11.80 (s, 1H, –CONH– and s, 1H, Ar($C_{2'}$)O–H): ^{13}C NMR (100 MHz, DMSO-d_6) δ 101.56 (O–CH_2–O), 106.42 and 106.82 (Ar(C_2)H), 108.57 and 108.66 (Ar(C_5)H), 115.22 and 117.61 (CH=CH–COOH), 116.05 and 116.36 (Ar($C_{3'}$)H), 118.75 and 120.31 (Ar($C_{1'}$)), 119.33 and 119.46 (Ar($C_{5'}$)H), 123.91 and 124.27 (Ar(C_6)H), 126.50 and 128.97 (Ar(C_1)), 129.30 and 129.37 (Ar($C_{6'}$)H), 130.95 and 131.31 (Ar($C_{4'}$)H), 140.83 and 141.91 (CH=CH–COOH), 146.82 and 148.03 (Ar(C_4)), 146.85 and 148.95 (Ar(C_3)), 156.31 and 157.34 (–N=CH–), 161.47 (Ar($C_{2'}$)), 166.02 (CH=CH–COOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3045 (ν O–H), 2900 (ν N–H), 1675 (ν C=O), 1500 (ν C=C), 1261 (ν C–O): MS (ESI) *m/z*: 309.1 (100%). Anal. Calcd for $C_{17}H_{14}N_2O_4$: C: 65.80; H: 4.55; N: 9.03. Found: C: 65.64; H: 4.56; N: 9.06.

4.5.17. (2E)-3-(1,3-Benzodioxol-5-yl)-N′-[(1E)-4-hydroxy-3-methoxy-5-nitrobenzylidene] acrylohydrazide (**19**)

83% yield; yellow solid, mp 255 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{18}H_{15}N_3O_7$: C: 56.11; H: 3.92; N: 10.91. Found: C: 55.98; H: 3.93; N: 10.87.

4.5.18. (2E)-N′-[(1E)-2-Hydroxybenzylidene]-3-(3,4,5-trimethoxyphenyl) acrylohydrazide (**20**)

82% yield; white solid, mp 222 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{19}H_{20}N_2O_5$: C: 64.04; H: 5.66; N: 7.86. Found: C: 64.19; H: 5.67; N: 7.89.

4.5.19. (2E)-N′-[(1E)-4-Hydroxy-3-methoxy-5-nitrobenzylidene]-3-(3,4,5-trimethoxyphenyl) acrylohydrazide (**21**)

83% yield; orange solid, mp 228 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.70 (s, 3H, Ar(C_4)OCH_3), 3.84 and 3.85 (s, 6H, Ar(C_3)OCH_3 and Ar(C_5)OCH_3), 3.96 and 3.99 (s, 3H, Ar($C_{3'}$)OCH_3), 6.66 (d, J = 16.0 Hz, 1H, CH=CH–COOH), 6.98 and 7.11 (s, 2H, Ar(C_2)H and Ar(C_6)H), 7.56–7.64 (m, 2H, CH=CH–COOH and Ar($C_{2'}$)H), 7.75–7.81 (s, 1H, Ar($C_{6'}$)H), 8.01 and 8.18 (s, 1H, –CONH–), 10.88 (s, 1H, Ar($C_{4'}$)O–H), 11.57 and 11.73 (s, 1H, –CONH–): ^{13}C NMR (100 MHz, DMSO-d_6) δ 55.82 and 55.92 (Ar(C_3)OCH_3 and Ar(C_5)OCH_3), 56.31 and 56.69 (Ar(C_4)OCH_3), 60.13 (Ar($C_{3'}$)OCH_3), 105.23 and 105.62 (Ar(C_2)H and Ar(C_6)H), 111.90 and 112.22 (Ar($C_{2'}$)H), 115.91 and 116.07 (Ar($C_{6'}$)H), 116.70 and 119.41 (CH=CH–COOH), 125.11 (Ar($C_{1'}$)), 130.23 and 130.42 (Ar(C_1)), 137.12 (Ar($C_{5'}$)), 139.04 (Ar(C_4)), 140.87 (CH=CH–COOH), 142.16 and 144.82 (–N=CH–), 143.84 and 144.10 (Ar($C_{4'}$)), 149.81 (Ar($C_{3'}$)), 153.11 (Ar(C_3) and Ar(C_5)), 161.54 and 166.16 (CH=CH–COOH) ppm: IR (KBr) ν_{max} cm^{-1}: 3333 (ν O–H), 2966 (ν N–H), 1695 (ν C=O), 1535 (ν C=C), 1260 (ν C–O): MS (ESI) *m/z*: 430.2 (100%). Anal. Calcd for $C_{20}H_{21}N_3O_8$: C: 55.68; H: 4.91; N: 9.74. Found: C: 55.82; H: 4.90; N: 9.70.

4.5.20. (2E)-3-(3,4-Dimethoxy-5-nitrophenyl)-N′-[(1E)-(2-hydroxybenzylidene)]acrylohydrazide (**22**)

87% yield; yellow solid, mp 206 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.88 (s, 3H, Ar(C_4)OCH_3), 3.95 and 3.98 (s, 3H, Ar(C_3)OCH_3), 6.74 (d, J = 16.0 Hz, 1H, CH=CH–COOH), 7.84–7.91 (m, 2H, Ar($C_{3'}$)H and Ar($C_{5'}$)H), 7.21–7.29 (t, 1H, Ar($C_{4'}$)H), 7.53–7.70 (m, 4H, CH=CH–COOH, Ar(C_2)H, Ar(C_6)H and Ar($C_{6'}$)H); 8.38 and 8.43 (s, 1H, –N=CH–), 10.07 and 11.14 (s, H, Ar($C_{2'}$)O–H), 11.51 and 11.96 (s, 1H, –CONH–); ^{13}C NMR (125 MHz, DMSO-d_6) δ 56.66 (Ar(C_3)OCH_3), 61.71 (Ar(C_4)OCH_3), 114.56 (Ar(C_6)H), 115.67 and 116.08 (Ar(C_2)H), 116.27 and 116.37 (Ar($C_{3'}$)H), 118.70 and 120.23 (Ar($C_{1'}$)), 119.35 and 119.43 (Ar($C_{5'}$)H), 121.66 (CH=CH–COOH), 126.53 and 129.37 (Ar($C_{6'}$)H), 131.01 and 131.09 (Ar(C_1)), 131.40 and 131.46 (Ar($C_{4'}$)H), 138.61 and 139.82 (CH=CH–COOH), 140.92 and 147.35 (–N=CH–), 142.01 and 142.29 (Ar(C_5)), 144.68 and 144.93 (Ar(C_4)), 153.53 and 153.66 (Ar(C_3)), 156.40 and 157.39 (Ar($C_{2'}$)), 160.86 and 165.55 (CH=CH–COOH); ^{13}C NMR DEPT (100 MHz, DMSO-d_6) δ 56.66 (Ar(C_3)OCH_3), 61.71 (Ar(C_4)OCH_3), 114.56 (Ar(C_6)H), 115.67 and 116.08 (Ar(C_2)H), 116.27 and 116.37 (Ar($C_{3'}$)H), 119.35 and 119.43 (Ar($C_{5'}$)H), 121.66 (CH=CH–COOH), 126.53 and 129.37 (Ar($C_{6'}$)H), 131.40 and 131.46 (Ar($C_{4'}$)H), 138.61 and 139.82 (Ar(C_3)),

140.92 and 147.35 (−N=CH−) ppm; IR (KBr) ν_{max} cm^{-1}: 3284 (ν O−H), 1676 (ν C=O), 1537 (ν C=C), 1355 (ν (N=O)$_2$), 1274 (ν C−O): MS (ESI) *m/z*: 370.3 (100%). Anal. Calcd for $C_{18}H_{17}N_3O_8$: C: 58.22; H: 4.61; N: 11.32. Found: C: C: 58.41; H: 4.60; N: 11.34.

4.5.21. (2E)-3-(3,4-Dimethoxy-5-nitrophenyl)-N′-[(1E)-(4-hydroxy-3-methoxy-5-nitro benzylidene)]acrylohydrazide (**23**)

87% yield; yellow solid, mp 253 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.89 (s, 3H, Ar(C$_4$)OCH$_3$), 3.95 and 3.98 (s, 6H, Ar(C$_3$)OCH$_3$ and Ar(C$_{3'}$)OCH$_3$), 6.77 (d, 1H, J = 16.0 Hz, CH=CH−COOH), 7.59–7.83 (m, 5H, CH=CH−COOH, Ar(C$_2$)H, Ar(C$_6$)H, Ar(C$_{2'}$)H and Ar(C$_{6'}$)H), 8.00 and 8.18 (s, 1H, −N=CH−); 11.66 and 11.78 (s, 1H, −CONH−); ^{13}C NMR (125 MHz, DMSO-d_6) δ 56.45 and 56.60 (Ar(C$_{3'}$)OCH$_3$), 56.70 (Ar(C$_3$)OCH$_3$), 61.77 (Ar(C$_4$)OCH$_3$), 112.07 and 112.19 (Ar(C$_{2'}$)H), 114.43 and 115.77 (Ar(C$_{6'}$)H), 115.40 and 115.57 (Ar(C$_2$)H), 116.02 and 116.26 (Ar(C$_6$)H), 119.46 and 122.14 (CH=CH−COOH), 124.87 and 124.97 (Ar(C$_{1'}$)), 131.12 and 131.31 (Ar(C$_1$)), 137.13 and 137.19 (Ar(C$_{5'}$)), 138.37 and 139.62 (CH=CH−COOH), 141.41 and 145.41 (−N=CH−), 142.20 (Ar(C$_5$)), 143.91 and 144.33 (Ar(C$_{4'}$)), 144.72 (Ar(C$_4$)), 149.81 and 149.88 (Ar(C$_{3'}$)), 153.60 and 153.67 (Ar(C$_3$)), 161.06 and 165.77 (CH=CH−COOH); ^{13}C NMR DEPT (125 MHz, DMSO-d_6) δ 56.45 and 56.60 (Ar(C$_{3'}$)OCH$_3$), 56.70 (Ar(C$_3$)OCH$_3$), 61.77 (Ar(C$_4$)OCH$_3$), 112.07 and 112.19 (Ar(C$_{2'}$)H), 114.43 and 115.77 (C Ar(C$_{6'}$)H), 115.40 and 115.57 (Ar(C$_2$)H), 116.02 and 116.26 (Ar(C$_6$)H), 119.46 and 122.14 (CH=CH−COOH), 138.37 and 139.62 (CH=CH−COOH), 141.41 and 145.41 (−N=CH−).ppm: IR (KBr) ν_{max} cm^{-1}: 3284 (ν O−H), 3012 (ν N−H), 1666 (ν C=O), ~1550 (ν C=C), ~1320 (ν (N=O)2), 1274 (ν C−O): MS (ESI) *m/z*: 445.3 (100%). Anal. Calcd for $C_{19}H_{18}N_4O_9$: C: 51.12; H: 4.06; N: 12.55. Found: C: 51.25; H: 4.05; N: 12.50.

4.5.22. (2E)-3-(3,4-Dimethoxyphenyl)-N′-[(1E)-(2-hydroxybenzylidene)] acrylohydrazide (**24**)

90% yield; beige solid, mp 193 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{18}H_{18}N_2O_4$: C: 66.25; H: 5.56; N: 8.58. Found: C: 66.19; H: 5.55; N: 8.60.

4.5.23. (2E)-3-(3,4-Dimethoxyphenyl)-N′-[(1E)-(4-hydroxy-3-methoxy-5-nitrobenzylidene)] acrylohydrazide (**25**)

85% yield; yellow solid, mp 265 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{19}H_{19}N_3O_7$: C: 56.86; H: 4.77; N: 10.47. Found: C: 57.04; H: 4.76; N: 10.44.

4.5.24. (2E)-3-(4-Hydroxy-3-methoxy-5-nitrophenyl)-N′-[(1E)-benzylidene]acrylohydrazide (**26**)

81% yield; yellow solid, mp 216 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{17}H_{15}N_3O_5$: C: 59.82; H: 4.43; N: 12.31. Found: C: 59.67; H: 4.44; N: 12.35.

4.5.25. N′-[(1E)-2-Hydroxybenzylidene]-3-phenylpropanohydrazide (**27**)

85% yield; white solid, mp 151 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.53 (m, 2H, CH$_2$−CH$_2$−COOH), 2.90 (m, 2H, CH=CH−COOH), 6.82–6.92 (m, 2H, Ar(C$_{3'}$)H and Ar(C$_{5'}$)H), 7.19 (m, 1H, Ar(C$_{4'}$)H), 7.24–7.31 (m, 5H, Ar(C$_2$)H, Ar(C$_3$)H, Ar(C$_4$)H, Ar(C$_5$)H and Ar(C$_6$)H), 7.49 (d, J = 8.0 Hz, 1H, Ar(C$_{6'}$)H), 8.25 and 8.32 (s, 1H, −N=CH−), 10.10 and 11.14 (s, 1H, Ar(C$_4$)O−H), 11.24 and 11.59 (s, 1H, −CONH−). ^{13}C NMR (100 MHz, DMSO-d_6) δ 30.86 and 31.41 (CH$_2$−CH$_2$−COOH), 34.72 and 36.26 (CH$_2$−CH$_2$−COOH), 117.12 and 117.29 (Ar(C$_{3'}$)H), 119.11 and 120.13 (Ar(C$_{1'}$)), 120.69 and 120.82 (Ar(C$_{5'}$)H), 127.09 and 127.16 (Ar(C$_{6'}$)H), 128.86 and 129.18 (Ar(C$_2$)H and Ar(C$_6$)H), 129.40 (Ar(C$_3$)H and Ar(C$_5$)H), 130.67 (Ar(C$_4$)H), 132.46 and 132.73 (Ar(C$_{4'}$)H), 141.43 and 141.79 (Ar(C$_1$)), 144.17 and 148.55 (−N=CH−), 157.20 and 157.93 (Ar(C$_{2'}$)), 169.84 and 174.95 (CH=CH−COOH) ppm; RMN-^{13}C DEPT (100 MHz, DMSO-d_6): 30.86 and 31.41 (CH$_2$−CH$_2$−COOH), 34.72 and 36.26 (CH$_2$−CH$_2$−COOH), 117.12 and 117.29 (Ar(C$_{3'}$)H), 120.69 and 120.82 (Ar(C$_{5'}$)H), 127.09 and 127.16 (Ar(C$_{6'}$)H), 128.86 and 129.18 (Ar(C$_2$)H and Ar(C$_6$)H), 129.40 (Ar(C$_3$)H and Ar(C$_5$)H), 130.67 (Ar(C$_4$)H), 132.46 and 132.73 (Ar(C$_{4'}$)H), 144.17 and 148.55 (−N=CH−), 157.20 and 157.93 (Ar(C$_{2'}$)) ppm: IR (KBr) ν_{max} cm^{-1}: 3275 (ν O−H), 3082 (ν N−H), ~1670 (ν C=O), ~1530 (ν C=C): MS (ESI) *m/z*: 291.1 (M^{+}[+Na]) (100%). Anal. Calcd for $C_{16}H_{16}N_2O_2$: C: 71.62; H: 6.01; N: 10.44. Found: C: 71.55; H: 5.99; N: 10.48.

4.5.26. N′-[(1E)-4-Hydroxy-3-methoxy-5-nitrobenzylidene]-3-phenylpropanohydrazide (**28**)

88% yield; orange solid, mp 197 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{17}H_{17}N_3O_5$: C: 59.47; H: 4.99; N: 12.24. Found: C: 59.60; H: 4.98; N: 12.21.

4.5.27. N′-[(1E)-2-Hydroxybenzylidene]-2-naphthohydrazide (**29**)

89% yield; beige solid, mp 209 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{18}H_{14}N_2O_2$: C: 74.47; H: 4.86; N: 9.65. Found: C: 74.61; H: 4.85; N: 9.62.

4.5.28. N′-[(1E)-4-Hydroxy-3-methoxy-5-nitrobenzylidene]-2-naphthohydrazide (**30**)

88% yield; yellow solid, mp 197 °C; Spectroscopic data are described in Supplementary Material. Anal. Calcd for $C_{19}H_{15}N_3O_5$: C: 62.46; H: 4.14; N: 11.50. Found: C: 62.33; H: 4.13; N: 11.52.

4.6. Activity against intracellular amastigote forms [36]

Rat skeletal muscle myoblasts (L6 cells) were seeded in 96-well microtiter plates at 2000 (cells/well)/100 μL RPMI 1640 medium with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. After 24 h, 5000 trypomastigotes of *T. cruzi* (Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene) was added. After 48 h, the medium was removed from the wells and replaced by 100 μL of fresh medium with or without a serial drug dilution. Seven 3-fold dilutions were used covering a range from 0.123 to 90 μg/mL. The plates were incubated at 37 °C in 5% CO_2 for 4 days. Then the substrate CPRG/Nonidet (50 mL) was added to all wells. The color reaction that developed during the following 2–6 h was read photometrically at 540 nm. From the sigmoidal inhibition curve, IC_{50} values were calculated. Bzn was used as the standard drug.

4.7. Activity against bloodstream trypomastigote forms [37]

Stock solutions of NAH derivatives were prepared in DMSO. Bloodstream trypomastigote forms of *T. cruzi* (Y strain) were isolated from infected Swiss mice and re-suspended with Dulbecco's modified Eagle medium plus 10% fetal calf serum (DMES) to a parasite concentration of 10 × 10^6 cells/mL. This suspension (100 μL) was added to the same volume of each NAH derivative, previously prepared at twice the desired concentrations in DMES. Stock solutions of the NAH derivatives were prepared in dimethylsulfoxide (DMSO) and they were assayed in the range of 0.5–2000 μg/mL[1], with the final concentration of DMSO never exceeding 0.5%, which has no deleterious effect on the parasites [38].

4.8. Cytotoxic effect on murine macrophages

Cellular viability in the presence and absence of the NAH derivatives (**3**–**30**) was determined by Mosmans assay using MTT (3-(4,5-dimethylthiazol-2yl)-2,5-dimethyl tetrazolium bromide; Merck) as previously described [39]. The macrophage cell line J774 was plated in flat-bottomed 96-well plates (2.5 × 10^6 cells/mL) for 1 h in controlled atmosphere (5% CO_2, 37 °C), and non-adherent cells were washed by gentle flushing with RPMI 1640. Adherent cells were

cultured in the presence of medium alone, 3% Tween 20 and the compounds (0.1–100 μg/mL) in a triplicate assay. After 18 h, 20 μL of 5 mg/mL MTT solution was added to each well and after 4 h later the supernatant was discharged and dimethylsulfoxide (DMSO) (100 μL/well) was added for solubilization of the formazan crystals and the absorbance was read at 540 nm in a plate reader (Bio-Rad-450).

4.9. Evaluation of cruzain inhibitory profile of NAH derivatives

Cruzain truncated in the C-terminal extension was obtained from *Escherichia coli* (strain M15 or DH5α containing the expression plasmid) [40]. The substrate Z–Phe–Arg–AMC and all reagents for buffer preparation were purchased from Sigma–Aldrich. Stock solutions of substrate and inhibitors candidates at 10 mM in neat DMSO were stored at −20 °C and at −4 °C, respectively.

The highly purified enzyme (0.64 nM), in 50 mM sodium phosphate, 100 mM sodium chloride, 5 mM EDTA, pH 6.5, containing 5 mM DTT, was incubated with the *N*-acylhydrazone derivatives for 5 min at room temperature followed by the addition of the fluorogenic substrate *Z*–Phe–Arg–AMC [14,19]. Fluorescence was monitored on a Wallac 1420-042 PerkinElmer spectrofluorometer and measurements were done using 355 nm as the excitation wavelength and 460 nm as the emission wavelength. Cruzain activity was measured as an increase of fluorescence intensity by cleavage of the substrate Z–Phe–Arg–AMC and release of aminocoumarin.

The IC_{50} values were calculated using nonlinear regression analysis employing the Sigma-Plot enzyme kinetics module and it was based on percentage of enzyme activity in the presence of inhibitor, relatively to the activity in the absence of inhibitor. At least seven inhibitor concentrations with inhibition range between 20 and 85% were used.

Acknowledgments

This work was supported by grants from CNPq (BR.), FAPERJ (BR.), and FIOCRUZ (BR.). The *in vitro* screening of trypanocidal activity of the amastigotes of *T. cruzi* was supported by DND*i* (SW.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.05.041.

References

[1] WHO, First WHO Report on Neglected Tropical Diseases: Working to Overcome the Global Impact of Neglected Tropical Diseases. Geneva, Switzerland, World Health Organization, Geneva, 2010 (WHO/HTM/NTD/2010.1).
[2] A. Rassi Jr., A. Rassi, J.A. Marin-Neto, Chagas disease, Lancet 375 (2010) 1388–1402.
[3] G.A. Schmunis, Epidemiology of Chagas disease in non-endemic countries: the role of international migration, Mem. Inst. Oswaldo Cruz 102 (2007) 75–85.
[4] M.N.C. Soeiro, S.L. deCastro, Screening of potential anti-*Trypanosoma cruzi* candidates: *in vitro* and *in vivo* studies, Open Med. Chem. J. 5 (2011) 21–30.
[5] J.J. Cazzulo, V. Stoka, V. Turk, The major cysteine proteinase of *Trypanosoma cruzi*: a valid target for chemotherapy of Chagas disease, Curr. Pharm. Des. 7 (2001) 1143–1156.
[6] J.C. Engel, I. Hsieh, J.H. McKerrow, Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection, J. Exp. Med. 188 (1998) 725–734.
[7] S.C. Barr, K.L. Warner, B.G. Kornreic, J. Piscitelli, A. Wolfe, L. Benet, J.H. McKerrow, A cysteine protease inhibitor protects dogs from cardiac damage during infection by *Trypanosoma cruzi*, Antimicrob. Agents Chemother. 49 (2005) 5160–5161.
[8] M. Sajid, S.A. Robertson, L.S. Brinen, J.H. McKerrow, Cruzain: the path from target validation to the clinic, Adv. Exp. Med. Biol. 712 (2011) 100–115.
[9] C.D. Duarte, E.J. Barreiro, C.A.M. Fraga, Privileged structures: a useful concept for the rational design of new lead drug candidates, Mini Rev. Med. Chem. 7 (2007) 1108–1119.
[10] C.A.M. Fraga, E.J. Barreiro, Medicinal chemistry of *N*-acylhydrazones: new lead-compounds of analgesic, antiinflammatory and antithrombotic drugs, Curr. Med. Chem. 13 (2006) 167–198.
[11] R. Li, X. Chen, B. Gong, P.M. Selzer, Z. Li, E. Davidson, G. Kurzban, R.E. Miller, E.O. Nuzum, J.H. McKerrow, R.J. Fletterick, S.A. Gillmor, C.S. Craik, I.D. Kuntz, F.E. Cohen, G.L. Kenyon, Structure-based design of parasitic protease inhibitors, Bioorg. Med. Chem. 4 (1996) 1421–1427.
[12] C.R. Rodrigues, T.M. Flaherty, C. Springer, J.H. McKerrow, F.E. Cohen, CoMFA and HQSAR of acylhydrazide cruzain inhibitors, Bioorg. Med. Chem. Lett. 12 (2002) 1537–1541.
[13] D.R. Ifa, C.R. Rodrigues, R.B. de Alencastro, C.A.M. Fraga, E.J. Barreiro, A possible molecular mechanism for the inhibition of cysteine proteases by salicylaldehyde N-acylhydrazones and related compounds, J. Mol. Struct. Theochem. 505 (2000) 11–17.
[14] R. Li, G.L. Kenyon, F.E. Cohen, X. Chen, B. Gong, J.N. Dominguez, E. Davidson, G. Kurzban, R.E. Miller, E.O. Nuzum, P.J. Rosenthal, J.H. McKerrow, *In vitro* antimalarial activity of chalcones and their derivatives, J. Med. Chem. 38 (1995) 5031–5037.
[15] J.C. Powers, J.L. Asgian, O.D. Ekici, K.E. James, Irreversible inhibitors of serine, cysteine, and threonine proteases, Chem. Rev. 102 (2002) 4639–4750.
[16] M. Sajid, J.H. McKerrow, Cysteine proteases of parasitic organisms, Mol. Biochem. Parasitol. 120 (2002) 1–21.
[17] P.V. Desai, A. Patny, Y. Sabnis, B. Tekwani, J. Gut, P. Rosenthal, A. Srivastava, M. Avery, Identification of novel parasitic cysteine protease inhibitors using virtual screening. 1. The ChemBridge database, J. Med. Chem. 47 (2004) 6609–6615.
[18] J.C. Aponte, M. Verástegui, E. Málaga, M. Zimic, M.A.J. Vaisberg, R.H. Gilman, G.B. Hammond, Synthesis, cytotoxicity, and anti-*Trypanosoma cruzi* activity of new chalcones, J. Med. Chem. 51 (2008) 6230–6234.
[19] D.M. Borchhardt, A. Mascarello, L.D. Chiaradia, R.J. Nunes, G. Oliva, R.A. Yunes, A.D. Andricopulo, Biochemical evaluation of a series of synthetic chalcone and hydrazide derivatives as novel inhibitors of cruzain from Trypanosoma cruzi, J. Braz. Chem. Soc. 21 (2010) 142–150.
[20] E. Knoevenagel, Condensation von malondiure mit aromatiachen aldehyden durch ammoniak und amine, Ber. Dtsch. Chem. Ges. 31 (1898) 2596–2619.
[21] O. Doebner, Synthesis of sorbic acid [translation], Chem. Ber. 33 (1900) 2140–2142.
[22] X. Zhang, M. Breslav, J. Grimm, K. Guan, A. Huang, F. Liu, C.A. Maryanoff, D. Palmer, M. Patel, Y. Qian, C. Shaw, K. Sorgi, S. Stefanick, D. Xu, A new procedure for preparation of carboxylic acid hydrazides, J. Org. Chem. 67 (2002) 9471–9474.
[23] P.C. Lima, L.M. Lima, K.C.M. Silva, P.H.O. Leda, A.L.P. Miranda, C.A.M. Fraga, E.J. Barreiro, Synthesis and analgesic activity of novel *N*-acylarylhydrazones and isosters, derived from natural safrole, Eur. J. Med. Chem. 35 (2009) 187–203.
[24] S.A. Carvalho, E.F. da Silva, C.A.M. Fraga, S.M.S.V. Wardell, J.L. Wardell, E.R.T. Tiekink, (2*E*)-*N*′-[(*E*)-4-Chlorobenzylidene]-3-phenylprop-2-enohydrazide monohydrate, Acta Cryst. E E66 (2010) o2410–o2411.
[25] A.E. Kummerle, J.M. Raimundo, C.M. Leal, G.S. DaSilva, T.M. Balliano, M.A. Pereira, C.A. DeSimone, R.T. Sudo, G. Zapata-Sudo, C.A.M. Fraga, E.J. Barreiro, Studies towards the identification of putative bioactive conformation of potent vasodilator arylidene *N*-acylhydrazone derivatives, Eur. J. Med. Chem. 44 (2009) 4004–4009.
[26] G. Palla, G. Predieri, P. Domiano, C. Vignali, W. Turner, Conformational behavior and E/Z isomerization of *N*-acyl- and *N*-aroylhydrazones, Tetrahedron 42 (1986) 3649–3654.
[27] S. Nwaka, A. Hudson, Innovative lead discovery strategies for tropical diseases, Nat. Rev. Drug Discov. 5 (2006) 941–955.
[28] A.J. Romanha, S.L. DeCastro, M.N. Soeiro, J. Lannes-Vieira, I. Ribeiro, A. Talvani, B. Bourdin, B. Blum, B. Olivieri, C. Zani, C. Spadafora, E. Chiari, E. Chatelain, G. Chaves, J.E. Calzada, J.M. Bustamante, L.H. Freitas-Junior, L.I. Romero, M.T. Bahia, M. Lotrowska, M. Soares, S.G. Andrade, T. Armstrong, W. Degrave, Z.A. Andrade, Experimental *in vitro* and *in vivo* models for drug screening and development for Chagas disease, Mem. Inst. Oswaldo Cruz 105 (2010) 233–238.
[29] I.A. Pearl, D.L. Beyer, Reactions of vanillin and its derived compounds. XI. Cinnamic acids derived from vanillin and its related compounds, J. Org. Chem. 16 (1951) 216–220.
[30] A.R. Katritzky, M. Wang, S. Zhang, One-pot synthesis of cinnamoyl hydrazides, Arkivoc (2001) 19–23.
[31] K. Tanaka, K. Matsuo, A. Nakanishi, T. Hatano, H. Izeki, Y. Ishida, W. Mori, Synthesis and anti-inflammatory and analgesic activities of hydroxamic acids and acid hydrazides, Chem. Pharm. Bull. 31 (1983) 2810–2819.
[32] A. Hasan, S. Gapil, I. Khan, Synthesis, characterization and antifungal activity of some new 5-substituted 1,3,4-oxadiazole-2-thiols, Asian J. Chem. 23 (2011) 2007–2010.
[33] K.M. Khan, M. Rasheed, Z. Ullah, S. Hayat, F. Kaukab, M.I. Choudhary, Atta-ur-Rahman, S. Perveen, Synthesis and in vitro leishmanicidal activity of some hydrazides and their analogues, Bioorg. Med. Chem. 11 (2003) 1381–1387.
[34] W.O. Godtfredsen, S. Vangedal, The reaction of hydrazine with cinnamic acid derivatives, Acta Chem. Scand. 9 (1955) 1498–1509.
[35] M.B. Devani, U.S. Pathak, J.N. Sane, Synthesis of some cinnamoylarylidene hydrazines, Indian J. Chem. 7 (1969) 1198–1199.
[36] I. Orhan, B. Sener, T. Atici, R. Brun, R. Perozzo, D. Tasdemir, Turkish freshwater and marine macrophyte extracts show in vitro antiprotozoal activity and inhibit FabI, a key enzyme of Plasmodium falciparum fatty acid biosynthesis, Phytomedicine 13 (2006) 388–393.

[37] K. Salomão, A.P. Dantas, C.M. Borba, L.C. Campos, D.G. Machado, F.R. Aquino-Neto, S.L. DeCastro, Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis, Lett. Appl. Microbiol. 38 (2004) 87–92.

[38] S.L. DeCastro, M.C. Pinto, A.V. Pinto, Screening of natural and synthetic drugs against *Trypanosoma cruzi*: I – establishing a structure/activity relationship, Microbios 78 (1994) 83–90.

[39] M.V. Carvalho, C. Penido, A.C. Siani, L.M.M. Valente, M.G.M.O. Henriques, Investigations on the anti-inflammatory and anti-allergic activities of the leaves of *Uncaria guianensis* (Aublet) J. F. Gmelin, Inflammopharmacology 14 (2006) 48–56.

[40] A.E. Eakin, A.A. Mills, G. Harth, J.H. McKerrow, C.S. Craik, The sequence, organization, and expression of the major cysteine protease (cruzain) from cruzi, J. Biol. Chem. 267 (1992) 7411–7420.