Bioorganic & Medicinal Chemistry 17 (2009) 641–652

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis, trypanocidal activity and docking studies of novel quinoxaline-*N*-acylhydrazones, designed as cruzain inhibitors candidates

Nelilma C. Romeiro [a], Gabriela Aguirre [b], Paola Hernández [b], Mercedes González [b], Hugo Cerecetto [b], Ignacio Aldana [c], Silvia Pérez-Silanes [c], Antonio Monge [c], Eliezer J. Barreiro [a], Lídia M. Lima [a,*]

[a] *Laboratório de Avaliação e Síntese de Substâncias Bioativas (LASSBio), Faculdade de Farmácia, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, PO Box 68024, RJ 21944-970, Brazil*
[b] *Departamento de Química Orgánica, Facultad de Química-Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay*
[c] *Unidad en Investigación y Desarrollo de Medicamentos, Centro de Investigación en Farmacobiología Aplicada (CIFA), Universidad de Navarra, c/Irunlarrea s/n, 31080 Pamplona, Spain*

ARTICLE INFO

Article history:
Received 27 August 2008
Revised 20 November 2008
Accepted 24 November 2008
Available online 3 December 2008

Keywords:
Quinoxaline-*N*-acylhydrazone (NAH) derivatives
Trypanosoma cruzi
Chagas disease
Docking
Cruzain

ABSTRACT

In this paper, we report the structural design, synthesis, trypanocidal activity and docking studies of novel quinoxaline-*N*-acylhydrazone (NAH) derivatives, planned as cruzain inhibitors candidates, a cysteine protease essential for the survival of *Trypanosoma cruzi* within the host cell. The salicylaldehyde *N*-acylhydrazones **7a** and **8a** presented IC_{50} values of the same magnitude order than the standard drug nifurtimox (Nfx), when tested in vitro against epimastigote forms of *Trypanosoma cruzi* (Tulahuen 2 strain) and were non-toxic at the highest assayed doses rendering selectivity indexes (IC_{50} (macrophages)/IC_{50} (*Trypanosoma cruzi*)) of >25 for **7a** and >20 for **8a**, with IC_{50} values in macrophages >400 μM.
© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Chagas' disease is one of the most important medical problems in South America. It is caused by the intracellular protozoan *Trypanosoma cruzi*, which infects about 9–12 million people in Central and South America.[1]

The World Bank estimated an annual loss of 2.74 million disability-adjusted life years, representing an economic loss to the endemic countries equivalent to US$ 6.5 billion per annum.[2]

The epidemiology of Chagas' disease, first described in 1909, still remains a challenge, given that the sylvatic transmission cycle of the parasite occurs in a complex trophic network that includes several mammalian species.[3] The complexity of the epidemiology is exemplified by the recently described new epidemiological features expressed by outbreaks of human disease probably due to the oral route described in Amazonia and Santa Catarina, a southern area of the Atlantic Coastal Rain Forest, where no domiciliation of triatomines had been reported up to now.[4,5]

Cysteine proteases are proteolytic enzymes which depend on the nucleophilic thiol group of a cysteine residue for their enzymatic activity and that are also functionally diverse and widely distributed.[6,7] Cruzain, the major cysteine protease in *Trypanosoma cruzi*, is a lysosomal hydrolase with significant homology to cathepsin L.[8] It is expressed throughout the life cycle of *Trypanosoma cruzi* and is known to be essential for the survival of the parasite within the host cell.[8] Selective inhibitors of this protease block the proliferation of both extracellular epimastigotes and intracellular amastigotes, and arrest metacyclogenesis (transformation of epimastigotes to metacyclic trypomastigotes) in vitro.[9] Therefore, cruzain represents an interesting target for the design of potential trypanocidal drugs.[9] The structures of current cruzain inhibitors are diverse including a series of vinyl-sulfones (1), α-hydroxyketones (2), thiosemicarbazones (3) and 2-hydroxy-aryl-*N*-acylhydrazone derivatives (4) (Chart 1).[10–15]

Recently, the *N*-acylhydrazone moiety (NAH, R**CONHN**=**C**HR) has been described as an interesting privileged structure, being largely used in the design of new bioactive compounds with distinct pharmacological profiles, such as *anti*-trypanosome (**5**, Chart 1 and **6**, Chart 2).[10,16–18]

In this paper, we report the design, synthesis, trypanocidal activity and docking studies of novel quinoxaline-*N*-acylhydrazone (NAH) derivatives (**7a–o** and **8a**, Chart 2), planned as cruzain inhibitors candidates. The design of these new derivatives was performed by applying the classical bioisosterism strategy[19] and taking into consideration the role of the quinoxaline nucleus as

* Corresponding author. Tel.: +55 2125626503; fax: +55 2125626644.
E-mail address: lidia@pharma.ufrj.br (L.M. Lima).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.11.065

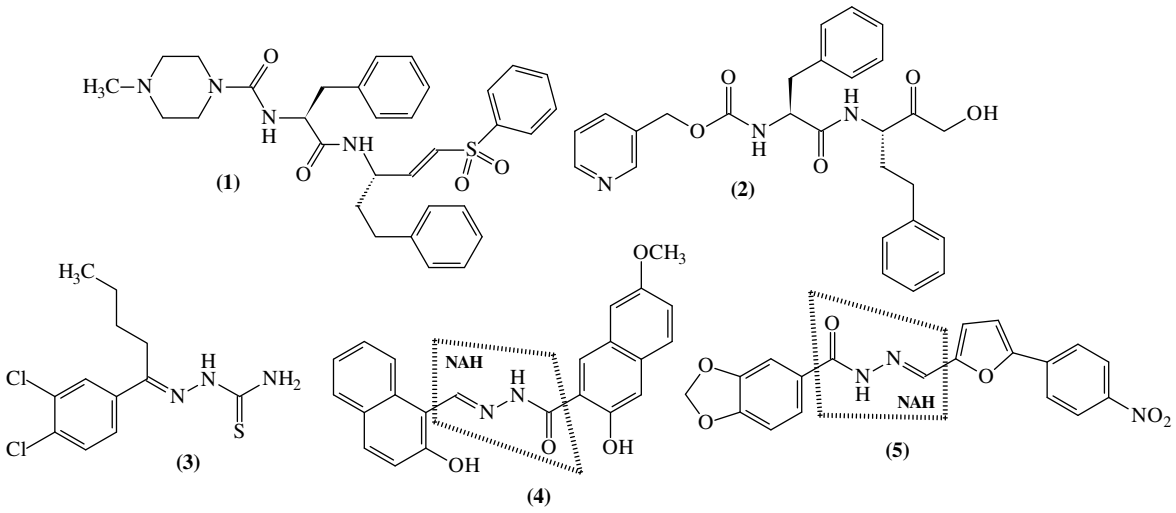


Chart 1.

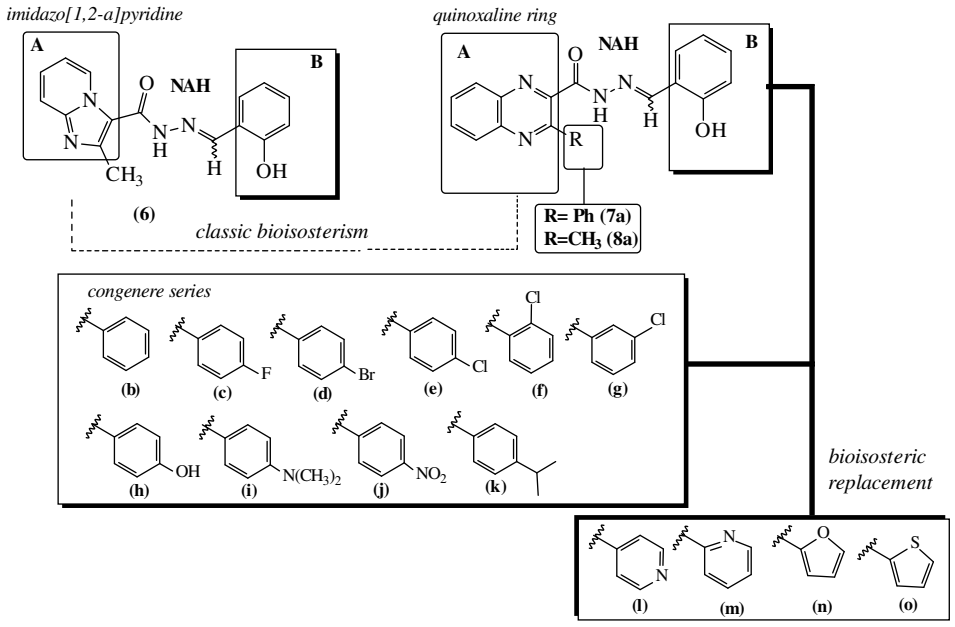


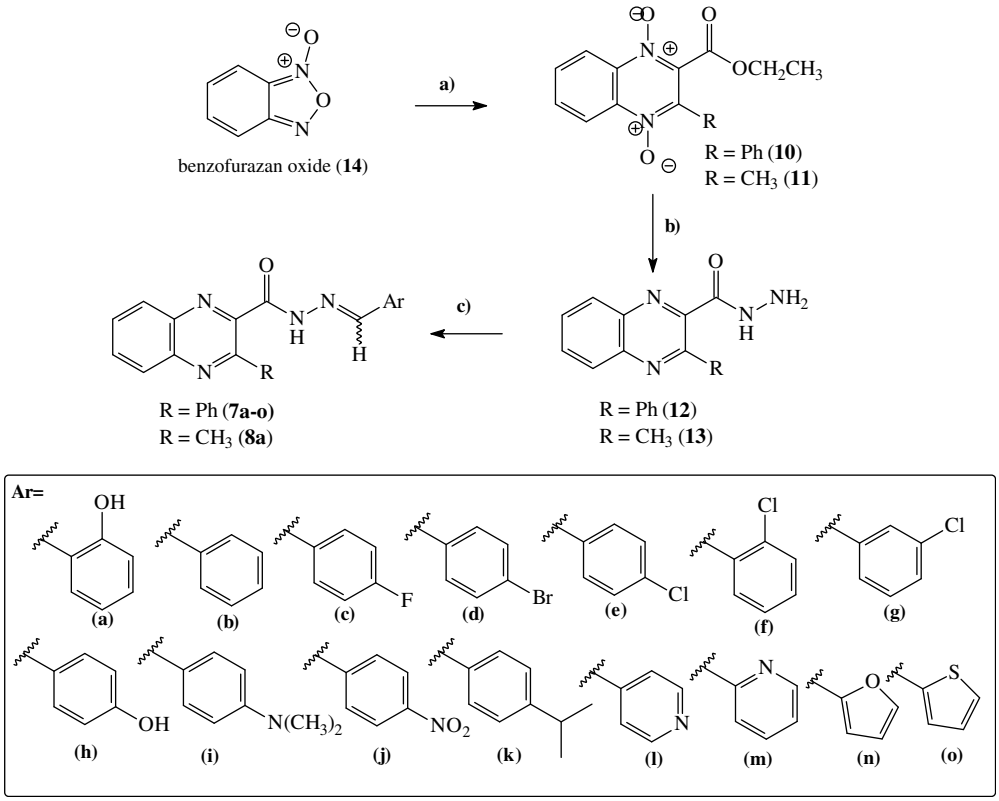
Chart 2.

an important biophore (scaffold), since some recently reported derivatives with this chemotype have demonstrated interesting trypanocidal activity.[20] In addition, it also taken into account the fact that the NAH subunit show an aza-vinylogue relationship with the amide group present in peptides, as the primary sites of hydrolysis catalyzed by proteases, and that iminic double bond can work like an hydrophobic anchor. The structural design of this new series was accomplished by replacing the imidazo[1,2-*a*]pyridine nucleus, present in the prototype (**6**), by a quinoxaline ring giving the structures of derivatives **7a–o** and **8a** (Chart 2). The nature of the *para*-substituent (F, Br, Cl, OH, $N(CH_3)_2$, NO_2, *i*Pr), present in the phenyl group of subunit-B of the new compounds (**7c–e**, **7h–k**), was defined in order to introduce an important variation in Hammett's σp values[21] (ranging from −0.83 ($N(CH_3)_2$) to +0.78 (NO_2)), aiming at investigating any eventual electronic contribution of this structural moiety in the trypanocidal activity. The hydrophobic parameter π (ranging from −0.61 (4-OH) to +1.43 (4-*i*Pr)) of the aromatic substituents was also considered.[21]

2. Results and discussion

2.1. Chemistry

The aforementioned compounds (**7a–o** and **8a**) were prepared according to synthetic process illustrated in Scheme 1. The synthesis of the esters **10** and **11** was carried out by performing the condensation of benzofurazan oxide (**14**) obtained by previously described methods,[22] with ethyl acetoacetate or ethyl benzoylacetate, in the presence of potassium carbonate, using acetone as the solvent, in a classical Beirut reaction. The synthesis of hydrazide derivatives (**12** and **13**) was performed in good yield, exploring the hydrazinolysis of the ethoxycarbonyl-group using hydrazine hydrate in ethanol at reflux, in 72% and 63% yields, respectively. Next, condensation of **12** and **13** with the appropriate aromatic aldehydes yielded the new series of quinoxaline *N*-acylhydrazone (**7a–o**, **8a**) derivatives in excellent yields. Analysis of the ^{1}H NMR spectra of the new derivatives (**7a–o**, **8a**) showed the presence of



Scheme 1. Reagents and conditions: (a) ethyl acetoacetate or ethyl benzoylacetate; K_2CO_3; acetone; rt; 8 h; 32% (**11**) and 50% (**10**); (b) $N_2H_4{\cdot}H_2O$; EtOH; reflux; overnight; 63% (**13**) and 72% (**12**); (c) Ar-CHO; EtOH; HCl (cat.); rt; 30 min.; 75–96% (**7a–o**, **8a**).

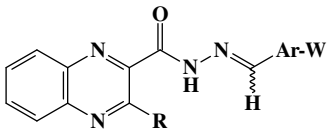
two signals referring to the ylidenic hydrogen (N=CH), attributed to a mixture of the *E*/*Z*-diastereomers. The assignment of (*E*) and (*Z*) isomers was made in agreement with previous results disclosed by Karabatsos and coworkers for the relative configuration of hydrazones and related compounds.[23,24] Moreover, we performed a brief study of the relative energy of both possible diastereomers by molecular modeling, using the semiempirical AM1 method[25] available in the PC SPARTAN´ 04 software,[26] indicating a minor difference between the heat of formation values for the (*E*) and (*Z*) diastereomers of all NAH-derivatives from this bioactive series (data not shown).

2.2. Trypanocidal activities

The primary screening data for all compounds (at 25 μM) are shown in Table 1. All compounds were tested in vitro against epimastigote forms of *Trypanosoma cruzi*, Tulahuen 2 strain. The new quinoxaline *N*-acylhydrazones derivatives (**7a–o**, **8a**) were incorporated into the media at 25 μM and their ability to inhibit the parasite growth was evaluated in comparison to the standard drug nifurtimox (Nfx).[27] The percentage of growth inhibition was calculated and the IC_{50} concentration determined only for the compounds with % inhibition superior to 80%.

The results shown in Table 1 demonstrate that seven, out of the sixteen evaluated compounds, presented percentage of growth inhibition superior to 30%, standing out the derivatives **7a** and **8a** with inhibitions of 96% and 81%, respectively. These compounds were selected and next the IC_{50} value determined. The quinoxaline *N*-acylhydrazones presented IC_{50} values of the same magnitude order than the standard drug Nfx. Also, unspecific cytotoxicity of the most active derivatives **7a** and **8a** and one inactive compound (**7o**) against mammalian cells was evaluated in vitro at 100, 200 and 400 μM, using J774 mice macrophages as the cellular model and terbinafine (Tbf) and ketoconazole (Ktz) as reference drugs.[27,28] The results demonstrated that compounds **7a**, **8a** and 7**o** were non-toxic at the highest assayed doses, rendering selectivity in-

Table 1
The in vitro trypanocidal activity of quinoxaline *N*-acylhydrazone derivatives (**7a–o**, **8a**)



Compound	W-Ar/R	PGI[a](%)[a,b,c]	IC_{50} (μM)[c]
Nifurtimox®	—	100	7.7
LASSBio-1008 (**7b**)	Ph/Ph	3	n.d.[d]
LASSBio-1009 (**7c**)	4-F–Ph/Ph	22	n.d.
LASSBio-1010 (**7d**)	4-Br–Ph/Ph	40	n.d.
LASSBio-1011 (**7e**)	4-Cl–Ph/Ph	53	n.d.
LASSBio-1012 (**7g**)	3-Cl–Ph/Ph	47	n.d.
LASSBio-1013 (**7f**)	2-Cl–Ph/Ph	35	n.d.
LASSBio-1014 (**7i**)	4-$N(CH_3)_2$–Ph/Ph	29	n.d.
LASSBio-1015 (**7j**)	4-NO_2–Ph/Ph	19	n.d.
LASSBio-1016 (**7a**)	2-OH–Ph/Ph	96	15.9
LASSBio-1017 (**7h**)	4-OH–Ph/Ph	27	n.d.
LASSBio-1018 (**7k**)	4-*i*Pr–Ph/Ph	36	n.d.
LASSBio-1019 (**7o**)	2-Thienyl/Ph	0	n.d.
LASSBio-1020 (**7n**)	2-Furyl/Ph	0	n.d.
LASSBio-1021 (**7m**)	2-Pyridinyl/Ph	0	n.d.
LASSBio-1022 (**8a**)	2-OH–Ph/CH_3	81	20.0
LASSBio-1025 (**7l**)	4-Pyridinyl/Ph	0	n.d.

[a] Percentage of growth inhibition.
[b] Inhibition of epimastigotes growth of Tulahuen 2 strain, doses = 25 μM.
[c] The results are the means of three independent experiments with a SD less than 10% in all cases.
[d] n.d.: Not determined.

dexes (IC_{50}(macrophages)/IC_{50}(*Trypanosoma cruzi*))[29] of >25 for **7a** and >20 for **8a**, with IC_{50}(macrophages) > 400 μM. (Table 2). This toxicity parameter, along with the trypanocidal activity, revealed quinoxalines **7a** and **8a** as promising lead-compounds.

Structurally, these compounds share as a common characteristic the presence of an *ortho*-hydroxyphenyl group linked to the hydrazone subunit. Salicylaldehyde *N*-acylhydrazones (or *ortho*-hydroxyarylaldehydehydrazones) are described as inhibitors of some cysteine proteases as in the case of *Plasmodium falciparum* trophozoite cysteine protease (TCP) and *Trypanosoma cruzi* cruzain.[10] Aiming to rationalize the inhibitory activity profile of salicylaldehyde *N*-acylhydrazones towards different proteases, Ifa and coworkers proposed a possible theoretical mechanism of interaction of compound **6** (Chart 2) with cysteine proteases.[30] The proposed mechanism involves the nucleophilic attack, through a Michael addition mechanism, of the sulfur atom of a negatively charged cysteine residue on a reactive *ortho*-quinonemethyde intermediate, generated from the tautomeric equilibrium of *ortho*-hydroxyarylaldehydehydrazone moiety.[30]

2.3. Docking studies

In an attempt to theoretically explain the difference found in the trypanocidal activity of the new quinoxaline *N*-acylhydrazone derivatives (**7a–o**, **8a**), docking studies using the enzyme cruzain were performed. The *ortho*-quinonemethyde-intermediates, the tautomers of compounds **7a** and **8a**, were also considered in these studies.

The molecular construction and docking analysis of quinoxaline derivatives LASSBio 1008–1025 were performed as described in Section 3. The binding modes of quinonemethyde-like tautomeric forms of LASSBio-1016 (**7a**) and LASSBio-1022 (**8a**), denominated **15a** and **16a** (Fig. 1), respectively, were also evaluated by docking into the active site of cruzain.

The docking analysis was carried out on the crystal structures of *Trypanosoma cruzi* cysteine protease cruzain, with different bound inhibitors,[31–33] using the flexible docking software FlexE.[34] Since all compounds have been synthesized as *Z*/*E* diastereomeric mixtures, the modeling has been based on the isomers separately. In this study, the proposed interaction modes of the quinoxaline derivatives into the active site of cruzain were determined as the highest scored conformations (best-fit ligand) among 30 conformational and binding modes generated according to FlexX scoring function, which correspond to the structure with the most favorable free energy of binding, that is ΔG_{bind} (kJ/mol) (Fig. 2). ΔG values shown in this study have been based on MMFF94 charges.[35]

The investigation of the top poses obtained by docking with FlexE has shown some interesting features, although this software does not consider covalent binding, which would be the case of interaction between Michael acceptors and cruzain. For instance, there is no obvious preference for binding of *Z* and *E* isomers, since these structures presented very close ΔG_{bind} values, except in the case of LASSBio-1022 (**8a**), (Fig. 2), whose (*E*)-isomer has shown a larger ΔG_{bind} value difference in comparison to its (*Z*)-isomer (ca. −7.00 kJ/mol, Fig. 2). Interestingly, the *E*/*Z* quinone adducts of LASSBio-1016 (**7a**) and LASSBio-1022 (**8a**) have the second most favorable ΔG_{bind} values, close to the ones observed for the (*E*)-isomers of these compounds (Fig. 2).

Figure 3 illustrates the top docking poses obtained for the adducts and (*E*)-isomers of LASSBio-1016 (**7a**) and LASSBio-1022 (**8a**) with amino acid residues in the active site of cruzain (Fig. 3A–D).

From the docking experiments, the best complex of the quinone adduct of LASSBio-1016 (Fig. 3A) with cruzain reveals that the carbon atom of the amide carbonyl group of LASSBio-1016 (**7a**) is within 2.69 Å of the sulfur atom of Cys25, the key nucleophilic amino acid residue in the active site of cruzain. The nitrogen atom in position 1 of the quinoxaline ring also establishes a hydrogen bond with His159; the oxygen atom of the hydrazide moiety hydrogen bonds to Gln19 and to the –NH group of the backbone of Cys25. One of the –NH groups of the molecule establishes a hydrogen bond with the –SH group of Cys25.

The visual inspection of the top pose obtained for the (*E*)-isomer of LASSBio-1016 (**7a**) (Fig. 3B) reveals that it is able to establish a hydrogen bond with His159 via the nitrogen atom of the imine group. Additional hydrogen bonds involve the –NH group and the oxygen atom of the backbone of Asp158 and the 2-OH–Ph group and the side chain of Gln19.

The docking analysis of the quinone adduct of LASSBio-1022 (**8a**) and its (*E*)-isomer (Fig. 3C and D) reinforces the hydrogen bonding pattern that had been observed for LASSBio-1016 (7**a**), its quinone adduct and the (*E*)-isomer, since these structures interact with the same amino acid residues (Fig. 3C and D). The putative binding modes of these compounds are reinforced by the closer proximity of the carbon atom of the amide pertaining to the acyl hydrazone function of the quinone adduct and the sulfur atom of Cys25, with a distance of 2.64 Å (Fig. 3C). Moreover, the β-keto carbon atom of the α,β-unsaturated ketone in the quinone adduct is 4.44 Å away from Cys25 (Fig. 3C). Interestingly, the carbon atom of the carbonyl group of the (*E*)-isomer of LASSBio-1022 (**8a**) (Fig. 3D) is closer in space to the sulfur atom of Cys25, with a distance of 2.09 Å, when compared to the distance observed in the top pose obtained for the (*E*)-isomer of LASSBio-1016 (**7a**), which is 2.69 Å (Fig. 3B).

Among compounds that bear lipophilic substituents in the phenyl ring, such as LASSBio-1010 (**7d**) LASSBio-1011 (**7e**), LASSBio-1012 (**7g**) and LASSBio-1013 (**7f**), the 4-Cl–Ph derivative **7e** has shown a good theoretical ΔG_{bind} (Fig. 2). Therefore, we have investigated the putative binding modes of its *E*/*Z* isomers with cruzain (Fig. 4A and B). The visual inspection of the top pose obtained for the (*Z*)-isomer of LASSBio-1011 (**7e**) and cruzain shows that this derivative establishes hydrogen bonds with Gln19, His159 and with Cys25. The observed interatomic distance between the carbon atom of the amide carbonyl and the sulfur atom of Cys25 is 2.75 Å, while the distance between the β-keto carbon atom of the α,β-unsaturated ketone in the quinone adduct and the same sulfur atom is 3.00 Å. No extra van der Waals interactions were observed with the 4-Cl–Ph group (Fig. 4A).

Also, the visual inspection of the top pose obtained for the (*E*)-isomer of LASSBio-1011 (**7e**) and cruzain shows that this compound interacts by hydrogen bonds to Gln156 and Leu157 via the nitrogen atom in position 1 of the quinoxaline ring and the nitrogen atoms of the hydrazide moiety. Worth of notice is the fact that the putative Michael acceptors in this molecule are very distant from Cys25, in a range of 10–12 Å (Fig. 4B).

Interestingly, LASSBio-1017 (**7h**), a quinoxaline derivative that bears a hydroxyl group in position 4 of the phenyl ring, showed a low predicted bioactivity. Docking studies of this compound and

Table 2
Cytotoxicity of quinoxaline derivatives against J-774 mouse macrophages

Compound	Pcyt.[a] at 400 (μM)[b]	$IC_{50,\ macrophage}$[b]	SI[c,d]
7a	14	>400	>25.2
7o	33	>400	—[e]
8a	12	>400	>20.0
Tbf	100	88.0	5.1
Ktz	100	<50.0	<5.0

[a] Pcyt = Percentage of cytotoxicity.
[b] The results are the means of two different experiments with a SD less than 10% in all cases.
[c] SI: Selectivity index.
[d] SI = $IC_{50,macrophage}/IC_{50,T2,epimastigote}$.
[e] Not determined.

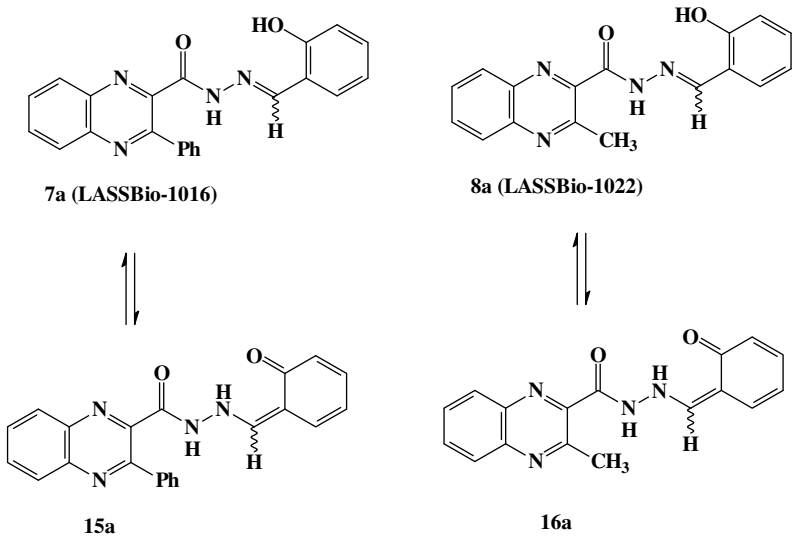


Figure 1. Possible Michael acceptor quinone adducts (**15a** and **16a**), tautomers of LASSBio-1016 (**7a**) and LASSBio-1022 (**8a**).

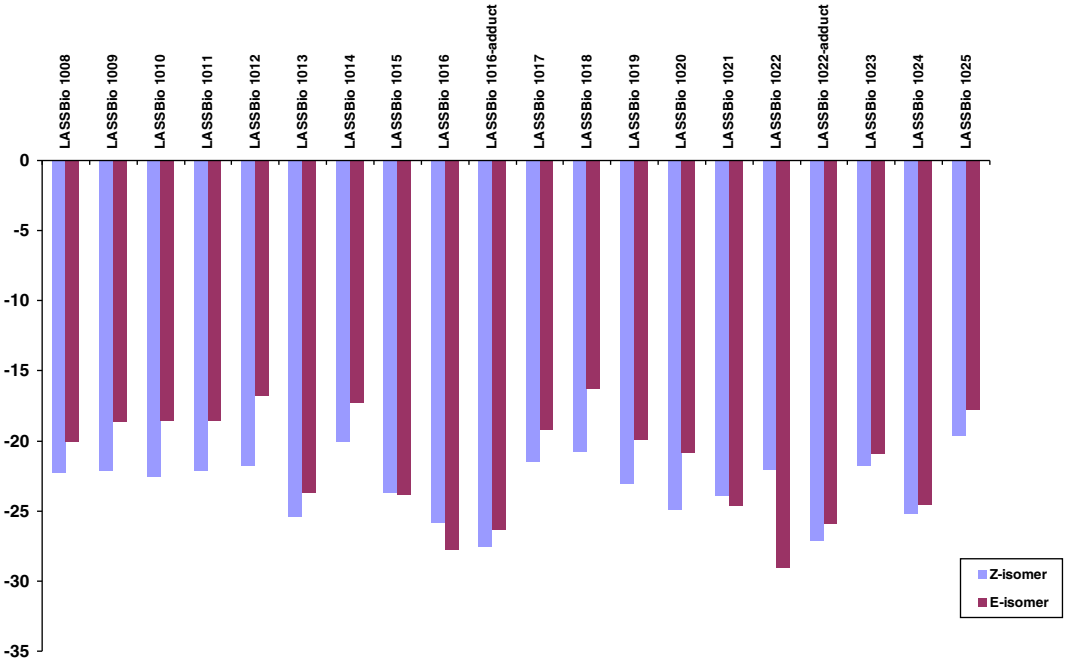


Figure 2. In silico ΔG_{bind} values (kJ/mol) obtained from docking of the quinoxaline derivatives with cruzain.

cruzain showed that, opposite to what had been observed for the other hydroxylated derivatives, LASSBio-1016 (**7a**) and LASSBio-1022 (**8a**), the (*Z*)-isomer of LASSBio-1017 (**7h**) presented a different binding pose in the enzyme active site (Fig. 4C). Most remarkable is the fact that the carbon atom of the carbonyl pertaining to the NAH moiety and the sulfur atom of Cys25 are 4.65 Å apart, even though this derivative is able to establish hydrogen bonds to Ser61 Gly66, Gln156 and with the backbone oxygen of the carbonyl group of Leu157, which may explain its lower bioactivity in comparison to the other *ortho*-hydroxylated quinoxaline derivatives, since the nucleophilic species is the sulfur atom from Cys25. In addition, the distance between the carbon atom of the imine group and the sulfur atom of Cys25 is 7.59 Å (Fig. 4C).

Finally, the visual inspection of the top pose obtained for the (*E*)-isomer of LASSBio-1017 (**7h**) and cruzain shows that this compound establishes hydrogen bonds to Gln156 and Leu157 via the nitrogen atom in position 1 of the quinoxaline ring and the nitrogen atoms of the hydrazide moiety (Fig. 4D). Again, the putative Michael acceptors in this molecule are very distant from Cys25, in a range of 10–12 Å, which reinforces the possible inability of this molecule to act as a Michael acceptor for the nucleophilic –SH group of Cys25 (Fig. 4D).

The docking results presented herein have shown a reasonable correlation with the structural design of the quinoxaline derivatives towards cruzain and orientate our studies towards the molecular mechanism of action of these substances against cysteine proteases, enzymes that are the target of a wide range of therapeutically active substances, including a few anti-chagasic compounds.[36]

2.4. Lipinski's rule of five

In addition to ligand–protein complex modeling, in vivo absorption capabilities of the designed molecules were tentatively as-

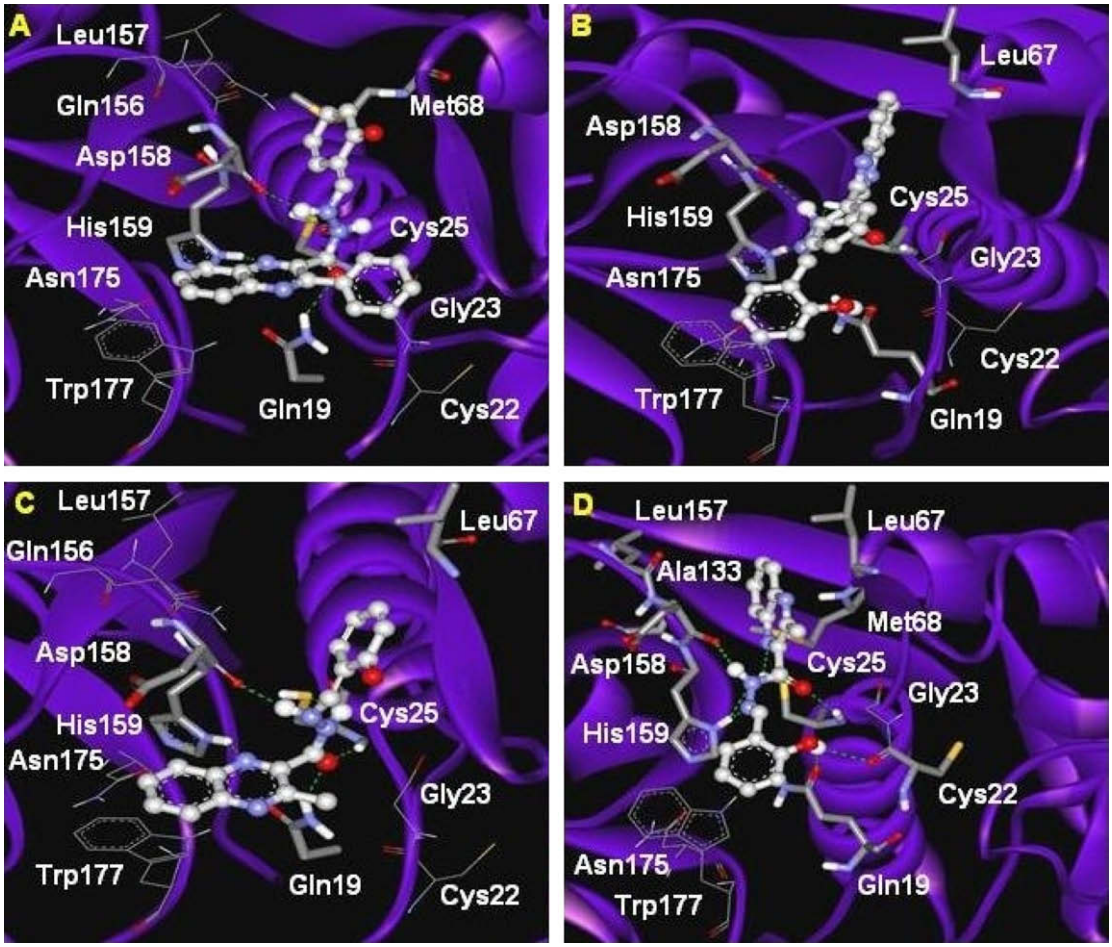


Figure 3. Top poses obtained by docking with FlexE of (A) the *E* isomer of the quinone adduct of LASSBio-1016 and (B) *E* isomer of LASSBio-1016; (C) the *E* isomer of the quinone adduct of LASSBio-1022 and (D) *E* isomer of LASSBio-1022, with cruzain. Hydrogen bonds are shown as green dashed lines. Only polar hydrogens are shown for clarity.

sessed by means of theoretical calculations following Lipinski's rule of five,[37] that predicts that a compound administered orally will more likely have good absorption or permeation if it satisfies the following criteria:

Hydrogen bond donors $\leqslant$ 5 (OH and NH groups)
Hydrogen bond acceptors $\leqslant$ 10 (N and O atoms)
Molecular weight < 500
Calculated log*P* (*C*log P) < 5

This approach has been widely used as a filter in the decision-making of which substances should be further developed in drug design programs. The results of the calculations for the molecules designed in this study show that all molecules have a potential for good in vivo absorption, since the majority of the compounds, except for LASSBio-1010 (**7d**) and LASSBio-1018 (**7k**), that did not satisfy only one requisite, satisfied Lipinski's rule of five with zero violations (Table 3).

2.5. Conclusions

A new series of trypanocidal quinoxaline-*N*-acylhydrazone (NAH) derivatives were discovered, outstanding the salicylaldehyde *N*-acylhydrazones **7a** and **8a** with IC_{50} of the same magnitude order than the standard drug nifurtimox (Nfx). These compounds were non-toxic at the highest assayed concentration rendering selectivity indexes (IC_{50}(macrophages)/IC_{50}(*Trypanosoma cruzi*)) of >25 for **7a** and >20 for **8a**, with IC_{50} (macrophages) >400 μM.

The docking results presented herein have shown a reasonable correlation with the molecular design of the quinoxaline derivatives towards cruzain, since the predicted in silico ΔG_{bind} values obtained through docking studies have pointed towards LASSBio-1016 (**7a**) and LASSBio-1022 (**8a**) as the most promising inhibitors, assuming that the molecular mechanism of these compounds involves this target. Also, these studies suggest that low bioactivity may be due to lack of adequate distances to the sulfur atom of Cys25 of cruzain. Finally, the application of parameters preconized by Lipinski's rule of five showed that all molecules have a potential for good in vivo absorption, since the designed compounds had zero or less than two rule violations.

3. Experimental

3.1. Chemistry

The synthesized compounds were chemically characterized by thin layer chromatography (TLC), infrared (IR), nuclear magnetic resonance (^{1}H NMR), mass spectra (MS) and elemental microanalysis (CHN). Alugram SIL G/UV254 (Layer: 0.2 mm) (Macherey-Nagel GmbH & Co. KG. Postfach 101352. D-52313 Düren, Germany) was used for TLC and Silica gel 60 (0.040–0.063 mm) for column flash chromatography (Merck). The ^{1}H NMR spectra

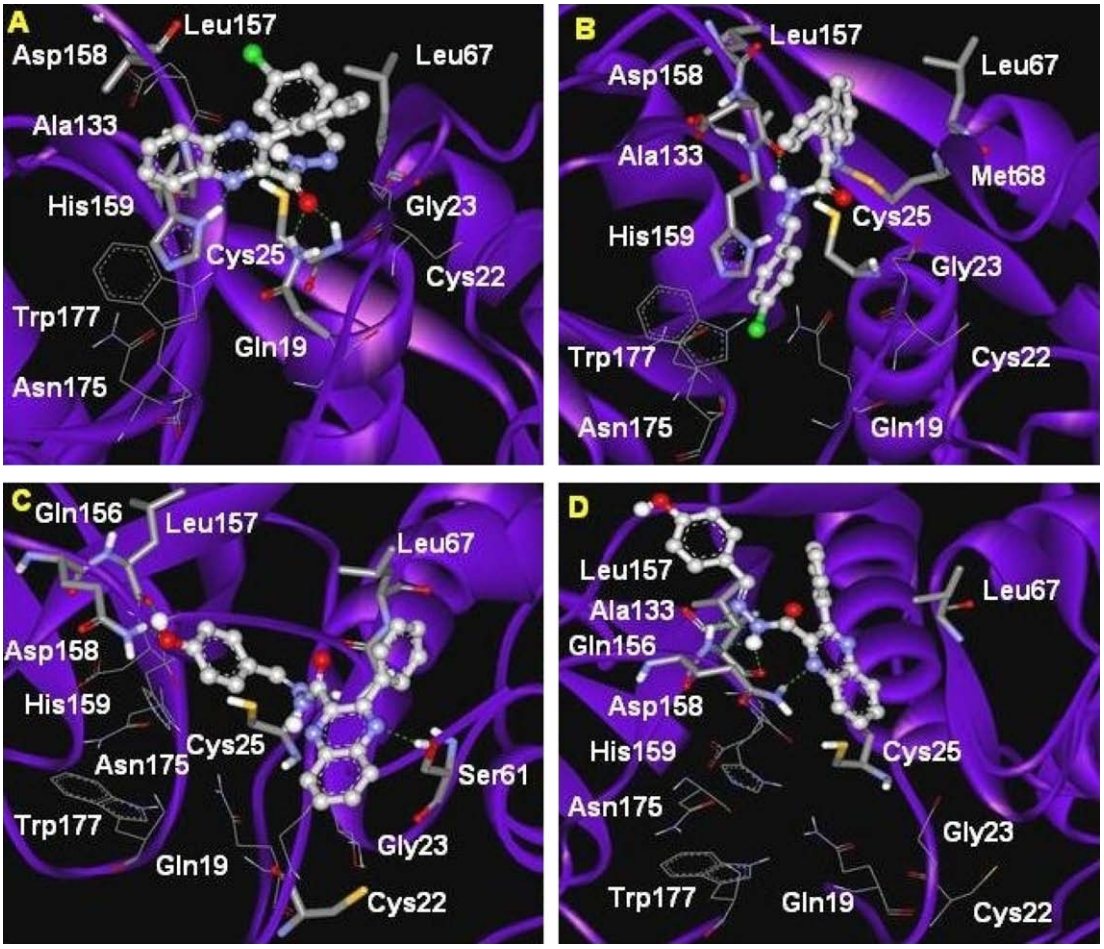


Figure 4. Top poses obtained by docking with FlexE of (A) and (B) LASSBio-1011 and (C) and (D) LASSBio-1017, *Z* and *E* isomers, respectively, with cruzain. Hydrogen bonds are shown as green dashed lines. Only polar hydrogens are shown for clarity.

Table 3
Compliance of compounds to computational parameters of bioavailability

Compound	CLog*P*	Molecular weight	Hydrogen bond donors	Hydrogen bond acceptors	Satisfies the rule of five?
LASSBio-1008 (**7b**)	4.24	352.391	1	4	Yes
LASSBio-1009 (**7c**)	4.38	370.381	1	4	Yes
LASSBio-1010 (**7d**)	5.10	431.287	1	4	No
LASSBio-1011 (**7e**)	4.95	386.836	1	4	Yes
LASSBio-1012 (**7g**)	4.95	386.836	1	4	Yes
LASSBio-1013 (**7f**)	4.35	386.836	1	4	Yes
LASSBio-1014 (**7i**)	4.70	395.459	1	5	Yes
LASSBio-1015 (**7j**)	3.98	397.388	1	6	Yes
LASSBio-1016 (**7a**)	4.77	368.390	2	5	Yes
LASSBio-1017 (**7h**)	4.14	368.390	2	5	Yes
LASSBio-1018 (**7k**)	5.67	394.471	1	4	No
LASSBio-1019 (**7o**)	3.89	358.419	1	4	Yes
LASSBio-1020 (**7n**)	3.41	342.353	1	5	Yes
LASSBio-1021 (**7m**)	2.96	353.379	1	5	Yes
LASSBio-1022 (**8a**)	3.29	306.320	2	5	Yes
LASSBio-1025 (**7l**)	2.76	353.379	1	5	Yes

were recorded on a Bruker 400 Ultrashield instrument (400 MHz), using TMS as the internal standard and with DMSO-d_6 and $CDCl_3$ as the solvents; the chemical shifts are reported in ppm (δ) and coupling constants (J) values are given in Hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), t (triplet), q (quadruplet), dd (double doublet), hp (septuplet) and m (multiplet). The IR spectra were performed on a Thermo Nicolet Nexus FTIR (Madison, USA) in KBr pellets; the frequencies are expressed in cm^{-1}. The mass spectra were measured on an Agilent Technologies Model MSD/DS 5973N (mod. G2577A) mass spectrometer with direct insertion probe (DIP) (Waldbronn, Germany) and the ionization method was electron impact (EI, 70 eV). Elemental microanalyses were obtained on an Elemental Analyzer (Leco CHN-900, Tres Cantos, Madrid, Spain) from vacuum-dried samples. The analytical results for C, H, and N, were within ±0.4 of the theoretical values.

3.1.1. General procedure for the preparation of 2-(Ethoxycarbonyl)-3-substituted-quinoxaline 1,4-dioxide derivatives (10–11)

7.34 mmol of the corresponding ester (ethyl acetoacetate or ethyl benzoylacetate) and 9.55 mmol of potassium carbonate were added to a solution of 7.34 mmol of benzofurazan oxide in 50 mL of acetone. The suspension was stirred at room temperature for 2 h. The quinoxaline 1,4-dioxide derivatives (**10 and 11**) were isolated by adding 50 mL of water, followed by extraction with dichlorometane (5×40 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated to dryness. The residue was purified by recrystallization from a mixture of methanol/ether/*n*-hexane (2:4:4).

3.1.2. 2-(Ethoxycarbonyl)-3-phenylquinoxaline 1,4-dioxide (10)

The derivative (**10**) was obtained by condensation of benzofurazan oxide with ethyl benzoylacetate, as a yellow powder in 50% yield.

^1H *NMR (CDCl₃):* δ 1.08 (t, *J* = 7.2 Hz, OCH_2CH_3); 4.25 (q, *J* = 7.2 Hz, OCH_2CH_3); 7.53 (m, H3′–H5′); 7.61 (m, H2′ and H6′); 7.91 (m, H6 and H7); 8.65 (m, H5 and H8) ppm.

^{13}C *NMR (CDCl₃):* δ 13.98 (OCH_2CH_3), 63.65 (OCH_2CH_3), 120.89 (C5), 121.08 (C8), 127.84 (C1′), 129.16 (C3′ and C5′), 130.15 (C2′ and C6′), 131.26 (C4′), 132.51 (C6), 132.53 (C7), 136.53 (C2), 137.73 (C10), 138.81 (C3), 140.08 (C9), 159.66 (CO_2Et) ppm.

Ir (KBr): 2978 (ArC-H), 1746 (C=O), 1352 (N-oxide), 701 and 666 (mono-substituted phenyl) cm^{-1}.

Mass: 310 (*m*/*z*, 100%), 294 (M^+, 6%), 249 (M^+, 51%), 221 (M^+, 46%), 77 (M^+, 46%).

Anal. Calcd for $C_{17}H_{14}N_2O_4$: C, 65.80; H, 4.52; N, 9.03. Found: C, 65.65; H, 4.57; N, 8.98.

3.1.3. 2-(Ethoxycarbonyl)-3-methylquinoxaline 1,4-dioxide (11)

The derivative (**11**) was obtained by condensation of benzofurazan oxide with ethylacetoacetate as a yellow powder in yield 30%.

^1H *NMR (400 MHz, CDCl₃)* δ*:* 1.52 (t, *J* = 7.1 Hz, OCH_2CH_3); 2.61 (s, $ArCH_3$); 4.60 (q, *J* = 7.1 Hz, OCH_2CH_3); 7.88 (m, H6 and H7); 8.62 (m, H5 and H8).

^{13}C *NMR (100 MHz, CDCl₃)* δ*:* 14.42 (OCH_2CH_3); 14.81 ($ArCH_3$); 64.09 (OCH_2CH_3); 120.55 (C5); 120.77 (C8); 131.90 (C6); 132.97 (C7); 136.02 (C2); 137.31 (C9); 138.30 (C10); 139.34 (C3); 160.26 ($CO_2CH_2CH_3$).

Ir (KBr): 1732 (C=O), 1334 (N-oxide), 1224 (C–O–C) cm^{-1}.

Anal. Calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.59; N, 12.95. Found: C, 66.66; H, 5.59; N, 12.95.

3.1.4. General procedure for the preparation of 3-substituted quinoxaline-2-hydrazide derivatives (12–13)

To a solution of 1.28 mmol of ester derivative **10** or **11** in 5 mL of ethanol, was added 2.5 mL of hydrazine monohydrate. The reaction mixture was maintained under reflux for 3 h, when TLC indicated the end of the reaction. Then, the media was poured on ice and the resulting precipitate was filtered out affording the title compounds in 60–79% yields.

3.1.5. 3-Phenylquinoxaline-2-hydrazide (12)

The derivative (**12**) was obtained as a white solid in 75% yield.

^1H *NMR (400 MHz, DMSO-d₆):* δ 4.64 (br, $CONHNH_2$); 7.53 (m, H3′–H5′); 7.86 (m, H2′ and H6′); 7.90 (m, H6 and H7); 8.17 (m, H5 and H8); 10.00 (br, $CONHNH_2$) ppm.

^{13}C *NMR (100 MHz, DMSO-d₆):* δ 129.32 (C3′ and C5′), 129.52 (C2′ and C6′), 129.62 (C4′), 129.85 (C6), 130.40 (C5), 131.65 (C7), 132.32 (C8), 138.17 (C1′), 140.30 (C9), 141.61 (C2), 149.57 (C10), 151.58 (C3), 166.63 ($CONHNH_2$) ppm.

Ir (KBr): 3241 (N–H), 3010 (C=H), 1703 (C=O), 768 (monosubstituted phenyl) cm^{-1}.

Mass: 264 (*m*/*z*, 67%), 248 (M^+, 70%), 205 (M^+, 100%), 77 (M^+, 59%).

Anal. Calcd for $C_{15}H_{12}N_4O$: C, 68.17; H, 4.58; N, 21.20. Found: C, 68.15; H, 4.57; N, 20.21.

3.1.6. 3-Methylquinoxaline-2-hydrazide (13)

The derivative (**13**) was obtained as a brown solid in 63% yield.

^1H *NMR (400 MHz, DMSO-d₆):* δ 2.54 ($ArCH_3$), 4.59 (br, $CONHNH_2$); 7.88 (m, H6 and H7), 8.08 (m, H5 and H8); 9.95 (br, $CONHNH_2$) ppm.

^{13}C *NMR (100 MHz, DMSO-d₆):* δ 23.36 ($ArCH_3$), 129.06 (C6), 129.75 (C5), 130.74 (C7), 132.01 (C8), 139.70 (C9), 142.35 (C2), 148.58 (C10), 151.98 (C3), 165.65 ($CONHNH_2$) ppm.

Mass: 202 (*m*/*z*, 21%), 171 (M^+, 5%), 143 (M^+, 100%), 90 (M^+, 23%).

Anal. Calcd for $C_{10}H_{10}N_4O$: C, 59.40; H, 4.98; N, 27.71. Found: C, 59.39; H, 4.98; N, 27.68.

3.1.7. General procedure for the preparation of 3-phenyl (or 3-methyl)-quinoxaline-2-arylidenehydrazides (7a–o, 8a)

An equimolar amount of appropriates aromatic aldehydes was added to a solution of hydrazide derivatives **12** or **13** (1.05 mmol) in 20 mL of ethanol, in the presence of catalytic amount of hydrochloric acid. The reaction was stirred for 0.5–1.0 h, at reflux, and the solvent was evaporated under reduced pressure. The colored precipitate was collected by filtration, washed with cold water and dried under vacuum to give the desired *N*-acylhydrazone derivatives (**7a–o** and **8a**), that were purified by recrystallization in ethanol, yielding compounds **7a–o** and **8a** as a mixture of *E* and *Z* stereoisomers. in good and excellent yields. The chemical shifts of the different isomers were tentatively assigned according to previously reported by Karabatsos and coworkers.[23,24] When mentioned isomer *Z* or *E* this means that most probably this signal corresponds to the first mention isomer.

3.1.8. *(E/Z)-N′*-(2-Hydroxybenzylidene)-3-phenylquinoxaline-2-hydrazide (7a)

Derivative **7a** was obtained as a white solid by condensation of **12** with 2-hydroxybenzaldehyde in 77% yield.

^1H *NMR (400 MHz, DMSO-d₆):* δ 6.67 (dd, *J* = 7.2 Hz and 7.6 Hz, H5″, isomer *Z* or *E*), 6.74 (d, *J* = 8.0 Hz, H3″, isomer *Z* or *E*); 6.93 (dd, *J* = 8.0 Hz and 8.4 Hz, H5″, isomer *E* or *Z*), 6.94 (d, *J* = 8.0 Hz, H3″, isomer *E* or *Z*); 7.01 (d, *J* = 8.0 Hz, H6″, isomer *E* or *Z*), 7.14 (dd, *J* = 8.4 Hz and 8.0 Hz, H4″, isomer *E* or *Z*), 7.33 (dd, *J* = 7.2 Hz and 8.0 Hz, H4″, isomer *Z* or *E*), 7.53 (m, H3′–5′, isomer *Z* or *E*), 7.55 (m, H3′–5′, isomer *E* or *Z*), 7.61 (d, *J* = 7.6 Hz, H6″, isomer *Z* or *E*), 7.84 (m, H2′ and H6′, isomer *Z* and *E*), 7.97 (m, H6 and H7, isomer *Z* and *E*), 8.16 (s, N=CH, isomer *Z* or *E*), 8.18 (m, H5 and H8, isomer *Z* or *E*), 8.23 (m, H5 and H8, isomer *E* or *Z*), 8.60 (s, N=CH, isomer *E* or *Z*), 9.80 (s, ArOH), 10.88 (ArOH), 12.18 (s, CONH, isomer *Z* or *E*), 12.56 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 368 (*m*/*z*, 17%), 248 (M^+, 29%), 206 (M^+, 71%), 191 (M^+, 36%), 57 (M^+, 100%).

Anal. Calcd for $C_{22}H_{16}N_4O_2$: C, 71.73; H, 4.38; N, 15.21. Found: C, 71.75; H, 4.37; N, 15.18.

3.1.9. *(E/Z)-N′*-Benzylidene-3-phenylquinoxaline-2-hydrazide(7b)

Derivative **7b** was obtained as a white solid by condensation of **12** with benzaldehyde in 96% yield.

^1H *NMR (400 MHz, DMSO-d₆):* δ 7.20 (m, H2″ and H6″, isomer *Z* or *E*), 7.26 (m, H3″–H5″, isomer *Z* or *E*); 7.55 (m, H3′–5′, isomer *Z* and *E*), 7.54 (m, H2″ and H6″, isomer *E* or *Z*), 7.75 (m, H3″ and H5″, isomer *E* or *Z*), 7.83 (m, H2′ and H6′, isomer *Z* and *E*), 7.85 (s, N=CH, isomer *Z* or *E*), 7.99 (m, H6 and H7, isomer *Z* and *E*), 8.18 (m, H5 and H8, isomer *Z* or *E*), 8.24 (m, H5 and H8, isomer *E* or *Z*), 8.36 (s, N=CH, isomer *E* or *Z*), 12.16 (s, CONH, isomer *Z* or *E*), 12.36 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 352 (*m*/*z*, 2%), 248 (M^+, 47%), 206 (M^+, 100%), 77 (M^+, 15%).

Anal. Calcd for $C_{22}H_{16}N_4O$: C, 74.98; H, 4.58; N, 15.90. Found: C, 74.97; H, 4.57; N, 15.88.

3.1.10. *(E/Z)-Ń*-(4-Fluorobenzylidene)-3-phenylquinoxaline-2-hydrazide (7c)

Derivative **7c** was obtained as a white solid by condensation of **12** with 4-fluorobenzaldehyde in 96% yield.

^1H *NMR (400 MHz, DMSO-d₆):* δ 7.11 (t, *J* = 8.8 Hz, H3″ and H5″, isomer *Z* or *E*), 7.25 (t, *J* = 8.8 Hz, H2″ and H6″, isomer *E* or *Z*); 7.31 (d, *J* = 8.4 Hz, H3″ and H5″, isomer *Z* or *E*), 7.48 (m, H3′–5′, isomer *Z* or *E*), 7.53 (m, H3′–H5′, isomer *E* or *Z*), 7.80 (m, H2' and H6′, isomer *Z* and *E*; and H2″ and H6″, isomer *E* or *Z*), 7.85 (s, N=CH, isomer *Z* or *E*), 7.98 (m, H6 and H7, isomer *Z* and *E*), 8.21 (m, H5 and H8, isomer *Z* or *E*), 8.23 (m, H5 and H8, isomer *E* or *Z*), 8.36 (s,

N=CH, isomer *E* or *Z*), 12.17 (s, CONH, isomer *Z* or *E*), 12.38 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 370 (*m*/*z*, 1%), 248 (M^+, 51%), 205 (M^+, 100%), 77 (M^+, 13%).

Anal. Calcd for $C_{22}H_{15}N_4OF$: C, 71.34; H, 4.08; N, 15.13. Found: C, 71.35; H, 4.08; N, 15.15.

3.1.11. *(E/Z)-N′*-(4-Bromobenzylidene)-3-phenylquinoxaline-2-hydrazide (7d)

Derivative **7d** was obtained as a white solid by condensation of **12** with 4-bromobenzaldehyde in 89% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 7.13 (d, *J* = 8.4 Hz, H2″ and H6″, isomer *Z* or *E*), 7.47 (d, *J* = 8.4 Hz, H2″ and H6″, isomer *E* or *Z*); 7.48 (m, H3′–5′, isomer *Z* or *E*), 7.53 (m, H3′–H5′, isomer *E* or *Z*), 7.67 (d, *J* = 8.4 Hz, H3″ and H5″, isomer *Z* or *E*), 7.70 (d, *J* = 8.4 Hz, H3″ and H5″, isomer *E* or *Z*), 7.78 (m, H2′ and H6′, isomer *Z* or *E*), 7.82 (s, N=CH, isomer *Z* or *E*), 7.83 (m, H2′ and H6′, isomer *E* or *Z*), 7.98 (m, H6 and H7, isomer *Z* and *E*), 8.19 (m, H5 and H8, isomer *Z* or *E*), 8.23 (m, H5 and H8, isomer *E* or *Z*), 8.34 (s, N=CH, isomer *E* or *Z*), 12.22 (s, CONH, isomer *Z* or *E*), 12.44 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 431 (*m*/*z*, 2%), 248 (M^+, 59%), 205 (M^+, 100%), 77 (M^+, 35%).

Anal. Calcd for $C_{22}H_{15}N_4OBr$: C, 61.27; H, 3.51; N, 12.99. Found: C, 61.30; H, 3.52; N, 12.96.

3.1.12. *(E/Z)-N′*-(4-Chlorobenzylidene)-3-phenylquinoxaline-2-hydrazide (7e)

Derivative **7e** was obtained as a white solid by condensation of **12** with 4-chlorobenzaldehyde in 96% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 7.21 (d, *J* = 8.4 Hz, H3″ and H5″, isomer *Z* or *E*), 7.34 (d, *J* = 8.4 Hz, H3″ and H5″, isomer *E* or *Z*); 7.48 (m, H3′–5′, isomer *Z* or *E*), 7.54 (m, H3′–H5′, isomer *E* or *Z*; H2′ and H6′, isomer *Z* or *E*), 7.77 (d, *J* = 8.4 Hz, H2″ and H6″, isomer *Z* or *E*), 7.79 (m, H2′ and H6′, isomer *E* or *Z*), 7.82 (d, *J* = 8.4 Hz, H2″ and H6″, isomer *E* or *Z*), 7.84 (s, N=CH, isomer *Z* or *E*), 7.99 (m, H6 and H7, isomer *Z* and *E*), 8.19 (m, H5 and H8, isomer *Z* or *E*), 8.23 (m, H5 and H8, isomer *E* or *Z*), 8.36 (s, N=CH, isomer *E* or *Z*), 12.22 (s, CONH, isomer *Z* or *E*), 12.43 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 386 (*m*/*z*, 2%), 248 (M^+, 51%), 234 (M^+, 23%), 205 (M^+, 100%), 77 (M^+, 10%).

Anal. Calcd for $C_{22}H_{15}N_4OCl$: C, 68.31; H, 3.91; N, 14.48. Found: C, 68.30; H, 3.88; N, 14.46.

3.1.13. *(E/Z)-N′*-(2-Chlorobenzylidene)-3-phenylquinoxaline-2-hydrazide (7f)

Derivative **7f** was obtained as a white solid by condensation of **4** with 2-chlorobenzaldehyde in 89% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ *7.12 (d, J* = 8.0 Hz, H3″, isomer *Z* or *E*), 7.20 (dd, *J* = 7.6 Hz and 7.2 Hz, H5″, isomer *Z* or *E*); 7.31 (dd, *J* = 8.0 Hz and 7.6 Hz, H4″, isomer *Z* or *E*), 7.41 (d, *J* = 8.0 Hz, H3″, isomer *E* or *Z*), 7.45 (dd, *J* = 7.6 Hz and 8.0 Hz, H5″, isomer *E* or *Z*); 7.49 (m, H3′–5′, isomer *Z* or *E*), 7.55 (m, H3′–H5′, isomer *E* or *Z*; H4″, isomer *E* or *Z*), 7.84 (m, H2′ and H6′, isomer *Z* and *E*), 7.94 (d, *J* = 7.2 Hz, H6″, isomer *Z* or *E*), 7.96 (m, H6 and H7, isomer *Z* and *E*), 8.19 (d, *J* = 7.2 Hz, H6″, isomer *E* or *Z*), 8.25 (s, N=CH, isomer *Z* or *E*), 8.26 (m, H5 and H8, isomer *Z* and *E*), 8.79 (s, N=CH, isomer *E* or *Z*), 12.34 (s, CONH, isomer *Z* or *E*), 12.64 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 386 (*m*/*z*, 1%), 248 (M^+, 46%), 234 (M^+, 20%), 206 (M^+, 100%), 77 (M^+, 19%).

Anal. Calcd for $C_{22}H_{15}N_4OCl$: C, 68.31; H, 3.91; N, 14.48. Found: C, 68.29; H, 3.89; N, 14.48.

3.1.14. *(E/Z)-N′*-(3-Chlorobenzylidene)-3-phenylquinoxaline-2-hydrazide (7g)

Derivative **7g** was obtained as a white solid by condensation of **12** with 3-chlorobenzaldehyde in quantitative yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 7.15 (d, *J* = 7.6 Hz, H4″, isomer *Z* or *E*), 7.22 (s, H6″, isomer *Z* or *E*), 7.30 (dd, *J* = 7.6 Hz and 8.0 Hz, H3″, isomer *Z* or *E*); 7.35 (d, *J* = 8.0 Hz, H4″, isomer *Z* or *E*), 7.49 (m, H3′–5′, isomer *Z* or *E*; H6″, isomer *E* or *Z*), 7.53 (m, H3′–H5′, isomer *E* or *Z*; H3″, isomer *E* or *Z*), 7.72 (d, *J* = 8.0 Hz, H2″, isomer *Z* or *E*), 7.80 (m, H2′ and H6′, isomer *Z* or *E*), 7.82 (m, H2′ and H6′, isomer *E* or *Z*, H2″, isomer *E* or *Z*), 7.83 (s, N=CH, isomer *Z* or *E*), 7.98 (m, H6 and H7, isomer *Z* and *E*), 8.20 (m, H5 and H8, isomer *Z* or *E*), 8.24 (m, H5 and H8, isomer *E* or *Z*), 8.37 (s, N=CH, isomer *E* or *Z*), 12.29 (s, CONH, isomer *Z* or *E*), 12.49 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 386 (*m*/*z*, 2%), 248 (M^+, 44%), 234 (M^+, 21%), 205 (M^+, 100%), 77 (M^+, 17%).

Anal. Calcd for $C_{22}H_{15}N_4OCl$: C, 68.31; H, 3.91; N, 14.48. Found: C, 68.31; H, 3.90; N, 14.48.

3.1.15. *(E/Z)-N′*-(4-Hydroxybenzylidene)-3-phenylquinoxaline-2-hydrazide (7h)

Derivative **7h** was obtained as a white solid by condensation of **12** with 4-hydroxybenzaldehyde in 90% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 6.63 (d, *J* = 8.4 Hz, H3″ and H5″, isomer *Z* or *E*), 6.84 (d, *J* = 8.4 Hz, H3″ and H5″, isomer *E* or *Z*); 7.02 (d, *J* = 8.4 Hz, H2″ and H6″, isomer *Z* or *E*), 7.45 (m, H3′–5′, isomer *Z* or *E*), 7.51 (m, H3′–H5′, isomer *E* or *Z*), 7.59 (d, *J* = 8.4 Hz, H2″ and H6″, isomer *E* or *Z*), 7.74 (s, N=CH, isomer *Z* or *E*), 7.79 (m, H2′ and H6′, isomer *Z* and *E*), 7.94 (m, H6 and H7, isomer *Z* and *E*), 8.15 (m, H5 and H8, isomer *Z* or *E*), 8.18 (m, H5 and H8, isomer *E* or *Z*), 8.20 (s, N=CH, isomer *E* or *Z*), 11.92 (s, CONH, isomer *Z* or *E*), 12.20 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 368 (*m*/*z*, 25%), 191 (M^+, 51%), 108 (M^+, 87%), 57 (M^+, 100%).

Anal. Calcd for $C_{22}H_{16}N_4O_2$: C, 71.73; H, 4.38; N, 15.21. Found: C, 71.70; H, 4.40; N, 15.19.

3.1.16. *(E/Z)-N′*-(4-Dimethylaminobenzylidene)-3-phenylquinoxaline-2-hydrazide(7i)

Derivative **7i** was obtained as a yellow solid by condensation of **12** with 4-dimethylaminobenzaldehyde in 84% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ *2.85 (s, N(CH*$_3$*)*$_2$, isomer *Z* or *E*), 2.97 (s, $N(CH_3)_2$, isomer *E* or *Z*), 6.54 (d, *J* = 8.8 Hz, H3″ and H5″, isomer *Z* or *E*), 6.75 (d, *J* = 8.8 Hz, H3″ and H5″, isomer *E* or *Z*); 7.00 (d, *J* = 8.8 Hz, H2″ and H6″, isomer *Z* or *E*), 7.48 (m, H3′–5′, isomer *Z* or *E*), 7.53 (m, H3′–H5′, isomer *E* or *Z*), 7.55 (d, *J* = 8.4 Hz, H2″ and H6″, isomer *E* or *Z*), 7.70 (s, N=CH, isomer *Z* or *E*), 7.81 (m, H2′ and H6′, isomer *Z* or *E*), 7.84 (m, H2′ and H6′, isomer *E* or *Z*), 7.98 (m, H6 and H7, isomer *Z* and *E*), 8.18 (m, H5 and H8, isomer *Z* or *E*), 8.19 (s, N=CH, isomer *E* or *Z*), 8.22 (m, H5 and H8, isomer *E* or *Z*), 11.86 (s, CONH, isomer *Z* or *E*), 12.05 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 395 (*m*/*z*, 67%), 248 (M^+, 22%), 205 (M^+, 87%), 146 (M^+, 100%), 133 (M^+, 87%), 77 (M^+, 48%).

Anal. Calcd for $C_{24}H_{21}N_5O$: C, 72.89; H, 5.35; N, 17.71. Found: C, 72.90; H, 5.35; N, 17.68.

3.1.17. *(E/Z)-N′*-(4-Nitrobenzylidene)-3-phenylquinoxaline-2-hydrazide (7j)

Derivative **7j** was obtained as a yellow solid by condensation of **12** with 4-nitrobenzaldehyde in 93% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 7.46 (m, H3′–5′, isomer *Z* or *E*; H2″ and H6″, isomer *Z* or *E*), 7.54 (m, H3′–H5′, isomer *E* or *Z*), 7.81 (m, H2′ and H6′, isomer *Z* or *E*), 7.83 (m, H2′ and H6′, isomer *E* or *Z*); 7.97 (s, N=CH, isomer *Z* or *E*), 7.99 (m, H2′ and H6′, isomer *E* and *Z*), 8.02 (d, *J* = 8.8 Hz, H2″ and H6″, isomer *E* or *Z*), 8.13 (d, *J* = 8.8 Hz, H3″ and H5″, isomer *Z* or *E*), 8.20 (m, H5 and H8, isomer *Z* or *E*), 8.24 (m, H5 and H8, isomer *E* or *Z*), 8.32 (d, *J* = 8.8 Hz, H3″ and H5″, isomer *E* or *Z*), 8.49 (s, N=CH, isomer *E* or *Z*), 12.46 (s, CONH, isomer *Z* or *E*), 12.67 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 397 (*m*/*z*, 2%), 248 (M^+, 31%), 205 (M^+, 100%), 77 (M^+, 26%).

Anal. Calcd for $C_{22}H_{15}N_5O_3$: C, 66.49; H, 3.80; N, 17.62. Found: C, 66.50; H, 3.82; N, 17.65.

3.1.18. *(E/Z)-N′*-(4-Isopropylbenzylidene)-3-phenylquinoxaline-2-hydrazide (7k)

Derivative **7k** was obtained as a white solid by condensation of **12** with 4-isopropylbenzaldehyde in 84% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 1.11 (d, J = 6.8 Hz, RCH(CH_3)$_2$, isomer *Z* and *E*), 1.22 (d, J = 6.8 Hz, RCH(CH_3)$_2$, isomer *E* and *Z*), 2.80 (hp, J = 6.8 Hz, RCH(CH_3)$_2$, isomer *Z* and *E*), 2.93 (hp, J = 6.8 Hz, RC*H*(CH_3)$_2$, isomer *E* and *Z*), 7.11 (d, J = 8.4 Hz, H3″ and H5″, isomer *Z* or *E*), 7.14 (d, J = 8.4 Hz, H2″ and H6″, isomer *Z* or *E*); 7.35 (d, J = 8.0 Hz, H3″ and H5″, isomer *E* or *Z*), 7.49 (m, H3′–5′, isomer *Z* or *E*), 7.54 (m, H3′–H5′, isomer *E* or *Z*), 7.67 (d, J = 8.0 Hz, H2″ and H6″, isomer *E* or *Z*), 7.82 (m, H2′ and H6′, isomer *Z* and *E*), 7.83 (s, N=CH, isomer *Z* or *E*), 7.98 (m, H6 and H7, isomer *Z* and *E*), 8.19 (m, H5 and H8, isomer *Z* or *E*), 8.23 (m, H5 and H8, isomer *E* or *Z*), 8.32 (s, N=CH, isomer *E* or *Z*), 12.11 (s, CONH, isomer *Z* or *E*), 12.29 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 394 (*m*/*z*, 1%), 239 (M^+, 57%), 206 (M^+, 100%), 77 (M^+, 34%).

Anal. Calcd for $C_{25}H_{22}N_4O$: C, 76.12; H, 5.62; N, 14.20. Found: C, 76.13; H, 5.65; N, 14.23.

3.1.19. *(E/Z)-N′*-(Pyridin-4-ylmethylene)-3-phenylquinoxaline-2-hydrazide (7l)

Derivative **7l** was obtained as a white solid by condensation of **12** with pyridine-4-carboxaldehyde in 90% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 7.11 (d, J = 5.6 Hz, H2″ and H6″, isomer *Z* or *E*), 7.47 (m, H3′–5′, isomer *Z* or *E*), 7.52 (m, H3′–H5′, isomer *E* or *Z*), 7.69 (d, J = 5.6 Hz, H2″ and H6″, isomer *E* or *Z*), 7.78 (m, H2′ and H6′, isomer *Z* or *E*), 7.82 (m, H2′ and H6′, isomer *E* or *Z*), 7.83 (s, N=CH, isomer *Z* or *E*), 8.0 (m, H6 and H7, isomer *Z* and *E*), 8.20 (m, H5 and H8, isomer *Z* or *E*), 8.23 (m, H5 and H8, isomer *E* or *Z*), 8.37 (s, N=CH, isomer *E* or *Z*), 8.45 (d, J = 5.6 Hz, H3″ and H5″, isomer *Z* or *E*), 8.66 (d, J = 5.6 Hz, H3″ and H5″, isomer *E* or *Z*), 12.65 (br, CONH, isomer *Z* or *E*), 12.68 (br, CONH, isomer *E* or *Z*) ppm.

Mass: 353 (*m*/*z*, 1%), 248 (M^+, 29%), 234 (M^+, 37%), 206 (M^+, 100%), 77 (M^+, 17%).

Anal. Calcd for $C_{21}H_{15}N_5O$: C, 71.38; H, 4.28; N, 19.82. Found: C, 71.35; H, 4.31; N, 19.78.

3.1.20. *(E/Z)-N′*-(Pyridin-2-ylmethylene)-3-phenylquinoxaline-2-hydrazide (7m)

Derivative **7m** was obtained as a beige solid by condensation of **12** with pyridine-2-carboxaldehyde in 75% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 7.10 (d, J = 7.6 Hz, H6″, isomer *Z* or *E*), 7.28 (dd, J = 6.4 Hz and 6.0 Hz, H4″, isomer *Z* or *E*), 7.48 (m, H3′–5′, isomer *Z* or *E*; H6″, isomer *E* or *Z*), 7.54 (m, H3′–H5′, isomer *E* or *Z*), 7.65 (dd, J = 7.6 Hz and 8.0 Hz, H4″, isomer *E* or *Z*), 7.78 (m, H2′ and H6′, isomer *Z* or *E*), 7.82 (m, H2′ and H6′, isomer *E* or *Z*), 7.90 (s, N=CH, isomer *Z* or *E*), 7.92 (m, H5″, isomer *Z* and *E*), 7.99 (m, H6 and H7, isomer *Z* and *E*), 8.20 (d, J = 4.0 Hz, H3″, isomer *Z* or *E*), 8.22 (m, H5 and H8, isomer *Z* and *E*), 8.41 (s, N=CH, isomer *E* or *Z*), 8.47 (d, J = 4.0 Hz, H3″, isomer *E* or *Z*), 12.40 (s, CONH, isomer *Z* or *E*), 12.70 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 353 (*m*/*z*, 1%), 248 (M^+, 34%), 234 (M^+, 29%), 206 (M^+, 100%), 77 (M^+, 15%).

Anal. Calcd for $C_{21}H_{15}N_5O$: C, 71.38; H, 4.28; N, 19.82. Found: C, 71.37; H, 4.28; N, 19.80.

3.1.21. *(E/Z)-N′*-(Furfurylidene)-3-phenylquinoxaline-2-hydrazide (7n)

Derivative **7n** was obtained as a beige solid by condensation of **12** with 2-furaldehyde in 89% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 6.47 (t, J = 4.0 Hz, H4″, isomer *Z* or *E*), 6.66 (t, J = 4.0 Hz, H4″, isomer *E* or *Z*), 6.67 (d, J = 4.4 Hz, H5″, isomer *Z* or *E*), 6.98 (d, J = 3.6 Hz, H3″, isomer *Z* or *E*), 7.49 (m, H3′–H5′, isomer *Z* or *E*), 7.53 (m, H3′–H5′, isomer *E* or *Z*), 7.62 (d, J = 4.4 Hz, H5″, isomer *E* or *Z*), 7.76 (s, N=CH, isomer *Z* or *E*), 7.78 (m, H2′ and H6′, isomer *Z* and *E*), 7.83 (m, H2′ and H6′, isomer *E* and *Z*), 7.91 (d, J = 3.6 Hz, H3″, isomer *E* or *Z*), 7.97 (m, H6 and H7, isomer *Z* and *E*), 8.21 (m, H5 and H8, isomer *Z* or *E*), 8.23 (m, H5 and H8, isomer *E* or *Z*), 8.24 (s, N=CH, isomer *E* or *Z*), 12.10 (s, CONH, isomer *Z* or *E*), 12.34 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 342 (*m*/*z*, 1%), 248 (M^+, 44%), 205 (M^+, 100%), 77 (M^+, 29%).

Anal. Calcd for $C_{20}H_{14}N_4O_2$: C, 70.17; H, 4.12; N, 16.37. Found: C, 70.19; H, 4.10; N, 16.38.

3.1.22. *(E/Z)-N′*-(2-Thenylidene)-3-phenylquinoxaline-2-hydrazide (7o)

Derivative **7o** was obtained as a beige solid by condensation of **12** with thiophene-2-carboxaldehyde in 92% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 6.97 (t, J = 4.0 Hz, H4″, isomer *Z* or *E*), 7.16 (t, J = 4.0 Hz, H4″, isomer *E* or *Z*), 7.23 (d, J = 3.2 Hz, H5″, isomer *Z* or *E*), 7.41 (d, J = 4.8 Hz, H3″, isomer *Z* or *E*), 7.48 (m, H3′–H5′, isomer *Z* or *E*), 7.50 (d, J = 3.2 Hz, H5″, isomer *E* or *Z*), 7.53 (m, H3′–H5′, isomer *E* or *Z*), 7.71 (d, J = 4.8 Hz, H3″, isomer *E* or *Z*), 7.81 (m, H2′ and H6′, isomer *Z* and *E*), 7.94 (m, H6 and H7, isomer *Z* or *E*), 7.96 (m, H6 and H7, isomer *E* or *Z*), 7.98 (s, N=CH, isomer *Z* or *E*), 8.19 (m, H5 and H8, isomer *Z* or *E*), 8.22 (m, H5 and H8, isomer *E* or *Z*), 8.57 (s, N=CH, isomer *E* or *Z*), 12.11 (s, CONH, isomer *Z* or *E*), 12.34 (s, CONH, isomer *E* or *Z*) ppm.

Mass: 358 (*m*/*z*, 2%), 248 (M^+, 19%), 206 (M^+, 100%), 77 (M^+, 23%).

Anal. Calcd for $C_{20}H_{14}N_4OS$: C, 67.02; H, 3.94; N, 15.63. Found: C, 67.03; H, 3.95; N, 15.65.

3.1.23. *(E/Z)-N′*-(2-Hydroxybenzylidene)-3-methylquinoxaline-2-hydrazide (8a)

Derivative **8a** was obtained as a yellow solid by condensation of **13** with 2-hydroxybenzaldehyde in 90% yield.

^1H *NMR (400 MHz, DMSO-d_6):* δ 2.72 (s, ArCH_3, isomer *Z* or *E*), 2.89 (s, Ar*C*H_3, isomer *E* or *Z*), 6.68 (dd, J = 7.2 Hz and 7.6 Hz, H5″, isomer *Z* or *E*), 6.75 (d, J = 8.0 Hz, H3″, isomer *Z* or *E*); 6.94 (dd, J = 7.2 Hz and 6.8 Hz, H5″, isomer *E* or *Z*), 6.95 (d, J = 8.8 Hz, H3″, isomer *E* or *Z*); 7.05 (d, J = 7.6 Hz, H6″, isomer *Z* or *E*), 7.14 (dd, J = 7.2 Hz and 7.6 Hz, H4″, isomer *Z* or *E*), 7.32 (dd, J = 7.2 Hz and 8.8 Hz, H4″, isomer *E* or *Z*), 7.58 (d, J = 6.8 Hz, H6″, isomer *E* or *Z*), 7.93 (m, H6 and H7, isomer *Z* or *E*), 8.08 (d, J = 7.6 Hz, H5, isomer *Z* and *E*), 8.18 (d, J = 7.6 Hz, H8, isomer *Z* and *E*), 8.4 (s, N=CH, isomer *Z* or *E*), 8.74 (s, N=CH, isomer *E* or *Z*), 11.07 (s, ArOH, isomer *Z* or *E*), 11.16 (ArOH, isomer *E* or *Z*), 12.39 (s, CONH, isomer *Z* or *E*), 12.51 (s, CONH, isomer *E* or *Z*) ppm.

Anal. Calcd for $C_{17}H_{14}N_4O_2$: C, 66.66; H, 4.61; N, 18.29. Found: C, 66.67; H, 4.59; N, 18.32.

3.2. Trypanocidal activities

Epimastigote forms of *Trypanosoma cruzi*, Tulahuen 2 strain, were grown in axenic BHI-Tryptose media complemented with 5% fetal bovine serum. The compounds dissolved in DMSO were incorporated into the media at 25 μM and their ability to inhibit the parasite growth was evaluated in comparison to the control (no drug added). No effect on epimastigotes growth was observed by the presence of up to 1% DMSO in the culture media. Nifurtimox (Nfx) was used as the reference trypanocidal drug. Growth of the parasite was followed by measuring the increase in absorbance at 600 nm, which had previously been shown to be proportional to the number of cells present. The percentage of inhibition was calculated as follows: $\% = \{1 - [(A_p - A_{0p})/(A_c - A_{0c})]\} \times 100$, where A_p is A_{600} of the culture containing the drug at day 5; A_{0p} is A_{600} of

the culture containing the drug right after addition of the inocula (day 0); A_c is A_{600} of the culture in the absence of any drug (control) at day 5; A_{0c} is A_{600} in the absence of the drug at day 0. The IC_{50} (50% inhibitory concentration) was determined only for compounds presenting high trypanocidal activity at the screening concentration (25 μM).[27]

3.3. Cytotoxicity to mice macrophages

J-774 mouse macrophages were seeded (100.000 cells/well) in 96 well flat bottom microplates (Nunclon) with 200 μL of RPMI 1640 medium supplemented with 20% heat inactivated foetal calf serum. Cells were allowed to attach for 48 h in a humidified 5% CO_2/95% air atmosphere at 37 °C. Then, cells were exposed to derivatives **7a** and **8a** compounds (100, 200, and 400 μM) for 48 h. Afterwards, the cells were washed with PBS and incubated (37 °C) with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma) 0.4 mg/mL for 3 h. Then, formazan was dissolved with DMSO (180 μL) and optical densities were measured. Each concentration was assayed three times and six growth controls were used in each test. Cytotoxicity percentages (%*C*) were determined as follows: $\%C = [100 - (ODd - ODdm)/(ODc - ODcm)] \times 100$, where ODd is the mean of OD_{595} of wells with macrophages and different concentrations of the compounds; ODdm is the mean of OD_{595} of wells with different compounds concentration in the medium; ODc is the growth control and ODcm is the mean of OD_{595} of wells with medium only.[28,29]

3.4. Docking studies

3.4.1. General procedures

All calculations have been performed on a PC running under Linux Red Hat Enterprise version 3.0 platform. Structural manipulations were performed using SYBYL 7.3.[38]

3.4.2. Preparation of the ligands

The preparation of the ligands for FLExE was performed as for FLExX,[39] using SYBYL version 7.3. The ligands coordinates have been generated using the program Sketcher, available in SYBYL version 7.3. Next, the correct atom types (including hybridization states) and correct bond types were defined, hydrogen atoms were added and charges were assigned to each atom. The structures have been modeled in their neutral states. Finally, the structures were energy-minimized, using the semiempirical AM1 method[25] available in the PC SPARTAN´04 software.[25] After this procedure, MMFF94[38] point charges were assigned to the ligands.

3.4.3. Selection of protein crystal structures

Ligand-bound crystallographic structures of cruzain are available in the Protein Data Bank.[40] In this study, 1EWL, 1F2C, 1ME3, and 1ME4 were evaluated and selected for docking.[30–32] The active recognition site of the ensemble has been defined as the collection of residues within 10.0 Å of the bound inhibitor and comprised the union of all ligands of the ensemble. All atoms located less than 10.0 Å from any ligand atom were considered. 1ME4 was used as a reference structure for the united protein preparation. All carboxylic acid and amino groups were modeled in their ionized forms. Proteins were prepared for the docking studies using the Biopolymer module of SYBYL 7.3. Amber7 FF99[38] charges were attributed to the protein atoms.

Next, Biopolymer protein analysis tool was used, in a stepwise process of analysis and correction of geometry parameters. For each structure, the description of an ensemble contains the definition of the protein atoms (via chain identifiers and hetero groups), the resolution of ambiguities in the PDB file (alternate location indicators, etc.), the location of hydrogen atoms at hetero atoms, and the definition of the active site atoms. The assignment of hydrogen positions has been made on the basis of default rules except for the definition of the hydrogen positions inside the histidine side-chain. The side-chains of lysine, arginine and the carboxylate groups of aspartic and glutamic acid have been modeled in their ionized states. Water molecules contained in the PDB file have been removed.

3.5. Lipinski's rule of five

CLOGP, the log of the octanol/water partition coefficient, was calculated with the Clog*p* software[41,42]; Molecular Weight calculations have been performed with the MOLPROP facility, all available in SYBYL 7.3.[38]

Acknowledgements

The authors wish to thank financial support from IM-INOFAR (CNPq-BR #420.015/05-1), PROSUL (CNPq-BR # 490.600/2007-8), FAPERJ (BR), and the 'Coordenação de Aperfeiçoamento de Pessoal de Nível Superior' (CAPES; BR) for the fellowship (to L.M.L.; BEX0520/04-7) received.

References and notes

1. de Souza, W. *Microbes and Infection* **2007**, *9*, 544–545.
2. World Bank, World Development Reports: Investing in Health; Oxford University Press, New York, 1993.
3. Briones, M. R. S.; Souto, R. P.; Stolf, B.; Zingales, B. *Mol. Biochem. Parasitol.* **1999**, *104*, 219–232.
4. Camandaroba, E. L.; Pinheiro Lima, C. M.; Andrade, S. G. *Rev. Inst. Med. Trop. São Paulo* **2002**, *44*, 97–103.
5. (a) Coura, J. T.; Junqueira, A. C.; Fernandes, O.; Valente, S. A.; Miles, M. A. *Trends Parasitol.* **2002**, *18*, 171–176; (b) Xavier, S. S. C.; Vaz, V. C.; D'Andrea, P. S.; Herrera, L.; Emperaire, L.; Alves, J. R.; Fernandes, O.; Ferreira, L. F.; Jansen, A. M. *Parasitol. Int.* **2007**, *56*, 119–128.
6. Otto, H. H.; Schirmeister, T. *Chem. Rev.* **1997**, *97*, 133–171.
7. Sajid, M.; McKerrow, J. H. *Mol. Biochem. Parasitol.* **2002**, *120*, 1–21.
8. (a) Cazzulo, J. J.; Stoka, V.; Turk, V. *Curr. Pharm. Des.* **2001**, *7*, 1143–1156; (b) Eakin, A. E.; Mills, A. A.; Harth, G.; McKerrow, J. H.; Craik, C. S. *J. Biol. Chem.* **1993**, *267*, 7411–7420.
9. (a) Bonaldo, M. C.; D'Escoffier, L. N.; Salles, J. M.; Goldenberg, S. *Exp. Parasitol.* **1991**, *73*, 44–51; (b) de Cazzulo, B. M. F.; Martínez, J.; North, M. J.; Coombs, G. H.; Cazzulo, J. J. *FEMS Microbiol. Lett.* **1994**, *124*, 81–86; (c) Cazzulo, J. J.; Stoka, V.; Turk, V. *Curr. Pharm. Des.* **2001**, *7*, 1143–1156; (d) Tomas, A. M.; Miles, M. A.; Kelly, J. M. *Eur. J. Biochem.* **1997**, *244*, 596–603; (e) Engel, J. C.; García, C. T.; Hsieh, I.; Doyle, P. S.; McKerrow, J. H. *J. Cell Sci.* **2000**, *113*, 1345–1354; (f) Caffrey, C. R.; Scory, S.; Steverding, D. *Curr. Drug Targets* **2000**, *1*, 155–162; (g) Klemba, M.; Goldberg, D. E. *Ann. Rev. Biochem.* **2002**, *71*, 275–305.
10. Li, R.; Chen, X.; Gong, B.; Selzer, P. M.; Li, Z.; Davidson, E.; Kurzban, G.; Miller, R. E.; Nuzum, E. O.; McKerrow, J. H.; Fletterick, R. J.; Gillmor, S. A.; Craik, C. S.; Kuntz, I. D.; Cohen, F. E.; Kenyon, G. L. *Bioorg. Med. Chem.* **1996**, *4*, 1421–1427.
11. Huang, L. H.; Brinen, L. S.; Ellman, J. A. *Bioorg. Med. Chem.* **2003**, *11*, 21–29.
12. Urbina, J. A.; Docampo, R. *Trends Parasitol.* **2003**, *19*, 495–501.
13. Choe, Y.; Brinen, L. S.; Price, M. S.; Engel, J. C.; Lange, M.; Grisostomi, C.; Weston, S. G.; Pallai, P. V.; Cheng, H.; Hardy, L. W.; Hartsough, D. S.; McMakin, M.; Tilton, R. F.; Baldino, C. M.; Craik, C. S. *Bioorg. Med. Chem.* **2005**, *13*, 2141–2156.
14. Fujii, N.; Mallari, J. P.; Hansell, E. J.; Mackey, Z.; Doyle, P.; Zhou, Y. M.; Gut, J.; Rosenthal, P. J.; McKerrow, J. H.; Guy, K. *Bioorg. Med. Chem.* **2005**, *15*, 121–123.
15. Siles, R.; Chen, S.-E.; Zhou, M.; Pinney, K. G.; Trawick, M. L. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 4405–4409.
16. Duarte, C. D.; Barreiro, E. J.; Fraga, C. A. M. *Mini Rev. Med. Chem.* **2007**, *7*, 1108–1119.
17. Caffrey, C. R.; Schanz, M.; Nkemgu-Njinkeng, J.; Brush, M.; Hansell, E.; Cohen, F. E.; Flaherty, T. M.; McKerrow, J. H.; Steverding, D. *Int. J. Antimicrob. Agents* **2002**, *19*, 227–231.
18. Maccari, R.; Ottanà, R.; Vigorita, M. G. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2509–2513.
19. Lima, L. M.; Barreiro, E. J. *Curr. Med. Chem.* **2005**, *12*, 23–49.
20. (a) Aguirre, G.; Cerecetto, H.; Di Maio, R.; González, M.; Montoya Alfaro, M. E.; Jaso, A.; Zarranz, B.; Ortega, M. A.; Aldana, I.; Monge-Vega, A. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3835–3839; (b) Monge, A.; Palop, J. A.; de Ceráin, A. D.; Senador, V.; Martínez-Crespo, F. J.; Sainz, Y.; Narro, S.; García, E.; de Miguel, C.; González, M.; Hamilton, E.; Barker, A. J.; Clarke, E. D.; Greenhow, D. T. *J. Med. Chem.* **1995**, *38*, 1786–1792; (c) Urquiola, C.; Vieites, M.; Aguirre, G.; Marin, A.; Solano, B.;

Arrambide, G.; Noblía, P.; Lavaggi, M. L.; Torre, M. H.; González, M.; Monge, A.; Gambino, D.; Cerecetto, H. *Bioorg. Med. Chem.* **2006**, *14*, 5503–5509.
21. (a) Jaffé, H. H. *Chem. Rev.* **1953**, *53*, 191–261; (b) Hammett, L. P. *Physical Organic Chemistry*, 2nd ed.; McGraw Hill: New York, 1970; (c) Topliss, J. G. *J. Med. Chem.* **1977**, *20*, 463–469; (d) Hansch, C.; Leo, A. J. *Substituent Constants for Correlation Analysis in Chemistry and Biology*; Wiley: New York, 1979.
22. Lima, L. M.; Zarranz, B.; Marin, A.; Solano, B.; Vicente, E.; Pérez-Silanes, S.; Aldana, I.; Monge, A. *J. Heterocycl. Chem.* **2005**, *42*, 1381–1385.
23. Karabatsos, G. L.; Graham, J. D.; Vane, F. M. *J. Am. Chem. Soc.* **1962**, *84*, 753–755.
24. Karabatsos, G. L.; Taller, R. A. *J. Am. Chem. Soc.* **1963**, *85*, 3624–3629.
25. Dewar, M. J. S.; Zoebisch, E. G.; Healy, E. F.; Stewart, J. J. P. *J. Am. Chem. Soc.* **1985**, *107*, 3902–3909.
26. SPARTAN 04, Wavefunction, Inc. 18401 Von Karman Avenue, Suite 370. Irvine, California 92612, USA.
27. Porcal, W.; Hernandez, P.; Boiani, M.; Aguirre, G.; Boiani, L.; Chidichimo, A.; Cazzulo, J. J.; Campillo, N. E.; Páez, J. A.; Castro, A.; Krauth-Siegel, R. L.; Davies, C.; Basombrío, M. A.; González, M.; Cerecetto, H. *J. Med. Chem.* **2007**, *50*, 6004–60015.
28. Muelas, S.; Di Maio, R.; Cerecetto, H.; Seoane, G.; Ochoa, C.; Escario, J. A.; Gómez-Barrio, A. *Folia Parasitol.* **2001**, *48*, 105–108.
29. Caterina, M. C.; Perillo, I. A.; Boiani, L.; Pezaroglo, H.; Cerecetto, H.; González, M.; Salerno, A. *Bioorg. Med. Chem.* **2008**, *16*, 2226–2234.
30. Ifa, D. R.; Rodrigues, C. R.; de Alencastro, R. B.; Fraga, C. A. M.; Barreiro, E. J. *J. Mol. Struct. (Theochem)* **2000**, *505*, 11–17.
31. http://www.rcsb.org/pdb/explore.do?structureId=1EWL.
32. Brinen, L. S.; Hansell, E.; Cheng, J.; Roush, W. R.; McKerrow, J. H.; Fletterick, R. J. *Struct. Fold. Des.* **2000**, *8*, 831–840.
33. Huang, L.; Brinen, L. S.; Ellman, J. A. *Bioorg. Med. Chem.* **2003**, *11*, 21–29.
34. Clauβen, H.; Buning, C.; Rarey, M.; Lengauer, T. *J. Mol. Biol.* **2001**, *308*, 377–395.
35. Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490–519.
36. Kosec, G.; Alvarez, V.; Cazzulo, J. J. *Biocell* **2006**, *30*, 479–490.
37. Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. *Adv. Drug Delivery Rev.* **1997**, *23*, 3–25.
38. SYBYL, Version 7.3. Tripos Associates: St. Louis, MO, 2007.
39. Rarey, M.; Kramer, B.; Lengauer, T.; Klebe, G. A. *J. Mol. Biol.* **1996**, *261*, 470–489.
40. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E. *Nucleic Acids Res.* **2000**, *28*, 35–242.
41. Case, D. A.; Pearlman, D. A.; Caldwell, J. W.; Cheatham, T. E.; Wang, J.; Ross, W. S.; Simmerling, C. L.; Darden, T. A.; Merz, K. M.; Stanton, R. V.; Cheung, A. I.; Vincent, J. J.; Crowley, M.; Tsui, V.; Gohike, H.; Radmer, R. J.; Duan, Y.; Pitera, J.; Massova, I.; Seibel, G. L.; Singh, U. C.; Weiner, P. K.; Kollman, P. A. *Amber 7*; University of California: San Francisco, 2002.
42. Chow, J.; Jurs, P. *J. Chem. Inf. Comput. Sci.* **1979**, *19*, 172–178.