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Article

Synthetic ligustrazine based cyclohexanone and oxime analogs as Anti-*Trypanosoma* and Anti-*Leishmanial* agents

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In the present study a series of 34 synthetic ligustrazine-containing α, β-Unsaturated carbonyl-based compounds and oximes, recognized as anticancer compounds were assessed against protozoa of the *Trypanosoma* and *Leishmania* species. Ligustrazine, chemically known as tetramethylpyrazine (TMP), was selected as the core moiety for the synthesis of α, β-Unsaturated carbonyl-based compounds and these compounds were selected as precursors for the synthesis of new oximes. Some derivates, including **5f** and **6i**, showed multiple activities against all tested strains. In particular compounds **5f** and **8o** are the most potent and they are, therefore, potential candidates for trypanosomiasis and leishmaniasis.

Keywords: Protozoan parasites. Sleeping sickness. α,β-Unsaturated carbonyl-based compounds; toxicity. Organic synthesis.

INTRODUCTION

Leishmaniasis and trypanosomiasis are among the most neglected diseases of animals and humans caused by kinetoplastid protozoa, such as genera *Leishmania* and *Trypanosoma* (Barrett, 2000; Olliaro *et al.*, 2002). Sleeping sickness, commonly called human African trypanosomiasis (HAT), is a medical condition caused by protozoa of the genus *Trypanosoma* (Barrett *et al.*, 2003; Büscher *et al.*, 2017). Sleeping sickness is spread by the tsetse fly, and caused by two subspecies of *Trypanosoma brucei brucei*, *T. b. gambiense* and *T. b. rhodesiense* (Büscher *et al.*, 2017). There are two stages of disease. Stage 1, commonly called hemolymphatic stage, involves the proliferation of the parasite in the blood and lymph. In Stage 2, referred to as the meningoencephalitic stage, the infection has crossed the blood brain barrier and reached the CNS (Bisser *et al.*, 2006; Blum *et al.*, 2006). Cognitive dysfunction, sleep disturbance, coma and death are the signs and symptoms of late-stage HAT. Except for diagnosis and treatment in the early stage, drugs should be able to cross the blood-brain barrier to have a therapeutic effect. The non-availability of a vaccine for African trypanosomiasis (La Greca, Magez, 2011) ensures that chemotherapy remains the only therapeutic option and requires a drive to discover and develop novel chemical entities into operational medicines for HAT and various other neglected diseases. Although the WHO suggests a decrease in HAT incidence over the last decade, it will still be necessary to develop novel and active agents to combat the disease (WHO Report. 2015).

Leishmaniasis is a disease caused by *Leishmania*, a genus of protozoan parasites. The reservoir hosts of the majority of *Leishmania* species are canids and rodents from which parasites are transferred to humans via phlebotomine sand flies; transmission from infected to non-infected humans also takes place via sand fly vectors but is a significant factor for only a few *Leishmania* species (Lipoldova, Demant, 2006). Substantial rates

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of morbidity and mortality around the world are caused by leishmaniasis, and it affects many tropical and subtropical countries, including most Mediterranean nations, putting around 350 million humans at infection risk. The total occurrence of leishmaniasis is assessed to be 12 million cases around the globe, and the worldwide annual occurrence of all clinical forms of disease is 1.3 million (WHO Report, 2017). The latest refugee crisis and conflicts in North Africa and Middle East resulted in a very serious leishmaniasis outbreak (Du *et al.*, 2016). Global warming and this outbreak are the key reasons behind the increasing spread of leishmaniasis to Europe (Hotez, 2016; Naucke *et al.*, 2008) and to North America (Gonzalez *et al.*, 2010). Yet, this disease also remains neglected and current treatment options are insufficient, undermining any efforts to contain its further spread. Although research is focussing on potential drug targets in Leishmania and Trypanosoma species, and the causes of drug resistance, an effective, safer and simple substitute therapy is immediately needed.

Based on our previous results (Alkhaldi *et al.*, 2015; Changtam *et al.*, 2010; Zha *et al.*, 2016), we have endeavoured to discover new compounds with anti-protozoal activities and in doing so we have extended our earlier reported work (Zha *et al.*, 2017) comprising 34 novel α,β-Unsaturated carbonyl-based compounds linked to ligustrazine (tetramethylpyrazine (TMP)). TMP is an important constituent of Chinese traditional medicinal plant chuanxiong (*Ligusticum chuanxiong Hort*). In this study, a series of novel α,β-Unsaturated carbonyl-based compounds coupled to one or two TMP moieties was evaluated for their activity against a number of *Leishmania* and *Trypanosoma* strains including the well-characterised multi-drug resistant *Trypanosoma brucei* clonal line B48. This clonal line B48 is extremely resistant to the two major classes of trypanocides, the diamidines and the melaminophenyl arsenicals, due to the loss of HAPT1 and TbAT1/P2 drug transporters (Bridges *et al.*, 2007).

MATERIAL AND METHODS

Material

Synthetic compounds were synthesized, characterized and reported by us previously (Zha *et al.*, 2017). Stock solutions in 100% DMSO for each compound were prepared and for the concentrations used in assay, the calculated amount of stock solution was taken and diluted with complete medium, ensuring that the final DMSO concentration did not exceed 1% in the final conditions.

Cell culture

Trypanosoma brucei *bloodstream forms (BSF) in-vitro*

In this research, two strains of the bloodstream forms of *Trypanosoma brucei* were utilised. The first was the wild-type strain of *Trypanosoma brucei brucei* (s427-WT) and the other was TbAT1-B48 that was acquired from the clone TbAT1-KO, itself derived from s427WT by deletion of the *TbAT1* gene (Matovu *et al.*, 2003), by exposure to pentamidine thus causing more resistance to pentamidine, diminazene and the melaminophenyl arsenicals. Consequently, these cells have neither TbAT1/P2 transporter nor the high affinity-pentamidine transporter genes (Bridges *et al.*, 2007; Munday *et al.*, 2014). Both strains were cultured in HMI-9 medium (pH 7.4) supplemented with 10% heat-inactivated Fetal Calf Serum (FCS, BioSera) and 14 µL/L of 13.4 M β-mercaptoethanol (Sigma), as described by Hirumi and Hirumi (Hirumi, Hirumi, 1989). The medium was sterilized by filtration (0.22 µm, Millipore) inside a flow cabinet. The *T. b. brucei* cultures were incubated at 37 ºC and 5% CO_2 and passaged in vented flasks three times a week.

Leishmania major and Leishmania mexicana *promastigotes*

Leishmania major strain Friedlin (LmjF) and strains of *Leishmania mexicana* (MNYC/BZ/62/M379) were propagated in essential medium (HOMEM) with a pH value of 7.4 and 10% FCS in plastic flasks, at a temperature of 25 ºC. The cultures were passed to fresh medium three times per week.

Alamar blue assay to determine the sensitivity to test compounds

Resazurin sodium salt (Alamar Blue) is commonly used as a cell metabolic function indicator. It is a non-fluorescent, blue dye that is mixed with cell cultures containing various drug concentrations, in order to determine the sensitivity of African trypanosomes or

Leishmania cultures to the test compounds *in vitro* (Fumarola *et al.*, 2004; Raz *et al.*, 1997). In case there are no toxic effects caused by the drug, the color of the living cells changes from blue to red and fluorescent. Preparation of Alamar Blue involves the dissolution of 12.5 milligrams of Resazurin sodium salt (Sigma) in 100 mL of phosphate-buffered saline (PBS) of pH 7.4, which is then filter-sterilized and stored in the dark at 4 ºC.

Drug sensitivity using Alamar Blue assay in *T. b. brucei* BSF

For each test compound a solution of 200 µM in HMI-9 medium + 10% FCS is prepared using a 20 mM stock solution in DMSO; 200 µL of this is added to a first well of a 96-well plate. Of this, 100 µL is transferred to the next well, containing 100 µL of the same medium, achieving a 1:1 dilution, initiating a doubling dilution series across 2 rows of the plate; each experiment was positively controlled using pentamidine and the final well for each compound received 100 microliters of HMI-9 medium as negative, drug-free control. To each well, 100 µL of a suspension of 2×10^5 cells/mL is added, amounting to 1×10^5 cells/mL as the final cell density. The plate is then incubated for 48 hours at 37 ºC/5% CO_2 *after which* 20 µL of the Alamar Blue solution is added, followed by another incubation of the plate for 24 hours. A fluorimeter (FluoStar Optima) is used to read the fluorescence of the plate at the wavelengths of 590 nm for emission and 530 nm for excitation and the data are analysed using the GraphPad Prism 5 software package, fitting the fluorescence to a sigmoid curve with variable slope to determine the EC_{50}value. A very similar protocol was followed using *Leishmania* promastigotes, as described (Al-Salabi *et al.*, 2003).

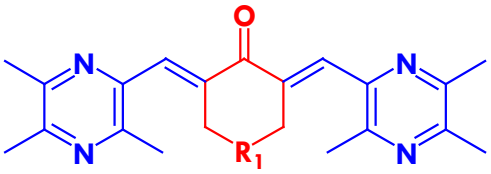
Cytotoxicity assay on Human Foreskin Fibroblast (HFF)

Toxicity of test compounds to mammalian cells was carried out using the resazurin assay previously described (Ebiloma *et al.*, 2018) with slight modifications. HFF human cells were grown in a culture medium containing 500 mL of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), 50 mL New-Born Calf Serum (NBCS) (Gibco), 5 mL Penicillin/Streptomycin (Gibco), and 5 mL of L-Glutamax (200 nM, Gibco). Mammalian cells were incubated at 37 ºC and 5% CO_2 and were passaged when they reached 80-85% of confluence in vented flasks. For the cytotoxicity assay cells were suspended at $3x10^5$ cell/mL and 100 µL was added to each well of a 96-well plate. The plate was incubated at 37 ºC and 5% CO_2 for 24 hours to allow cell adhesion. Serial test compounds dilution was performed in a different 96-well plate and 100 µL was transferred to each well containing cells. Pentamidine was used as positive control. The plates were incubated at 37 ºC and 5% CO_2 for an additional 30 hours before 10 µL of resazurin solution (125 mg/mL in PBS) was added to each well, followed by a final incubation at 37 ºC and 5% CO_2 for 24 hours. The plates were read using a FLUOstar Optima (BMG Labtech, Durham, NC, USA) at wavelength of 540 nm for the excitation, 590 nm for the emission. EC_{50} values were calculated by non-linear regression using an equation for a sigmoidal dose-response curve with variable slope using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

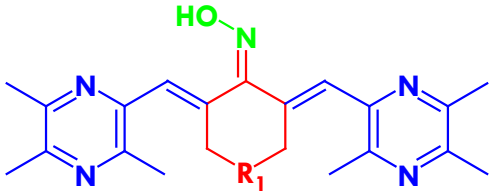
RESULTS AND DISCUSSION

In the present study, we demonstrated the effect of synthetic compounds (Table I) on the growth of promastigotes of *L. major, L. mexicana* and different strains of *Trypanosoma brucei brucei in vitro*. The antitrypanosomal and leishmanicidal activities of compounds are summarized in Table II.

TABLE I – Structures of tested synthetic α, β-Unsaturated carbonyl based compounds (Zha et al., 2017)

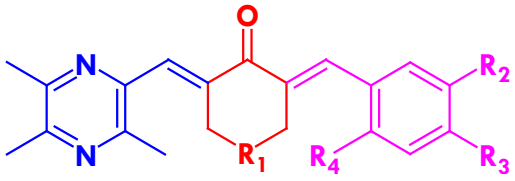


(5a-g)

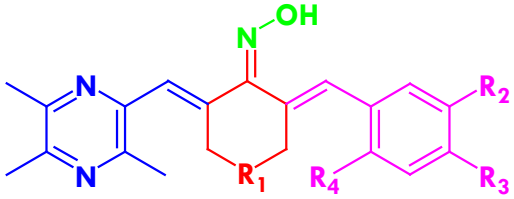


7(b,e,f)

No.	Comp.	R_1
	5a	CH_2
	5b/7b	$CH{-}CH_3$
	5c	$CHCH(CH_3)_2$
	5d	NH
	5e/7e	$N{-}CH_3$
	5f/7f	$NCH(CH_3)_2$
	5g	O



(6a-u)



(8f,o,r)

	R_1	R_2	R_3	R_4
6a	CH_2	H	H	Cl
6b	CH_2	H	OCH_3	Cl
6c	CH_2	OCH_3	OCH_3	Br
6d	$CH{-}CH_3$	H	H	Cl
6e	$CH{-}CH_3$	H	OCH_3	Cl
6f/8f	$CH{-}CH_3$	OCH_3	OCH_3	Br

(continuing)

TABLE I – Structures of tested synthetic α, β-Unsaturated carbonyl based compounds (Zha et al., 2017)

6g	$CHCH(CH_3)_2$	H	H	Cl
6 h	$CHCH(CH_3)_2$	H	OCH_3	Cl
6i	$CHCH(CH_3)_2$	OCH_3	OCH_3	Br
6j	NH	H	H	Cl
6k	NH	H	OCH_3	Cl
6l	NH	OCH_3	OCH_3	Br
6m	$N—CH_3$	H	H	Cl
6n	$N—CH_3$	H	OCH_3	Cl
6o/8o	$N—CH_3$	OCH_3	OCH_3	Br
6p	$NCH(CH_3)_2$	H	H	Cl
6q	$NCH(CH_3)_2$	H	OCH_3	Cl
6r/8r	$NCH(CH_3)_2$	OCH_3	OCH_3	Br
6s	O	H	H	Cl
6t	O	H	OCH_3	Cl
6u	O	OCH_3	OCH_3	Br

TABLE II – Effect of synthetic compounds on *Trypanosoma brucei brucei* and *Leishmania Mexicana* strains

No.	**Compounds**	***Trypanosoma brucei brucei*** EC_{50} μM		***Leishmania* promastigotes** EC_{50} μM	
		(WT)	**(B48)**	***L. major***	***L. mexicana***
1.	5a	>100	>100	>100	>100
2.	5b	30.7±1.2	31.5±0.3	>100	>100
3.	5c	>100	>100	>100	>100

(continuing)

TABLE II – Effect of synthetic compounds on *Trypanosoma brucei brucei* and *Leishmania Mexicana* strains

No.	Compounds	*Trypanosoma brucei brucei* EC_{50} µM		*Leishmania* promastigotes EC_{50} µM	
		(WT)	**(B48)**	***L. major***	***L. mexicana***
4.	5d	14.2±0.4	14.8±1.4	>100	>100
5.	5e	61.0±1.2	56.3±1.3	>100	>100
6.	5f	11.2±2.9	8.0±0.8	46.6±2.1	42.8±1.5
7.	5g	>100	>100	>100	>100
8.	6a	>100	>100	>100	>100
9.	6b	>100	>100	>100	>100
10.	6c	>100	>100	>100	>100
11.	6d	>100	>100	>100	>100
12.	6e	74.8±8.0	74.4±5.4	>100	>100
13.	6f	16.4±1.3	19.2±2.8	>100	>100
14.	6g	82.3±3.7	86.8±0.5	>100	89.3±3.0
15.	6 h	>100	>100	>100	>100
16.	6i	32.5±2.8	34.9±4.6	75.7±6.9	90.9±4.9
17.	6j	>100	>100	>100	>100
18.	6k	>100	>100	>100	>100
19.	6l	>100	>100	>100	>100
20.	6m	61.4±4.0	61.7±1.4	>100	>100
21.	6n	>100	>100	>100	>100
22.	6o	>100	>100	>100	>100
23.	6p	>100	>100	>100	>100
24.	6q	>100	>100	>100	>100
25.	6r	>100	>100	>100	>100
26.	6s	>100	>100	49.1±4.6	73.9±2.8

(continuing)

TABLE II – Effect of synthetic compounds on *Trypanosoma brucei brucei* and *Leishmania Mexicana* strains

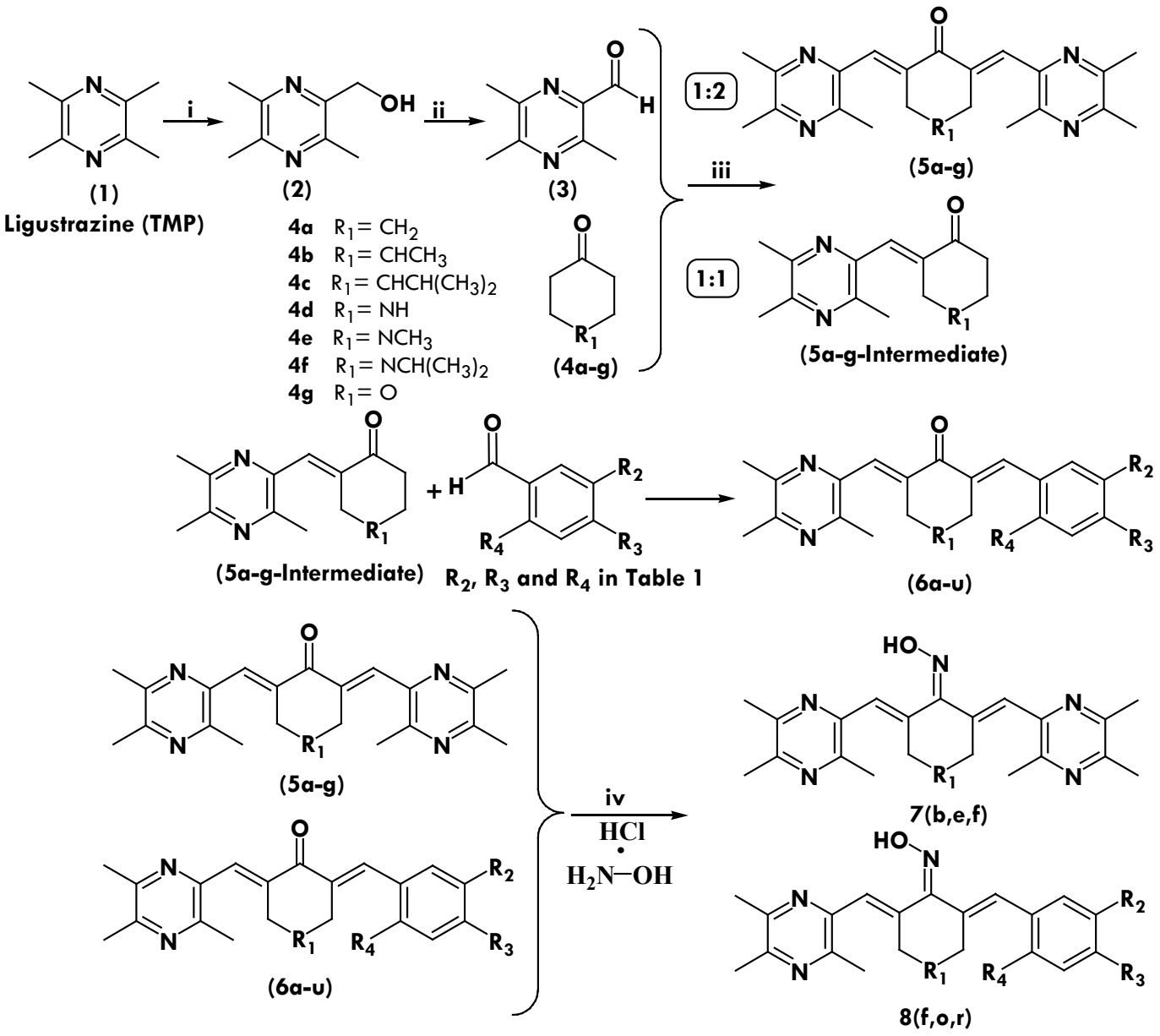
No.	Compounds	*Trypanosoma brucei brucei* EC_{50} µM		*Leishmania* promastigotes EC_{50} µM	
		(WT)	**(B48)**	***L. major***	***L. mexicana***
27.	6t	54.7±2.4	61.8±1.4	>100	>100
28.	6u	>100	>100	>100	>100
29.	7b	>100	>100	>100	>100
30.	7e	>100	>100	>100	>100
31.	7f	>100	>100	>100	>100
32.	8f	54.9±1.6	56.1±2.2	>100	>100
33.	8o	7.2±0.3	6.9±0.0	>100	>100
34.	8r	>100	>100	>100	>100
	Pentamidine	0.005±0.00	0.37±0.01	4.3±0.2	1.2±0.1

All EC_{50} values were obtained using the Alamar blue assay and are given as averages in µM (±SEM), of 3 independent evaluations. WT = wild-type sensitive control strain; B48 is a multi-drug resistant clone; Resistance Factor = EC_{50} (resistant clone)/EC_{50} (WT).

Antitrypanosomal activity of synthetic compounds

All 34 synthetic α,β-Unsaturated carbonyl-based compounds and their oxime derivatives were assessed for *in vitro* trypanocidal properties on two different types of *Ttrypanosoma brucei brucei* including wild type (WT) and the highly resistant *Trypanosoma brucei* clonal line B48 (Table II). Twelve compounds inhibited both strains, with almost identical EC_{50} values. The highest inhibitory activity was exhibited by oxime derivative **8o** (EC_{50} = 6.9 µM) for clonal line B48 and second most strong inhibitor was cyclohexanone derivative **5f** with EC_{50} = 8.0 µM.

All compounds bear a central cyclohexanone linker that was substituted with seven different types of functional groups. On both sides of central linker aldehydes were attached through an unsaturated bond. These compounds can be divided in two main groups on the basis of their chemical backbones for detailed explanation of their biological activities. The first group contains α,β-Unsaturated carbonyl-based cyclohexanone compounds (**5a-g**) and (**6a-u**). While the other group contains oximes (**7b,e,f**) and (**8f,o,r**) that were synthesized from α,β-Unsaturated carbonyl-based compounds. This first group of cyclohexanone derivatives can also be further subdivided in two classes as compounds **5a-g** contain a tetramethylpyrazine (TMP) moiety on both sides of the central cyclohexanone linker while **6a-u** contain TMP on only one side of the linker and a different type of substituted aldehyde on the other side (see scheme 1).



Scheme 1 – Synthesis scheme of α, β-Unsaturated carbonyl based compounds, oxime and oxime ether analogs. Reagents and conditions: (**i**-a) 30% H_2O_2, acetic acid, 70 ºC, 8 h.; (**i**-b) acetic anhydride, reflux, 2 h; (**i**-c) 20% NaOH; (**ii**) IBX, DMSO, room temperature, 0.5 h; (**iii**) NaOH, EtOH, Room temperature **(iv)** $NH_2OH.HCl$, pyridine, ethanol, anhyd., reflux (Zha et al., 2017).

In comparison of trypanocidal properties (Table II) of tested compounds it was observed that the first class of cyclohexanone derivatives (**5a-g**), containing a tetramethylpyrazine (TMP) moiety on both sides of central cyclohexanone linker, were more active than respective oximes (**7b,e,f**). As (**5b, d, e** and **f**) showed inhibition of *Ttrypanosoma brucei brucei* including wild type (WT) and the highly resistant *Trypanosoma brucei* clonal line B48 with EC_{50} ranging from 11.2 to 61.0 µM but respective oximes (**7b,e,f**) displayed EC_{50} above 100 µM and should be considered inactive against *T. brucei*. On the other hand, one oxime **8o** was found to be a strong inhibitor for both strains although its carbonyl precursor **6o** was completely inactive. It was also interesting observation that oximes were found active while they have TMP moiety on only one side of central cyclohexanone linker. These variations showed that central linkers and substitutions on the both sides of aromatic rings played a vital role in the biological

properties of these compounds and different combinations of cyclohexanone substitutions and functional groups on aldehyde rings exhibited different patterns of trypanocidal properties. Compound **8o** was the most active against *Trypanosoma brucei* but its carba-isoster, compound **8f** was around 7 times less potent, so clearly the basic nitrogen seems important for activity. Curiously though, compounds **6m**, **6n** and **6o**, all bearing the same R1 group as **8o**, were not active at all, suggesting that the oxime is even more important than the basic Nitrogen. And, to make everything more interesting and complex, compound **8r**, was inactive, despite having a basic nitrogen and the oxime group. The result with **8r** made clear that there is a size limit for the alkyl group bonded to the basic nitrogen. All this seem to change in the symmetric series of compounds **5a-g**, where **5f** was the most active, bearing the bulkier substituent at the basic nitrogen.

All compounds showed similar effects on *Trypanosoma brucei* wild type and B48, which is a multi-drug resistant line with *in vitro* resistance to pentamidine, proving that there is no cross-resistance between this TMP/cyclohexanone/cyclohexoxime scaffold and the most important classes of trypanocides currently in clinical use. Indeed, in our previous study we reported on an extensive series of curcumin-related α,β-Unsaturated carbonyl-based compounds with various chemical modifications to the corresponding demethylated, methylated and higher alkylated analogs (Changtam *et al*., 2010) and the result with the B48 line was unexpected, as this line displayed hypersensitivity to some of the most promising curcuminoids (Changtam *et al*., 2010).

Antileishmanial activity of synthetic compounds

The same 34 synthetic α,β-Unsaturated carbonyl-based compounds and their oxime derivatives were tested on promastigotes of *L. major* and *L. mexicana* (Table II). Only three new compounds (**5f, 6i** and **6s**) showed any inhibition, with EC_{50} values in the range 46.6 to 75.6 μM for *L. major*. The same three compounds, in addition to compound **6g**, exhibited some inhibition of *L. mexicana*. However, the activity was generally below that displayed for *Ttrypanosoma brucei brucei*. In fact, we found only weak correlation between the trypanocidal and antileishmanial activity of these compounds, with **8o** diaplaying no activity at all against either species of *Leishmania*, and **6s** having no effect against *T. brucei* at 100 μM. The fact that **5f** was active against both *Trypanosoma* and *Leishmania* indicates that maybe it has a molecular target that is common to both parasites, or at least is the same kind of receptor or enzyme. Therefore at least some of the compounds appear to act on a highly species-specific target, explaining the absence of activity against human cell lines (see below).

Effect of synthetic ligustrazine-based compounds on a human cell line

To assess whether the antiprotozoal activity depicted for tested compounds should be attributed to general toxicity, rather than specific antiprotozoal activity, some of the most active analogs (**5d, 6f** and **8o**) were also tested for their effect on human foreskin fibroblast (HFF) cells. The toxicity to HFF cells was very low and the selected test compounds exhibited high EC_{50} values. The highest concentration tested on HFF (human cells) was 300 μM. Cyclohexanone derivatives **5d** and **6f** showed the value > 300 μM and oxime derivative **8o** showed the EC_{50} value 280.6 ± 8.05 μM that was also very high. Phenylarsine oxide (PAO or PhAsO), an organometallic compound, was used as control and it was found to be strongly toxic with EC_{50} =1.17±0.12 μM.

CONCLUSION

In this study some synthetic cyclohexanone and oxime derivatives have been identified with potential applications against trypanosomiasis and leishmaniasis, based on their sturdy antiparasitic activity and low toxicity. The results obtained herein revealed that some compounds including **5f** and **8o** can be effective against trypanosomiasis as they showed effects on both tested strains and they can serve as starting points for structure-based lead optimization and development against *T. brucei*.

CONFLICTS OF INTEREST

The authors declare no conflict of interests.

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