Antileishmanial and antitrypanosomal activity of symmetrical dibenzyl-substituted $\alpha,\beta$-unsaturated carbonyl-based compounds

**Background:** Human African Trypanosomiasis (HAT) and leishmaniasis are two of the most neglected challenging tropical diseases, caused by the kinetoplastid parasites *Trypanosoma* and *Leishmania* species, respectively. For both of these complex disease spectra, treatment options are limited and threatened by drug resistance, justifying urgent new drug discovery efforts.

**Purpose:** In the present study we investigated the antitrypansomonal and antileishmanial activity of a series of 21 symmetrical $\alpha,\beta$-unsaturated carbonyl-based compounds, each featuring two 3-methoxybenzyl groups attached to a central cyclohexanone, tetrahydro-4-pyranone scaffold or 4-piperidone ring. Structure-activity relationships were explored with respect to substitution on positions 3, 4 and 6 of the terminal 3-methoxybenzyl groups, and seven types of central ring.

**Results:** Compounds 3a, 3o, 3s and 3t, showed broad anti-kinetoplastid activity against all species and strains tested.

**Conclusion:** Compound 3o featuring N-methyl-4-piperidone was found to be the most potent analog and therefore can serve as a potential lead for the development of new drug candidates for trypanosomiasis and leishmaniasis.

**Keywords:** protozoan parasites, sleeping sickness, $\alpha,\beta$-unsaturated carbonyl-based compounds, cyclohexanone, piperidine

**Introduction**

Leishmaniasis and trypanosomiasis are arthropod-borne diseases of humans and animals caused by infection with protozoan hemoflagellates of the genus *Leishmania* and *Trypanosoma*, respectively. The two genera are incorporated into the family Trypanosomatidae, order Kinetoplastida. The most recent information released by the World Health Organization (WHO) shows that the extraordinary efforts at control, implemented at the peak of the epidemic in the early 2000s is reducing the number of new cases of HAT. However, HAT has been close to eradication half a century before now, and the subsequent discontinuation of control programs allowed the disease to re-emerge to epidemic levels throughout Africa, which had then to be combated with the same drugs that were introduced in the 1930s and before. Combination of nifurtimox and efornithine (NECT) is presently suggested for the treatment of the late stage HAT because NECT treatment is simpler, safer, shorter and less expensive than single-agent efornithine (DFMO). Moreover, animal trypanosomiasis is a very important disease of livestock causing billions of dollars in economic damage and spreading from Africa to areas from South America to East Asia.
Leishmaniasis is caused by approximately 20 *Leishmania* species, which are transmitted by phlebotomine sandflies. According to the World Health Organization (WHO) around 1 million new cases, which cause around 30,000 deaths, occur every year around the world, mostly in the tropics or sub-tropics. The disease is still spreading geographically and is far from being controlled, due in large part to ecological and climate changes, and affects predominantly poor populations but, increasingly also eastern and southern Europe. Among individual infectious diseases as the ninth largest disease burden, leishmaniasis should not be overlooked in deliberations of tropical disease urgencies.

In recent years new α,β-unsaturated carbonyl-based compounds have been extensively investigated as multifunctional drug candidates. Previously we synthesized and evaluated several series of such compounds for anti-inflammatory, anticancer, immunomodulatory, and anti-Alzheimer, identifying potent activities. Here, we have selected a series of such α,β-unsaturated carbonyl-based compounds for evaluation of their effects against a number of *Leishmania* and *Trypanosoma* strains including the well-characterized multi-drug resistant *Trypanosoma brucei* clonal line B48, which displays very high levels resistance to the two major classes of trypanocides, the diamidines and the melaminophenyl arsenicals, due to the loss of HAPT1 and TbAT1/P2 drug transporters.

**Materials and methods**

**Materials**

Synthetic α,β-unsaturated carbonyl-based compounds with cyclohexanone, 4-piperidone or tetrahydro-4-pyranone moiety were synthesized (Scheme 1), characterized and reported by us previously. Stock solutions were prepared for each tested compound and diluted with complete medium for antiparasite testing, ensuring that the final DMSO concentration did not exceed 1% in the final conditions. Efomithine (Sigma-Aldrich Ltd., Gillingham, UK) and pentamidine (Sigma-Aldrich Ltd., Gillingham, UK), the most widely used drugs for the treatment of HAT were selected as positive control.

**Cell culture**

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

The selected compounds were tested on bloodstream forms of two strains of *Trypanosoma brucei*: a standard drug-sensitive wild-type strain of *Trypanosoma brucei* (Lister s427-WT) and B48, which was adapted from the clone TbAT1-KO by deletion of the TbAT1 drug transporter, itself derived from s427WT by removal of the *TbAT1* gene by prolonged exposure to pentamidine in vitro. Thus, these cells have neither TbAT1/P2 transporter nor the high affinity-pentamidine transporter (HAPT1) genes. 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. In a flow cabinet through filtration medium was sterilized. *T. b. brucei* cultures were incubated at 37 ºC and 5% CO2 and passaged in vented flasks three times a week.

**Leishmania major and leishmania mexicana promastigotes**

*Leishmania major* strain Friedlin (LmjF) and *Leishmania mexicana* (strain MNYC/BZ/62/M379) were cultured in essential medium (HOMEM) and 10% FCS, pH 7.4, in plastic flasks at 25 ºC. The cultures were passed to fresh medium three times per week, exactly as described previously.

**Alamar blue assay**

Resazurin (Alamar Blue) is commonly used as a metabolic function indicator. It is a non-fluorescent, blue dye that is mixed with cell cultures containing various drug concentrations, to determine the sensitivity of African trypanosomes or *Leishmania* cultures to the test compounds in vitro. Living cells metabolize the resazurin to red and fluorescent resoruﬁn. Preparation of Alamar Blue reagent 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. For *T. b. brucei* bloodstream forms the assays were performed as described in 96-well plates with 1 × 10⁵ cell/well in the presence of 23 doubling dilutions of test compound, and one well for each dilution series receiving growth medium only, for 48 h at 37 ºC/5% CO₂. The Alamar blue solution was added for a further 24 h incubation before fluorescence was measured in a FLUOstar Optima plate reader (BMG Labtech, Durham, NC, USA), λex 540 nm, λem 590 nm. EC50 values were calculated by non-linear regression to a sigmoidal curve with variable slope using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). For *Leishmania* promastigotes the procedure was slightly adapted and the final density was...
Results and discussion
In the present study, the effect of synthetic α,β-unsaturated carbonyl-based compounds on the growth of promastigotes of *L. major*, *L. mexicana* and different strains of *Trypanosoma brucei* was evaluated in vitro. Structures of all tested compounds are shown in Table 1 while the antitrypanosomal and leishmanicidal activities of compounds are shown in Table 2.

Antitrypanosomal activity
In vitro trypanocidal properties of synthetic α,β-unsaturated carbonyl-based compounds were assessed for two different types of *Trypanosoma brucei* where one was WT and second was the highly resistant *T. b. brucei* clonal line B48 (Table 2). In this assay, reference drugs efloverine and pentamidine were used as positive controls. Pentamidine was found very potent with EC50=0.005 µM for wild type and 0.37µM for clonal line B48 (P<0.001), while EC50=19.47 and 22.6 uM were determined for efloverine-treated WT and B48 (P>0.05) parasites, respectively. Among the tested compounds, twelve derivatives inhibited both strains, with EC50 values within 2-fold different for the two strains, as compared to a 74-fold (increase) difference for pentamidine, showing that this class of compounds is not cross-resistant with diamidines and melaminophenyl arsenicals (Bridges et al, 2007). The highest inhibitory activity was exhibited by 3o (EC50=1.7 µM) for clonal line B48 and second most strong inhibitor was synthetic compound 3a with an EC50 value of 2.8 µM, closely followed by 3s showing inhibition with an EC50 of 3.55 µM.

An interesting structure-activity relationship was observed for synthetic α,β-unsaturated carbonyl-based derivatives. Based on their chemical structure all 21 compounds can be divided into seven main groups, with three compounds in each group. Seven groups are suggested on the basis of R1 substitution in the central ring that linked the two substituted 3-methoxybenzene moieties. In each of the 7 groups the aromatic ring substitutions R1’, R3’, R4’ were kept the same [(i) H/H/Cl; (ii) H/OCH3/Cl; (iii) OCH3/OCH3/Br], allowing direct deduction of the role of the central ring modifications for each set of aromatic ring substitutions.

It is immediately clear that the R1 position substitution in the central linker played a vital part in the biological properties of these types of compounds, as we have previously reported for anti-inflammatory activities. However, in the case of anti-inflammatory and anticancer activities different substitutions correlated with highest potency.

All three compounds with a tetrahydro-4-pyranone linker, 3s, 3t and 3u, exhibited good activity, meaning that oxygen at the R1 position was favorable for activity against both strains of *T. b. brucei*. Within this group, 3s displayed the highest activity, indicating that addition of R3 and R4 methoxy groups were not favored with this central ring. Among the other six linkers, the one with N-CH3 at R1 (3m-3o) exhibited the strongest trypanocidal activity and 3o was the most active compound in the entire series (EC50(WT) =1.83±0.06). However, for the 1-methyl-4-piperidones the activity dramatically improved with the sequential addition of methoxy groups at R3 and R4, which appears to indicate that these compounds may have a different target than the tetrahydro-4-pyranones, where the methoxy groups had a deleterious effect. This notion is strengthened by the understanding that the tetrahydropyran oxygen is a strong H-bond acceptor, whereas the ring nitrogen of 1-methylpiperidone is unable to engage in similar H-bonding; similar reasoning can be applied to the methoxy substitution. Thus, the potential interactions of the most active compounds in these groups, 3s and 3o, respectively, with a cellular target would be very different, yet their trypanocidal activities are similar.
Table 1 Structures of synthetic α,β-unsaturated carbonyl based compounds (cyclohexanone, tetrahydro-4-pyranone scaffold and 4-piperidone derivatives)

| No. | Compound | R₁ | R'₄ | R'₃ | R'₁ |
|-----|----------|----|-----|-----|-----|
| 3a  |          | CH₂ | H   | H   | Cl  |
| 3b  |          | CH₂ | H   | OCH₃| Cl  |
| 3c  |          | CH₂ | OCH₃| OCH₃| Br  |
| 3d  |          | CH—CH₃| H     | H   | Cl  |
| 3e  |          | CH—CH₃| H     | OCH₃| Cl  |
| 3f  |          | CH—CH₃| OCH₃| OCH₃| Br  |
| 3g  |          | CH⁻ | H   | H   | Cl  |
| 3h  |          | CH⁻ | H   | OCH₃| Cl  |
| 3i  |          | CH⁻ | OCH₃| OCH₃| Br  |
| 3j  |          | NH  | H   | H   | Cl  |
| 3k  |          | NH  | H   | OCH₃| Cl  |
| 3l  |          | NH  | OCH₃| OCH₃| Br  |
| 3m  |          | N—CH₃| H     | H   | Cl  |
| 3n  |          | N—CH₃| H     | OCH₃| Cl  |
| 3o  |          | N—CH₃| OCH₃| OCH₃| Br  |
| 3p  |          |     | H   | H   | Cl  |
| 3q  |          |     | H   | OCH₃| Cl  |
| 3r  |          |     | OCH₃| OCH₃| Br  |
| 3s  |          | O   | H   | H   | Cl  |
| 3t  |          | O   | H   | OCH₃| Cl  |
| 3u  |          | O   | OCH₃| OCH₃| Br  |

Indeed, the favorability of the methoxy substitutions appeared to differ depending on the identity of R₁, probably because this influenced the 3D structure through the orientation/rotation of the aromatic rings relative to the central structure and each other (Figure 1). These observations mean that further extension of the SAR in this series should focus on one central group, optimizing the substituents on the aromatic rings, especially as the importance of the 2’ methoxy and the 1’ halogen for antitypanosomal activity cannot be assessed from the current as they are present in all derivatives. However, the present data (1) reveal which central rings are optimal, (2) provide a limited SAR starting point for
Table 2: Effect of α,β-unsaturated carbonyl based compounds on *Trypanosoma brucei brucei* bloodstream forms and *Leishmania mexicana* and *Leishmania major* strains

| Compounds | *Trypanosoma brucei brucei* bloodstream forms | | | *Leishmania* promastigotes |
|-----------|-----------------------------------------------|-----------------|-----------------|-----------------|
|           | *EC₅₀ (µM)* | *EC₅₀ (µM)* | *EC₅₀ (µM)* | *EC₅₀ (µM)* |
|           | **WT** | **B48** | **L. major** | **L. mexicana** |
| 3a        | 2.81±0.71 | 5.70±0.66 | 12.13±1.67 | 10.03±0.67 |
| 3b        | 17.92±1.12 | 23.42±2.34 | 19.36±4.06 | 15.32±2.44 |
| 3c        | >100 | >100 | >100 | >100 |
| 3d        | >100 | >100 | >100 | >100 |
| 3e        | 4.35±0.15 | 4.45±0.09 | >100 | >100 |
| 3f        | >100 | >100 | >100 | >100 |
| 3g        | 35.93±1.01 | 31.49±0.37 | >100 | >100 |
| 3h        | >100 | >100 | >100 | >100 |
| 3i        | >100 | >100 | >100 | >100 |
| 3j        | >100 | >100 | >100 | >100 |
| 3k        | 5.59±0.08 | 8.47±0.79 | 41.55±2.46 | >100 |
| 3l        | >100 | >100 | >100 | >100 |
| 3m        | >100 | >100 | >100 | >100 |
| 3n        | 15.15±1.60 | 11.60±0.62 | >100 | >100 |
| 3o        | 1.83±0.06 | 1.75±0.05 | 3.02±0.10 | 4.12±0.69 |
| 3p        | 7.94±0.24 | 7.47±0.09 | 16.85±2.80 | 25.57±4.93 |
| 3q        | >100 | >100 | >100 | >100 |
| 3r        | 17.36±2.75 | 8.22±1.20 | >100 | >100 |
| 3s        | 3.36±0.59 | 3.55±0.18 | 10.56±0.96 | 22.63±0.75 |
| 3t        | 4.12±0.12 | 4.04±0.11 | 4.89±0.42 | 6.37±0.36 |
| 3u        | 10.27±2.15 | 6.66±0.67 | 38.40±8.69 | 89.04±3.95 |
| Efflornithine | 19.47±2.45 | 22.60±1.13 | - | - |
| Pentamidine | 0.005±0.000 | 0.37±0.01 | 4.34±0.17 | 1.25±0.11 |

Notes: All EC₅₀ values were obtained using the Alamar blue assay and are given as averages in µM (±SEM), of 3 independent determinations.

Abbreviations: WT, wild-type sensitive control strain; B48, multi-drug resistant clone.

Figure 1: Energy-minimized 3D structures of compounds 3s and 3o, produced using Chem3D Ultra 10.0 (CambridgeSoft).
these scaffolds and (3) establish that even with the very limited exploration of this chemical space several trypanocides with EC50 values in the range 1–5 µM could be identified. This activity is obviously much superior to efornithine (Table 2) and close to that of the standard veterinary trypanocide diminazene aceturate as well.21 Moreover, the α,β-unsaturated carbonyl-based compounds were not cross-resistant with diamidines and arsenical trypanocides as they displayed the same activities against the multi-drug resistant clone B48.

**Antileishmanial activity**

The extracellular insect-infective promastigote form of *Leishmania* is addressed in many drug screening assays against *Leishmania* which can be performed readily, thus being additional suitable for automation even though the *Leishmania* parasite exists in the human host in its intramacrophage amastigote form.29 In the current study, all synthetic α,β-unsaturated carbonyl-based compounds were tested on promastigotes of *L. major* and *L. mexicana* (Table 2). Eight synthetic compounds showed inhibition, with EC50 values in the range 3 to 41.5 µM for *L. major* and very similar activities against *L. mexicana*. The most active compound was again 3o (EC50 3.02±0.10 µM), which performed superior to the positive control pentamidine (EC50 4.34±0.17 µM). The activity was generally better than that displayed for *T. b. brucei* but in general the compounds most active against the trypanosome strains were also the most active against both *Leishmania* species (*mexicana & major*). In fact, we found very strong correlation between the trypanocidal and antileishmanial activity of these compounds, as 3a, 3b, 3o, 3p, 3s, 3t and 3u displayed activities against two trypanosoma strains as well as two distinct Leishmania species, and some compounds like 3c, 3f, 3h, 3i, 3j, 3m and 3q have no effect against any of the parasites. For antileishmanial activity, again tetrahydro-pyran-4-one backbone was found best inhibitor among all other linkers as all three compounds bearing this backbone were found to have various levels of activity - yet the best activity was by 3o, with a 1-methyl 4-piperidone backbone.

**Conclusion**

In this study some synthetic α,β-unsaturated carbonyl-based compounds with three types of substitution pattern and seven types of central linker have been identified with potential applications against trypanosomiasis and leishmaniasis. Total twenty-one derivatives were evaluated and twelve of them exhibited good trypanocidal and/or antileishmanial activities. The most promising results were obtained for compound 3o that showed the best inhibition of *Trypanosoma brucei brucei* wild type (WT) as well as *T. b. brucei* clonal line B48 and strong activity against *Leishmania* promastigotes, exceeding the efficacy of the positive control. Further studies should be conducted to further explore the SAR and better understand the mechanism of action of these molecules. Thus, these series of new α,β-unsaturated carbonyl-based could be considered as promising hit compounds against leishmaniasis and trypanosomiasis requiring further hit optimization and safety evaluations.

**Disclosure**

The authors report no conflicts of interest in this work.

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