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# Methoxylated 2'-hydroxychalcones as antiparasitic hit compounds

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**Abstract**

Chalcones display a broad spectrum of pharmacological activities. Herein, a series of 2'-hydroxy methoxylated chalcones was synthesized and evaluated towards *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania infantum*. Among the synthesized library, compounds **1, 3, 4, 7** and **8** were the most potent and selective anti-T. *brucei* compounds ($EC_{50}$ = 1.3 – 4.2 μM, selectivity index >10-fold). Compound **4** showed the best early-tox and antiparasitic profile. The pharmacokinetic studies of com-

pound **4** in BALB/c mice using hydroxypropil-β-cyclodextrins formulation showed a 7.5 times increase in oral bioavailability.

**Keywords**. Chalcones, *Trypanosoma brucei*, *Trypanosoma cruz*i, *Leishmania infantum*, Early Toxicity studies, cyclodextrin formulations.

### 1. Introduction

The kinetoplastid parasites *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania spp*. are responsible for potentially fatal insect-borne diseases namely, respectively, Human African Trypanosomiasis (HAT), Chagas disease and Leishmaniasis [1-3]. Despite the serious health, economic and social consequences of these parasitic infections, there are no available vaccines. Therefore, disease control relies only on chemotherapy and prophylaxis. The available drugs suffer from many drawbacks including toxicity, poor efficacy and drug resistance [4]. Hence, there is an urgent requirement for new, safe and effective drugs. Phenotypic screening approaches have been successfully used in the field of neglected diseases, particularly for the treatment of HAT [5]. Two compounds, discovered through a phenotypic screening, have recently been progressed into clinical trials by DNDi: fexinidazole, a nitroimidazole, and SCYX-7158, an oxaborole [6]. In the drug discovery process for new antileishmanial and antitrypanosomal drugs, phenotypic screening and follow up optimization were largely applied and provided a wide range of chemical structures, including chalcones (1,3-diphenyl-2-propen-1-one derivatives) [7-14]. Chalcones exist as E- or Z-isomers. The E-isomer is the thermodynamically most stable form; thus, chalcones are usually isolated as E- stereoisomers after recrystallization of a Z-E mixture [15]. Chalcones display a broad spectrum of pharmacological activities including anti-oxidant, antifungal, anti-infective, anti-inflammatory, anticancer and antinociceptive properties, and have been shown to affect a great variety of parasitic targets [16-20]. The chalcone scaffold has been widely explored for the antileishmanial activity [21,22], while a limited number of compounds bearing the 1,3-diphenyl-2-

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propen- 1-one moiety and active towards T. *brucei* has been reported in literature. One of the most studied antileishmanial chalcones is licochalcone A (Figure 1, compound A), a natural chalcone, that inhibits the in-vitro growth of L. major promastigotes ($IC_{50}$ = 21 μM) and L. donovani amastigotes ($IC_{50}$ = 2.7 μM). Intraperitoneal administration of licochalcone A prevents the development of lesions in BALB/c mice infected with L. *major* and reduces the parasite load in the spleen and liver of hamsters infected with L. *donovani* [23]. Licochalcone A was found to inhibit the activity of the parasite mitochondrial dehydrogenase and to alter the ultrastructure of mithocondria [24]. Very recently, flavokawin B, a 2'-hydroxy methoxylated natural chalcone (Figure 1, compound B), has been reported to inhibit T. *brucei* parasite growth (T. *brucei* strain 427 $IC_{50}$ = 6.2 μM) [25]. In our previous work, methoxylated flavonols (Figure 1, scaffold C) proved to be active against T. *brucei* and T. *cruzi* showing $EC_{50}$ in the range of 1-8 μM [11]. The best compounds had shown a potency analogue or higher than flavokavin B against T. *brucei*. Based on the mentioned results, we focused our attention on the open analogues of the flavone heterocycle (Figure 1, scaffold D). Thirteen 2'- hydroxy methoxylated chalcones (**1-13**, Table 1) were synthesized and evaluated in-vitro towards cultured T. *brucei,* T. *cruzi* and L. *infantum* aiming to develop novel antiparasitic compounds. We evaluated the cytotoxicity of the compounds on THP-1 cells and we assessed a panel of in-vitro toxicological properties in order to identify safe hits. We selected compound 4 for pharmacokinetic studies in BALB/c mice and we investigated the use of cyclodextrins as drug delivery system.

## 2. Results and discussion

### 2.1. Chemistry

Herein, we synthesized a library of thirteen 2'-hydroxy chalcones bearing methoxy groups (1-13, Table 1). Eight compounds (**1-3, 5, 6, 9-11**) are intermediates of the previously published flavonols11, while five additional chalcones (**4, 7, 8, 12** and **13**) were synthesized to explore the Structure Activity Relationship (SAR) of chalcones showing methoxy groups in different positions of ring A and B. Com-

pounds **1-3, 5-7, 9-12, 13** have been previously described in literature [11, 26-28], while compounds **4** and **8** are novel structures. The chalcones (**1-13**) were synthesized by aldol condensation of substituted acetophenones and benzaldehydes in presence of NaOH (3M) as previously reported in literature [29]. The general synthetic procedure is reported in Scheme 1. LogP was calculated with ALOGPS 2.1 to evaluate a possible effect of the lipophylicity on the biological activity. All compounds showed analogue logP (~3.5, Table 1).

### 2.2. Biological evaluation

All the synthesized compounds were assessed for their antiparasitic activity against the bloodstream form of T. *brucei* (Table 1, Figure 2 and Table S1) and the intracellular T. *cruzi* at 10 μM (Figure 2 and Table S3). The library was tested against L. *infantum* amastigotes (Figure 2 and Table S2) at a higher concentration (50 μM) since it is usually more difficult to identify anti-leishmanial hits. Eight compounds (**1-4**, **7**, **8**, **12** and **13**) showed an antiparasitic activity towards T. *brucei* higher than 85%, thus dose-response studies were performed (Table 1 and Table S1 Supporting Information). The eight compounds showed $EC_{50}$ values against T. *brucei* L427 WT bloodstream form in the low micromolar range (1.3-7.4 μM). In 3-methoxy substituted chalcones, the presence of methoxy groups on ring A did not remarkably influence the activity towards T. *brucei*. In fact, compounds **1-4** showed a comparable activity ($EC_{50}$ = 1.3, 3.4, 2.1 and 4.4 μM, respectively), with compound **1** bearing no methoxy groups on ring A being the most potent. It is worth noting that while the -$OCH_3$ group at position 3 of ring B provides activity, the same group at position 4 renders the molecule inactive, as highlighted by comparing the activity of compounds **2** and **3** with that of their corresponding 4-methoxy chalcones (compounds **5** and **6**). In addition, the presence of -$OCH_3$ group at both 3 and 4 positions on ring B makes compounds **9** and **10** almost inactive. A clear effect of the methoxy group on compounds antiparasitic activity could not be observed for 3,4,5-trimethoxy-substituted compounds: compound **11** was inactive, while compounds **12** and **13** showed low micromolar activity against T. *brucei* ($EC_{50}$ = 4.4 and 7.4 μM, re-

spectively). Compounds **7** and **8** bearing methoxy groups at both positions 2 and 6 on ring B showed $EC_{50}$ in the low micromolar range ($EC_{50}$ = 3.4 and 2.1 μM, respectively). With the exception of compounds **11-13**, the activity against *T. brucei* of methoxylated chalcones is guided by the position of methoxy groups on ring B, while the presence of a methoxy group in position 4' or 5' of ring A does not significantly influence the chalcones activity. We evaluated the cytotoxicity of methoxy-substituted 2'-hydroxy chalcones on PMA-differentiated THP1 cells and $CC_{50}$ (or NOAEL) was determined (Table 1). Compounds **1**, **3**, **4**, **7** and **8** showed a selectivity index (SI = $CC_{50}/EC_{50}$) higher than **10**, with compound **7** being the most selective (29-fold). Most of the tested compounds were active against T. *brucei*, while only compounds **11**, **12** and **13**, bearing a 3,4,5-trimethoxy substituted ring, had moderate activity towards L. *infantum* (Figure 2). The $EC_{50}$ values towards L. *infantum* of compounds **11**, **12** and **13** were 30.3, 42.2 and 22.2 μM, respectively (Table S2, Supporting Information). Seven compounds (**1**, **2**, **4**, **5**, **8**, **9**, **11**) were able to reduce the parasite load of T. *cruzi*-infected HG39 cells by more than 40% at 10 μM, with compound **2** being the most potent (Figure 2). Compounds **2**, **8**, **9** and **11** showed $EC_{50}$ towards T. *cruzi* lower than 10 μM (Table S3, Supporting Information). These results indicate that methoxylated 2'-hydroxy chalcones showed interesting antiparasitic activity in particular towards the bloodstream form T. *brucei*.

2.3.Early toxicology studies

The chalcones library was assessed at 10 μM in a panel of assays including hERG, Aurora B kinase, cytochromes (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4), cytotoxicity (A549 and W1-38 cell lines) and mitochondrial toxicity. Among the 13 compounds evaluated for hERG liability, only compounds 6 and 11 decrease hERG activity by more than 25%. Most of the compounds inhibited at least one cytochrome isoform. None of the compounds evaluated towards Aurora B kinase showed inhibitory activity at the tested concentration. None of the compounds turned out to be either cytotoxic or mitotoxic. These data have been summarised using a traffic light system in Figure 3 with absolute

values being reported in Table S4 of the Supporting Information. After evaluating the data obtained from the different assays, we selected compound **4** for further studies. The activity of compound **4** towards hERG ($IC_{50}$ >100 μM) was more than 20-fold greater than T. *brucei* $EC_{50}$ ($EC_{50}$ = 4.2 μM), thus consistent with the parameters defined for progressing a compound from in-vitro studies to early pharmacokinetic studies in mice (target-product profile) [11]. Compound **4** did not inhibit two cytochrome isoforms (CYP2C9 $IC_{50}$ = 42.1 μM and CYP3A4 $IC_{50}$ = 27.4 μM), while it strongly inhibited CYP1A2 ($IC_{50}$ = 1.5 μM) and moderately inhibited CYP2C19 and CYP2D6 ($IC_{50}$ = 7.7 and 6.8 μM, respectively). The toxicological profile of pentamidine, the reference compound for *T. brucei* infections, is reported in Table S4 of the Supporting Information.

### 2.4. Solubilization of compound 4 with cyclodextrins and snapshot PK

The solubility of compound **4** was evaluated using UV-Vis spectroscopy. Pharmacokinetics of compound **4** in BALB/c mice (1 mg/kg, IV) was characterized by low plasma concentrations (Figure 4A). Thus, compound **4** was complexed with hydroxypropil-β-cyclodextrins (50%, w/v). The bioavailability of compound **4** alone and formulated with cyclodextrins was determined in BALB/c mice using LC-MS. The data are reported in Table S5 and S6 of the Supporting Information. Compound **4** administered by IV injection showed a biphasic elimination behavior. The half life (t ½) of the free compound after IV administration was 62.5 min (Figure 4A), while after oral administration was 12.6 min (Figure 4B). The t ½ of compound **4** in cyclodextrins after oral administration was 36.7 min and the cyclodextrins increased by ca. 7.5 times the oral bioavailability of compound **4** (Figure 4B).

### 3. Conclusion

Herein, we discovered methoxylated 2'-hydroxychalcones as potent anti-T. *brucei* agents. The best compounds (**1-4**, **7**, **8**, **12**, **13**) showed similar or higher potency ($EC_{50}$ = 1.3- 7.4 μM) with respect to the recently isolated natural compound flavokawin B (*T. brucei* strain 427 $IC_{50}$ = 6.2 μM) [25], with compound **1** being over 4 times more potent than flavokawin B. To our knowledge none of our meth-

oxylated 2'-hydroxychalcones has been previously reported in literature as anti-trypanosomatidic agent. A panel of *in-vitro* early toxicological properties was evaluated. Chalcones showed a toxicological profile similar to that of the previously identified flavonols [11] showing some effects on CYP isoforms. The selectivity index of compound **1** is almost 5 times higher than that of the corresponding flavonol (Table S6 of the Supporting Information). Compound 4 ($EC_{50}$ T. *brucei* = 4.2 μM, SI = 12) was selected for pharmacokinetic studies. The formulation of compound **4** with cyclodextrins led to an increase of the oral bioavailability of the molecule by 7.5 fold and to a half-life of 36.7 min. Hence, chemical modifications together with further optimization of the drug delivery systems and the understanding of the molecular targets could lead to fully exploit the anti-parasitic potential of methoxylated 2'-hydroxychalcones.

## 4. Material and methods

### 4.1. Synthesis

The general procedure for the synthesis of (2E)-1-(2-hydroxyphenyl)- 3-phenylprop-2-en-1-ones is reported in the Supporting Information together with the spectral data of all remaining compounds. ALOGPS 2.1 program (http://www.vcclab.org/lab/alogps/) was used to calculate logP.

**(2E)-1-(2-hydroxy-4,5-dimethoxyphenyl)-3-(3-methoxyphenyl)prop-2-en-1-one (4)** was isolated as an orange solid in a 40% yield. Mp. [119-120 °C]. $^{1}$H NMR (CDCl$_3$, 400 MHz) δ: 13.18 (s,1H, OH), 7.88 (d, 1H, J = 15.4 Hz, H$_B$), 7.51 (d, 1H, J = 15.4 Hz, H$_A$), 7.38 (dd, 1H, J = 7.9 Hz, H-5), 7.28 (m, 1H, H-6), 7.27 (s, 1H, H-6') 7.19 (m, 1H, H-2), 7.00 (ddd, 1H, J $_{4,5}$ = 8.2 Hz, J = 2.6, 0.9 Hz, H-4), 6.53 (s, 1H, H-3'), 3.96 (s, 1H, 4'-OCH$_3$), 3.94 (s, 1H, 5'-OCH$_3$), 3.89 (s, 3H, 3-OCH$_3$). $^{13}$C NMR (CDCl$_3$, 100 MHz) δ: 190.47, 161.87, 159.99, 157.19, 144.43, 142.00, 136.22, 130.03, 121.12, 120.74, 116.08, 114.00, 112.02, 111.10, 100.87, 57.01, 56.23, 55.43. ESI-HRMS calcd. for C$_{18}$H$_{19}$O$_5$ [M+H]$^{+}$ 315.1227, found 315.1228.

**(2E)-3-(2,6-dimethoxyphenyl)-1-(2-hydroxy-5-methoxyphenyl)prop-2-en-1-one (8)** was isolated as an orange solid in a quantitative yield. Mp. [151-152°C].^{1}H NMR (CDCl$_3$, 400 MHz) δ: 12.75 (br s,1H, OH), 8.41 (d, 1H, J $_{B,A}$= 15.7 Hz, H$_B$), 8.11 (d, 1H, J $_{A,B}$ = 15.7 Hz, H$_A$), 7.40 (d, 1H, J $_{4',6'}$ = 2.9 Hz, H-6'), 7.34 (t , 1H, J = 8.4 Hz, H-4), 7.13 (dd, J $_{4',3'}$ = 9.1, J $_{4',6'}$ = 2.9 Hz, H-4'), 6.98 (d, 1H, J $_{3',4'}$ = 9.1 Hz, H-3'), 6.61 (d, 1H, J = 8.4 Hz, H-2 + H-5), 3.96 (s, 3H, -OCH$_3$), 3.85 (s, 3H, -OCH$_3$). $^{13}$C NMR (CDCl$_3$, 100 MHz) δ: 195.11, 160.60, 157.84, 151.53, 136.57, 132.12, 123.31, 122.76, 120.13, 119.08, 113.12, 112.73, 103.80 (2C), 55.94 (3C). ESI-HRMS calcd. for C$_{18}$H$_{19}$O$_5$ [M+H]$^+$ 315.1227, found 315.1227.

4.2. In vitro Antiparasitic evaluation

4.2.1.   *In vitro* evaluation of activity against *L. infantum* intramacrophage amastigotes

The efficacy of compounds **1-13** against L. *infantum* intracellular amastigotes at 10 μM was determined according to literature [30]. The Operetta high-content automated imaging system was used to acquire images and the Harmony Software was optimized quantifying host cells number, infection ratio and number of parasites per infected cell. The ratio between infected cells and total number of cells was then calculated, and defined as the Infection Ratio (IR) [31].

4.2.2.   *In vitro* evaluation of activity against *T. brucei*

The efficacy of compounds against T. *brucei* bloodstream forms was evaluated using a modified resazurin-based assay previously described in literature [32]. The reported EC$_{50}$ are the arithmetic average of at least two independent determinations done in triplicate.

4.2.3.   *In-vitro* evaluation of activity against *T. cruzi*

Infections were performed in 6-well plates (3 x 106 HG39 cells/well). Confluent HG39 cells were infected with sanguineous trypomastigotes of T. *cruzi* Y strain at a 1:1 ratio. The method used to assess the growth inhibition effect is based on the LGC Genomics, Berlin kit for the gDNAs detection [33]. TaqMan™ probe-based quantitative real-time PCR was performed.

### 4.3. Early *In vitro* Toxicology study

#### 4.3.1. Cytotoxicity assessment against THP-1 macrophages

The effect of compounds **1-13** on THP-1-derived macrophages was assessed by the colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. The reported $CC_{50}$ and NOAEL are the arithmetic average of at least two independent determinations done in triplicate.

#### 4.3.2. hERG assay

This assay made use of Invitrogen's Predictor™ hERG Fluorescence Polarisation Assay. The assay uses a membrane fraction containing hERG channel (Predictor™ hERG Membrane) and a high-affinity red fluorescent hERG channel ligand, or "tracer" (Predictor™ hERG Tracer Red), whose displacement by test compounds can be determined in a homogenous, Fluorescence Polarisation (FP) based format [11].

#### 4.3.3. Cytochrome P450 1A2, 2C9, 2C19, 2D6 and 3A4 assays.

These assays made use of the PromegaP450-Glo™assayplatform. Each CYP450 assay made use of microsomal preparations of cytochromes from baculovirus infected insect cells. Action of the CYP450 enzymes upon each substrate ultimately resulted in the generation of light and a decrease in this was indicative of inhibition of the enzymes [11].

#### 4.3.4. Cytotoxicity assay against A549 and WI38 cells

The assays were performed using the Cell Titer-Glo assay from Promega. The assay detects cellular ATP content with the amount of ATP being directly proportional to the number of the present cells. The A549 cells were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and WI38cells were obtained from ATCC (ATCC® CCL- 75™) and were grown in DMEMwith FCS (10% v/v), streptomycin (100 µg/ml) and penicillin G (100 U/ml) [11].

#### 4.3.5. Aurora B kinase assay

This assay made use of the ADP-Glo Kinase Enzyme System from Promega, which is a bioluminescent assay that employs firefly luciferase in a coupled-enzyme assay format to enable detection of ADP levels from ATPase assays [11].

### 4.3.6. Assessment of mitochondrial toxicity

This assay made use of MitoTracker® Red chloromethyl-Xrosamine (CMXRos) uptake and High Content Imaging to monitor compound mediated mitochondrial toxicity in the 786-O (renal carcinoma) cell line. Cells were maintained using RPMI-1640 medium containing 2 mM glutamine, FCS (10% v/v), streptomycin (100 μg/ml) and penicillin G (100 U/ml) [11].

### 4.4. Pharmacokinetic evaluation

### 4.4.1. Solubility of compound 4 using UV-Vis spectroscopy

UV-visible spectroscopy was used to measure the solubility of compound 4. The experiments were carried out with the instrument CARY 50. Six different solutions (3.125, 6.25, 12.5, 25, 50 and 100 μM) were prepared. The maximum of absorbance was found at 312 nm and epsilon was calculated. Four saturated solutions were prepared: 100 μM in water, 100 μM in PBS, 200 μM in PBS + 10 % DMSO and 10 mM in PBS + 50 % DMSO. The suspensions were left in incubation at 25°C for 18 hours, then filtered (0.2 μm). Maximum solubility in water, phosphate buffered saline (PBS) and PBS with 10% or 50% of DMSO were, respectively, 0.91, 2.37 μM, 11.41 μM and 0.6 mM.

### 4.4.2. Cyclodextrins solubilization of compound **4**

Hydroxypropil-β-cyclodextrin (50%, w/v) was used to solubilize compound **4** as previously described [11].

### 4.4.3. Pharmacokinetics of compound 4 using LC-MS

The snapshot PK studies were carried out according to literature [34]. BALB/c mice were treated with compound **4** administered alone (8% DMSO) (1 mg/kg IV or 20 mg/kg *per os*), compound **4** solubilized

with hydroxypropil-β-cyclodextrin (50%) (20 mg/kg per os) or with a mixture of cyclodextrins (25%) plus polyethylene glycol 400 (Quimidroga) (50%) (20 mg/kg per os). Plasma samples were analyzed by LC-MS. Chromatographic separation was carried out using a Shimadzu LC system consisting of two pumps, column oven, degasser and autosampler. Attached to this system was an analytical column (C18, Gemini 5 μm 110 A Phenomenex, 150 x 2 mm). The HPLC system was connected to a triplequadrupole

mass spectrometer equipped with a turboionspray source operated with unit resolution in the positive ion mode (ESI-QQQMS, Shimadzu LCMS-30). Under these conditions a retention time of approximately 4 minutes for the molecule was obtained. Mobile phase consisted of a mixture (80:20) of acetonitrile: purified water flow at a 0.2 ml/min rate in isocratic mode. Run time was 10 minutes and the injection volume was 5 μl. The mass transition of m/z was: Quantifier (m/z): 315.0 > 161.0 CE: - 20; Qualifier (m/z): 315.0 > 132.9 CE: -34. Plasma samples of mice (NMRI and BALB/c) were obtained by serial sampling from submandibular vein and stored at -20 ℃ until analyzed.

**Ethics Statement**

The experimental design and housing conditions at UCM were approved by the Committee of Animal Experimentation (Universidad Complutense de Madrid) and regional authorities (Community of Madrid) (Ref. PROEX 169/15). Experiments were carried out at the animal house with official identification code ES280790001164 following the 3Rs principles. Animal handling and sampling were performed by trained and officially qualified personnel.

**Supporting Information.**

Antiparasitic activity against *Trypanosoma bruc*ei, *Leishmania infantum* and *Trypanosoma cruzi* (Table S1, S2 and S3); ADME-Tox data (Table S4); Concentration of compound 4 in BALB/c mice plasma at different sampling times after intravenous injection (IV) (1 mg/kg) (Table S5); Concentration of compound 4 in BALB/c mice plasma at different sampling times after oral administration of 20 mg/kg

compound 4 formulated with cyclodextrins, cyclodextrins + PEG 400 or administered free (Table S6); Comparison of activity and selectivity between eight 2'- hydroxy methoxylated chalcones and their corresponding methoxylated flavonols already published (Table S7); Experimental data of the synthesized compounds (pp. S11- S16); 1H-NMR and 13C-NMR spectra of compounds 4 and 8 (pp. S17-S20).

Molecular formula strings (CSV).

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**Abbreviations used**

NTDs, Neglected Tropical diseases; HAT, Human African trypanosomiasis; DNDi, Drugs for Neglected Diseases initiative; *L. infantum*, *Leishmania infantum*; *L. donovani*, *Leishmania donovani*; *T. brucei, Trypanosoma brucei*; *T. cruzi, Trypanosoma cruzi*; $CDCl_3$, deuterated trichloromethane, EtOH, Ethanol; NaOH, sodium hydroxide; TMS, Trimethylsilane; IV, intravenous; PBS, phosphate-buffered saline; ACN, acetonitrile; FBS, fetal bovine serum; $EC_{50}$, half maximal effective concentration; $CC_{50}$, half maximal cytotoxicity concentration; THP1, human monocytic cell line; A549, human lung adenocarcinoma epithelial cell line; WI-38, fetal lung fibroblasts cell lines; NOAEL, no adverse effect level.

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**Scheme 1**. Synthesis of the compounds **1**-**13**.
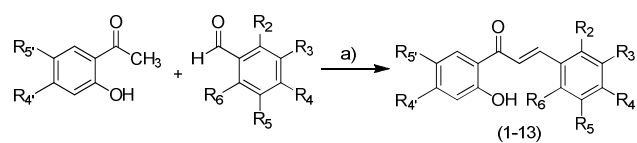


Reaction conditions: (a) NaOH (3 M), EtOH, rt.

**Table 1**. Chemical structure of the 13 synthesized 2'-hydroxy chalcones bearing methoxy groups, logP, $EC_{50}$ towards *T. brucei*, NOAEL (THP-1 cells) and selectivity index. * LogP licochalcone A = 4.74, LogP flavokawin B = 3.90.
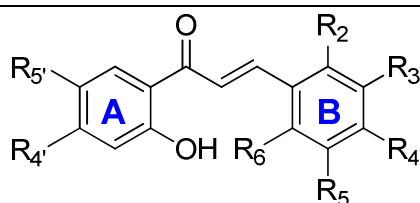


| Comp. | $R_{4'}$ | $R_{5'}$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | $R_6$ | LogP* | $EC_{50}$ *T. brucei* ($\mu$M) | $CC_{50} \pm$ SD | Selectivity index ($CC_{50}/EC_{50}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | H | H | H | $OCH_3$ | H | H | H | 3.94 | $1.3 \pm 0.2$ | >25 | >19 |
| 2 | $OCH_3$ | H | H | $OCH_3$ | H | H | H | 3.86 | $3.4 \pm 0.8$ | >25 | >7 |
| 3 | H | $OCH_3$ | H | $OCH_3$ | H | H | H | 3.87 | $2.1 \pm 0$ | >25 | >12 |
| 4 | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | H | H | H | 3.59 | $4.2 \pm 0.2$ | $51 \pm 7$ | 12 |
| 5 | $OCH_3$ | H | H | H | $OCH_3$ | H | H | 3.88 | - | >100 | - |
| 6 | H | $OCH_3$ | H | H | $OCH_3$ | H | H | 3.87 | - | >100 | - |
| 7 | $OCH_3$ | H | $OCH_3$ | H | H | H | $OCH_3$ | 3.51 | $3.4 \pm 0.1$ | >100 | >29 |
| 8 | H | $OCH_3$ | $OCH_3$ | H | H | H | $OCH_3$ | 3.52 | $2.1 \pm 0.4$ | >25 | >12 |
| 9 | $OCH_3$ | H | H | $OCH_3$ | $OCH_3$ | H | H | 3.53 | - | >100 | - |
| 10 | H | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | H | H | 3.52 | - | >50 | - |
| 11 | $OCH_3$ | H | H | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | 3.49 | - | >100 | - |
| 12 | H | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | 3.47 | $4.4 \pm 0.4$ | >25 | >6 |
| 13 | $OCH_3$ | $OCH_3$ | H | $OCH_3$ | $OCH_3$ | $OCH_3$ | H | 3.50 | $7.4 \pm 1.2$ | >25 | >3 |

(C) $R_{5'}$, $R_{4'}$, $R_3$, $R_4$, $R_5$ = H, $OCH_3$
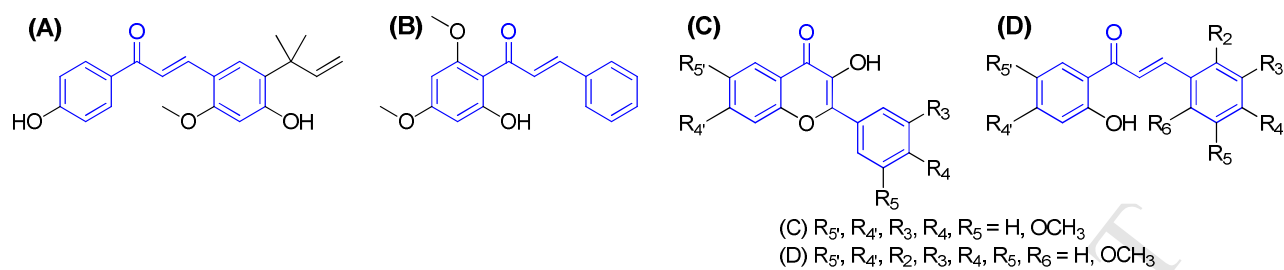(D) $R_{5'}$, $R_{4'}$, $R_2$, $R_3$, $R_4$, $R_5$, $R_6$ = H, $OCH_3$

**Figure 1.** Chemical structure of licochalcone A (A) and flavokawin B (B), general chemical structure of the compounds studied in the present paper (C) and in our previously published paper (D). The common chemical features are shown in blue.
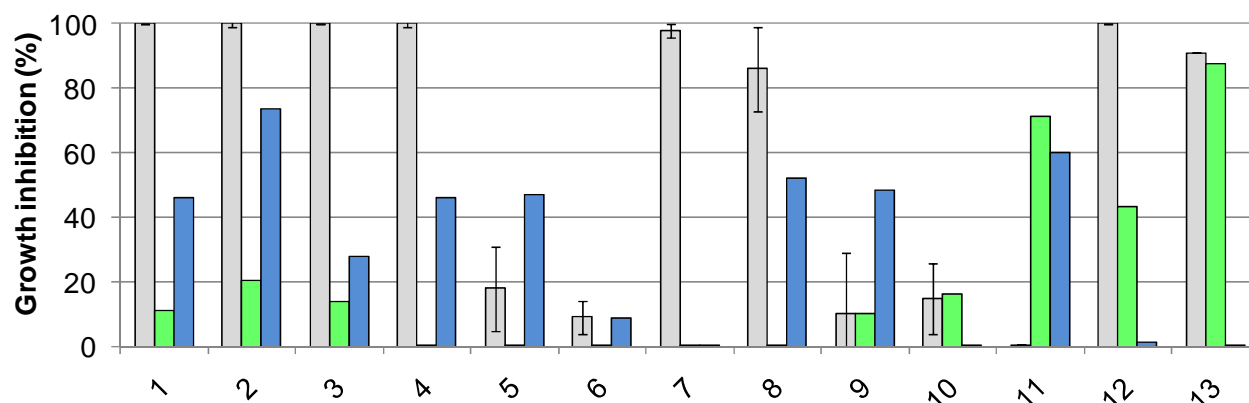
**Figure 2.** Percentage of growth inhibition of the synthesized compounds against *T. brucei* at 10 µM (gray bar)*, T. cruzi* at 10 µM (blue bar) and *L. infantum* at 50 µM (green bar)*. The reference compounds were pentamidine (EC$_{50}$ = 1.55 ± 0.24 nM) for *T. brucei* and miltefosine for *L. infantum* (EC$_{50}$ = 2.65 ± 0.4 µM), nifurtimox for *T. cruzi* (EC$_{50}$= 2.2 ± 0.3 µM). The data are reported in Table S1, S2 and S3 of the Supporting Information.

**Figure 3.** Early toxicological data of the thirteen synthesized compounds. The data are reported as a traffic light system. An ideal compound (I. comp.) should have all the parameters green. The cells are colored in green when the percentage of inhibition of *T. brucei* is between 60 and 100, while the percentage of inhibition of CYP isoforms, hERG, Aurora B kinase and mitochondrial toxicity is between 0 and 30. Cells are colored in red when data indicates toxicity (percentage of inhibition of CYP isoforms, hERG, Aurora B kinase and mitochondrial toxicity ≥ 60) or inactivity (percentage of inhibition of *T. brucei* is ≤ 30). Yellow stands for a borderline value (> 30 and < 60%): moderately active or slightly toxic compound. Compounds are not cytotoxic (green) when the percentage of A549 and W1-38 cell growth is between 60 and 100, cytostatic (yellow) when it is between 0 and 60 and cytotoxic (red) when it is below 0. Cells are colored in green when the selectivity index (CC$_{50}$ THP1/EC$_{50}$ *T. brucei*) is higher than 10. Gray: not evaluated.
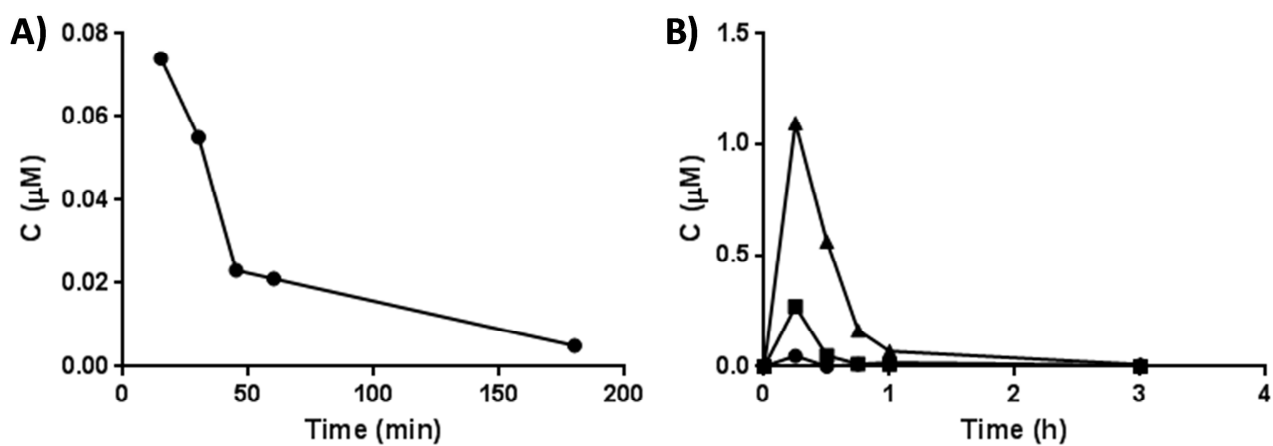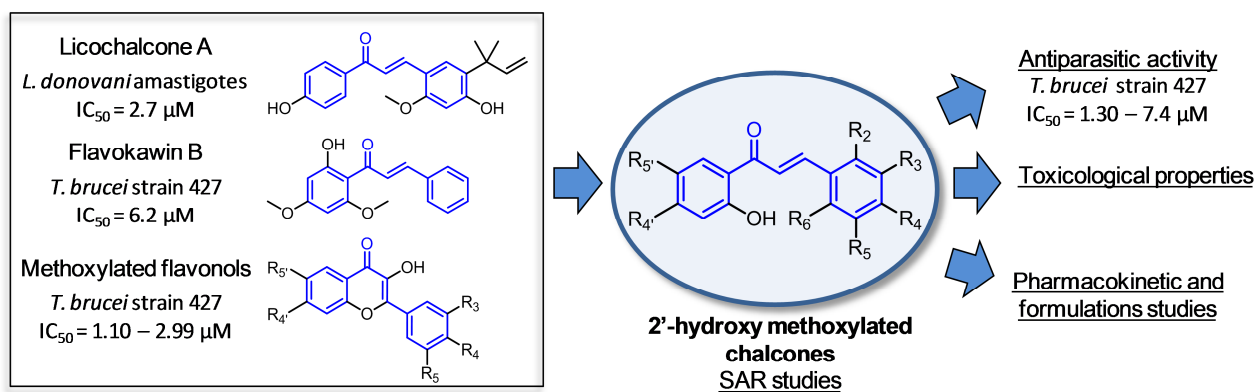
**Figure 4. A)** Plasma concentration in BALB/c mice of free compound **4** administered IV (1 mg/kg). **B)** Plasma concentration in BALB/c mice of compound **4** after oral administration of 20 mg/kg formulated with cyclodextrins (triangles), cyclodextrins + PEG 400 (circles) or free compound (squares).

## Graphical abstract



Licochalcone A
*L. donovani* amastigotes
$IC_{50}$ = 2.7 µM

Flavokawin B
*T. brucei* strain 427
$IC_{50}$ = 6.2 µM

Methoxylated flavonols
*T. brucei* strain 427
$IC_{50}$ = 1.10 − 2.99 µM

**2'-hydroxy methoxylated chalcones**
SAR studies

Antiparasitic activity
*T. brucei* strain 427
$IC_{50}$ = 1.30 − 7.4 µM

Toxicological properties

Pharmacokinetic and formulations studies

**Highlights**

- Thirteen 2'-hydroxy methoxylated chalcones were synthesized.
- Compounds 1, 3, 4, 7 and 8 showed higher potency than flavokawin B towards *T. brucei*.
- A panel of in-vitro early toxicological properties was evaluated.
- The formulation of compound 4 with cyclodextrins increased the oral bioavailability by 7.5 fold.