Letter

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SAR Studies and Biological Characterization of a chromen-4-one derivative as an Anti-*Trypanosoma brucei* Agent

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KEYWORDS: Trypanosoma brucei, flavonol-like compounds, SAR studies, ADME-tox properties, neglected tropical diseases.

ABSTRACT: chemical modulation of the flavonol 2-(benzo[d][1,3]dioxol-5-yl)-chromen-4-one (**1**), a promising anti-Trypanosomatid agent previously identified, was evaluated through a phenotypic screening approach. Herein, we have performed structure-activity relationship studies around hit compound **1**. The pivaloyl derivative (**13**) showed significant anti-*T. brucei* activity (EC_{50}= 1.1 μM) together with a selectivity index higher than 92. The early *in vitro* ADME-tox properties (cytotoxicity, mitochondrial toxicity, cytochrome P450 and *h*ERG inhibition) were determined for compound **1** and its derivatives and these led to the identification of some liabilities. The 1,3-benzodioxole moiety in the presented compounds confers better in vivo pharmacokinetic properties than those of classical flavonols. Further studies using different delivery systems could lead to an increase of compound blood levels.

Neglected tropical diseases (NTDs) are a group of infections that affect more than 1.4 billion people worldwide and mainly thrive among the poorest populations in tropical and subtropical areas.[1] Kinetoplastid parasites are responsible for the potentially fatal insect-borne diseases, namely Chagas disease, Human African Trypanosomiasis (HAT) and Leishmaniasis.[2] HAT, also known as sleeping sickness, is caused by infection with the *gambiense* and *rhodesiense* subspecies of the extracellular protozoan parasite *Trypanosoma brucei (T. brucei)*.[3] The tsetse fly, *Glossina spp*., is the vector of the sleeping sickness disease.[4] According to the World Health Organization (WHO), HAT continues to be a public health issue with an estimated number of new cases per year around 20000 and an estimated population at risk of 65 million people.[5] Despite the serious health, economic and social consequences of *T. brucei* infections, effective vaccines are lacking and the limited existing drug therapy presents drawbacks including toxicity, poor efficacy and serious side effects. Most of the available drugs have been used for over half a century, thus problems of drug resistance are emerging. Therefore, there is an urgent need for new, safe and effective drugs.[6] A phenotypic approach is a useful tool for drug discovery with the advantage of identifying compounds which are active against the whole cell. Membrane permeability, cell uptake and cell efflux are taken into account in the selection of new hits through phenotypic screening.[7] Phenotypic approaches to drug discovery have been successfully used in the field of neglected diseases, particularly for the treatment of HAT.[8-9] Two compounds discovered through phenotypic screening have recently been progressed into clinical trials by DNDi (Drugs for Neglected Diseases initiative): fexinidazole, a nitroimidazole and SCYX-7158, an oxaborole.[10] A wide range of chemical structures, including flavonols (3-hydroxy-2-phenylchromen-4-one), have been investigated in drug discovery programs with the aim of identifying novel antileishmanial and anti-trypanosomatid agents.[11-15] Very recently, we had replaced the phenyl ring of classical flavonols with heteroaromatic rings and biphenyl rings and we had synthesized a series of flavonol-like compounds with improved antiparasitic activity with respect to classical flavonols (Figure 1). Compound **1** bearing a 1,3-benzodioxole was identified as the most active and selective molecule towards *T. brucei* (EC_{50} = 0.4 μM, Selectivity Index (SI) = 250) (Figure 1).[16] According to the biological activity profile, compound **1** was suitable for progression in the drug discovery path. Moreover, the 1,3-benzodioxole represents a crucial pharmacophore with diverse biological activities and has been exploited in bioactive compounds with a wide range of medical applications, including cancer[17,18], tuberculosis[19], hepatitis B[20], fungal infections[21] and parasitic diseases[22,23].

The aims of our study were to validate compound **1** through Structure Activity Relationship (SAR) studies, discover follow-up hits and characterize their biological profile for potential liabilities identifications. The synthetic procedure followed for the synthesis of the compounds (**1-21**) is shown in Scheme 1 and the chemical structures are depicted in Table 1-3. The chalcones (**22-34**) were synthesized by Claisen-Schmidt condensation using substituted acetophenones and benzaldehydes in presence of NaOH as base. The reaction was carried out in ethanol as previously reported.[15] The chalcones were converted into the corresponding flavonol-like compounds (**1-10**, **19-21**) using the Flynn-Algar-Oyamada method for epoxidation and subsequent intramolecular cyclization of the open-chain structure (Scheme 1A). For the synthesis of esters (**11-15**) and carbamate **16**, compound **1** was treated with an excess of acyl chloride in dry DCM and in presence of triethylamine. The reaction was carried out at room temperature overnight. For the synthesis of ethers **17** and **18**, alkyl halide was added to a solution of compound **1** in dry DMF and in presence of K_2CO_3. The reaction was carried out under microwave irradiation (Scheme 1B).

The novel library of flavonol-like compounds (**2-21**) was evaluated towards *T. brucei* bloodstream form. The series was assessed for cytotoxicity on THP1 macrophage-like cells to estimate the CC_{50}. For compounds showing a percentage of parasite growth inhibition higher than 70%, the dose-response curve (DRC) was performed. The percentages of parasite growth inhibition at 10 μM are reported in Table S1 of the Supporting Information.

We started the SAR investigation of this scaffold by modifying the substituents on ring A (Table 1). Nine compounds (**2-10**) were synthesized introducing different substituents in position 6 and 7 of ring A. Five compounds (**2**, **4**, **8-10**) showed a significant activity towards *T. brucei* with EC_{50} lower than 5 μM. When the OCH_3 in position 7 of compound **1** was replaced with a methyl group and a chlorine or fluorine (**8**, **9** and **10**, respectively), the compounds maintained a meaningful anti-*T. brucei* activity. Moving the methoxy group from position 7 to 6 (compound **3**), we observed a huge drop of the antiparasitic activity. Compound **2**, bearing unsubstituted ring A, and compound **4**, with a methyl group in position 6 showed activity towards *T. brucei*, while compounds bearing halogen in position 6 (**5**-bromide; **6**-chlorine; **7**-fluorine) did not significantly inhibit *T. brucei* cells growth. Compound **8** (EC_{50} = 0.4 μM) displayed a potency comparable to that of the starting hit **1**, however it presented a reduced selectivity index (SI = 31).

Following this, our SAR was focused on modifications of the hydroxyl group in position 3 of the chromen-4-one scaffold (Table 2). The presence of an ester instead of a hydroxyl group in position 3 (**11-15**) led to significant activity on *T. brucei* (EC_{50} <1.1 μM) together with a SI >20. Among the esters, the 3-pivaloyl derivative of compound **1** (**13**) showed the most interesting profile with an EC_{50} towards *T. brucei* of 1.1 μM and SI >92. On the contrary, the presence of a carbamate (**16**) or an ether (**17** and **18**) led to inactivity towards *T. brucei*. These data suggested that the hydroxyl group in position 3 should be free in order to have a meaningful anti-*T. brucei* activity. The activity of esters can be related to an easier hydrolysis with respect to ethers and carbamates. We enlarged the SAR study modifying the 1,3-benzodioxole ring of compound **1** (compounds **19-21**, Table 3). Compound **19**, with two fluorine atoms instead of two hydrogens linked to the dioxolane ring, was less active than the starting compound **1**. The anti-*T. brucei* activity decreased replacing the dioxolane ring of **1** with a dioxane (compound **20**), while it was maintained in compound **21**, bearing a tetrahydrofurane. Compound **21** presented an EC_{50} towards *T. brucei* equal to 3.1 μM, but SI = 8. Overall, six compounds (**8**, **11-15**) showed a low micromolar EC_{50} and SI >20. Compound **13**, the 3-pivaloyl derivative of **1**, was the most selective among the novel synthesized molecules.

The synthesized library was assessed at 10 μM in a panel of early *in vitro* ADME-tox assays including cytotoxicity (A549 cell line), mitochondrial toxicity, cytochrome P450 (CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 isoforms) and *h*ERG inhibition. The data are reported in Figure 2 using a traffic light system. Compound **1** and all of its derivatives exhibited no liability towards *h*ERG and mitochondrial toxicity. Some compounds were shown to be cytostatic, with two compounds (**9** and **12**) being cytotoxic (<0% A549 cell growth). Most of the compounds displayed varying degrees of CYP450 liability. The IC_{50} towards *h*ERG and CYP isoforms were measured for compound **1**. The *h*ERG IC_{50} (>100 μM) was over 250-fold higher than the EC_{50} towards the parasite, thus in accord with the Target Product Profile (TPP) for hit prioritization. Compound **1** IC_{50} towards CYP1A2 and CYP2D6 were 0.4 and 0.05 μM, respectively, whereas for CYP2C9, CYP2C19 and CYP3A4 the IC_{50} were equal to 1.6, 1.5 and 6.0 μM, respectively. Compound **1** was the most optimal for its anti-trypanosomatid activity and ADME-tox profile and progressed to *in vivo* pharmacokinetic studies.

In vivo bioavailability and half-life were evaluated in BALB/c mice treated IV with 1 mg/kg and orally 20 mg/kg. Compound **1** displayed a half-life of 19 hrs after iv administration and of 45 hrs after oral (os) administration (Table 4). Both AUC and C_{max} values were similar despite the much higher dose administered per os. T_{max} for IV administration was reached after 1h this suggesting the possible intravascular aggregation of compound **1** given its low solubility.

The aggregation behavior of compound **1** in aqueous solution was investigated spectroscopically and the albumin sequestration assay performed. As compound **1** concentration is increased, both the absorption and the emission spectra show an increase of bands due to aggregates relative to the monomer bands (Figure 3). The absorption data were well fitted in terms of a monomer/dimer equilibrium, with a 1.8 (±0.3) x 10^5 M^{-1} equilibrium constant at 20 °C (see the Supporting Information). The fact that the aggregate absorption band is found at shorter wavelengths and its emission band at longer wavelengths than the corresponding bands of the monomeric form indicates the aggregates to be of H-type (as opposed to a J-type), i.e., with the monomers stacked on top of each other with a small slip angle.[24-25] Subsequent additions of human serum albumin (HSA) caused a progressive recovery of the monomer absorption band and the replacement of both aggregate and free monomer emission bands by a single new band that we assign to a compound **1**/HSA complex. Therefore, the latter represents a stable state with respect to the monomeric and dimeric states. Emission data analysis provided in the Supporting Information allowed us to estimate the **1**/HSA binding equilibrium constant, 2.5

(±1) x 10^5 M^{-1}. These results indicate that compound **1** has a tendency to aggregate in aqueous solution that can be reverted by albumin binding. We expect this behavior to occur in blood where albumin binding should help compound solubilization. Chemical changes enhancing solubility are expected to avoid aggregate formation and increase the blood levels of compound **1**, thus producing
testing.
Although removal of systemic infection may be beneficial to host survival, in the second stage HAT (which represents 90% of the total cases), the parasites colonize the central nervous system. To understand the suitability of compound **1** to pass the BBB we evaluated molecular descriptors, such as lipophilicity (cLogP), molecular weight (MW) and polar surface area (PSA) that provide insight into the factors that govern BBB penetration. Compound **1** fulfils the requirements for BBB penetration, i.e., cLogP in the range 1.5-2.7 (2.19 for compound **1**), MW < 400 (312.3 for compound **1**) and PSA < 90 $Å^2$ (74.22 $Å^2$ for compound **1**). Additionally, the 10^5 order of magnitude of the **1**/HSA binding equilibrium constant is consistent with that of CNS drugs that do cross the BBB (6 x10^4 M^{-1}).Therefore, we expect compound **1** to be sufficiently lipophilic to be transported by HSA and pass the CNS barrier.[26]

In summary, we have validated compound **1** bearing a 1,3-benzodioxole moiety as a potent anti-Trypanosomatid agent *in vitro*.[16] SAR studies around compound **1** have confirmed its profile as a valuable hit to progress to animal studies. We have synthesized twenty derivatives (**2-21**), compounds **10-21** are novel structure and have not been previously reported. The pivaloyl derivative (**13**) was the best compound of the hit-to-lead optimization process. Compound **13** has significant anti-*T. brucei* activity (EC_{50} = 1.1 μM) together with SI >92 and a reduced toxicity, thus showing a biological profile similar to **1**. The pharmacokinetic (PK) studies on **1** have demonstrated the ability of the 1,3-benzodioxole flavonol derivative to reach plasma concentrations >EC_{50} for *T. brucei* with oral administration, thus increasing classical flavonols half-life.[15] Compound **1** blood exposure was probably limited due to its low solubility and sequestration by albumin, as shown in aqueous solution experiments. Compound **1** is an interesting scaffold for anti-Trypanosomatid drug development that can be further exploited using drug delivery systems such as β-cyclodextrins which have a proven capacity to improve solubility of flavonoids.[27]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Antiparasitic activity towards *Trypanosoma brucei* (Table S1); Early ADME-tox data (Table S2); General information and Experimental data of synthesized compounds (pp. S6-S16). Molecular formula strings (CSV).

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

ADME-tox, Absorption, Distribution, Metabolism, and Excretion-tox; A549, human lung adenocarcinoma epithelial cell line; CC_{50}, half maximal cytotoxicity concentration; DCM, dichloromethane; DMF, dimethylformamide; DRC, dose-response curve; EC_{50}, half maximal effective concentration; EtOH, Ethanol; HAT, Human African trypanosomiasis; *h*ERG, human ether-a-go-go-related gene; HAS, human serum albumin; NaOH, sodium hydroxide; SI, Selectivity Index; *T. brucei*, *Trypanosoma brucei*; THP1, human monocytic cell line.

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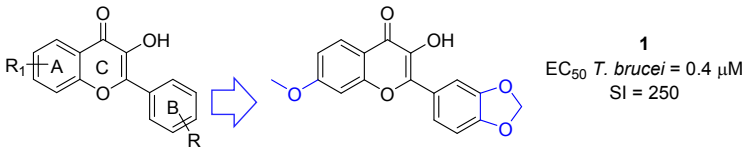


Figure 1. SAR studies on flavonol-like compounds and identification of compound **1**.

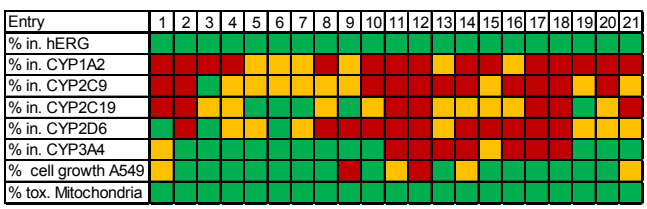
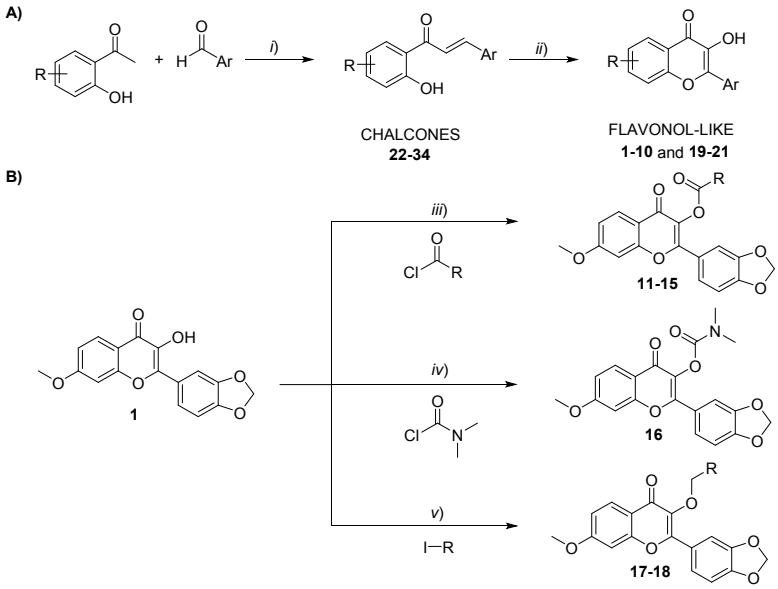


Figure 2. Early *in vitro* ADME-tox properties of compounds **1-21**. All the assays were performed at 10 μM. The data are reported as a traffic light system. An ideal compound would be expected to be associated with a green color (yielding <30% effect). For

CYP450, *h*ERG and mitochondrial toxicity, the cell is colored green when the value is 0-30%, yellow for values 31- 60% and red for values ≥61%. Compounds are non-cytotoxic (green) when A549 cell growth value is 60-100%, cytostatic (yellow) for values 0-59% and cytotoxic (red) for values <0%.



Scheme 1. A) Synthesis of the compounds **1-10** and **19-21**. Reaction conditions: (i) NaOH (3 M), EtOH, r.t.; (ii) H_2O_2, NaOH (1 M), EtOH, r.t. **B)** Synthesis of the compounds **11-18**. Reaction conditions: (iii) acyl chloride, dry DCM, N_2, r.t.; (iv) carbamoyl chloride, dry DCM, r.t.; (v) alkyl halide, dry DMF, MW 80°C, 0.5 h.

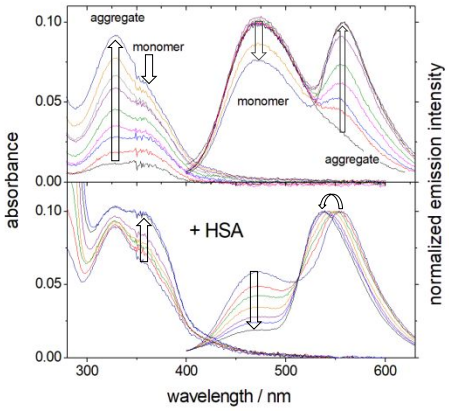
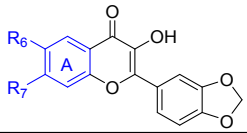


Figure 3. Absorption (left) and fluorescence emission spectra of compound 1 in phosphate buffer at pH 8 in the absence (top) and in the presence of human serum albumin (HSA). Top: effect of increasing concentration of compound **1**: 1.25, 2.5, 3.75, 5, 6.25, 7.5, 8.75, 10, 11.25 μM. Bottom: the arrows indicate the effect of the subsequent additions of HSA (1.68, 2.72, 4.11, 6.65, 9.65, 13.86 μM) to the 11.25 μM solution of compound **1**. Absorption maxima: free and HSA-complexed monomer, ≈ 360 nm; aggregate, 325 nm. Emission maxima: free monomer, 475 nm; aggregate, 560 nm, HSA-complexed monomer, 540 nm. λ_{exc} = 320 nm. The emission spectra were normalized to their maximum values for ease of presentation.

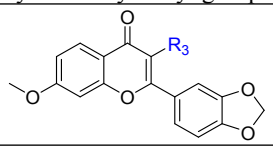
Table 1. SAR study on ring A of the cromen-4-one scaffold.

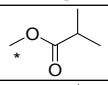
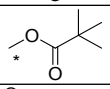
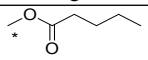
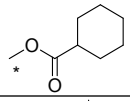
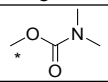
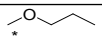


Comp.	R_3	R_6	R_7	EC_{50} ± SD (μM)	CC_{50} (μM)	SI
1	OH	H	OCH_3	0.4± 0.1	>100	250
2	OH	H	H	2.9 ± 0.4	12.5<CC_{50}<25	4
3	OH	OCH_3	H	-	<12.5	-
4	OH	CH_3	H	4.1 ± 2.1	<12.5	3*
5	OH	Br	H	-	12.5<CC_{50}<25	-
6	OH	Cl	H	-	<12.5	-
7	OH	F	H	-	12.5<CC_{50}<25	-
8	OH	H	CH_3	0.4 ± 0.1	12.5<CC_{50}<25	31
9	OH	H	Cl	3.8 ± 4.0	<12.5	3*
10	OH	H	F	2.4 ± 0.3	<12.5	8*

* Only estimations as the lower threshold of toxicity was not determined, EC_{50} >10μM. The reference compound for *T. brucei* was pentamidine (IC_{50} = 1.55 ± 0.24 nM). The synthesis of compounds **1**[28], **2**[29], **3**[30], **4**[31], **5**[30], **6**[30], **7**[30], **8**[32] and **9**[33] has been already published in literature. Compound **10** is a novel structure and has not been previously reported in literature.

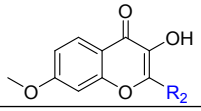
Table 2. SAR study on the hydroxyl group in position 3 of the cromen-4-one scaffold.

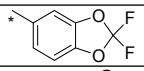
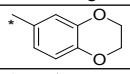
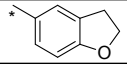


Comp.	R_3	EC_{50} ± SD (μM)	CC_{50} (μM)	SI
11		0.3 ± 0.3	<12.5	46*
12		0.5 ± 0.1	<12.5	24*
13		1.1 ± 0.2	>100	>92
14		0.6 ± 0.2	<12.5	22*
15		0.5 ± 0.1	12.5<CC_{50}<25	25
16		-	>100	-
17		-	50<CC_{50}<100	-
18		-	12.5<CC_{50}<25	-

*Only estimations as the lower threshold of toxicity was not determined, EC_{50} >10μM. The reference compound for *T. brucei* was pentamidine (IC_{50} = 1.55 ± 0.24 nM). Compounds **11-18** are novel structures and have not been previously reported in literature.

Table 3. SAR study modifying the 1,3-benzodioxole ring of compound **1**.



Comp.	R_2	EC_{50} ± SD (μM)	CC_{50} (μM)	SI
19		-	>100	-
20		-	50<CC_{50}<100	-
21		3.1 ± 0.5	25<CC_{50}<50	8

- EC_{50} >10μM. The reference compound for *T. brucei* was pentamidine (IC_{50} = 1.55 ± 0.24 nM). Compounds **19-21** are novel structures and have not been previously reported in literature.

Table 4. Pharmacokinetic parameters of compound **1**.

Comp.	Dose (mg) and route	Cmax (ng/mL)	Cmax (μM)	Tmax (h)	AUCtot (ng/mL h)	AUCtot (nmol/mL h)	Half life (h)
1	1 (IV)	340	1.08	1.00	3120	9.99	19.8
1	20 (per os)	290	0.91	0.50	2700	8.65	45.4

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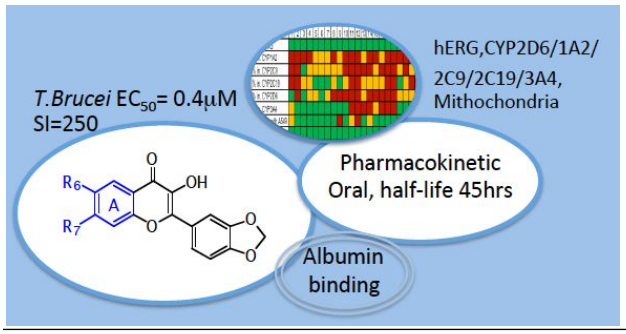
T.Brucei EC50= 0.4μM
SI=250
hERG,CYP2D6/1A2/
2C9/2C19/3A4,
Mithochondria
Pharmacokinetic
Oral, half-life 45hrs
Albumin
binding
R6
R7
A
O
OH
O
O
O