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Original article

Synthesis and in vitro anti-protozoal activity of a series of benzotropolone derivatives incorporating endocyclic hydrazines

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Abstract

The preparation and evaluation as potential anti-protozoal agents of molecules bearing an endocyclic hydrazine moiety is presented. The synthetic route to this new series of compounds is straightforward, involving a hetero Diels–Alder reaction between different benzotropolone esters and diethyl azodicarboxylate (DEAD). While they show limited or no in vitro activity against *Leishmania donovani*, *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense*, several of the compounds have activities against *Trypanosoma cruzi* in the 15.8–41.0 μM range. These activities are comparable to that of benznidazole (IC_{50} 6.0 μM), the main chemotherapy employed in the treatment of *T. cruzi* infections. In addition, all but one of the new bicyclic hydrazine esters are virtually non-toxic, one of the most important drawbacks of currently available trypanocidal drugs.

Keywords: Benzotropolone derivatives; Endocyclic hydrazines; Hetero Diels–Alder reaction; Anti-protozoal activity

1. Introduction

Parasitosis such as malaria, sleeping sickness, Leishmaniasis and Chagas disease are a threat to half of the world population, and constitute one of the most important public health concerns in tropical and subtropical regions. Despite the efforts made in the past 50 years to control transmission vectors, parasitic diseases are still endemic in most areas of incidence, with the number of infected individuals growing yearly [1,2]. Furthermore, the development of effective vaccines against them has been hampered by the complex biology and high adaptability through antigenic variation of the protozoan causative agents [3]. Thus, the main line of defense against parasitic infections has been through chemotherapy. However, and with the exception of artemisinin and its derivatives for the treatment of malaria [4], most of the drugs currently used for this purpose are old and non-specific, have significant toxicity, and present adverse side effects [5]. In view of this, the development of new classes of readily accessible compounds with anti-protozoal activity and improved pharmacological properties is imperative.

In a recent report we presented the synthesis and biological evaluation against *P. falciparum* and *T. cruzi* of a series of novel oxazine esters (Fig. 1a) [6]. The preparation of these compounds was straightforward, involving the esterification of benzotropolones followed by a high-yielding hetero Diels–Alder cycloaddition in which nitrosobenzene was used to incorporate an endocyclic –N–O– moiety into the seven-membered tropolone ring.

While these compounds displayed only limited activity against *P. falciparum*, several showed activities against *T. cruzi* comparable to that of benznidazole (Fig. 1b), one of the current therapies against this parasite. Unfortunately, the most active molecules had significant cytotoxicity due to their time-dependent thermal decomposition into starting materials with concomitant release of nitrosobenzene [7]. These results showed, however, that a new class of compounds with a completely novel molecular framework and potential

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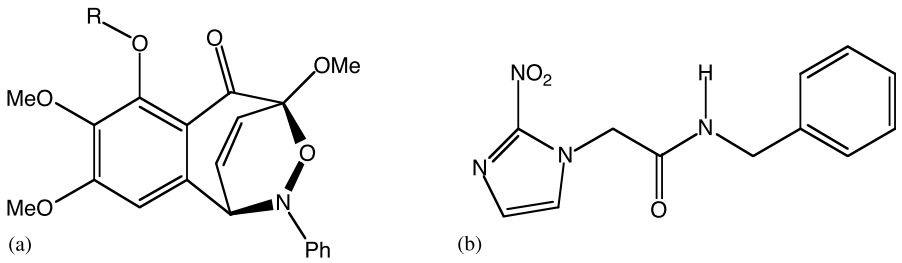

Fig. 1. General structure of the oxazines mentioned in the text (a), and of the antiparasitic drug benznidazole (b).

antiparasitic activity could be obtained from easily accessible starting materials using well established chemical transformations [8–12].

One of the attractive features of our approach is that the flexibility of the hetero Diels–Alder reaction can be exploited to incorporate moieties other than –N–O– into the final products, while at the same time maintaining a similar molecular scaffold. Thus, compounds bearing bridged –N–N–, –N–S–, or –O–O– fragments can be obtained by simply changing the dienophile used in the cycloaddition step [9–12]. This allows for a large number of compounds based on common precursors to be rapidly synthesized and tested, increasing the structural diversity and efficiency of the lead identification process. In this report we wish to present the evaluation as anti-protozoal agents of a series of benzotropolone derivatives bearing an endocyclic hydrazine moiety which were prepared through this approach. As described below, several of the new compounds have activities against *T. cruzi* approaching that of benznidazole, and, in contrast to our original oxazine esters and several antiparasitic drugs, display very low cytotoxicity.

2. Chemistry

As stated in the Introduction, our strategy for the preparation of the different series of exploratory compounds relies on a hetero Diels–Alder reaction between benzotropolone derivatives and diverse dienophiles. In order to incorporate a bridged hydrazine moiety, dienophiles bearing an electron-deficient azo functional group are required. A compound that has these characteristics is diethyl azodicarboxylate (DEAD). Apart from being a commercially available reagent, the use of DEAD in cycloaddition reactions is well documented [9], and it was therefore ideal for our purposes.

The first step towards the Diels–Alder adducts is the synthesis of the benzotropolone precursors, which has been described by us in previous reports [6,13]. Briefly, the C4′-position hydroxyl group of trimethylpurpurogallin (**1**) was acylated by treatment with the appropriate acyl chloride in the presence of triethylamine and catalytic dimethyl aminopyridine (DMAP) using CH_2C1_2 as solvent. The selection of the acyl chlorides employed in the acylations was guided by several factors. Namely, they correspond with those used in our earlier work [6], are readily available commercially, and provide a small but representative series of alkyl and aryl substitutions at the C4′-position of the benzotropolone. Ten aliphatic and aromatic esters were prepared in yields ranging from 55 to 93% following this procedure (**2a**–**2j**, Fig. 2).

The final bicyclic hydrazines were then obtained by treatment of esters **2a**–**2j** with 2 equiv. of DEAD in refluxing toluene. The reaction proceeded smoothly giving exclusively products **3a**–**3j**, with no starting materials detectable by TLC after 16–32 hours. In addition to these compounds, the DEAD Diels–Alder adducts of tetramethylpurpurogallin (**2k**) and **1**, compounds **3k** and **3l**, were also prepared following this procedure.

In all cases, the success of the Diels–Alder reaction was confirmed by inspection of the spectroscopic data, particularly the ^{1}H-NMR spectra. Apart from the signals corresponding to two distinct ethylcarbamate groups in the DEAD adducts, considerable differences for the chemical shifts of protons in the tropolone ring are observed before and after the cycloaddition. While protons on carbons C5, C6, and C7 in the starting materials display signals at approximately 6.20, 6.50 and 6.90 ppm, respectively, their resonances shift to roughly 5.95, 7.25 and 5.70 ppm in the products. These changes are consistent with the conversion of the planar pseudo-aromatic tropolone ring system into a bicyclic framework in which the C5–C6 double bond is not co-planar with the remaining aromatic ring.

It is also worth noting that when we attempted to record the ^{1}H- or ^{13}C-NMR spectra of compounds **3a**–**3l** at 9.4 Tesla, most signals corresponding to nuclei in the vicinity of the bridged nitrogen atoms were severely broadened due to the effects of the ^{14}N quadrupoles on ^{1}H and ^{13}C relaxation times. Although still observable, these effects were not as dramatic at lower magnetic fields, and all NMR spectra for compounds **3a**–**3l** were recorded at 2.1 Tesla.

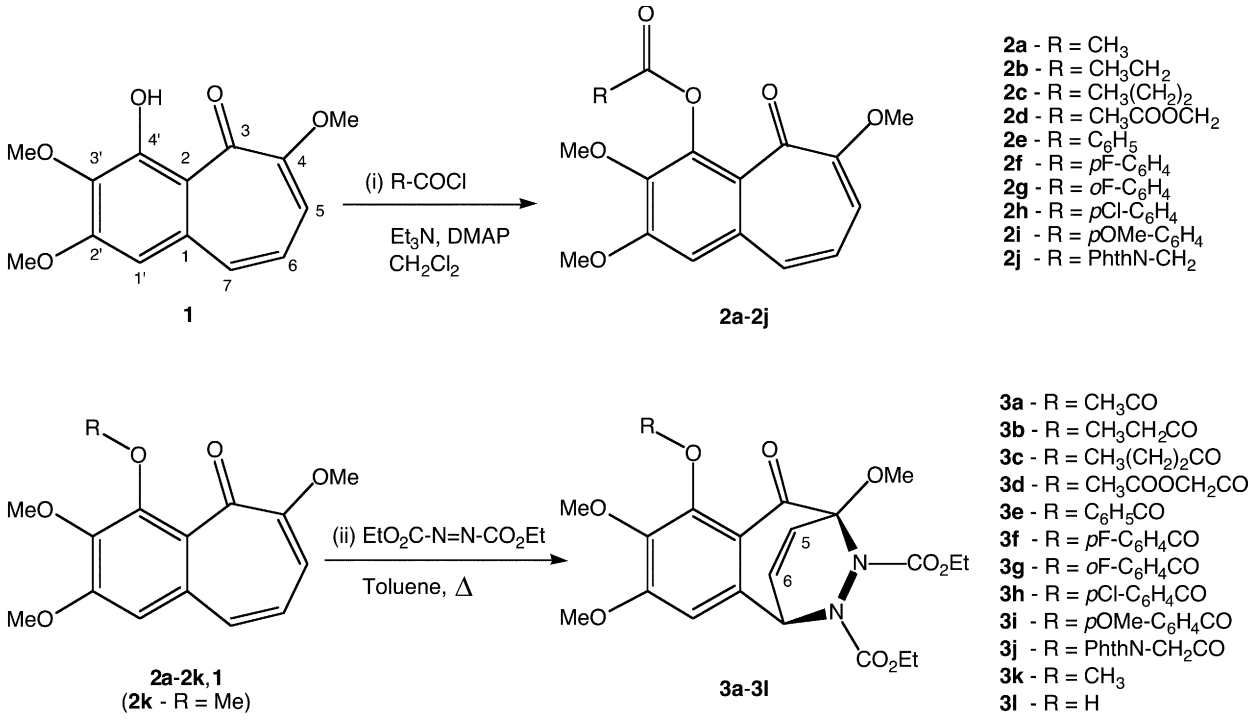

Fig. 2. Synthetic route to hydrazines **3a**–**3l**. (i) R–COC1 (1.5 equiv.), Et_3N (1.5 equiv.), DMAP (0.5 equiv.), CH_2C1_2, RT, 8–16 h. (ii) $EtCO_2$–N=N–CO_2Et (2.0 equiv.), toluene (reflux), 16–32 h. PhthN represents the phthalinido group (compounds **2j** and **3j**).

3. Biological results and discussion

The in vitro biological activity of compounds **3a**–**3l** against *L. donovani*, *T. brucei rhodesiense*, *P. falciparum* and *T. cruzi* is presented in Table I. Our data indicate that the compounds are ineffective against *L. donovani*, with all IC_{50} being at least higher than 48.2 μM. Hydrazines **3b** and **3f** display only limited activity against *P. falciparum*, with IC_{50} more than three orders of magnitude larger than that measured for chloroquine. Similar low activities against *T. brucei* were found for compounds **3e**, **3g**, **3h** and **3j**, their IC_{50} in this case being at least four orders of magnitude higher than that of the control pentamidine.

The results obtained in the *T. cruzi* assays were more encouraging. The IC_{50} for hydrazines **3a**, **3c**, **3d**, **3f**, and **3k** against this parasite were in the 15.8–41.0 μM range. These IC_{50} are less than one order of magnitude higher than the one of benznidazole, found to be 6.0 μM in the same assay, with compound **3d** being only 2.5 times less

Table I
In vitro activity of hydrazines **3a**–**3l** against protozoan parasites

Compound	IC_{50} (μM)				Toxicity (ED_{50} μg mL^{-1})
	L. donovani	*P. falciparum*	*T. brucei*	*T. cruzi*	
3a	> 62.8	> 62.8	> 62.8	41.0	> 300
3b	> 61.0	51.4	> 61.0	> 61.0	> 300
3c	> 59.3	> 59.3	> 59.3	26.3	> 300
3d	> 55.9	> 55.9	> 55.9	15.8	> 300
3e	> 55.6	> 55.6	30.6	> 55.6	> 300
3f	> 52.6	39.1	> 52.6	23.0	> 300
3g	> 52.3	> 52.3	17.9	> 52.3	> 300
3h	> 53.8	> 53.8	44.9	> 53.8	> 300
3i	> 53.8	> 53.8	> 53.8	> 53.8	< 0.3
3j	> 48.2	> 48.2	38.4	> 48.2	> 300
3k	> 68.8	> 68.8	> 68.8	37.4	> 300
31	> 66.7	> 66.7	> 66.7	> 66.7	> 300

The following controls were used: Pentostam (IC_{50} 4.0 μg Sb^V mL^{-1}, *L. donovani*); Chloroquine (IC_{50} 3.0 nM, *P. falciparum*); Pentanidine (IC_{50} 35 nM, *T. brucei*); Benznidazole (IC_{50} 6.0 μM, *T. Cruzi*). Toxicity was assayed against KB cells using podophyllotoxin (ED_{50} 0.0001 μg mL^{-1}) as standard.

active than the control. Although no clear correlation between the structure of the substituent on the C4′ hydroxyl and activity can be established, the presence of aliphatic chains at this position appears to be beneficial, with *p*-anisoate ester **3f** being the exception. From the compounds in this subset, acetoacetate ester **3d** deserves further attention. This molecule is the most active against *T. cruzi*, and, as shown above, is ineffective against other parasites. Interestingly, the corresponding acetoacetate ester from our earlier oxazines had no trypanocidal activity, but proved to be the most active compound from that series against *P. falciparum* [6]. Although preliminary, these data seem to indicate that the nature of the endocyclic moiety could be involved in specificity, and to some extent the parasite targeted by these compounds.

Cytotoxicity against KB cells is also reported in Table I. Except for *p*-fluorobenzoate ester **3i**, which incidentally was one of the two compounds inactive in all antiprotozoal assays performed, the molecules were non-toxic to cells in concentrations of up to 300 μg mL^{-1}. These results contrast those obtained for our earlier oxazine derivatives, which were cytotoxic at concentrations of 60.2 μg mL^{-1} or lower [6]. As mentioned earlier, the benzotropolone derivatives containing a bridged –N–O– moiety undergo a time-dependent thermal decomposition through a retro Diels–Alder reaction. This process, driven by the return to stable aromatic starting materials [6,7], releases highly toxic nitrosobenzene. Thus, all of the compounds from our original series with activity against *T. cruzi* were significantly cytotoxic, and none of them had therapeutic indexes (TI) above 7.5. For example, the most active oxazine in the *T. cruzi* assay, with an IC_{50} of 4.3 μM, was toxic to KB cells at concentrations of 0.3 μg mL^{-1} or higher ($TI < 0.19$). These molecules also displayed considerable activity against *P. falciparum*, indicating that they were non-specific. On the other hand, hydrazines **3a**, **3c**, **3d**, **3f** and **3k** have TI values ranging from 15.3 to 35.3, and, with the exception of *p*-anisoate **3f** which shows limited activity in the *P. falciparum* assay, are specific against *T. cruzi*.

The results presented above allow us to draw several conclusions regarding the biological properties of the compounds reported here. First, comparison of activity and cytotoxicity data obtained for the Diels–Alder adducts bearing –N–N– and –N–O– groups indicates that the endocyclic fragment determines the specificity of the benzotropolone derivatives towards different parasites. Biological data for structurally related compounds bearing other endocyclic moieties, which, as mentioned earlier, can be obtained by varying the structure of the dienophiles used in the hetero Diels–Alder reaction, are required to further prove this hypothesis. Second, aliphatic substituents at the C4′-position were shown to improve the in vitro trypanocidal activity of the compounds, most likely due to an enhancement of their membrane permeability. This would explain why compound **3l**, which has a free phenolic hydroxyl group at the C4′-position, lacks activity against all four parasites studied. In the case of compound **3d**, the acetoacetate ester could initially confer permeability through the cell membrane, only to be hydrolyzed to a free hydroxyl group after absorption. The resulting decrease in lipophilicity could prevent the molecule from escaping the trypanosomatids, explaining why this derivative displayed the highest activity in the *T. cruzi* assay. A similar prodrug behavior has been proposed for chloroquine and other aminoquinolone derivatives [14], and this could be exploited to further improve the pharmacodynamic properties and activity of these compounds.

Although the mode of action of these molecules is unknown at this time, we believe that their activity against *T. cruzi* is linked to the presence of the endocyclic hydrazine moiety. The single N–N bond in this group has a bond dissociation energy (BDE) of only 38.4 Kcal mol^{-1} [15], making it prone to undergo homolytic cleavage. The resulting free radicals should be capable of disrupting oxygen metabolism processes specific to trypanosomatids, which are known to be highly sensitive to oxidative stress [16]. This mechanism of action has been proposed for benznidazole, nifurtimox, and other nitroaromatic compounds [17,18]. Due to their low in vitro toxicity and unique structure, it is worth investigating if the novel compounds described in this report have a mode of action similar to that of the aforementioned nitroaromatic drugs.

4. Conclusions

The preparation and biological evaluation of a series of benzotropolone derivatives incorporating bridged hydrazine fragments was presented. Our results show that the compounds are virtually non-toxic, and despite they posses limited or no activity against *L. donovani*, *P. falciparum* and *T. brucei rhodesiense*, they display activities against *T. cruzi* which approach that of benznidazole. Comparison of these results with biological data obtained for an earlier series of structurally related oxazine derivatives indicates that the activity of the new compounds stems from the presence of the endocyclic hydrazine moiety, while the substituent at the C4′-position is likely to modulate their pharmacodynamic properties. Experiments aimed at improving the activity of this novel class of compounds and determining their mode of action are underway, and the results from these investigations will be presented in due course.

5. Experimental

5.1. Synthesis

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. When appropriate, products were purified by column chromatography using 60–200 mesh silica gel (EM Science, NJ, USA). IR spectra were obtained on a Perkin–Elmers spectrum 1000 FT-IR instrument. NMR spectra were recorded on Bruker Avance 400 and Anazasi EFT-90 spectrometers at fields of 9.4 and 2.1 Tesla, respectively, using $CDC1_3$ as a solvent unless otherwise specified. Chemical shifts (δ) are in ppm relative to tetramethylsilane (TMS), and coupling constants (J) are reported in Hz. ESI-MS spectra were recorded on a Micromass Trio-2000 single quadrupole mass spectrometer. Purpurogallin (m.p. 276–277 °C, dec., lit. 271–272 °C [19]) was prepared in 75% yield by oxidation of pyrogallol with aqueous KIO_3 following reported procedures [19]. Trimethylpurpurogallin (**1**, m.p. 174–176 °C, lit. 174–177 °C [20]) and tetramethylpurpurogallin (**2k**, m.p. 90–91 °C, lit. 92–93 °C [7]) methylethers were obtained in 42 and 82% yield, respectively, by treatment of purpurogallin with dimethyl sulfate in aqueous NaOH using the protocols of Barltrop and Nicholson [20].

5.1.1. General procedure for the preparation of 4,2′,3′-trimethylpurpurogallin derivatives (*2a*–*2j*)

The appropriate acyl chloride (15.2 mmol) in CH_2C1_2 (5 mL) was added gradually to a solution of trimethylpurpurogallin (**1**, 7.6 mmol), DMAP (3.8 mmol), and triethylamine (15.2 mmol) in CH_2C1_2 (20 mL) at 0 °C under a nitrogen atmosphere. The resulting solution was stirred for a period of 8–16 h at room temperature (r.t.) until TLC showed no starting material. The reaction mixture was then washed with 1 N HC1, saturated aqueous $NaHCO_3$, and water, the organic extracts were dried with $MgSO_4$, and the solvent was evaporated under vacuum. The crude solid was recrystallized from ether–hexanes, and further purified by column chromatography using CH_2C1_2–EtOAc (10:1–1:1) as eluting solvent.

5.1.1.1. 4,2′,3′-Trimethyl-4′-acetyl-purpurogallin (*2a*).

Obtained in 57% yield. M.p. 141–143 °C. IR (KBr disc, cm^{-1}): 1625 (s, C=O, ketone), 1773 (s, C=O, ester), 2950 (w, Ar–H). ^{1}H-NMR (400 MHz): δ 2.41 (3H, s), 3.80 (3H, s), 3.89 (3H, s), 3.95 (3H, s), 6.23 (1H, d, $J = 8.8$), 6.52 (1H, dd, $J = 8.8$, $J = 11.6$), 6.87 (1H, bs), 6.91 (1H, bd, $J = 11.6$). ^{13}C-NMR (100 MHz): δ 21.2, 56.4, 56.5, 61.5, 106.9, 110.3, 124.1, 124.2, 129.9, 133.7, 142.9, 143.9, 155.5, 159.4, 169.7, 183.0. ESI-MS:305.2 ([M]+H).

5.1.1.2. 4,2′,3′-Trimethyl-4′-propionyl-purpurogallin (*2b*).

Obtained in 67% yield. M.p. 114–116 °C. IR (KBr disc, cm^{-1}): 1595 (s, C=O, ketone), 1764 (s, C=O, ester), 2942 (w, Ar–H). ^{1}H-NMR (400 MHz): δ 1.32 (3H, t, $J = 7.6$), 2.74 (2H, q, $J = 7.6$), 3.80 (3H, s), 3.89 (3H, s), 3.96 (3H, s), 6.23 (1H, d, $J = 8.8$), 6.53 (1H, dd, $J = 8.8$, $J = 11.6$), 6.88 (1H, bs), 6.91 (1H, bd, $J = 11.6$). ^{13}C-NMR (100 MHz): δ 9.4, 27.8, 56.4, 56.5, 61.5, 106.9, 110.1, 124.1, 124.4, 129.9, 133.7, 142.9, 144.0, 155.5, 159.4, 173.1, 183.1. ESI-MS: 319.4 ([M]+H).

5.1.1.3. 4,2′,3′-Trimethyl-4′-butyryl-purpurogallin (*2c*).

Obtained in 67% yield. M.p. 116–119 °C. IR (KBr disc, cm^{-1}): 1624 (s, C=O, ketone), 1769 (s, C=O, ester), 2950 (m, Ar–H). ^{1}H-NMR (400 MHz): δ 1.09 (3H, t, $J = 7.6$), 1.86 (2H, sex, $J = 7.6$), 2.69 (2H, t, $J = 7.6$), 3.81 (3H, s), 3.89 (3H, s), 3.97 (3H, s), 6.23 (1H, d, $J = 8.8$), 6.54 (1H, dd, $J = 8.8$, $J = 11.6$), 6.88 (1H, bs), 6.93 (1H, bd, $J = 11.6$). ^{13}C-NMR (100 MHz): δ 14.0, 18.7, 36.3, 56.4, 56.5, 61.4, 106.8, 110.1, 124.1, 124.4, 129.9, 133.6, 142.8, 143.9, 155.5, 159.4, 172.2, 183.1. ESI-MS: 331.1 ([M]+H).

5.1.1.4. 4,2′,3′-Trimethyl-4′-acetoxyacetyl-purpurogallin (*2d*).

Obtained in 55% yield. M.p. 142–144 °C. IR (KBr disc, cm^{-1}): 1624 (s, C=O, ketone), 1750, 1786 (s, C=O, ester), 2943 (w, Ar–H). ^{1}H-NMR (400 MHz): δ 2.22 (3H, s), 3.84 (3H, s), 3.93 (3H, s), 4.00 (3H, s), 5.04 (2H, bs), 6.29 (1H, d, $J = 8.8$), 6.59 (1H, dd, $J = 8.8$, $J = 11.6$), 6.94 (1H, bs), 6.98 (1H, bd, $J = 11.6$). ^{13}C-NMR (100 MHz): δ 21.0, 56.4, 56.6, 61.3, 61.7, 107.3, 110.7, 124.1, 124.3, 130.0, 133.7, 143.1, 143.2, 155.7, 159.5, 166.7, 170.7, 182.6. ESI-MS: 363.0 ([M]+H).

5.1.1.5. 4,2′,3′-Trimethyl-4′-benzoyl-purpurogallin (*2e*).

Obtained in 63% yield. M.p. 158–160 °C. IR (KBr disc, cm^{-1}): 1598 (s, C=O, ketone), 1742 (s, C=O, ester), 2942 (w, Ar–H). ^{1}H-NMR (400 MHz): δ 3.77 (3H, s), 3.91 (3H, s), 4.00 (3H, s), 6.25 (1H, d, $J = 8.8$), 6.56 (1H, dd, $J = 8.8$, $J = 11.6$), 6.94 (1H, bs), 6.97 (1H, bd, $J = 11.6$), 7.52 (2H, dd, $J = 7.6$, $J = 8.3$), 7.63 (1H, tt, $J = 1.3$, $J = 7.6$), 8.26 (2H, dd, $J = 1.3$, $J = 8.3$). ^{13}C-NMR (100 MHz): δ 56.4, 56.5, 61.6, 107.1, 110.3, 124.2, 124.6, 128.9, 129.9, 130.0, 130.9, 133.6, 133.7, 143.0, 144.0, 155.6, 159.5, 165.3, 183.0. ESI-MS: 367.4 ([M]+H).

5.1.1.6. 4,2′,3′-Trimethyl-4′-(*p*-anisoyl)-purpurogallin (*2f*).

Obtained in 85% yield. M.p. 126–130 °C. IR (KBr disc, cm^{-1}): 1601 (s, C=O, ketone), 1732 (s, C = 0, ester). ^{1}H-NMR (400 MHz): δ 3.76 (3H, s), 3.90 (3H, s), 3.91 (3H, s), 4.00 (3H, s), 6.24 (1H, d, $J = 8.8$), 6.56 (1H, dd, $J = 8.8$, $J = 11.6$), 6.93 (1H, bs), 6.97 (1H, bd, $J = 11.6$), 6.99 (2H, AA′XX′), 8.21 (2H, AA′XX′). ^{13}C-NMR (100 MHz): δ 55.9, 56.4, 56.5, 61.6, 107.0, 110.1, 114.2, 122.4, 124.1, 124.8, 129.8, 133.1, 133.5, 143.1,

144.1, 155.5, 159.5, 164.2, 165.0, 183.1. ESI-MS: 397.4 ([M]+H).

*5.1.1.7. 4,2′,3′-Trimethyl-4′-(p-chlorobenzoyl)-purpurogallin (**2g**).* Obtained in 55% yield. M.p. 150–152 °C. IR (KBr disc, cm^{-1}): 1599 (s, C=O, ketone), 1744 (s, C=O, ester). ^{1}H-NMR (400 MHz): δ 3.87 (3H, s), 3.91 (3H, s), 4.01 (3H, s), 6.26 (1H, d, $J=8.8$), 6.58 (1H, dd, $J=8.8$, $J=11.6$), 6.95 (1H, bs), 6.98 (1H, bd, $J=11.6$), 7.49 (2H, AA′XX′), 8.19 (2H, AA′XX′). ^{13}C-NMR (100 MHz): δ 56.4, 56.5, 61.6, 107.1, 110.4, 124.3, 124.4, 128.5, 129.2, 129.9, 132.3, 133.7, 140.2, 143.0, 143.8, 155.6, 159.5, 164.5, 182.9. ESI-MS: 401.1 ([M]+H).

*5.1.1.8. 4,2′,3′-Trimethyl-4′-(o-flurobenzoyl)-purpurogallin (**2h**).* Obtained in 77% yield. M.p. 174–176 °C. IR (NaCl film, cm^{-1}): 1600 (s, C=O, ketone), 1752 (s, C=O, ester), 2944 (w, Ar–H). ^{1}H-NMR (400 MHz): δ 3.78 (3H, s), 3.94 (3H, s), 4.00 (3H, s), 6.26 (1H, d, $J=8.8$), 6.57 (1H, dd, $J=8.8$, $J=11.6$), 6.94 (1H, bs), 6.97 (1H, bd, $J=11.6$), 7.20 (1H, dddd, $J=0.5$, $J=1.0$, $J=8.3$, $^3J_{HF}=10.6$), 7.28 (1H, ddd, $J=1.0$, $J=7.3$, $J=8.0$), 7.59 (1H, dddd, $J=2.0$, $J=7.3$, $J=8.3$, $^4J_{HF}=4.8$), 8.21 (1H, dddd, $J=0.5$, $J=2.0$, $J=8.0$, $^4J_{HF}=7.3$). ^{13}C-NMR (100 MHz): δ 56.4, 56.5, 61.6, 107.1, 110.4, 117.4 ($^2J_{CF}=22.0$), 118.6 ($^2J_{CF}=9.5$), 124.2, 124.4, 124.5 ($^3J_{CF}=4.4$), 129.9, 133.4, 133.7, 135.2 ($^3J_{CF}=8.8$), 143.0, 143.7, 155.6, 159.5, 162.7 ($^3J_{CF}=3.7$), 162.8 ($^1J_{CF}=261.3$), 182.9. ESI-MS: 385.4 ([M]+H).

*5.1.1.9. 4,2′,3′-Trimethyl-4′-(p-fluorobenzoyl)-purpurogallin (**2i**).* Obtained in 93% yield. M.p. 155–157 °C. IR (NaCl film, cm^{-1}): 1600 (s, C=O, ketone), 1744 (s, C=O, ester), 2945 (w, Ar–H). ^{1}H-NMR (400 MHz): δ 3.78 (3H, s), 3.91 (3H, s), 4.02 (3H, s), 6.26 (1H, d, $J=8.8$), 6.58 (1H, dd, $J=8.8$, $J=11.6$), 6.96 (1H, bs), 6.99 (1H, bd, $J=11.6$), 7.19 (2H, dd, $J=8.8$, $^3J_{HF}=8.8$), 8.27 (2H, dd, $J=8.8$, $^4J_{HF}=5.1$). ^{13}C-NMR (100 MHz): δ 56.4, 56.5, 61.6, 103.3, 107.0, 110.3, 116.0 ($^2J_{CF}=22.0$), 124.2, 124.5, 126.3 ($^4J_{CF}=2.9$), 129.8, 133.5 ($^3J_{CF}=9.5$), 133.6, 143.0, 155.6, 159.5, 164.4, 166.5 ($^1J_{CF}=253.9$), 182.9. ESI-MS: 385.4 ([M]+H).

*5.1.1.10. 4,2′,3′-Trimethyl-4′-phtaloylglycil-purpurogallin (**2j**).* Obtained in 71% yield. M.p. 98–100 °C. IR (NaCl film, cm^{-1}): 1600 (m, C=O, ketone), 1723, 1772 (s, C=O, ester), 2945 (w, Ar–H).^{1}H-NMR (400 MHz): δ 3.81 (3H, s), 3.94 (3H, s), 3.96 (3H, s), 4.87 (2H, bs), 6.25 (1H, d, $J=8.8$), 6.53 (1H, dd, $J=8.8$, $J=11.6$), 6.89 (1H, bs), 6.91 (1H, bd, $J=11.6$), 7.73 (2H, AA′MM′), 7.89 (2H, AA′MM′). ^{13}C-NMR (100 MHz): δ 39.4, 56.4, 56.6, 61.7, 107.4, 110.7, 123.9, 124.0, 124.3, 129.9, 132.6, 133.6, 134.5, 142.9, 143.3, 155.6, 159.5, 166.4, 167.7, 182.6. ESI-MS: 452.2 ([M]+H).

*5.1.2. General procedure for the preparation of the diethyl azodicarboxylate (DEAD) Diels–Alder adducts **3a–3l***

DEAD (7.0 mmol) was added to a solution of the appropriate trimethylpurpurogallin derivative (**2a–2k** and **1**, 3.5 mmol) in toluene (25 mL), and the resulting mixture heated to reflux until no benzotropolone was detectable by TLC analysis (16–32 h). The solvent was then removed under reduced pressure, and the resulting oily solid was triturated with ether–hexanes to eliminate unreacted DEAD. The crude products were further purified by column chromatography using CH_2Cl_2–EtOAc (10:1–1:1) as eluting solvent.

*5.1.2.1. 4,2′,3′-Trimethyl-4′-acetyl-purpurogallin·DEAD Diels–Alder adduct (**3a**).* Obtained from **2a** in 82% yield. M.p. 116–118 °C. IR (KBr disc, cm^{-1}): 1597 (s, C=O, ketone), 1720, 1774 (s, C=O, ester), 2983 (m, Ar–H). ^{1}H-NMR (90 MHz): δ 1.01 (3H, bt, $J=6.8$), 1.32 (3H, t, $J=7.1$), 2.40 (3H, s), 3.53 (3H, s), 3.81 (3H, s), 3.95 (3H, s), 4.06 (2H, bq, $J=6.8$), 4.30 (3H, q, $J=7.1$), 5.70 (1H, bdd, $J=0.7$, $J=6.0$), 5.99 (1H, dd, $J=0.7$, $J=9.1$), 6.88 (1H, bs), 7.26 (1H, dd, $J=6.0$, $J=9.1$). ^{13}C-NMR (22.5 MHz): δ 13.3, 13.8, 20.0, 51.5, 55.8, 60.5, 61.4, 62.6, 62.8, 92.4, 111.1, 117.5, 127.5, 132.2, 142.9, 144.2, 146.7, 156.0, 156.8, 157.0, 169.3, 185.7. ESI-MS: 479.4 ([M]+H).

*5.1.2.2. 4,2′,3′-Trimethyl-4′-propionyl-purpurogallin·DEAD Diels–Alder adduct (**3b**).* Obtained from **2b** in 63% yield. M.p. 142–143 °C. IR (KBr disc, cm^{-1}): 1596 (m, C=O, ketone), 1708, 1720, 1762 (s, C=O, ester), 2983 (w, Ar–H). ^{1}H-NMR (90 MHz): δ 1.01 (3H, bt, $J=7.0$), 1.28 (3H, t, $J=7.3$), 1.30 (3H, t, $J=7.0$), 2.72 (2H, ABX), 3.51 (3H, s), 3.76 (3H, s), 3.94 (3H, s), 4.05 (2H, bq, $J=7.0$), 4.28 (2H, q, $J=7.3$), 5.67 (1H, bdd, $J=0.8$, $J=5.8$), 5.97 (1H, dd, $J=0.8$, $J=9.2$), 6.84 (1H, bs), 7.24 (1H, dd, $J=5.8$, $J=9.2$). ^{13}C-NMR (22.5 MHz): δ 8.3, 13.4, 13.9, 26.6, 51.6, 55.8, 60.6, 61.5, 62.7, 62.9, 92.5, 111.1, 120.9, 127.6, 132.3, 143.0, 144.2, 147.0, 156.1, 156.8, 157.2, 172.8, 185.8. ESI-MS: 493.5 ([M]+H).

*5.1.2.3. 4,2′,3′-Trimethyl-4′-butyryl-purpurogallin·DEAD Diels–Alder adduct (**3c**).* Obtained from **2c** in 91% yield. M.p. 173–175 °C. IR (KBr disc, cm^{-1}): 1598 (m, C=O, ketone), 1717, 1735, 1768 (s, C=O, ester), 2983 (w, Ar–H). ^{1}H-NMR (90 MHz): δ 1.06 (3H, bt, $J=7.1$), 1.32 (3H, t, $J=6.9$), 1.80 (2H, ABX_5), 2.68 (2H, ABX_2), 3.52 (3H, s), 3.79 (3H, s), 3.95 (3H, s), 4.05 (2H, bq, $J=7.1$), 4.31 (3H, t, $J=6.9$), 5.69 (1H, bdd, $J=0.9$, $J=5.9$), 5.98 (1H, dd, $J=0.9$, $J=9.1$), 6.85 (1H, bs), 7.25 (1H, dd, $J=5.9$, $J=9.1$). ^{13}C-NMR (22.5

MHz): δ 13.1, 13.5, 13.9, 17.6, 35.2, 51.6, 55.9, 60.6, 61.6, 62.8, 63.0, 92.6, 111.1, 125.9, 127.8, 132.4, 143.0, 144.3, 147.0, 156.1, 156.9, 157.3, 171.9, 185.7. ESI-MS: 507.2 ([M]+H).

*5.1.2.4. 4,2′,3′-Trimethyl-4′-acetoxyacetyl-purpurogallin·DEAD Diels–Alder adduct (**3d**).* Obtained from **2d** in 67% yield. M.p. 167–169 °C. IR (NaCl film, cm^{-1}): 1598 (m, C=O, ketone), 1716, 1749, 1790 (s, C=O, ester), 2983 (w, Ar–H). ^{1}H-NMR (90 MHz): δ 1.00 (3H, bt, $J=6.8$), 1.30 (3H, t, $J=7.1$), 2.17 (3H, s), 3.51 (3H, s), 3.80 (3H, s), 3.95 (3H, s), 4.00 (2H, bq, $J=6.8$), 4.28 (2H, q, $J=7.1$), 5.00 (2H, AB), 5.67 (1H, bdd, $J=0.8$, $J=5.9$), 5.97 (1H, dd, $J=0.8$, $J=8.9$), 6.85 (1H, bs), 7.23 (1H, dd, $J=5.9$, $J=8.9$). ^{13}C-NMR (22.5 MHz): δ 13.4, 13.9, 19.9, 51.6, 56.0, 60.4, 60.9, 61.5, 62.7, 63.0, 92.4, 111.5, 117.3, 127.6, 132.4, 143.2, 144.4, 145.9, 156.3, 156.8, 157.2, 166.5, 170.4, 185.9. ESI-MS: 537.2 ([M]+H).

*5.1.2.5. 4,2′,3′-Trimethyl-4′-benzoyl-purpurogallin·DEAD Diels–Alder adduct (**3e**).* Obtained from **2e** in 92% yield. M.p. 218–220 °C. IR (KBr disc, cm^{-1}): 1597 (s, C=O, ketone), 1716, 1730 (s, C=O, ester), 2982 (m, Ar–H). ^{1}H-NMR (90 MHz): δ 1.05 (3H, bt, $J=7.0$), 1.30 (3H, t, $J=7.0$), 3.48 (3H, s), 3.80 (3H, s), 3.96 (3H, s), 4.12 (2H, bq, $J=7.0$), 4.28 (2H, t, $J=7.0$), 5.72 (1H, bd, $J=5.8$), 5.96 (1H, bd, $J=9.0$), 6.90 (1H, bs), 7.24 (1H, dd, $J=5.8$, $J=9.0$), 7.50 (3H, m), 8.19 (2H, dd, $J=1.8$, $J=8.0$). ^{13}C-NMR (22.5 MHz): δ 13.6, 14.0, 51.6, 56.0, 60.8, 61.7, 62.8, 63.0, 92.6, 111.3, 126.4, 127.8, 128.5, 129.4, 130.6, 132.4, 133.4, 143.3, 144.2, 147.2, 156.3, 157.0, 157.4, 165.3, 185.5. ESI-MS: 541.2 ([M]+H).

*5.1.2.6. 4,2′,3′-Trimethyl-4′-(p-anisoyl)-purpurogallin·DEAD Diels–Alder adduct (**3f**).* Obtained from **2f** in 76% yield. M.p. 200–202 °C. IR (KBr disc, cm^{-1}): 1604 (m, C=O, ketone), 1721, 1736, 1750 (s, C=O, ester), 2976 (w, Ar–H). ^{1}H-NMR (90 MHz): δ 1.06 (3H, bt, $J=6.9$), 1.31 (3H, t, $J=7.1$), 3.49 (3H, s), 3.80 (3H, s), 3.86 (3H, s), 3.96 (3H, s), 4.09 (2H, bq, $J=6.9$), 4.30 (2H, q, $J=7.1$), 5.73 (1H, bd, $J=5.9$), 5.76 (1H, bd, $J=9.2$), 6.90 (1H, bs), 6.97 (2H, AA′XX′), 7.25 (1H, dd, $J=5.9$, $J=9.2$), 8.15 (2H, AA′XX′). ^{13}C-NMR (22.5 MHz): δ 13.5, 13.9, 51.5, 55.2, 55.9, 60.6, 61.6, 62.7, 62.9, 92.5, 111.2, 113.7, 117.9, 121.7, 127.6, 132.3, 132.6, 143.2, 144.1, 147.3, 156.1, 156.8, 157.2, 164.0, 164.7, 185.4. ESI-MS: 571.2 ([M]+H).

*5.1.2.7. 4,2′,3′-Trimethyl-4′-(p-chlorobenzoyl)-purpurogallin·DEAD Diels–Alder adduct (**3g**).* Obtained from **2g** in 84% yield. M.p. 224–226 °C. IR (KBr disc, cm^{-1}): 1598 (m, C=O, ketone), 1749, 1734, 1749 (s, C=O, ester), 2983 (w, Ar–H). ^{1}H-NMR (90 MHz): δ 1.03 (3H, bt, $J=7.1$), 1.30 (3H, t, $J=7.2$), 3.48 (3H, s), 3.79 (3H, s), 3.95 (3H, s), 4.11 (2H, bq, $J=7.1$), 4.29 (2H, q, $J=7.2$), 5.72 (1H, bd, $J=5.9$), 5.96 (1H, bd, $J=9.2$), 6.90 (1H, bs), 7.25 (1H, dd, $J=5.9$, $J=9.2$), 7.46 (2H, AA′XX′), 8.13 (2H, AA′XX′). ^{13}C-NMR (22.5 MHz): δ 13.6, 14.0, 51.6, 56.0, 60.8, 61.6, 62.7, 63.0, 92.5, 111.4, 117.8, 127.7, 127.9, 128.8, 132.0, 132.4, 139.8, 143.2, 144.2, 146.8, 156.1, 156.9, 157.2, 164.3, 185.6. ESI-MS: 575.3 ([M]+H).

*5.1.2.8. 4,2′,3′-Trimethyl-4′-(o-fluorobenzoyl)-purpurogallin·DEAD Diels–Alder adduct (**3h**).* Obtained from **2h** in 88% yield. M.p. 196–197 °C. IR (KBr disc, cm^{-1}): 1598 (m, C=O, ketone), 1719, 1730 (s, C=O, ester), 2982 (w, Ar–H). ^{1}H-NMR (90 MHz): δ 1.04 (3H, bt, $J=7.0$), 1.30 (3H, t, $J=7.0$), 3.49 (3H, s), 3.82 (3H, s), 3.96 (3H, s), 4.09 (2H, bq, $J=7.0$), 4.28 (2H, q, $J=7.0$), 5.72 (1H, bd, $J=5.9$), 5.97 (1H, bd, $J=9.1$), 6.91 (1H, bs), 7.21 (3H, m), 7.57 (1H, m), 8.14 (1H, m). ^{13}C-NMR (22.5 MHz): δ 13.4, 13.8, 51.5, 55.9, 60.7, 61.6, 62.8, 62.9, 92.5, 111.4, 116.8 ($^2J_{CF}=21.8$), 117.7, 117.9 ($^2J_{CF}=9.2$), 124.1 ($^3J_{CF}=4.1$), 127.5, 132.5, 132.9, 135.0 ($^3J_{CF}=9.1$), 143.0, 144.3, 146.5, 156.2, 156.8, 157.2, 162.3, 162.4 ($^1J_{CF}=262.4$), 185.6. ESI-MS: 559.1 ([M]+H).

*5.1.2.9. 4,2′,3′-Trimethyl-4′-(p-fluorobenzoyl)-purpurogallin·DEAD Diels–Alder adduct (**3i**).* Obtained from **2i** in 87% yield. M.p. 203–204 °C. IR (NaCl film, cm^{-1}): 1599 (s, C=O, ketone), 1719, 1744 (s, C=O, ester), 2983 (m, Ar–H). ^{1}H-NMR (90 MHz): δ 1.03 (3H, bt, $J=7.1$), 1.31 (3H, t, $J=7.1$), 3.48 (3H, s), 3.80 (3H, s), 3.96 (3H, s), 4.10 (2H, bq, $J=7.1$), 4.29 (2H, q, $J=7.1$), 5.72 (1H, bd, $J=5.8$), 5.97 (bd, $J=9.1$), 6.90 (1H, bs), 7.20 (3H, m), 8.22 (2H, dd, $J=8.9$, $^4J_{HF}=5.4$). ^{13}C-NMR (22.5 MHz): δ 13.6, 13.9, 51.6, 55.9, 60.7, 61.6, 62.6, 63.0, 92.5, 111.3, 115.6 ($^2J_{CF}=22.6$), 117.9, 125.7 ($^4J_{CF}=2.5$), 127.7, 132.4, 133.2 ($^3J_{CF}=9.2$), 143.2, 144.2, 146.9, 156.2, 156.9, 157.2, 164.1, 166.2 ($^1J_{CF}=255.7$), 185.6. ESI-MS: 559.1 ([M]+H).

*5.1.2.10. 4,2′,3′-Trimethyl-4′-phtaloylglycil-purpurogallin·DEAD Diels–Alder adduct (**3j**).* Obtained from **2j** in 68% yield. M.p. 212–215 °C. IR (NaCl film, cm^{-1}): 1598 (m, C=O, ketone), 1724, 1774 (s, C=O, ester), 2982 (w, Ar–H). ^{1}H-NMR (90 MHz): δ 1.00 (3H, bt, $J=7.0$), 1.29 (3H, t, $J=7.1$), 3.53 (3H, s), 3.84 (3H, s), 3.95 (3H, s), 4.03 (2H, bq, $J=7.0$), 4.27 (2H, q, $J=7.1$), 4.66 (1H, d, $J=17.5$), 5.07 (1H, d, $J=17.5$), 5.67 (1H, bdd, $J=0.8$, $J=5.9$), 6.00 (1H, dd, $J=0.8$, $J=9.2$), 6.87 (1H, bs), 7.23 (1H, dd, $J=5.9$, $J=9.2$), 7.82 (4H, AA′BB′). ^{13}C-NMR (22.5 MHz): δ 13.6, 14.0, 38.6, 51.7, 56.1, 61.6, 62.9, 63.1, 92.6, 111.6, 117.1, 123.7, 127.8, 132.2, 132.3, 134.4, 143.2, 144.1, 146.1, 156.4, 156.9, 157.3, 166.3, 167.5, 185.7. ESI-MS: 623.5 ([M]+H).

*5.1.2.11. 4,2′,3′,4′-Tetramethylpurpurogallin·DEAD Diels–Alder adduct (**3k**).* Obtained from **2k** in 59% yield. M.p. 162–164 °C. IR (NaCl film, cm^{-1}): 1589 (m, C=O, ketone), 1716 (s, C=O, ester), 2983 (m, Ar–H). ^{1}H-NMR (90 MHz): δ 0.95 (3H, bt, $J = 6.9$), 1.31 (3H, t, $J = 7.1$), 3.52 (3H, s), 3.84 (3H, s), 3.87 (3H, s), 3.94 (3H, s), 4.01 (2H, bq, $J = 6.9$), 4.28 (2H, q, $J = 7.1$), 5.63 (1H, bdd, $J = 0.9$, $J = 5.9$), 6.07 (1H, dd, $J = 0.9$, $J = 9.0$), 6.71 (1H, bs), 7.23 (1H, dd, $J = 5.9$, $J = 9.0$). ^{13}C-NMR (22.5 MHz): δ 12.6, 13.0, 51.2, 55.5, 60.4, 61.1, 61.5, 62.2, 62.7, 92.9, 109.4, 120.4, 129.1, 132.9, 138.0, 144.4, 146.2, 157.2, 158.6, 158.5, 188.6. ESI-MS: 451.1 ([M]+H).

*5.1.2.12. 4,2′,3′-Trimethylpurpurogallin·DEAD Diels–Alder adduct (**3l**).* Obtained from **1** in 85% yield. M.p. 178–180 °C. IR (KBr disc, cm^{-1}): 1656 (m, C=O, ketone), 1716, 1742 (s, C=O, ester), 2988 (m, Ar–H), 3448 (s, O-H). ^{1}H-NMR (90 MHz): δ 0.96 (3H, bt, $J = 7.2$), 1.33 (3H, t, $J = 7.2$), 3.56 (3H, s), 3.84 (3H, s), 3.97 (3H, s), 4.06 (2H, bq, $J = 7.2$), 4.30 (2H, q, $J = 7.2$), 5.70 (1H, bdd, $J = 0.8$, $J = 6.0$), 6.07 (1H, dd, $J = 0.8$, $J = 9.1$), 6.63 (1H, s), 7.31 (1H, dd, $J = 6.0$, $J = 9.1$), 11.86 (1H, s). ^{13}C-NMR (22.5 MHz): δ 13.2, 13.7, 51.4, 55.7, 60.1, 61.5, 62.4, 62.8, 91.3, 106.2, 108.2, 127.0, 131.8, 137.5, 144.9, 156.6, 156.7, 157.0, 158.8, 194.3. ESI-MS: 437.1 ([M]+H).

5.2. Biological assays

Stock solutions of the test compounds, plus control drugs, were prepared at a concentration of 20 μg mL^{-1} in DMSO (Sigma, UK), and diluted to the appropriate concentration prior to the assays. IC_{50} values were calculated with MSXLFIT (IDBS, UK).

5.2.1. L. donovani

MHOM/ET/67/L82 *L. donovani* amastigotes were harvested from an infected hamster (*Mesocricetus auratus*) spleen and used to infect murine peritoneal exudate macrophages (PEM) at a ratio of 7:1. In brief, infected cells were exposed to drug for a total of 5 days [21]. The percentage of infected cells was evaluated microscopically and the percentage inhibition in comparison to untreated controls was calculated. The standard drug, sodium stibogluconate, has an IC_{50} of around 5 μg Sb^V mL^{-1}.

5.2.2. P. falciparum

Chloroquine-sensitive *P. falciparum* strain 3D7 was maintained in human A^+ erythrocytes in RPMI1640 medium (Sigma, UK) supplemented with Albumax II at 37 °C in a 5% CO_2–air mixture. *P. falciparum* intraerythrocytic cultures were set up as above, with 1% ring stage parasitemia, 2.5% hematocrit, in triplicate in 100 μL of medium in 96-well, flat-bottomed Microtest III tissue culture plates. Drugs were added in threefold dilution series and cultures incubated for a total of 48 h at 37 °C in a 5% CO_2–air mixture. After 24 h, [^{3}H]-hypoxanthine (0.2 mCi) was added to each well [22,23]. At the end of the assay, plates were rapidly freeze–thawed, harvested using a Tomtec Mach III cell harvester (Tomtec, CT) onto a 96-well format filtermat and Meltilex™ solid scintillant (both Wallac, Finland) added prior to reading in a Microbeta 1450 scintillation counter (Wallac, Finland) at 1 min per well.

5.2.3. T. brucei rhodesiense

STIB900 blood stream form *T. brucei rhodesiense* trypomastigotes were maintained in HMI-18 medium [24], with 15% heat-inactivated fetal calf serum (Harlan-SeraLab, UK) at 37 °C in a 5% CO_2–air mixture. Prior to drugging, trypomastigotes were washed and resuspended in fresh medium at a concentration of 2×10^5 mL^{-1}, and 100 μL of this suspension was added to the drug dilutions. The top concentration for the test compounds was 30 μg mL^{-1}. Pentamidine was included as the standard drug. Plates were incubated for 72 h at 37 °C in a 5% CO_2–air mixture. At 72 h Alamar Blue was added to the plates [25]. Plates were read after 5–6 h on a Gemini fluorescent plate reader (Sofimax Pro. 3.1.1, Molecular Devices, UK) at EX/EM 530/585 nm with a filter cut-off at 550 nm.

5.2.4. T. cruzi

Peritoneal exudate macrophages were infected with *T. cruzi* Tulahuen LAC-Z trypomastigotes [26], harvested from cultured L6 myoblast feeder-cell layers, at a ratio of 5:1. Infected cells were exposed to the drugs for 72 h. Anti-trypanosomal activity was evaluated using a β-galactosidase assay [26], and calculating the percent inhibition in comparison to untreated controls. The standard drug benznidazole was included in the assay.

5.2.5. Cytotoxicity assays

96-Well plates were seeded with KB cells at a concentration of 2×10^4 mL^{-1} (200 μL per well). Drugs at 300, 30, 3 and 0.3 μg mL^{-1} were added in fresh overlay after 24 h, in triplicate at each concentration. The plates were incubated for a further 72 h and then washed three times with PBS, and 100 μL PBS and 10 μL Alamar Blue added. This was followed by incubation of the plates for 2 h before reading EX/EM 530/585 nm on a Gemini plate reader. IC_{50} values were calculated against blanks and control samples.

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