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Design, synthesis and biological evaluation of (1-phenyl-9*H*-pyrido[3,4-*b*]indol-3-yl)(4-phenylpiperazin-1-yl)methanone derivatives as anti-leishmanial agents

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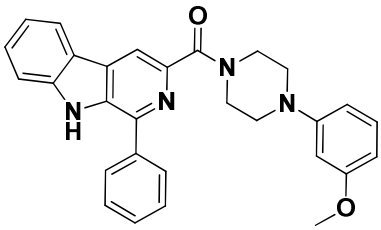
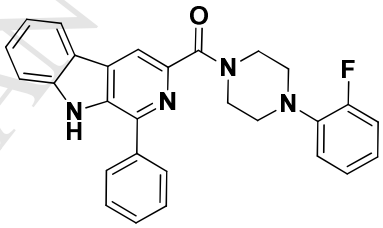
Graphical Abstract



Compound **7e**, activity against *L. infantum*
EC_{50} = 2.89 ± 0.34 μM (Promastigoes)
= 2.80 ± 0.13 μM (Axenic amastigotes)



Compound **7k**, activity against *L. donovani*
EC_{50} = 3.47 ± 0.17 μM (Promastigoes)
= 2.80 ± 0.10 μM (Axenic amastigotes)
= 4.00 ± 0.60 μM (Intracellular amastigotes)

Design, synthesis and biological evaluation of piperazinyl-β-carbolinederivatives as anti-leishmanial agents

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ABSTRACT

Molecular hybridization is a ligand based drug design approach is well known recent medicinal chemistry to design anti-parasitic agents. In the present study, we have designed a series of (1-phenyl-9*H*-pyrido[3,4-*b*]indol-3-yl)(4-phenylpiperazin-1-yl)methanone derivatives using molecular hybridization approach. Designed analogues were evaluated for cytotoxicity and inhibition activity against *Leishmania infantum* and *Leishmania donovani.* Among these reported analogues **7b**, **7d**, **7e**, **7f** and **7m** displayed potent inhibition of both *L. infantum* and *L. donovani*. Compounds **7i** and **7k** exhibited selective potent inhibition of *L. donovani*. Especially, compounds **7e** and **7k** showed most potent anti-leishmanial activity against *L. infantum* and *L. donovani* respectively. Anti-leishmanial activity of these compounds is comparable with standard drugs miltefosine and pentamidine. SAR studies revealed that, electron donating group substitution on phenyl ring recommended for potent anti-leishmanial activity.

Keywords: Molecular hybridization, β-carboline, anti-leishmanial activity, *Leishmania infantum, Leishmania donovani*

1. Introduction

Leishmaniasis is one of the most neglected diseases, caused by protozoan parasite *Leishmania spp.* and is present throughout the tropics and sub-tropics, putting a fifth of the world's population at risk [1]. Leishmaniasis has been classified into Cutaneous Leishmaniasis (CL), Mucocutaneous Leishmaniasis (MCL), Visceral Leishmaniasis (VL) and post-kala-azar dermal

leishmaniasis (PKDL) [2]. Among these, visceral Leishmaniasis is considered as severe form and is caused by *Leishmania donovani* and *Leishmania infantum.* Visceral leishmaniasis affects internal organs such as bone marrow, liver, and spleen [3]. Un-fortunately, treatment of leishmaniasis is dependent upon very old drugs such as antimonials. Pentavalent antimonials such as sodium stibogluconate and meglumine antimoniate have been used for more than 60 years, however increasing incidence of resistance has been reported. Despite of its adverse effects, Amphotericin B is the drug of choice, where antimonial resistance is prevalent. Second line drugs such as diamidine, pentamidine and paromomycin have limited use in leishmaniasis chemotherapy [4, 5]. Miltefosine is the only oral anti-leishmanial drug that has been approved by FDA / WHO to date [6]. Miltefosine has restricted use in pregnancy and high risk of resistance due to long half-life (150 h). Clinical efficacy of miltefosine has been decreasing in countries like India, where it is used extensively [7]. Hence, there is an urgent need of new anti-leishmanial agents with better therapeutic profiles.

β-Carbolines and derivatives thereof have a privileged position in medicinal chemistry, with diverse biological activity spectrum, such as, anti-malarial [8, 9], anti-leishmanial [10-12], anti-tubercular [13], anti-bacterial [14], anti-viral [15-17], anti-cancer [18] and anti-thrombotic [19]. Natural β-carboline alkaloids such as, harmine [20], buchtienine [11], annomontine [21] and manzamine A (Fig. 1) displayed potent anti-leishmanial activity. Manzamine a group of β-carboline alkaloids, have exhibited significant anti-leishmanial activity [22] and we recently published a review on their anti-leishmanial activity [23]. In addition to these natural alkaloids, large number of synthetic β-carboline derivatives also display potent anti-leishmanial activity. Synthetic β-carboline derivatives with substitution on position 1, 2, 3 and 9 of β-carboline skeleton have shown significant anti-leishmanial activity [24-26]. Piperazine moiety is well known in medicinal chemistry, to be the second most prevalent heterocyclic ring present in all FDA approved drugs up to 2013 [27]. Piperazine derivatives exhibited wide range of biological activities including anti-bacterial [28, 29], anti-protozoal [30, 31], anti-leishmanial [32], anti-tubercular, anti-viral [33] and anti-cancer [34] activities (Fig. 1). This is especially true for bisarylpiperazine derivatives (two different heterocyclic rings attached to piperazine scaffold) which show potent anti-leishmanial activity [35, 36]. Based upon these collective facts and through a molecular hybridization approach, we have designed a new series of β-carboline and

piperazine hybrid molecules (Fig. 1). In the present study, design, synthesis and anti-leishmanial activity of piperazinyl-β-carboline derivatives have been reported.

Fig. 1. Structure of reported and designed β-carboline-piperazine hybrid molecules

2. Results and Discussion

2.1. Chemistry

The synthetic protocol of the reported 1-phenyl-9*H*-pyrido[3,4-*b*]indole-3-carboxamide derivatives is illustrated in scheme 1. The compounds were synthesized in a sequence of reactions using DL-Tryptophan **(1)** as starting material. Initially DL-Tryptophan **(1)** was converted to DL-tryptophan ethyl ester **(2)** in a esterification reaction using thionylchloride and ethanol followed by Pictet-Spengler reaction to obtain ethyl 2,3,4,9-tetrahydro-1-phenyl-1*H*-pyrido[3,4-*b*]indole-3-carboxylate **(3)** upon reaction with benzaldehydein presence of trifluoroacetic acid and dichloro methane as solvent. Continued by oxidation of ethyl 2,3,4,9-tetrahydro-1-phenyl-1*H*-pyrido[3,4-*b*]indole-3-carboxylate **(3)** with potassium permanganate and tetrahydrofuran as solvent to get ethyl-1-phenyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylate **(4)** [37] and key intermediate 9-methyl-1-phenyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid **(5)** was obtained by alkaline ester hydrolysis using sodium hydroxide. The carboxylic acid group containing key intermediate **(5)** was further treated with substituted aryl-piperazines **(6a-p)** in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and hydroxybenzotriazole (HOBt) to obtain the desired products **(7a-p)** in excellent to good yields [38, 39].

Scheme 1: Reagents and conditions: i) thionylchloride, ethanol, reflux, 30 min, 76 %; ii) benzaldehyde, trifluoroaceticacid, DCM, rt, 3 h, 82 %; iii) $KMnO_4$, THF, rt, 24 h, 68 %; iv) 50 % aq. NaOH, reflux, 30 min, 78 %; v) EDCI, HOBt, THF, 0 ºC-rt 6 h, 62-82 %.

All the synthesized compounds were characterized by IR, NMR, Mass spectral and elemental analysis. IR spectra of the reported compounds showed *C=O* stretching at 1641 to 1606 cm^{-1}, aromatic *C-H* stretching at 3275 to 3157 cm^{-1}, *C-O* stretching (methoxy) at 1255 to 1242 cm^{-1}, *N-O* asymmetric stretching at 1319 cm^{-1} and *C-Cl* absorption band at 734 to 742 cm^{-1}. ^{1}H NMR

spectrum of the compounds showed, characteristic singlet around δ ~8.50 due to position-4 proton of β-carboline ring, eight piperazine protons appeared as two multiplets around δ value 4.19 to 4.02 ($O=CN(CH_2)_2$) and 3.64 to 2.62 ($CN(CH_2)_2$). Piperazine protons were shifted to down field (δ ~4.20 and 3.64) on nitro substitution whereas, shifted up field in benzyl derivatives (δ ~4.0 and 2.62). Methoxy protons appeared as singlet at δ ~3.80 and aromatic methyl protons as singlet at δ ~2.30. In mass spectral analysis, $[M+H]^+$ peak appeared as parent ion peak.

2.2. Biological evaluation

2.2.1. Cytotoxicity Evaluation

Reported (1-phenyl-9*H*-pyrido[3,4-*b*]indol-3-yl)(4-phenylpiperazin-1-yl)methanone derivatives were initially evaluated for cytotoxicity against HeLa cell lines at 500 µM concentration using an Alamar blue assay. Most of these analogues were non-toxic to HeLa cell lines at 500 µM concentration. Compounds which showed any significant cytotoxicity against HeLa cells, were further evaluated to determine their cytotoxic concentration (CC_{50}) values.

2.2.2. *Anti-leishmanial Screening*

Anti-leishmanial activity of these analogues was determined by evaluating their inhibition activity against both promastigote, amastigote forms of *Leishmania infantum* and *Leishmania donovani* strains. Compounds were screened against promastigote forms of the *Leishmania* strains to determine their effective concentration (EC_{50}) values. Finally, compounds which exhibited potent inhibition of promastigote (EC_{50}<20 µM) were further evaluated against amastigote forms of respective strain using axenic and intracellular amastigote assay methods. Anti-leishmanial drugs miltefosine and pentamidine were used as standards for comparison purpose.

2.2.2.1. Leishmania infantum

2.2.2.1.1. Anti-promastigote activity

Promastigote inhibition assay was used to evaluate *in-vitro* anti-leishmanial activity of these titled β-carboline derivatives. Most of these reported β-carboline derivatives displayed significant inhibition of *L. infantum* promastigotes. Among these compounds, **7b**, **7d**, **7e**, **7f** and **7m** exhibited potent anti-promastigote activity (EC_{50} 9.63, 9.07, 2.89, 3.35 and 10.70 µM, respectively) than standard drugs miltefosine and pentamidine (EC_{50} 8.31 and 12.6 µM).

Especially compounds **7e** and **7f** showed 4 times potent inhibition than standard drugs with better selectivity, while compounds **7b**, **7d** and **7m** displayed comparable equal potency as that of standard drugs. Compounds **7i**, **7j**, **7k** and **7l** displayed significant inhibition of promastigote (EC_{50} 15.70, 14.10, 14.90 and 19.80 µM, respectively) and are comparable to standard drugs (Table 1). Compounds such as **7c**, **7g** and **7o** displayed moderate inhibitory activity (EC_{50} 36.2, 33.0and 34.0 µM, respectively) against promastigotes. Structure activity relationship analysis emphasized that, electron donating groups, especially methoxy substitution on phenyl ring favored the activity, while replacement of phenyl with pyridine and benzyl rings significantly decreased the inhibition activity against *L. infantum* promastigotes.

2.2.2.1.2. Anti-amastigote activity

Compounds which displayed significant inhibitory activity (EC_{50}<20 µM) against promastigotes (insect stage) were further screened against amastigote forms (human life stage form) to establish their activity in the intracellular stage of the parasite. Anti-amastigote activity of selected β-carboline derivatives was evaluated using axenic amastigote assay method. Axenic amastigotes are the amastigotes that are grown in a medium outside host cells that mimic intracellular conditions. Axenic amastigotes have the advantage of high similarity with the human parasite stage than promastigotes and are commonly used as an easy *in-vitro* screening method to evaluate amastigote inhibition activity.[40]

Amastigote inhibition assay results suggested that these β-carboline derivatives exhibited potent inhibition of *L. infantum* amastigotes than promastigote forms (Table 1). Especially, compounds such as **7b**, **7d**, **7e**, **7f**, **7k** and **7m** exhibited promising anti-amastigote activity with EC_{50} values at uni-micro molar concentration (EC_{50} 6.20, 5.20, 2.80, 4.90, 9.40 and 2.90 µM, respectively). These compounds displayed 2-5 times potent inhibition of *L. infantum* amastigotes than promastigotes. Compounds **7i** and **7j** displayed significant inhibition (EC_{50} 12.20 and 11.30 µM) of *L. infantum* amastigotes and inhibition potency is comparable against both the forms.

*Anti-amastigote activity Structure Activity Relationship (SAR)***:** Among these reported β-carboline derivatives, un-substituted phenyl derivative **7a** showed significant inhibition of *L. infantum* amastigotes. Electron donating methoxy substitution on *meta* position of phenyl ring has considerably increased the anti-amastigote activity as well as reduced the cytotoxicity. Substitution of methoxy on ortho-para position of phenyl ring has not affected the activity but

decreased the toxicity. Para substitution with methyl group has also not altered the amastigote activity. Electron withdrawing chloro, fluoro, nitro group substitution on phenyl ring has negatively affected the anti-amastigote activity of these analogues. Although, meta, chloro substitution has increased the potency, selectivity index has been decreased to 5.6, due to increased cytotoxicity. Di-chloro substitutions at *ortho* and *meta* positions of phenyl ring affords increased amastigote inhibition activity. More interestingly, additional chloro substitution on *ortho* position marginally increased the activity and vanished the toxicity of the *meta* chloro derivative (**7h**). SAR study revealed that, *meta* position is highly responsive, substitution with either electron donating and withdrawing group has significantly enhanced anti-amastigote potency, but it also increased the cytotoxicity. Interestingly, additional substitution on *ortho* position has reversed the cytotoxicity of these meta derivatives. These results suggested that, selective substitutions on *ortho* / *para* along with meta substitution (preferably electron donating groups) can be explored to develop potent anti-leishmanial agents with good selectivity against *L. infantum*.

Table 1 *In-vitro* assay results of the titled compounds against *Leishmania infantum*

ND – Not Determined; a = $\boldsymbol{CC_{50}}$ / $\boldsymbol{EC_{50}}$

2.2.2.2. Leishmania donovani

In *Leishmania donovani* promastigote inhibition assay, compounds **7b**, **7d**, **7e**, **7f**, **7i**, **7k**, **7m** and **7o** exhibited potent inhibition (EC_{50} 4.91, 6.32, 4.85, 3.49, 6.98, 3.47, 6.95 and 7.5 µM respectively) with good selectivity. These compounds showed equal potency as that of standard anti-leishmanial drugs pentamidine and miltefosine (EC_{50} 6.40 and 3.12 µM). Compounds **7c**, **7j** and **7l** displayed significant anti-promastigote activity (EC_{50} 12.60 13.20 and 12.80 µM, respectively). Compound **7g** showed moderate inhibition activity (EC_{50} 38.60 µM) against *L. donovani* promastigotes. Structure activity relationship study suggested that, substitution of electron donating group on phenyl ring and electron withdrawing group (chloro and fluoro) on *ortho* position of phenyl ring favored promastigote inhibition (Table 2) activity.

Compounds displayed significant inhibition of *L. donovani* promastigotes (EC_{50} <20 µM) were further evaluated for anti-amastigote activity using axenic and intracellular amastigote assay methods. Most of these screened analogues displayed significant activity against amastigote

forms (Table 2). Especially, compounds **7b**, **7c**, **7d**, **7e**, **7f**, **7i**, **7k, 7m** and **7o** exhibited potent inhibition of axenic amastigotes (EC_{50} 3.30, 6.00, 2.80, 1.90, 2.30, 5.80, 2.80, 5.70 and 5.10 µM, respectively) with good selectivity. Especially, compounds **7d**, **7e**, **7f** and **7k** showed excellent potency against axenic amastigotes. Compounds **7j** and **7l** showed significant inhibition (EC_{50} 12.60 and 11.80 µM) of axenic amastigotes with good selectivity. Although, compounds **7a**, **7h** and **7p** displayed anti-leishmanial activity, they also showed a lack of selectivity. Structure activity relationship studies suggested that, electron donating group especially methoxy substitution on phenyl ring enhanced amastigote activity significantly. Methyl group substitution has increased axenic amastigote inhibition considerably, more preferably on para position than *ortho* position of phenyl ring. Electron withdrawing group substitution on phenyl ring has variable effect on amastigote activity. *Ortho* substitution on phenyl ring has significantly increased the activity, especially fluoro substitution, while para substitution has not affected the inhibition activity. Furthermore, replacement of the phenyl ring with a heterocyclic pyridine ring favored inhibition of *L. donovani* axenic, but not the intracellular amastigotes (compare **7a** to **7n).**

However, axenic amastigote assay is one of the most commonly used assay method to determine anti-leishmanial activity. Literature suggested that, axenic amastigotes are different from intracellular amastigotes in terms of protein expression and drug susceptibility [40, 41]. Hence, compounds displaying excellent potency against promastigote and axenic amastigotes were further screened against intracellular amastigotes to ensure their activity in the truly relevant stage of the parasite in the search of potent anti-leishmanial agents with good selectivity. Intra-macrophage assay report suggested that, macrophage amastigotes are highly susceptible to these β-carboline derivatives. These β-carboline derivatives exhibited similar anti-amastigote activity in both the assays with minor variations in potency. Most of these analogues **7b**, **7c**, **7d**, **7e**, **7f**,**7i**, **7k,7m** and **7o** exhibited potent inhibition of intracellular (EC_{50} 5.70, 9.60, 9.20, 6.90, 8.00, 8.50, 4.00, 6.40 and 11.90 µM, respectively) amastigotes with good selectivity. Compounds **7j** and **7l** displayed significant inhibition (EC_{50} 22.30 and 21.50 µM) against intracellular amastigotes. Compounds **7a**, **7h** and **7p** showed cytotoxicity against HeLa cell lines (CC_{50} 38.10, 18.40 and 28.90 µM respectively). However, electron withdrawing and donating substitutions has not affected the anti-leishmanial activity, instead showed significant effect on cytotoxicity of these molecules. SAR studies suggested that, electron donating group substitution favored anti-

leishmanial activity. Electron withdrawing groups such as chloro and fluoro on *ortho* substitution was recommended for anti-leishmanial activity. Meta substitution with methoxy and chloro has increased the activity as well as cytotoxicity, interestingly cytotoxicity has been reduced by additional substitution on *ortho* substitution. Hence, these di-substituent derivatives can be studied for further lead optimization process.

Table 2 *In-vitro* assay results of the titled compounds against *Leishmania donovani*

ND- Not Determined; a = $\boldsymbol{CC_{50}}$ / $\boldsymbol{EC_{50}}$

This new series of β-carboline and piperazine hybrid molecules displayed significant anti-leishamanial activity against both the tested *L. infantum* and *L. donovani* strains. Exact mechanism of action for their potent anti-leishmanial activity is yet to be identified. We hypothesize that known DNA topoisomerases or DNA synthesis inhibition abilities of these β-carboline derivatives might played a role in their anti-leishmanial activity [42]. Further exhaustive studies are required to understand the exact mechanism of action and would be useful for further lead optimization of these analogues to develop potential clinical candidates in the fight against leishmaniasis.

3. Conclusion

In summary, we have successfully applied a molecular hybridization technique to design new anti-leishmanial agents. Designed piperazinyl-β-carboline-3-carboxamide derivatives were evaluated for inhibition activity against *L. infantum* and *L. donovani*. Compounds that exhibited significant anti-promastigote activity were further screened against amastigote forms of respective species using axenic and intracellular amastigote cell cultures. Among these reported analogues, **7b**, **7d**, **7e**, **7f** and **7m** displayed potent inhibition of both promastigote and amastigote forms of *L. infantum* with good selectivity index. Compounds **7e** and **7m** showed potent activity against both forms of *Leishmania*, with potency just as good as, and in some case slightly better than miltefosine, while still having a workable therapeutic window. These β-carboline derivatives were also screened against intracellular amastigotes to ensure that their activity was not influenced detrimentally by the issue of the host cell, which might prevent an effective concentration getting to the parasite. Compounds **7b**, **7d**, **7e**, **7f, 7i, 7k** and **7m** were exhibited potent activity against both promastigotes and amastigotes of *L. donovani* with good selectivity.

Especially, compound **7k** displayed most potent activity against *L. donovani* and potency was comparable with miltefosine and pentamidine. SAR studies revealed that, electron donating substituents favored anti-leishmanial activity while electron withdrawing substitution on *ortho* position is recommended for activity. *Meta* position of phenyl ring is highly responsive, electron donating and withdrawing group substitution has significantly enhanced anti-leishmanial activity and cytotoxicity. Interestingly, additional substitution on *ortho* position has reversed the cytotoxicity of these *meta* derivatives. Replacement of phenyl ring has increased cytotoxicity of these analogues. Further exhaustive studies are required to understand the exact molecular mechanism of action and would be useful in lead optimization studies to develop potential clinical candidates to fight against leishmaniasis.

4. Experimental Protocols

4.1. Chemistry

All solvents and reagents purchased from Sigma or Merck companies were used as received without further purification. Solvent system used throughout the experimental work for running Thin Layer Chromatography (TLC) was Ethyl acetate and Hexane Mixture (6:4) in order to monitor the reaction. Column chromatography was performed using silica gel (100-200 mesh, SRL, India) as stationary phase, mixture of ethyl acetate and hexane as mobile phase. Melting point was determined in open capillary tube on a Precision Buchi B530 (Flawil, Switzerland) melting point apparatus containing silicon oil and are uncorrected. IR spectra of the synthesized compounds were recorded using FTIR spectrophotometer (Shimadzu IR Prestige 21, India). ^{1}H NMR spectra was recorded on a Bruker DPX-400 spectrometer (Bruker India Scientific Pvt. Ltd., Mumbai) using TMS as an internal standard (chemical shifts in δ). Elemental analysis was performed on Vario EL III M/s Elementar C, H, N and S analyzer (Elementar Analysen systeme GmbH, Germany). The ESMS was recorded on MICROMASS Quattro-II LCMS system (Waters Corporation, Milford, USA).

*4.2. General procedure for the preparation of **7***

To the stirred solution of 1-phenyl-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid **5** (0.29 g, 0.001 mol) in dry THF, HOBt (0.16 g, 0.012 mol) and EDC. HCl (0.23 g, 0.012 mol) were added and continued stirring for 30 min. To the reaction mixture, substituted phenyl piperazine (**6a-p**) (0.001 mol) was added under ice cold temperature and the reaction mixture was further stirred at

room temperature for 6 h. After completion of reaction as monitored by TLC, solvent was evaporated under vacuum. Reaction mixture was extracted with ethyl acetate (2 x 20 mL), collected organic layer was dried over anhydrous Na_2SO_4, concentrated under vacuum and passed through small bed of silica gel (60-120) using mobile phase (ethyl acetate: hexane; 3:7) to obtain analytically pure final product **7**.

(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)(4-phenylpiperazin-1-yl)methanone (7a)

White Solid; % Yield 74; mp 158-160 °C; IR νmax/cm (KBr): 3157, 1614, 1556, 1489, 738; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.53 (s, 1H), 8.21 (d, J = 7.8 Hz, 1H), 7.64-7.58 (m, 3H), 7.54-7.52 (m, 3H), 7.48-7.46 (m, 1H), 7.37-7.32 (m, 1H), 7.31-7.26 (m, 2H), 6.95-6.89 (m, 3H), 4.10-4.01 (m, 4H), 3.23-3.21 (m, 4H); MS m/z 433.4 $[M + H]^+$.

(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)(4-p-tolylpiperazin-1-yl)methanone (7b)

White solid; % Yield 74; mp 208-210 °C; IR νmax/cm (KBr): 3228, 1620, 1587, 1490, 1433, 746; 1H NMR (400 MHz, CDCl3) δ 8.77 (s, 1H), 8.51 (s, 1H), 8.19 (d, J = 7.9 Hz, 1H), 7.98 (d, J = 7.2 Hz, 2H), 7.65-7.52 (m, 7H), 7.37 (t, J = 7.3 Hz, 1H), 7.15 (d, J = 7.1 Hz, 2H), 4.15-4.03 (m, 4H), 3.35-3.26 (m, 4H), 2.32 (s, 3H); MS m/z 447.4 $[M + H]^+$.

(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)(4-o-tolylpiperazin-1-yl)methanone (7c)

White solid; % Yield 68; mp 122-124 °C; IR νmax/cm (KBr): 3201, 1622, 1556, 1490, 1431, 740; 1H NMR (400 MHz, CDCl3) δ 8.95 (s, 1H), 8.46 (s, 1H), 8.17 (d, J = 7.9 Hz, 1H), 8.05-7.96 (m, 2H), 7.67-7.55 (m, 4H), 7.52 (t, J = 7.4 Hz, 1H), 7.38-7.34 (m, 1H), 7.27-7.18 (m, 2H), 7.12-7.00 (m, 2H), 4.10-4.00 (m, 4H), 3.10-3.01 (m, 4H), 2.38 (s, 3H).

(4-(4-methoxyphenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7d)

White solid; % Yield 80; mp 182-184 °C; IR νmax/cm (KBr): 3234, 1625, 1546, 1510, 1255, 746; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.49 (s, 1H), 8.16 (d, J = 7.9 Hz, 1H), 7.95 (d, J = 7.2 Hz, 2H), 7.62-7.48 (m, 7H), 7.37-7.32 (m, 1H), 6.88 (d, J = 8.9 Hz, 2H), 4.25-4.10 (m, 4H), 3.79 (s, 3H), 3.32-3.26 (m, 4H); MS m/z 463.4 $[M + H]^+$.

(4-(3-methoxyphenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7e)

White solid; % Yield 74; mp 118-120 °C; IR νmax/cm (KBr): 3221, 1606, 1536, 1492, 1251, 741; 1H NMR (400 MHz, CDCl3) δ 8.78 (s, 1H), 8.51 (s, 1H), 8.19 (d, J = 7.9 Hz, 1H), 8.00 (d,

J = 7.2 Hz, 2H), 7.65-7.52 (m, 5H), 7.37 (t, J = 7.3 Hz, 1H), 7.22 (t, J = 8.2 Hz, 1H), 6.60-6.58 (m, 1H), 6.51-6.48 (m, 2H), 4.10-4.06 (m, 4H), 3.82 (s, 3H), 3.37-3.30 (m, 4H).

(4-(2-methoxyphenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7f)

White solid; % Yield 64; mp 110-112 °C; IR νmax/cm (KBr): 3244, 1624, 1558, 1498, 1242, 740; 1H NMR (400 MHz, CDCl3) δ 8.82 (s, 1H), 8.47 (s, 1H), 8.17 (d, J = 7.9 Hz, 1H), 8.01-7.98 (m, 2H), 7.64-7.51 (m, 5H), 7.38-7.34 (m, 1H), 7.08-7.04 (m, 1H), 7.01-6.90 (m, 3H), 4.20-4.10 (m, 4H), 3.91 (s, 3H), 3.25-3.16 (m, 4H).

(4-(4-chlorophenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7g)

White solid; % Yield 76; mp 186-188 °C; IR νmax/cm (KBr): 3203, 1610, 1588, 1481, 738; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.67 (s, 1H), 8.44 (s, 1H), 8.24 (d, J = 7.9 Hz, 1H), 8.10-8.06 (m, 2H), 7.67-7.60 (m, 5H), 7.30-7.26 (m, 1H), 7.19 (dd, J = 9.8, 6.4 Hz, 2H), 6.94 (d, J = 9.1 Hz, 2H), 3.99-3.95 (m, 4H), 3.30-3.25 (m, 4H); MS m/z 468.2 $[M + H]^+$, 470.2 $[M + H3]^+$.

(4-(3-chlorophenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7h)

White solid; % Yield 72; mp 138-140 °C; IR νmax/cm (KBr): 3201, 1618, 1558, 1481, 734; 1H NMR (400 MHz, CDCl3) δ 8.75 (s, 1H), 8.52 (s, 1H), 8.19 (d, J = 7.9 Hz, 1H), 8.00 (d, J = 7.0 Hz, 2H), 7.66-7.53 (m, 5H), 7.39-7.35 (m, 1H), 7.21 (t, J = 8.1 Hz, 1H), 6.93 (t, J = 2.1 Hz, 1H), 6.88-6.82 (m, 2H), 4.12-4.06 (m, 4H), 3.38-3.31 (m, 4H).

(4-(2-chlorophenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7i)

White solid; % Yield 64; mp 108-110 °C; IR νmax/cm (KBr): 3203, 1622, 1546, 1488, 742; 1H NMR (400 MHz, CDCl3) δ 8.74 (s, 1H), 8.50 (s, 1H), 8.19 (d, J = 7.9 Hz, 1H), 8.01-7.99 (m, 2H), 7.65-7.54 (m, 5H), 7.42-7.35 (m, 2H), 7.27-7.24 (m, 1H), 7.09-7.01 (m, 2H), 4.12-4.02 (m, 4H), 3.24-3.16 (m, 4H).

(4-(4-nitrophenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7j)

Yellow solid; % Yield 62; mp 134-136 °C; IR νmax/cm (KBr): 3184, 1624, 1595, 1489, 1319, 752; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.76 (s, 1H), 8.55 (s, 1H), 8.19-8.14 (m, 3H), 7.97 (d, J = 7.1 Hz, 2H), 7.64-7.52 (m, 5H), 7.36 (t, J = 7.3 Hz, 1H), 6.84 (d, J = 9.4 Hz, 2H), 4.20-4.06 (m, 4H), 3.64-3.56 (m, 4H).

(4-(4-fluorophenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7k)

White solid; % Yield 72; mp 178-180 °C; IR νmax/cm (KBr): 3209, 1614, 1550, 1508, 744; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.72 (s, 1H), 8.49 (s, 1H), 8.17 (d, J = 7.9 Hz, 1H), 7.98-7.96 (m, 2H), 7.63-7.40 (m, 5H), 7.37-7.33 (m, 1H), 7.01-6.89 (m, 4H), 4.09-4.06 (m, 4H), 3.26-3.18 (m, 4H); MS m/z 451.3 $[M + H]^+$.

(4-(2-fluorophenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone(7l)

White solid; % Yield 68; mp 104-106 °C; IR νmax/cm (KBr): 3182, 1610, 1556, 1498, 750; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (s, 1H), 8.50 (s, 1H), 8.18 (d, J = 7.9 Hz, 1H), 8.02-7.98 (m, 2H), 7.65-7.51 (m, 5H), 7.39-7.35 (m, 1H), 7.12-7.05 (m, 2H), 7.02-6.97 (m, 2H), 4.11-4.10 (m, 4H), 3.28-3.20 (m, 4H).

(4-(2,3-dichlorophenyl)piperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7m)

White solid; % Yield 62; mp 124-126 °C; IR νmax/cm (KBr): 3174, 1616, 1562, 1491, 742; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.44 (s, 1H), 8.14 (d, J = 7.9 Hz, 1H), 7.95 (d, J = 7.2 Hz, 2H), 7.60-7.47 (m, 4H), 7.49 (t, J = 7.4 Hz, 1H), 7.35-7.31 (m, 1H), 7.20-7.14 (m, 2H), 6.96 (dd, J = 7.5, 2.0 Hz, 1H), 4.07 (m, 4H), 3.19-3.12 (m, 4H).

(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)(4-(pyridin-4-yl)piperazin-1-yl)methanone (7n)

Pale yellow solid; % Yield 66; mp 130-132 °C; IR νmax/cm (KBr): 3162, 1641, 1541, 1419; ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.70 (s, 1H), 8.47 (s, 1H), 8.26-8.19 (m, 2H), 8.09-8.06 (m, 2H), 7.69-7.53 (m, 6H), 7.30-7.27 (m, 1H), 6.84 (t, J = 11.7 Hz, 2H), 4.03-3.93 (m, 4H), 3.57-3.51 (m, 4H).

(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)(4-(pyridin-2-yl)piperazin-1-yl)methanone (7o)

Pale yellow solid; % Yield 62; mp 110-112 °C; IR νmax/cm (KBr): 3275, 1635, 1581, 1435, 734; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.49 (s, 1H), 8.23 (d, J = 3.6 Hz, 1H), 8.18 (d, J = 7.9 Hz, 1H), 7.98 (d, J = 7.1 Hz, 2H), 7.62-7.50 (m, 6H), 7.36 (t, J = 7.3 Hz, 1H), 6.71-6.67 (m, 2H), 4.07-4.03 (m, 4H), 3.80-3.65 (m, 4H).

(4-benzylpiperazin-1-yl)(1-phenyl-9H-pyrido[3,4-b]indol-3-yl)methanone (7p)

White solid; % Yield 72; mp 160-162 °C; IR νmax/cm (KBr): 3174, 1626, 1556, 1487, 1431, 738; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (s, 1H), 8.43 (s, 1H), 8.16 (d, J = 7.9 Hz, 1H), 7.96 (d, J

= 7.2 Hz, 2H), 7.66-7.47 (m, 5H), 7.35-7.28 (m, 6H), 4.00-3.92 (m, 4H), 3.59 (s, 2H), 2.62-2.20 (m, 4H); MS m/z 447.6 $[M + H]^+$.

4.3. Biological Evaluation

4.3.1. Cytotoxicity assay

HeLa cell cytotoxicity studies were carried out as described previously [43]. Briefly, the cells were cultured in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were plated at initial cell concentration of 2×10^4 cells/well and incubated with the compounds for 65 h prior to addition of Alamar Blue solution for a further 5 h.

4.3.2. Promastigote assay

L. donovani promastigotes were cultured at 26°C in M199 medium supplemented with 10% heat-inactivated fetal calf serum. *L. infantum* JPCM5 MCAN/ES/98/LLM-87 promastigotes were cultured at 26 °C in modified Eagle's medium (HOMEM) [44]. Parasites were incubated with serial dilutions of compounds for 72 h, followed by Alamar blue-based assay as previously described [40].

4.3.3. Axenic amastigote assay

L. donovani LdBOB axenic amastigotes or *L. infantum* clone JPCM5 MCAN/ES/98/LLM-87 were cultured at 26°C in modified Eagle's medium (HOMEM; *L. infantum*) and axenic amastigotes were incubated for 72 h with compounds, followed by Alamar blue-based assay as previously described [40].

4.3.4. Intra macrophage *L. donovani* (amastigote) assay

THP-1 (human monocytic leukemia) cells were a kind gift from Dr Susan Wylie (Dundee) and maintained in minimal essential medium plus 10% (vol/vol) FBS. An intracellular *Leishmania* assay using LdBOB amastigotes were performed utilizing PMA differentiated THP-1 cells infected overnight with axenic amastigotes, prior to compounds being added and incubated for further 72 h, subsequent microscopy-based readout was used to determine EC_{50} values [40]. Briefly, THP-1 cells (20,000 per well, 200 µl) were plated into 96 well plates in presence of 10 nM PMA and incubated at 37 ^oC in 5% CO_2 atmosphere for 75 h. The adhered cells were then washed with phosphate buffered saline supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin and using LdBOB amastigotes added to all wells at a multiplicity of

infection of 5 (100,000 amastigotes per well) and incubated for 18 h. Any remaining extracellular amastigotes were removed and the adhered cells were washed with the same supplemented PBS solution as above. Pre-aliquoted compounds in a serial dilution were added from another plate, such that the total well volume was 200 μL and incubated for further 72 h. Cells were fixed with 100% methanol, stained with Giemsa and examined microscopically. Number of intracellular amastigotes (~200 macrophages per well) were determined and the percentage infection was established compared to an untreated control (100%) allowing EC_{50} values to be calculated.

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6. Conflict of Interest

The authors have no conflicts of interest to declare.

7. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at xxxxxxxxxxxx.

8. References

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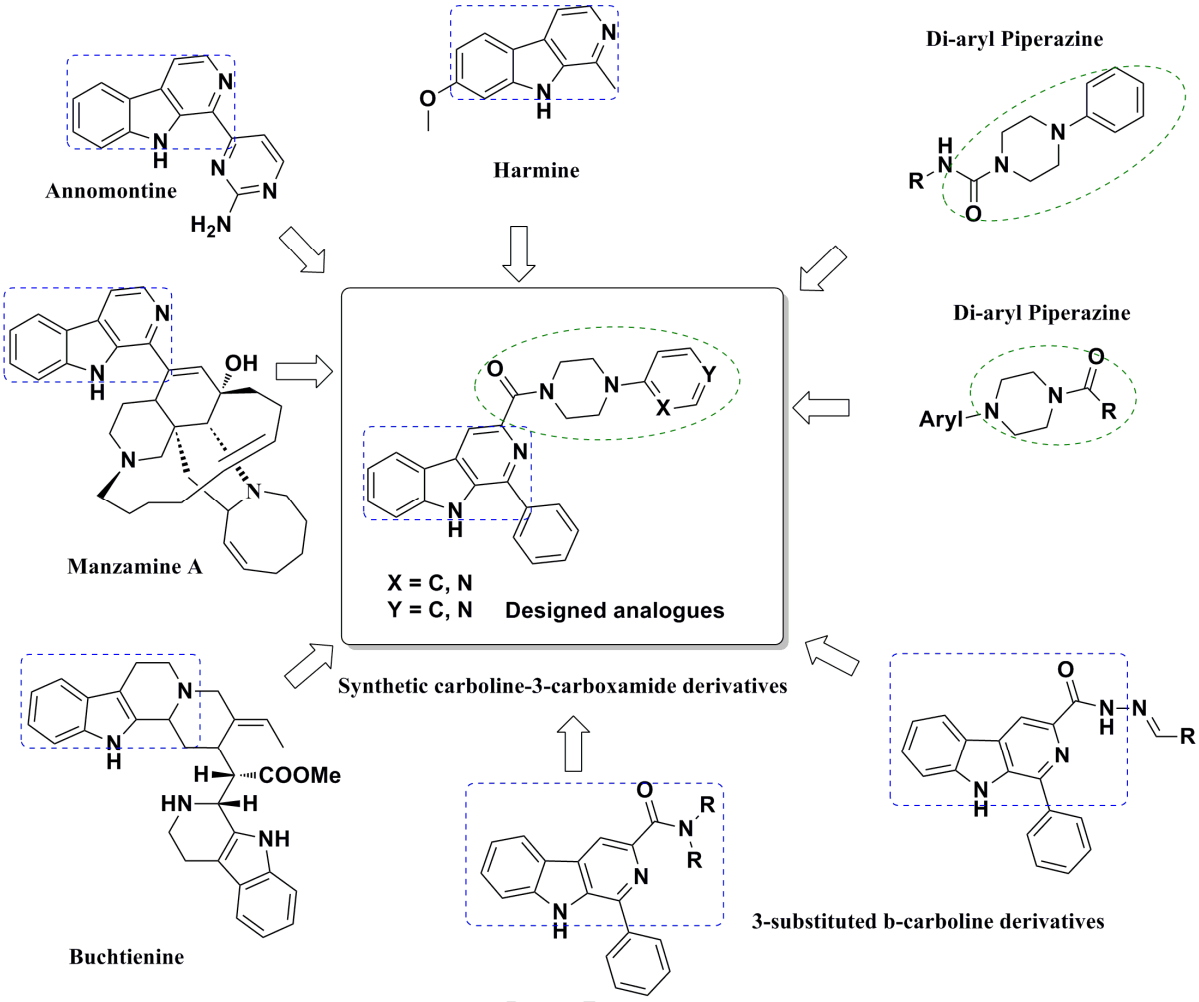
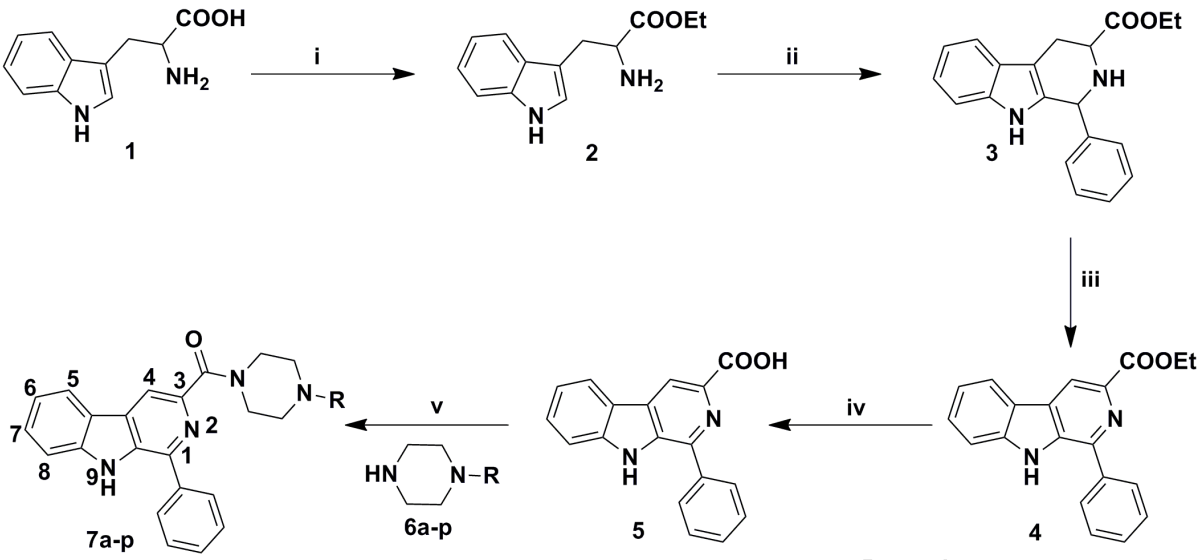
Table 1

Compound code	**R**	**HeLa CC_{50} (µM)**	***L. infantum* promastigotes EC_{50} *(µM)***	**S.I.[a]**	***L. infantum* axenic amastigotes EC_{50} *(µM)***	**S.I.[a]**
7a	$-C_6H_5$	38.1 ± 1.4	12.5 ± 1.7	3.0	4.5 ±2.1	8.5
7b	$-4CH_3C_6H_5$	>500	9.63 ± 0.72	>51.9	6.2±0.5	>80.6
7c	$-2CH_3C_6H_5$	>500	36.2 ± 2.5	>13.8	ND	-
7d	$-4OCH_3C_6H_5$	>500	9.07 ± 0.42	>55.1	5.2 ± 0.3	>96.2
7e	$-3OCH_3C_6H_5$	205.3 ± 9.6	2.89 ± 0.34	71.0	2.8±0.13	73.3
7f	$-2OCH_3C_6H_5$	204.8 ± 14.8	3.35 ± 0.25	61.1	4.9 ±0.4	41.8
7g	$-4ClC_6H_5$	>500	33.0 ± 1.7	>15.2	ND	-
7h	$-3ClC_6H_5$	18.4 ± 0.6	15.9 ± 1.4	1.2	3.3±0.4	5.6
7i	$-2ClC_6H_5$	>500	15.7 ± 1.5	>31.8	12.2±2.3	>41.0
7j	$-4FC_6H_5$	>500	14.1 ± 0.3	>35.5	11.3±0.7	>44.2
7k	$-2FC_6H_5$	>500	14.9 ± 0.9	>33.6	9.4±0.8	>53.2
7l	$-4NOC_6H_5$	>500	19.8 ± 0.9	>25.5	24.8± 1.7	>20.2
7m	$-2,3-ClC_6H_5$	>500	10.7 ± 1.0	>46.7	2.9±0.3	>172.4
7n	$-4C_5H_5N$	78.7 ± 4.5	66.7 ± 3.9	1.2	ND	-
7o	$-2C_5H_5N$	>500	34.0 ± 0.9	>14.7	ND	-
7p	$-CH_2C_6H_5$	28.9 ± 1.2	24.3 ± 0.8	1.2	ND	-
Pentamidine	-	-	8.31 ± 0.18	-	2.7 ± 0.4	-
Miltefosine	-	-	12.6±1.1	-	4.8 ± 0.8	-

Table 2

Compound code	**HeLa *CC*$_{50}$ (µM)**	***L. donovani* promastigotes *EC*$_{50}$ (µM)**	**S.I.[a]**	***L. donovani* axenic amastigotes *EC*$_{50}$ (µM)**	**S.I.[a]**	***L. donovani* intracellular amastigotes *EC*$_{50}$ (µM)**	**S.I.[a]**
7a	38.1 ± 1.4	16.3 ± 0.9	2.3	12.5±0.3	3.0	7.3±1.4	5.2
7b	>500	4.91 ± 0.38	>101.8	3.3±0.1	>151.5	5.7±0.6	>87.7
7c	>500	12.6 ± 0.9	>39.7	6.0±0.2	>83.3	9.6±2.2	>52.1
7d	>500	6.32 ± 0.59	>79.1	2.8±0.2	>178.6	9.2±1.5	>54.4
7e	205.3 ± 9.6	4.85 ± 0.28	42.3	1.9±0.1	108.1	6.9±0.4	29.7
7f	204.8 ± 14.8	3.49 ± 0.18	58.7	2.3±0.1	89.0	8.0±0.7	25.6
7g	>500	38.6 ± 3.5	>12.9	ND	-	ND	-
7h	18.4 ± 0.6	13.2 ± 0.95	1.4	10.2±0.4	1.8	6.8±2.4	2.7
7i	>500	6.98 ± 0.67	>71.6	5.8±0.3	>86.2	8.5±0.6	>58.8
7j	>500	17.5 ± 1.3	>28.6	12.6±1.1	>39.7	22.3±3.0	>22.4
7k	>500	3.47 ± 0.17	>144.1	2.8±0.1	>178.6	4.0±0.6	>125
7l	>500	12.8 ± 0.7	>39.1	11.8±0.9	>42.4	21.5±2.4	>23.3
7m	>500	6.95 ± 0.57	>71.9	5.7±0.4	>87.7	6.4±0.4	>78.1
7n	78.7 ± 4.5	68.5 ± 2.9	1.2	ND	-	ND	-
7o	>500	7.5 ± 0.69	>66.7	5.1±0.2	>98.0	11.9±1.6	>42.0
7p	28.9 ± 1.2	16.4 ± 1.7	1.8	12.6±0.4	2.3	8.8±2.7	3.3
Pentamidine	-	6.40 ± 0.11	-	1.6 ± 0.1	-	23.7 ± 1.8	-
Miltefosine	-	3.12±0.16	-	2.8 ± 0.4	-	6.4 ± 0.3	-





Design, synthesis and biological evaluation of piperazinyl-β-carboline derivatives as anti-leishmanial agents

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Highlights

- Novel β-carboline derivatives were designed based on molecular hybridization approach
- Cytotoxicity was evaluated against HeLa cell lines using an Alamar blue method
- Anti-leishmanial activity was determined against both *L. infantum* & *L. donovani*
- 7e displayed EC_{50} 2.89 & 2.80 µM against *L. infantum* promastigotes, axenic amastigotes
- 7k showed EC_{50} 3.47 & 2.80 µM against promastigotes, axenic amastigotes of *L. donovani*