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Graphical abstract:

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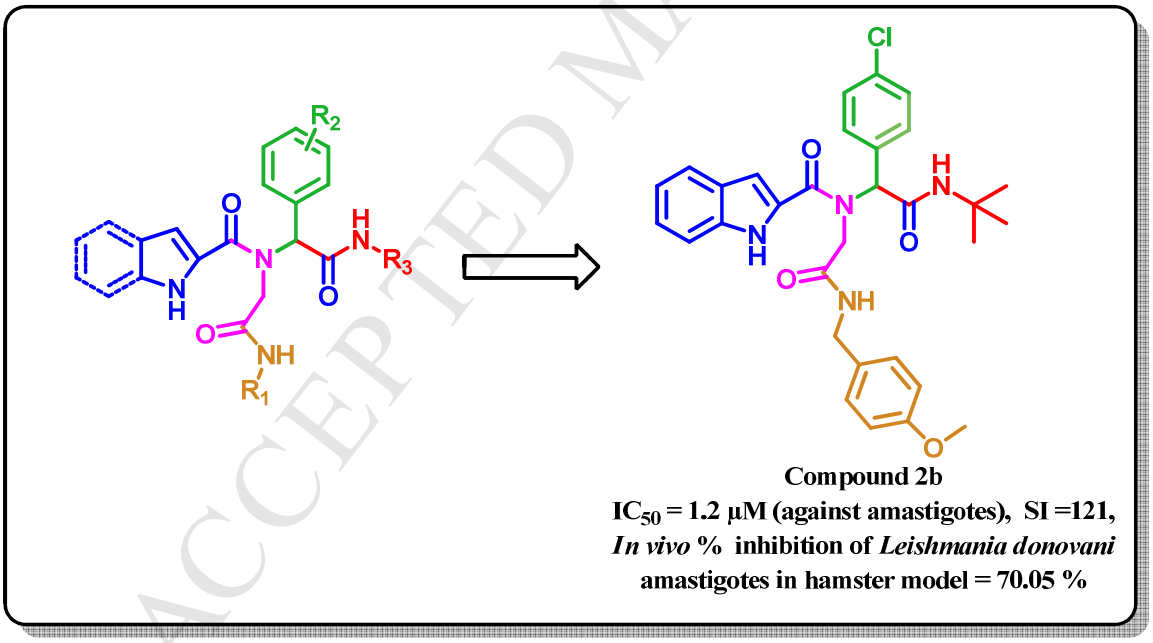

Compound 2b

IC_{50} = 1.2 µM (against amastigotes), SI =121,
***In vivo* % inhibition of *Leishmania donovani* amastigotes in hamster model = 70.05 %**

Identification of a diverse Indole-2-carboxamides as a potent antileishmanial chemotypes

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Abstract: A novel series of highly diverse indole-2-carboxamides was synthesized utilizing the isocyanide based multicomponent reaction (IMCR)-post modification approach and were identified as potential antileishmanial chemotype. Among the synthesized 18 analogues, 12 analogues exhibited better antileishmanial activity against intracellular amastigotes form of *Leishmania donovani* (IC_{50} values of 0.6 - 7.5 μM) as compared to standard drugs miltefosine and sodium stibogluconate. The compounds were also non-toxic towards Vero cells. Compounds **2b**, **2m** and **2p** with significant *in vitro* activity were then evaluated for their *in vivo* efficacy following intraperitoneal route. These three compounds at a concentration of 50 mg/kg/day for 5 consecutive days showed 70.0, 63.5 and 63.4 % inhibition of *Leishmania* amastigotes, respectively at day 7 post treatment in hamster model of visceral leishmaniasis.

Keywords: *Leishmania donovani*, Hamster model, Indole-2-Carboxamide, Multicomponent reaction.

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1. Introduction

Leishmaniasis is one of the most devastating neglected tropical diseases representing the serious public health problems in developing countries [1]. Leishmaniasis is second only to malaria in terms of the worldwide burden inflicted by protozoan parasitic diseases [2]. According to WHO estimations, there are almost 1.3 million new cases of leishmaniasis and 20,000 to 30,000 deaths annually [3]. This disease is caused by the protozoan parasites of the genus *Leishmania* and transmitted to the humans by the bite of female phlebotomine sandfly. It may manifest in three clinical forms, cutaneous, muco-cutaneous and visceral leishmaniasis (VL). Among all, VL (also known as Kala-azar) caused by *Leishmania donovani* (India and Near East) and *L. infantum* (around the Mediterranean Sea), is the most severe form of the disease in which vital organs of the body viz. liver, spleen and bone marrow are affected [4]. Currently, no effective vaccines are available [5], therefore the current treatment of leishmaniasis still relies on handful number of drugs such as, antimony based drugs, meglumine antimoniate (Glucantime), sodium stibogluconate (Pentostam), paromomycin, amphotericin B, and miltefosine [6]. Since its discovery, pentavalent antimonials have been used extensively as first line drug for the treatment of VL. However, requirement of high dose of parenteral administration, resistance issues, long-term treatment and severe side effects including cardiac arrhythmia and pancreatitis restricted the use of first line therapy in affected regions [7]. Furthermore, the second line drugs, pentamidine and amphotericin-B also suffer from moderate to severe side effects [8]. In the past decades, antileishmanial chemotherapy has been noticeably improved by the introduction of new liposomal formulation of amphotericin B, paromomycin, and serendipitous invention of miltefosine.

Although, single dose liposomal amphotericin B is a highly effective alternative with almost no side effect but its high cost makes this formulation unaffordable for poor people of

developing nations [9-10]. Moreover, miltefosine, a phosphocholine analogue, is introduced as first orally active drug for the treatment of VL. Despite the fact that this drug is effective, it has a long half-life (150-200 h), consequently prone to resistance development and has teratogenic effect in the women of child-bearing age [11]. The aforementioned limitations of the existing drugs along with the development of drug resistance especially in HIV−*Leishmania* co-infected patients [12] as well as the dearth of new antileishmanial drug candidates clearly reflects toward the efforts to hunt for novel drugs based on new molecular scaffolds capable of meeting the unmet needs in antileishmanial chemotherapy. In this regard, there is a need to develop new medicinal chemistry approaches to find out novel lead compounds that might populate a pipeline of new therapeutics.

Additionally, medicinal chemistry deliberately depends on robust synthetic methods capable of creating chemical diversity to satisfy the demand for the number and the quality of compounds for drug discovery. In this regard, multicomponent reactions (MCR) especially IMCRs provide versatile synthetic platforms for the preparation of libraries of diverse and complex 'drug-like' heterocyclic compounds. Among IMCRs, Ugi reaction has achieved profound attention for the synthesis and discovery of new lead structures and different types of biologically active scaffolds [13-15].

Indoles are omnipresent in natural products as well as in numerous biologically active compounds [16-17]. Literature search revealed that various indole containing natural products have shown potent antileishmanial activity [18-19]. Also, various indole containing scaffolds like [1,2,4]triazinoindole-[1,3,5]triazines [20], [1,2,4]triazinoindole-pyrimidine [20], pentamidine-aplysinopsin hybrids [21], triazino indole-quinolines [22] have been previously synthesized in our lab that have shown potential antileishmanial activity. Moreover, we have also synthesized analogues of coscinamide (indole containing natural products) i.e. 8,9-

Dihydrocoscinamide B [23], indolyl glyoxylamides [24] and explored their antileishmanial activity. While the naturally occurring coscinamides have not been reported to show any antileishmanial activity, the synthesized analogues showed potent antileishmanial activity [23-24].

Recently, we have developed an efficient and facile multicomponent based methodology which involves Ugi-post modification for the preparation of diverse *1H*-indole-2-carboxamide and *1H*-pyrrole-2-carboxamide scaffold [25]. Furthermore, *1H*-indole-2-carboxamide and *1H*-pyrrole-2-carboxamide templates are present in natural products [26] as well as in numerous biologically active synthetic compounds. These scaffolds are also associated with broad range of biological activities [27-29]. However, to the best of our knowledge; the antileishmanial activity of these bioactive pharmacophores has not been reported to date.

Inspired by these encouraging facts and as a part of our ongoing interest toward the design and synthesis of novel heterocycles as antileishmanial agent, [19-24, 30-31] we have synthesized a series of diverse indole-2-carboxamide derivatives and screened them for antileishmanial activity.

2. Results and discussion

2.1. Chemistry

The highly functionalized indole-2-carboxamides and pyrrole-2-carboxamides were synthesized using our previously developed [25] two-step procedure as outlined in Scheme 1. In first step, triethylamine was added to a solution of amino ester hydrochloride in methanol and the resulting solution was stirred at room temperature for 10 min. To the stirred solution, corresponding aldehyde, 1*H*-indole-2-carboxylic acid and isocyanide were successively added that produced corresponding indole-fused diketopiperazines **1(a-f)** in good to excellent yields. In the next step, compound **1** was refluxed with various amines in ethanol to afford highly functionalized

carboxamides **2(a-r)** in good to excellent yields. It is noteworthy here that the final products were obtained by subsequent regioselective ring opening of diketopiperazine unit **1** *via* an intermolecular transamidation reaction under mild condition. Moreover, our synthetic strategy is amenable for the quick and easy synthesis of a library of highly diverse analogues.

2.2. Biological assay

2.2.1. *In vitro* antileishmanial activity and cytotoxicity

In the endeavor to identify the activity of indole-2-carboxamides, we have synthesized a series of 18 compounds and screened them against intracellular amastigote form of *L. donovani* and their cytotoxicity was assessed on mammalian kidney fibroblast cells (Vero cell line). The results are summarized in Table 1. We have also tested the compounds for their cytotoxicity on murine macrophages (J-774A.1 cell line) and results are depicted in supplementary Table S1. Most of the synthesized compounds displayed excellent antiamastigote activity that was manifold better than the activity of standard drug, miltefosine. Initially, we synthesized derivatives **2(a-d)** with R_1 as *p*-chloro and **2(e-g)** with R_1 as 3, 4, 5-trimethoxy and different substitutions at R_2. Among these two groups of synthesized compounds, compound **2b** with *p*-chloro substituent at R_1 was found to be most active. Encouraged with the good activity profile of compound **2b**, we undertook the synthesis of a variety of structural analogues with *p*-chloro group retained at R_1 and variation at R and R_2 in the hope to improve the activity profile. The structure-activity relationship studies of these compounds suggested that the activity of these compounds were greatly influenced by the substituent at R_2. In case of aromatic substitution at R_2, the activity of compounds strongly depended on the substitution of benzyl ring at R_2. Analogue **2a** with unsubstituted benzyl ring and analogues **2c** and **2d**, with *p*-chloro and 3,4 dichloro substituted benzyl ring showed moderate antiamastigote activity, while analogue **2b** with an *p*-methoxybenzyl group showed potent activity with IC_{50} = 1.2 µM. In case of aliphatic substituent**,**

the introduction of ethanol group at R_2 in compound **2i** resulted in complete loss in antileishmanial activity, whereas analogue **2j** having 3-propyl morpholine at R_2 exhibited potent antileishmanial activity against amastigotes with IC_{50} = 2.1 µM which was 4 fold more active than standard drug miltefosine. Moreover, both the compounds (**2i** and **2j**) were non-toxic towards Vero cells (CC_{50} = 225.5 and 211.8 µM, respectively). These results suggested that presence of free polar group (here hydroxyl) in **2i** had detrimental effect on the *in vitro* antiamastigote activity. Introduction of 4-methylpyridine as heterocyclic aromatic moiety at R_2 in analogue **2k** demonstrated a slight decreased antileishmanial activity. Further, we replaced the 4-methoxy group in **2b** by 3-methoxy group in **2l** and we found that the compound **2l** also showed good activity with IC_{50} = 1.9 µM. Our SAR studies indicated that the introduction of methoxy substituent at benzyl ring of R_2 plays a crucial role in the antiamastigote activity.

Instigated with the potent activity profile and selectivity index of halogen containing analogues having *p*-chloro group at position R_1, we synthesized analogues **2m** and **2n** with *p*-bromo group at position R_1. Both these synthesized compounds also showed excellent antiamastigote activity with IC_{50} values of 1.0 and 1.5 µM, respectively as compared to standard drug miltefosine and were non-toxic towards Vero cells (CC_{50} > 400 µM). Furthermore, these compounds also showed very high selective index (SI = >400 and >266, respectively) that was 66 and 44 fold more than standard drug miltefosine. Next, replacement of tertiary butyl group of **2b** with 1,1,3,3-tetramethyl butyl group at position R in **2o** resulted in slight decrease in antileishmanial activity. In view of the fact, that the compounds **2b, 2n** and **2o** having 4-methoxy benzyl group at R_2 showed potent activity with high selectivity, we designed compounds **2p-2r** with 4-methoxy intact at position R_1 and variations at R_2. Interestingly, among all the synthesized analogues in present series, compound **2p** with R_2 as 4-methoxybenzyl group, was found to be the most potent with IC_{50} value of 0.6 µM which is 14-fold more active than

miltefosine (IC_{50} = 8.4 μM). Additionally, compound **2p** was non-toxic to Vero cells (CC_{50} = >400 μM) and has high selectivity (SI >666). Compound **2q** with 4-chlorobenzyl group exhibited moderate antiamastigote activity (IC_{50} = 7.50 μM) while compound **2r** with pyridine-4-ylamine group showed potent antiamastigote activity (IC_{50} = 1.4 μM)**.**

2.2.2. *In vivo* antileishmanial activity in hamster model

Encouraged with the results of these initial set of analogous, we selected the best three compounds (**2b, 2m** and **2p)** on the basis of their *in vitro* potency for further evaluation accordingly; *in vivo* tests were performed in the *L. donovani*/golden hamster model. The aqueous solutions of test compounds were administered for five consecutive days at 50 mg/kg/day by intraperitoneal (ip) route. The post-treatment (p.t.) splenic biopsies were done on day 7 of the last dose administration and amastigote counts were assessed by Giemsa staining. All the test compounds **2b, 2m** and **2p** showed good percentage inhibition of 70.0 ± 13.3%, 63.5 ± 10.3 and 63.4 ± 8.2 %, respectively in *Leishmania* parasite multiplication (Figure 1). Furthermore, we have not observed any abnormal behavior of animals at the time of dosing.

2.2.3. Cell death analysis

In order to confirm the mode of killing of *Leishmania* parasite by the action of best active compound **2b,** we have performed cell death analysis experiment using annexin V- FITC (stained apoptotic cells) and propidium iodide (stained necrotic cells). In our results, we have found that this derivative has potential to kill the parasite by the apoptotic way of death. *Leishmania* promastigotes were incubated with compound **2b** for various time points (12-72h), labeled with annexin V- FITC and propidium iodide and analyzed by flow cytometry.

Flow cytometric analysis revealed that after 12, 24 and 48 h incubation with compound **2b** (at IC_{50} concentration 0.9 μM against promastigotes) [32], 49.10, 56.17 and 73.30% of

promastigotes, respectively, stained positive for annexin V as shown in lower right quadrant (Figure 2B-D) as compared to untreated promastigotes, where cells were unstained with both the dyes (Figure 2A). The maximum phosphatidylserine exposure was observed at 72 h incubation (Figure 2E), where 79.07% promastigotes were in early apoptotic phase. Almost negligible cells were stained with propidium iodide, indicating that killing mode of promastigotes by compound **2b** *via* apoptosis.

2.2.4. Preliminary pharmacokinetic studies

The compelling *in vitro* and *in vivo* antileishmanial activity exhibited by compound **2b** encouraged us to perform *in vivo* pharmacokinetic studies of compound **2b**. Pharmacokinetic studies revealed that the animals tolerated the treatment, as no peculiarities in the behavior of animals were observed. Calculations of the pharmacokinetic parameters were based on the mean serum concentrations and were performed by use of the noncompartmental approaches using Phoenix WinNonlin, version 6.3 (Certara Inc, Missouri, USA). Following per oral (po, 50 mg/kg) and intravenous (5 mg/kg) administration, the compound was detected in serum upto 24 h. After po dose, it was rapidly absorbed ($t_{max1,}$ 1 h) and slowly eliminated (MRT, 8.5 h) with multiple peak phenomenon (Figure 3 and Table 2). It may be due to the delayed gastric emptying, enterohepatic recirculation and variability of absorption in different regions of gastrointestinal tract [33]. The compound possesses adequate oral bioavailability (34.9 %) with an average C_{max} 2852.6 ng/ml. The volume of distribution (4.4 L/kg) is higher than the total blood volume (0.064 L/kg; Nagarjun and Lal) of the rat and systemic clearance (0.6 L/h/Kg) is lower than the total hepatic blood flow in rats (2.9 L/h/kg) [34] indicating extravascular distribution with negligible extrahepatic elimination. Hence, the pharmacokinetic parameters support the good oral exposure of the compound to exhibit antileishmanial activity.

3. Conclusion

In conclusion, we have synthesized highly functionalized indole-2-carboxamide *via* Ugi-post transformation approach. Biological evaluation of these new chemical entities revealed that most of the synthesized compounds exhibited better potency against the intracellular amastigote form of *L. donovani* than the standard drug miltefosine and SSG in the *in vitro* system and were not found to be cytotoxic. The lead compound **2b** was found to be 7-fold more active than miltefosine against the amastigotes and eradicated the parasites *via* apoptotic mode of killing. Significant inhibition of parasites in hamster model of VL and pharmacokinetic parameters also supports the candidature of compound **2b** as a potential antileishmanial lead. Our findings underscore the importance of the indole-2-carboxamides as an important lead scaffold in the design and synthesis of antileishmanial agents.

4. Experimental Methods

4.1. General information: All commercially available starting materials and solvents were reagent grade, and used without further purification. Reactions were carrying out under dry glassware with magnetic stirring. IR spectra were recorded on a FTIR spectrophotometer Shimadzu 8201 PC and are reported in terms of frequency of absorption (cm^{-1}). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Supercon Magnet Avance 400 and 300 spectrometers using TMS as an internal reference and the samples were dissolved in suitable deuterated solvents (Chemical shifts (δ) are given in ppm relative to TMS and coupling constants (*J*) in Hz). HR-DART MS were recorded on JEOL, JMS T100LC Accu TOF. Melting points were determined in open capillary tubes on an electrically heated block and were uncorrected. Thin-layer chromatography (TLC) was carried out with silica gel plates (silica gel 60 F254), that were visualized by exposure to ultraviolet light. Purity of final compounds was determined by analytical HPLC, which was carried out on a Waters HPLC system (model pump: 515, detector

PDA-2998). HPLC analysis conditions: Thermo ODS-2 Hypersil C18 (5.0µM), 4.6x250 mm column flow rate 1.0 mL/min. All evaluated compounds are ≥ 95% pure**.**

4.1.1. General Procedure for the Preparation of Compound 1(a-f)

To a solution of amino ester hydrochloride (1 mmol) in methanol (3 mL) was added triethylamine (1.1 mmol) and the resulting solution was stirred at room temperature for 10 min. To the stirred solution were successively added corresponding benzaldehdye (1 mmol), 1H-indole-2-carboxylic acid 4 (1 mmol) and isocyanide (1 mmol). The mixture was stirred at room temperature for 30 min. The progress of the reaction was monitored by TLC. After completion of the reaction, solvent was evaporated at reduced pressure and purified through column chromatography (eluent: $CHCl_3$/MeOH) using 100−200 mesh silicagel to afford the desired product [25].

4.1.2. General procedure for the preparation of compound 2(a-r)

To a stirred solution of required fused diketopiperazine (1 mmol) in ethanol (5mL) corresponding amine (1.2 mmol) was added and the reaction mixture was heated at reflux for 4-8 h. The progress of the reaction was monitored by TLC. After completion of the reaction, solvent was evaporated at reduced pressure and purified through column chromatography (eluent: $CHCl_3$/ MeOH) using 100−200 mesh silica gel to afford the desired product **2(a-r)**. Characterization of Compounds **2(a-h)** [25].

4.2. Characterization of Compounds 2(i-r)

4.2.1. N-(2-(tert-butylamino)-1-(4-chlorophenyl)-2-oxoethyl)-N-(2-((2-hydroxyethyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2i)

White solid; Yield 84%; Mp: 112-113 °C; IR (KBr): 3421, 3019, 1657, 1521 cm^{-1}; HPLC–PDA: t_r =5.04 min (% area = 99.10%); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.63(br s, 2H), 7.66 (d, 1H, *J* =

7.9 Hz), 7.40-7.36(m, 4H), 7.31-7.27(m, 1H), 7.13-7.10 (m, 1H), 6.90 (s, 1H), 6.09-5.52(m, 2H), 4.34-3.25(m, 8H), 1.27 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 170.2, 169.9, 164.4, 136.1, 135.9, 131.4, 131.2, 129.7, 127.8, 127.6, 125.3, 122.5, 120.8, 111.7, 107.0, 66.3, 61.7, 52.2, 51.1, 42.7, 28.3: HRMS (ESI) calcd for [$C_{25}H_{29}ClN_4O_4$ + H^+] 485.1950; found 485.1950

4.2.2. N-(2-(tert-butylamino)-1-(4-chlorophenyl)-2-oxoethyl)-N-(2-((3-morpholinopropyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2j)

White solid; Yield 81%; Mp: 98-100 °C; IR (KBr): 3441, 3030, 1647, 1516 cm^{-1}; ; HPLC–PDA: t_r =5.64 min (% area = 95.01%); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.66(s, 1H), 9.44 (br s, 1H), 7.66 (d, 1H, *J* = 8.0 Hz), 7.41-7.26 (m, 5H), 7.13-7.09 (m, 1H), 6.92 (s, 1H), 5.93(s, 1H), 5.49(s, 1H), 4.40-4.29 (m, 1H), 3.90-3.82(m, 1H), 3.59(s, 4H), 3.36(s, 2H), 2.28(m, 5H), 1.93(s, 2H), 1.68-1.65(m, 2H), 1.31 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 170.1, 169.7, 164.0, 153.9, 138.9, 138.2, 135.9, 128.3, 128.1, 128.0, 127.9, 127.0, 125.2, 122.8, 120.6, 111.5, 107.1, 107.0, 66.8, 60.8, 56.0, 51.9, 50.3, 43.4, 28.3; HRMS (ESI) calcd for [$C_{30}H_{38}ClN_5O_4$ + H^+] 568.2685; found 568.2684.

4.2.3. N-(2-(tert-butylamino)-1-(4-chlorophenyl)-2-oxoethyl)-N-(2-oxo-2-((pyridin-4-ylmethyl) amino)ethyl)-1H-indole-2-carboxamide (2k)

White solid; Yield 74%; Mp:132-134 °C; IR (KBr): 3434, 2921, 1647, 1516 cm^{-1}; ; HPLC–PDA: t_r =6.01 min (% area = 96.33%); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.35(br s, 1H), 9.55(s, 1H), 8.37(s, 2H), 7.64 (d, 1H, *J* = 8.0 Hz), 7.42(d, 1H, *J* = 8.3 Hz), 7.35-7.29(m, 3H), 7.21(d, 2H, *J* = 8.0 Hz), 7.15-7.11(m, 3H), 6.88(s, 1H), 5.81(s, 1H), 5.53(s, 1H), 4.55-4.39(m, 3H), 3.80(br s, 1H), 1.27 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.8, 163.9, 149.6, 147.1, 136.0, 135.9, 131.1, 129.8, 127.8, 127.7, 125.5, 122.8, 122.7, 120.9, 111.6, 107.1, 66.4, 52.2, 50.6, 42.3, 28.3: HRMS (ESI) calcd for [$C_{29}H_{30}ClN_5O_3$ + H^+] 532.2110 ; found 532.2110.

4.2.4. N-(2-(tert-butylamino)-1-(4-chlorophenyl)-2-oxoethyl)-N-(2-((3-methoxybenzyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2l)

White solid; Yield 80%; Mp: 156-158 °C; IR (KBr) : 3414, 1656, 1591, 1397 cm^{-1}; ; HPLC–PDA: t_r = 5.38 min (% area = 96.26%); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.96(br s, 1H), 9.46(s, 1H), 7.64(d, 1H, *J* = 7.9 Hz), 7.40(d, 1H, *J* = 8.2 Hz), 7.31-7.28(m, 3H), 7.17-7.10(m, 4H), 6.87-6.76(m, 4H), 5.69(s, 1H), 5.49(s, 1H), 4.60-4.36(m, 3H), 3.76-3.70 (m, 4H), 1.25 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.5, 169.3, 164.1, 135.9, 131.3, 129.7, 129.4, 127.8, 125.3, 122.7, 120.7, 113.7, 112.9, 111.6, 107.2, 66.23, 55.05, 52.0, 50.8, 43.5, 28.3; HRMS (ESI) calcd for [$C_{31}H_{33}ClN_4O_4$ + H^+] 561.2263; found 561.2261.

4.2.5. N-(1-(4-bromophenyl)-2-(tert-butylamino)-2-oxoethyl)-N-(2-((3-morpholinopropyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2m)

White solid; Yield 82%; Mp:134-136 °C; IR (KBr): 3425, 3020, 1661, 1398 cm^{-1}; HPLC–PDA: t_r = 6.72 min (% area = 99.16 %); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.66(s, 1H), 9.43(br s, 1H), 7.66 (d, 1H, *J* = 8.0 Hz), 7.56(d, 2H, *J* = 7.3 Hz), 7.41(d, 1H, *J* = 8.2 Hz), 7.30-7.27(m, 2H), 7.13-7.09 (m, 1H), 6.92 (s, 1H), 5.93(s, 1H), 5.48(s, 1H), 4.30-4.26 (m, 1H), 3.85-3.83(m, 1H), 3.59(s, 4H), 3.36(s, 2H), 2.28(s, 5H), 1.88(s, 2H), 1.70-1.65(m, 2H), 1.31 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.3, 164.1, 135.9, 132.57, 131.5, 127.9, 125.3, 124.0, 122.7, 120.7, 111.6, 107.0, 66.9, 66.6, 56.4, 53.5, 52.1, 51.1, 38.0, 28.4, 25.6; HRMS (ESI) calcd for [$C_{30}H_{38}BrN_5O_4$ + H^+] 612.2180 ; found 612.2181.

4.2.6. N-(1-(4-bromophenyl)-2-(tert-butylamino)-2-oxoethyl)-N-(2-((4-methoxybenzyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2n)

White solid; Yield 88%; Mp:128-130 °C; IR (KBr): 3427, 3020, 1660, 1516 cm^{-1}; HPLC–PDA: t_r = 5.49 min (% area = 99.34%) ^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.82 (br s, 1H), 9.55 (s, 1H),

7.63 (d, 1H, *J* = 7.8 Hz), 7.43-7.38(m, 3H), 7.31-7.27(m, 1H), 7.22(d, 2H, *J* = 6.7 Hz), 7.14-7.06 (m, 3H), 6.85 (br s, 1H), 6.78 (d, 2H, *J* = 8.4 Hz), 5.78 (s, 1H), 5.49 (s, 1H), 4.53-4.29 (m, 3H), 3.75-3.73 (m, 4H), 1.24 (s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 169.4, 169.1, 164.4, 158.7, 136.0, 132.5, 131.6, 130.3, 129.6, 127.8, 127.6, 125.1, 123.9, 122.5, 120.6, 113.7, 111.7, 107.0, 65.8, 55.1, 51.8, 50.8, 42.9, 28.2; HRMS (ESI) calcd for [$C_{31}H_{33}BrN_4O_4$ + H^+] 605.1758; found 605.1757.

4.2.7. N-(1-(4-chlorophenyl)-2-oxo-2-((2,4,4-trimethylpentan-2-yl)amino)ethyl)-N-(2-((4-methoxybenzyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2o)

White solid; Yield 78%; Mp: 205-207 °C; IR (KBr): 3422, 3019, 1662, 1384 cm^{-1}; HPLC–PDA: t_r = 5.49 min (% area = 97.16%); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.89 (br s, 1H), 9.32 (s, 1H), 7.64 (d, 1H, *J* = 8.0 Hz), 7.39(d, 1H, *J* = 8.2 Hz), 7.32-7.28 (m, 3H), 7.25-7.19 (m, 4H), 7.14-7.11 (m, 1H), 6.85 (s, 1H), 6.79(d, 1H, *J* = 8.3 Hz), 5.53(s, 1H), 5.31 (br s, 1H), 4.54-4.32 (m, 3H), 3.89-3.76 (m, 4H), 1.71(d, 1H, *J* = 13.4 Hz), 1.53(d, 1H, *J* = 14.7 Hz), 1.39(d, 6H, *J* = 14.3 Hz), 0.89(s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ = 168.9, 168.6, 163.8, 158.8, 135.8, 131.3, 130.5, 129.7, 127.9, 125.2, 122.7, 120.7, 113.8, 111.5, 107.1, 67.0, 56.3, 52.7, 51.4, 42.9, 31.4, 31.3, 28.6, 27.9; HRMS (ESI) calcd for [$C_{35}H_{41}ClN_4O_4$ + H^+] 617.2889; found 617. 2888.

4.2.8. N-(2-(tert-butylamino)-1-(4-methoxyphenyl)-2-oxoethyl)-N-(2-((4-methoxybenzyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2p)

White solid; Yield 92%; Mp: 198-200 °C; IR (KBr): 3422, 3019, 1654, 1513 cm^{-1}; HPLC–PDA: t_r = 6.71 min (% area = 100.00%). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.1(br s, 1H), 9.53 (s, 1H), 7.63 (d, 1H, *J* = 7.9 Hz), 7.40 (d, 1H, *J* = 8.2 Hz), 7.30(d, 1H, *J* = 7.5 Hz), 7.24(d, 2H, *J* = 7.9 Hz), 7.13-7.09 (m, 3H), 6.85 (br s, 1H), 6.81-6.74(m, 4H), 5.68 (s, 1H), 5.54 (s, 1H), 4.59-4.54 (m, 1H), 4.40-4.29 (m, 2H), 3.79-3.74(m, 7H), 1.24(s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ =

170.3, 169.4, 164.2, 160.3, 158.6, 135.9, 131.3, 130.6, 129.6, 128.1, 127.8, 124.9, 124.5, 122.6, 120.5, 114.8, 113.6, 111.6, 106.9, 66.0, 55.1, 51.7, 50.4, 42.8, 28.2; HRMS (ESI) calcd for [$C_{32}H_{36}N_4O_5$ + H^+] 557.2758; found 557.2758.

4.2.9. N-(2-(tert-butylamino)-1-(4-methoxyphenyl)-2-oxoethyl)-N-(2-((4-chlorobenzyl)amino)-2-oxoethyl)-1H-indole-2-carboxamide (2q)

White solid; Yield 89 %; Mp: 130-132 °C; IR (KBr): 3420, 3020, 1658, 1610, 1516 cm^{-1}; HPLC–PDA: t_r = 5.47 min (% area =96.69 %); ^{1}H NMR (400 MHz, $CDCl_3$) δ = 10.35 (br s, 1H), 9.53 (s, 1H), 7.63 (d, 1H, *J* = 8.0 Hz), 7.42 (d, 1H, *J* = 8.3 Hz), 7.31-7.27 (m, 1H), 7.24-7.22 (m, 2H), 7.18-7.09 (m, 5H), 6.84 (s, 1H), 6.81 (d, 2H, *J* = 8.3 Hz), 5.69 (s, 1H), 5.56(br s, 1H), 4.61-4.56 (m, 1H), 4.42-4.29 (m, 2H), 3.80-3.67(m, 4H), 1.24(s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ = 170.4, 169.7, 164.1, 160.4, 136.9, 135.9, 132.8, 131.2, 129.7, 128.3, 128.0, 127.8, 125.1, 124.3, 122.6, 120.6, 114.8, 111.6, 106.9, 66.1, 55.2, 51.8, 50.4, 42.7, 28.2; HRMS (ESI) calcd for [$C_{31}H_{33}ClN_4O_4$ + H^+] 561.2263; found 561.2263.

4.2.10. N-(2-(tert-butylamino)-1-(4-methoxyphenyl)-2-oxoethyl)-N-(2-oxo-2-((pyridin-4-ylmethyl) amino)ethyl)-1H-indole-2-carboxamide (2r)

White solid; Yield 76 %; Mp: 212-213 °C; IR (KBr): 3421, 3019, 1650, 1513 cm^{-1}; HPLC–PDA: t_r = 7.45 min (% area = 95.05 %); ^{1}H NMR (400 MHz, DMSO-d_6) δ = 11.69(br s, 1H), 9.20-8.77(m, 1H), 8.46-8.25(m, 2H), 7.58-7.46(m, 2H), 7.25-7.21(m, 3H), 7.08-6.70(m, 5H), 6.08-5.85(m, 1H), 4.37-4.19(m, 2H), 3.96-3.97(m, 1H), 3.78(s, 3H), 1.28(s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ = 169.3, 163.2, 158.7, 148.8, 147.2, 135.5, 130.9, 130.1 129.2, 127.8, 126.4, 126.1, 123.3, 121.7, 121.1, 119.4, 113.5, 111.7, 103.6, 62.9, 54.6, 49.9, 49.2, 40.7, 27.8; HRMS (ESI) calcd for [$C_{30}H_{33}N_5O_4$ + H^+] 528.2605; found 528.2605.

4.3. Biological assay

4.3.1. **In vitro *antiamastigote assay***

For assessing the activity of compounds against the amastigote stage of parasite, mouse macrophage cell line (J-774A.1) infected with WHO reference strain (MHOM/IN/80/Dd8) of promastigote (expressing luciferase firefly reporter gene) was used. Macrophages were seeded in a 96 well plate (4 x 10^4/mL/100µL/well) in RPMI-1640 containing 10% Foetal calf serum and the plates were incubated at 37 ºC in a 5% CO_2 incubator. After 24 h, the medium was replaced with fresh medium containing stationary phase promastigotes (4 x 10^5/mL/100µL/well). Promastigotes invade the macrophage and are transformed into amastigotes. Each well of the plate was washed with plain RPMI medium after 24 h of incubation to remove the un-internalized promastigotes. The test compounds were added at two fold dilutions up to 7 points in complete medium starting from 40µM concentration after replacing the previous medium and the plates were incubated at 37ºC in a CO_2 incubator for 72 h. After incubation, the drug containing medium was aspirated and 50 µL PBS was added in each well and mixed with an equal volume of Steady Glo reagent. After gentle shaking for 1–2 min, the reading was taken in a luminometer[32]. The values are expressed as relative luminescence units (RLU). Data were transformed into a graphic program (Excel). IC_{50} of antileishmanial activity was calculated by nonlinear regression analysis of the concentration response curve using the four parameter Hill equations.

4.3.2. Cytotoxicity assay

The cell viability was determined using the MTT (3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [35]. As described earlier, mammalian fibroblast cells (Vero cell line) and murine macrophage cells (J-774A.1) (1 x 10^5 cells/mL/100µL/well) were incubated with test compounds at 7 concentrations starting from 400µM. After 72 h of incubation, 25 µL

of MTT reagent (5mg/mL) in PBS medium was added to each well and incubated at 37°C for 2 h. At the end of the incubation, the supernatant were removed and 150 µL of pure DMSO was added to each well. After 15 min of incubation, the readings were recorded as absorbance at 544 nm on a micro plate reader. Fifty percent cytotoxic concentration (CC_{50}) values were estimated as described by Huber & Koella (1993) [36]. The selectivity index (SI) for each compound was calculated as ratio between, cytotoxicity on Vero cells (CC_{50}) and activity (IC_{50}) against *Leishmania* amastigotes.

4.3.3. In vivo *trial in L. donovani / hamster model*

The *in vivo* antileishmanial activity was determined in golden hamsters (*Mesocricetus auratus*) infected with MHOM/IN/80/Dd8 strain of *Leishmania donovani* [37]. Golden hamsters (Inbred strain) of either sex weighing 40–45g were infected intracardiacally with 1x 10^7 amastigotes per animal. After establishment of infection in 15 days, pre-treatment spleen biopsy in all the animals was carried out to assess the degree of infection. The animals with +1 infection (5–10 amastigotes/ 100 spleen cell nuclei) were included in the studies. Five to six infected animals were randomized into several groups used for each test compound. Drug treatment by intraperitoneal or oral route was initiated after two days of biopsy and continued for five consecutive days. SSG and miltefosine are used as reference drugs. Post-treatment biopsies were done on day 7 of the last dose administration and amastigote counts were assessed by Giemsa staining method [36]. Intensity of infection in both, treated and untreated animals, and also the initial count in treated animals was compared and the efficacy was expressed in terms of percentage inhibition (PI) using the following formula:-

PI = 100 – [(ANAT x 100)/ (INAT x TIUC)]

Where PI is Per cent Inhibition of amastigotes multiplication, ANAT is Actual Number of Amastigotes in Treated animals, INAT is Initial Number of Amastigotes in Treated animals and TIUC is Time Increase of parasites in Untreated Control animals.

4.3.4. Double staining with annexin V-FITC and propidium iodide (Death cell analysis)

For the detection of mode of killing by annexin V-FITC and propidium iodide (PI) double staining in *Leishmania* promastigotes incubated with compound, the apoptosis detection kit (Sigma Aldrich) was used as per manufacturer's instructions. Briefly, log phase promastigotes (1 x 10^6/ mL / well) were incubated with test compound for 12, 24, 48 and 72 h time points. After incubation, washed and centrifuged promastigotes were incubated with 5 µL of annexin V- FITC and 10 µL of propidium iodide for 30 min at room temperature. Fluorescence of cells was determined with Cell Quest FACSCalibur (Becton Dickinson). At least 10,000 cells were analyzed for each sample. Cells stained with the dyes, annexin V-FITC and propidium iodide were analysed by flow cytometry using 488 nm and 536 nm excitation wavelengths and 530 nm and 617 nm emission wavelengths on FL1 and FL2 channel, respectively [32].

4.2.5. Pharmacokinetics of 2b in rats

The pharmacokinetic studies of the compound **2b** was carried out in young and healthy male *Sprague-Dawley* rats weighing 250 ± 25 g. The rats were obtained from Laboratory Animal Division of the Institute and were housed in plastic cages under standard laboratory conditions with a regular 12 h day-night cycle. Standard pelleted laboratory chow (Goldmohar Laboratory Animal Feed, Lipton India Ltd, Chandigarh, India) and water were allowed *ad libitum.* The rats were acclimatized to this environment for at least two days before conducting the experiments. The oral dose was administered after overnight fasting (12-16 h). The study was conducted in

three rats per time point. In all the experiments, euthanasia and disposal of carcasses were carried out as per the guidelines of Local Ethics Committee for animal experimentation.

Suspension formulation of the compound was prepared by triturating the compound in 2% (v/v) tween 80 in water in a pestle with mortar. A single 50 mg/kg dose was given to conscious rats by oral gavage in a volume of approximately 1 mL/250 g rat and the time of dosing was recorded. Blood samples were collected at 0.5, 1, 2, 4, 6, 8, 10, 18, 24 and 48 h post dose. Solution formulation containing 20 mg compound was prepared by dissolving the compound in dimethyl sulfoxide, ethanol, tween 80, propylene glycol (10:20:2:48, % v/v) and normal saline (q.s.) to obtain 10 mg/ml of **2b**. The formulation was administered intravenously to conscious rats (via the femoral vein) at a dose of 5 mg/kg. Blood was collected at 0.083, 0.33, 0.5,1, 2, 4, 6, 8, 10, 24 and 48 h post dose from each rat in microtubes (Axygen, CA, USA).

Two blood samples were withdrawn from each animal. An initial 0.5 mL blood sample was drawn by cardiac puncture under light anesthesia followed by a sample drawn from the inferior vena cava (terminal sample) from the dosed rats using a 24G needle and a syringe in a clean and dry test tube. The total volume of blood collected from each rat for the first sample was not more than 10% of the total body volume. The blood was allowed to clot, by keeping the tube on a slant for approximately 45 min. Then it was centrifugated at 3000 rpm for 10 min at 4°C and the serum was separated into clean and neatly labeled tubes. All samples were stored at -80°C until analysis.

Chromatographic separations and quantification of the compound was achieved by a reverse phase HPLC method on a Discovery HS C-18 column (5 μm, 150 x 4.6 mm id) preceded with a guard column (5 μm, 20 x 4.0 mm, id) packed with the same material under isocratic condition at a flow rate of 1 ml/min. The HPLC system used in this study consisted of a pump (LC-10AT VP with FCV-10AL VP), degasser (DGU-14A) and auto-injector (SIL-HTc, fixed

with a 100 µl loop) (Shimadzu, Japan). Eluents were monitored at 295 nm with UV-Vis multiple wavelength detector (Shimadzu, Japan) and chromatograms were integrated using Class-VP (version 6.12 SP5) software. The mobile phase composition was aqueous ammonium acetate buffer (0.01M) and acetonitrile (35:65, %v/v) and it was degassed by ultrasonication for 10 min before use. The HPLC system was equilibrated for approximately 30 min before commencement of analysis and chromatography was carried out at ambient temperature. The lower limit of quantification for the analytical method was 25 ng/ml of test analyte in serum. The mean and SEM of the serum concentrations of the compound at each time point was calculated using Microsoft Excel for Windows. Due to multiple peak phenomenons, all pharmacokinetic parameters were calculated by noncompartmental model using WinNonlin program, version 6.3 (Certara Inc, Missouri, USA).

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Captions

Table 1. *In vitro* antileishmanial activity against *L. donovani* amastigotes and cytotoxicity of indole-2-carboxamides **2(a-r**).

Table 2. Pharmacokinetic parameters of **2b** in male *Sprague Dawley* rats.

Figure 1. *In vivo* antileishmanial efficacy in *L. donovani* / golden hamster model.

Figure 2. Cell death analysis using annexin V-FITC and propidium iodide (PI) double staining in *Leishmania* promastigotes incubated with compound **2b**.

Figure 3. Plasma concentration-time profile of **2b** after single oral (50 mg/kg) and intravenous dose (5 mg/kg) in male *Sprague Dawley* rats (n=3). Bar represents SEM.

Table 1. *In vitro* antileishmanial activity against *L. donovani* amastigotes and cytotoxicity of indole-2-carboxamides **2(a-r)**

Compound	Antiamastigote activity IC_{50}[a] (in µM)	Cytotoxicity[b] on Vero cells CC_{50} (in µM)	Selectivity Index[c] SI
2a	8.8 ± 1.0	248.4 ± 9.6	28
2b	1.2 ± 0.3	145.6 ± 9.1	121
2c	10.9 ± 1.3	>400	>36
2d	7.5 ± 1.1	52.2 ± 6.1	7
2e	>40	176.1 ± 8.4	<4
2f	9.2 ± 1.2	70.3 ± 8.1	7
2g	6.3 ± 0.9	72.9 ± 5.9	11
2h	14.5 ± 1.4	73.0 ± 6.8	5
2i	>40	225.5 ± 10.3	<5
2j	2.1 ± 0.4	211.8 ± 5.2	101
2k	6.4±1.0	>400	>62
2l	1.9 ± 0.6	>400	>210
2m	1.0 ± 0.3	>400	>400
2n	1.5 ± 0.4	>400	>266
2o	3.1 ± 0.8	>400	>129
2p	0.6 ± 0.2	>400	>666
2q	7.5 ± 0.9	399.6 ± 0.5	53
2r	1.4 ± 0.6	109.1 ± 6.7	78
SSG[d]	56.1 ± 3.2	>400	>7

MF[e]	8.4 ± 1.2	53.1 ± 4.5	6

[a]IC_{50} (50 % maximum inhibitory concentration) and [b]CC_{50} (50 % maximum cytotoxic concentration) values are the average (mean ± standard deviation) of two independent assays done in duplicates, [c]Selectivity Index (SI) is defined as the ratio of CC_{50} on vero cells to IC_{50} against *L. donovani* intracellular amastigotes. [d]SSG (sodium stibo-gluconate) and [e]MF (miltefosine) were used as reference drugs.

Table 2. Pharmacokinetic parameters of **2b** in male *Sprague Dawley* rats[a]

Parameters		**Oral**	**Intravenous**
C_{max} (ng/ml)	1	1589.5 ± 146.6	2280 ± 23.2
	2	1633.1 ± 138.8	1390 ± 42.4
	3	2852.6 ± 424.4	-
t_{max} (h)	1	1	0.083
	2	6	0.5
	3	10	-
AUC_{last} (ng h/ml)		27190	7786
MRT (h)		8.5	7.4
V_{ss} (L/kg)		12.8	4.4
Clearance (L/h/kg)		1.5	0.6
Bioavailability (%)		34.9	-

[a]Each value represents the average of three rats dosed orally (50 mg/kg) and intravenously (5 mg/kg); Values of C_{max} are mean ± SEM; AUC_{last} = area under the serum concentration-time curve up to last observation, C_{max} = serum peak concentration, t_{max} = time to C_{max}, MRT = mean residence time, V_{ss} = volume of distribution at steady-state

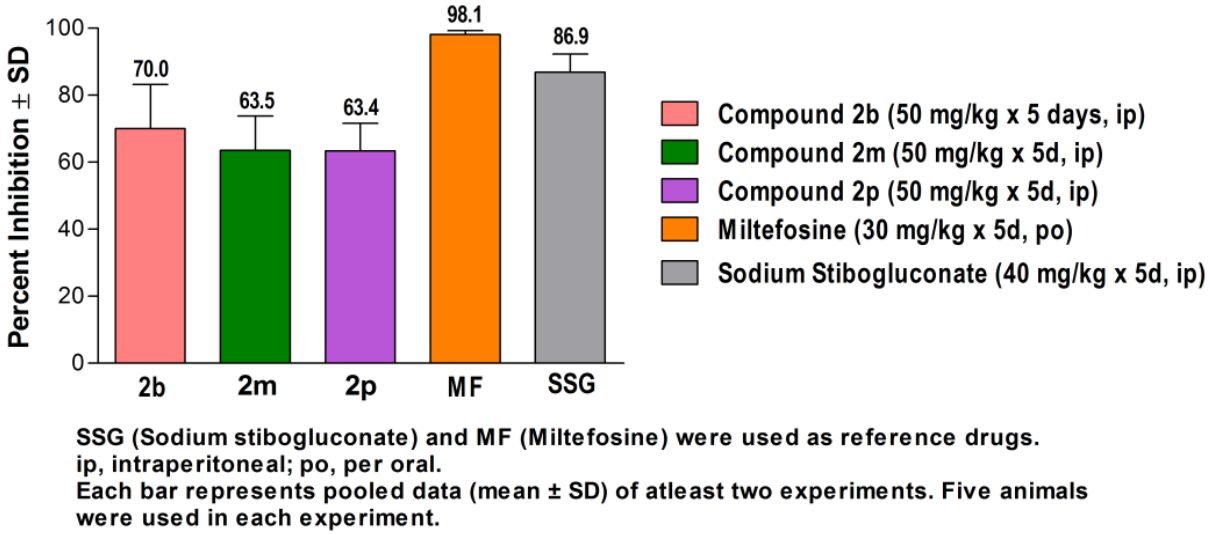


Figure 1: *In vivo* antileishmanial efficacy in *L. donovani* / golden hamster model.

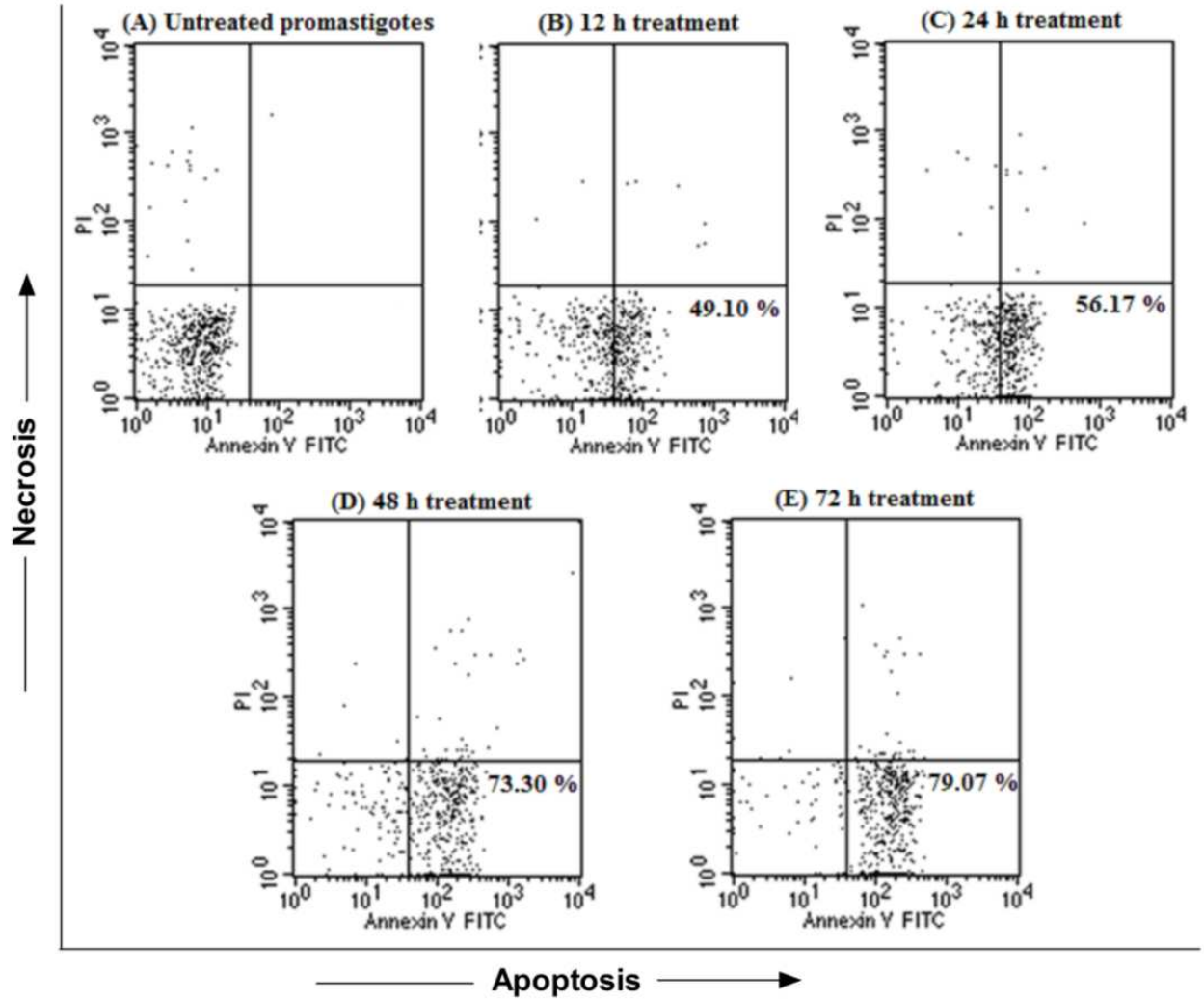


Figure 2. Cell death analysis using annexin V-FITC and propidium iodide (PI) double staining in *Leishmania* promastigotes incubated with compound **2b**. (A) Untreated promastigotes, (B) 12 h incubation, (C) 24 h incubation, (D) 48 h incubation and (E) 72 h incubation with compound **2b**. The lower left quadrant shows unstained living cells (Annexin-V and PI negative), the upper left shows necrotic cells (Annexin-V negative and PI positive), the upper right indicates late apoptotic cells (both Annexin-V and PI positive), and the lower right shows early apoptotic cells (Annexin-V positive and PI negative). The data are representative of two independent experiments done in duplicates

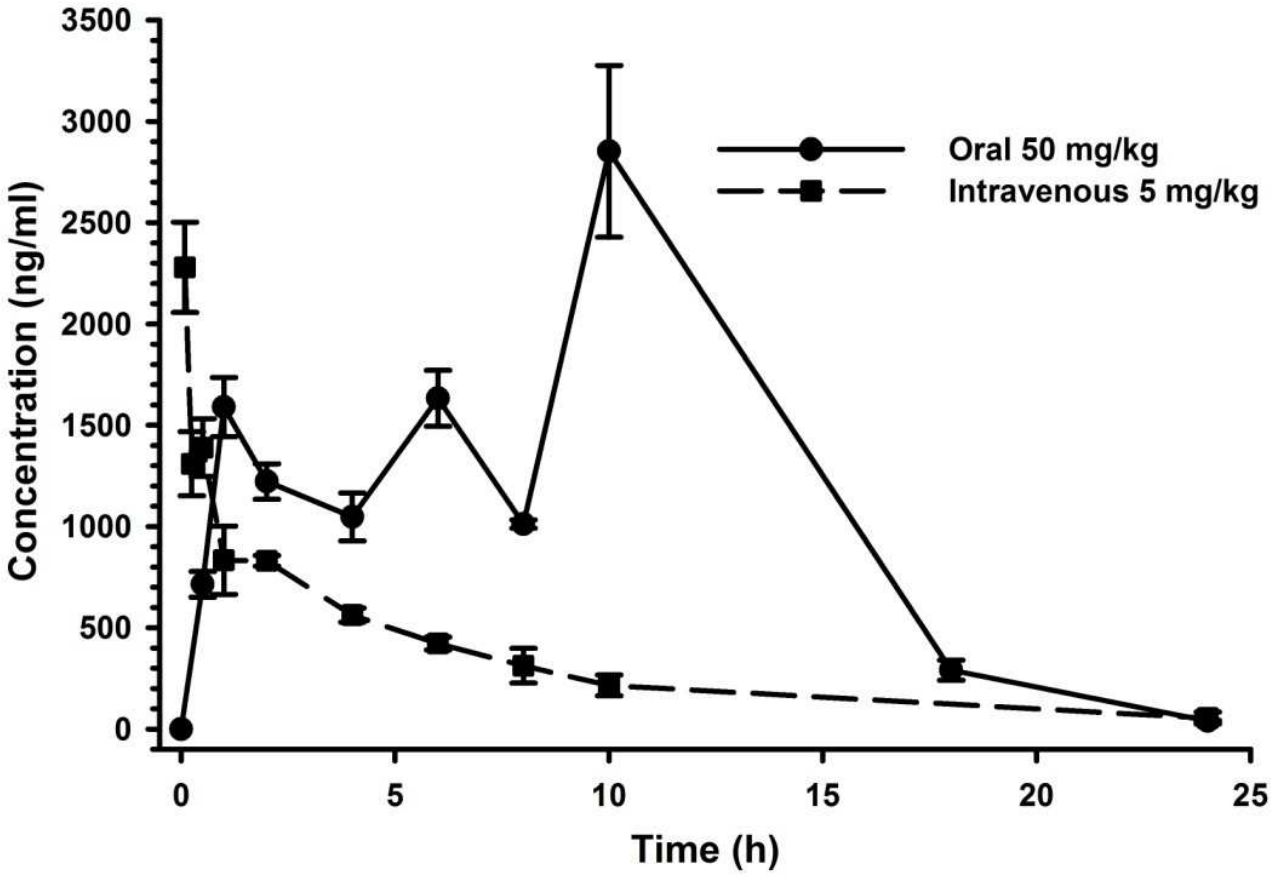
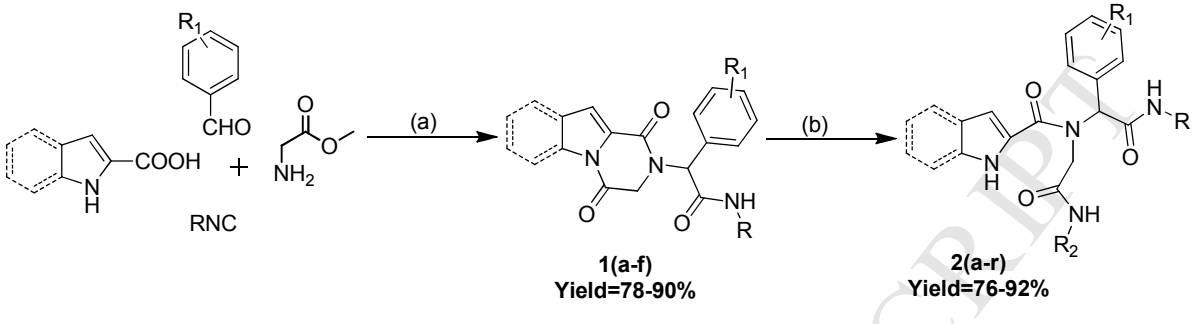
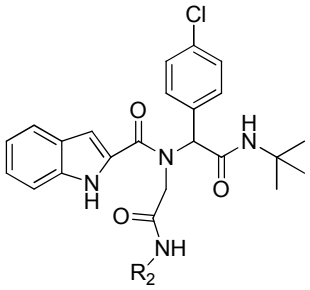


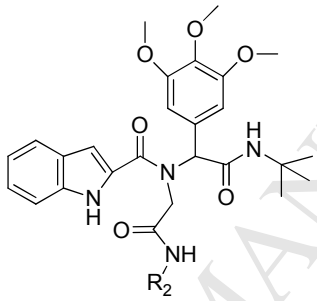
Figure 3. Plasma concentration-time profile of **2b** after single oral (50 mg/kg) and intravenous dose (5 mg/kg) in male *Sprague Dawley* rats (n=3). Bar represents SEM.

Scheme 1. Synthesis of *1H*-indole-2-carboxamide and *1H*-pyrrole-2-carboxamide derivatives

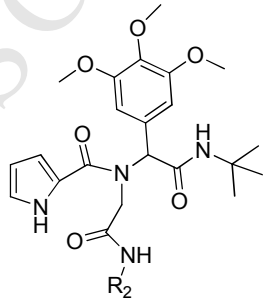




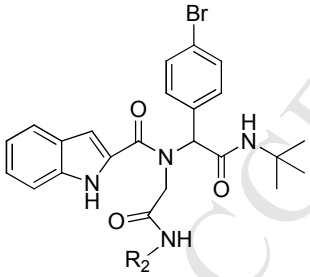
2a, R_2 = benzyl
2b, R_2= 4-methoxybenzyl
2c, R_2 = 4-chlorobenzyl
2d, R_2 = 3,4-dichlorobenzyl
2i, R_2 = ethanol
2j, R_2 = 3-propyl morpholine
2k, R_2 = 4-methyl pyridine
2l, R_2 = 3-methoxybenzyl



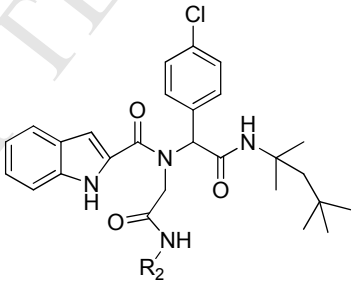
2e, R_2 = benzyl
2f, R_2 = 4-methoxybenzyl
2g, R_2 = 3,4-dichlorobenzyl



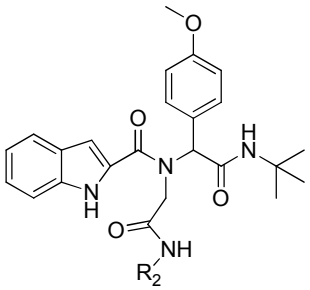
2h, R_2= 4-methoxybenzyl



2m, R_2 = 3-propyl morpholine
2n, R_2= 4-methoxybenzyl



2o, R_2= 4-methoxybenzyl



2p, R_2= 4-methoxybenzyl
2q, R_2 = 4-chlorobenzyl
2r, R_2 = 4-methyl pyridine

Reagents and conditions: (a) Et_3N, MeOH, rt, 1-2 h; (b) R_2NH_2, EtOH, reflux, 4-8 h.

Research Highlights:

Identification of a diverse Indole-2-carboxamides as a potent antileishmanial chemotypes

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- A series of highly diverse indole-2-carboxamides were synthesized by IMCR-post modification approach.
- Most of the compounds exhibited excellent antileishmanial activity against amastigotes form.
- The lead compound **2b** was found 7-fold more active than miltefosine against amastigotes *in vitro*.
- Analogs **2b**, **2m** and **2p** showed significant *in vivo* inhibition against *L. donovani* in hamster model.