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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · SEPTEMBER 2013

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European Journal of Medicinal Chemistry 69 (2013) 527–536

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Original article

Design, synthesis and biological evaluation of 2-substituted quinolines as potential antileishmanial agents

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ARTICLE INFO

Article history:
Received 10 June 2013
Received in revised form
19 August 2013
Accepted 25 August 2013
Available online 15 September 2013

Keywords:
2-Substituted quinolines
Antileishmanial activity
Luciferase assay
Liver microsomes
Metabolic stability

ABSTRACT

An analogous library of 2-substituted quinoline compounds was synthesized with the aim to identify a potential drug candidate to treat visceral leishmaniasis. These molecules were tested for their *in vitro* and *in vivo* biological activity against *Leishmania donovani*. Metabolic stability of these compounds was also improved through the introduction of halogen substituents. Compound (**26g**), found to be the most active; exhibited an IC_{50} value of 0.2 μM and >180 fold selectivity. The hydrochloride salt of (**26g**) showed 84.26 ± 4.44 percent inhibition at 50 mg/kg × 5 days (twice daily, oral route) dose in *L. donovani*/hamster model. The efficacy was well correlated with the PK data observed which indicating that the compound is well distributed.

1. Introduction

In 1903, Leishman and Donovan separately described a protozoan parasite found in the splenic tissue of patients in India. Their simultaneous discovery of the protozoan, now called *Leishmania donovani*, first alerted the scientific community to the life-threatening disease of Visceral Leishmaniasis (VL) [1]. VL is a parasitic disease spread by the bite of an infected female phlebotomine sand fly [2]. It is a geographically widespread prevalent disease in many parts of the tropical and subtropical world causing significant morbidity or mortality [3]. This disease is a severe public health problem in many developing countries of East Africa, the Indian subcontinent and Latin America. According to the World Health Organization (WHO), the pathogen is endemic in 88 countries and the magnitude of the disease is estimated to be 12 million infected people with 350 million people at risk. The rate of new cases per annum is estimated to be 2 million worldwide [4]. Many names correspond to this group of diseases: kala-azar, dum-dum fever, white leprosy, espundia or pian bois [5].

In recent years, new compounds with antileishmanial activity have been developed. Pentavalent antimonials, although first introduced 70 years ago, remain the first-line treatment in numerous countries [6]. Amphotericin B [7], originally identified as a systemic polyene antifungal, is currently used as an efficient second-line antileishmanial and its most recent liposomal formulation (Ambisome®) tends to become a first line treatment despite its high cost. The inclusion of miltefosine (Impavido®) [8] into the therapeutic armamentarium of VL is a landmark event for the therapy of VL, as it is the only oral treatment available to date. Pentamidine [9] (e.g. PentamR), a diamidine, has been used in the treatment of antimonial-resistant VL. However, treatment with these existing drugs suffers from several limitations such as cost, toxicity, parenteral administration, emergence and spread of drug resistance, and relapses in HIV–Leishmania co-infected patients. A low cost injectable version of paromomycin [10] has been registered in India for VL treatment and this drug is further evaluated both as mono and combination therapies. Sitamaquine (**1**) [11]

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http://dx.doi.org/10.1016/j.ejmech.2013.08.028

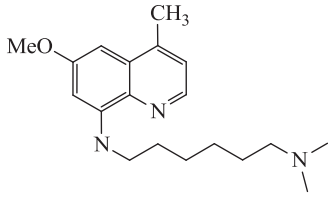


Fig. 1. Sitamaquine (**1**).

Fig. 1 (WR6026), an 8-aminoquinoline derivative, has been shown to have antileishmanial activity in Phase II studies, but confirmation of such activity during Phase III studies is still lacking. Allopurinol [12] and rifampicin [13] showed activity in experimental systems, but proved disappointing in clinical trials. Various other compounds, such as atovaquone [14], licochalcone A [15], Ilmofosine [16], formycin B [17] or Camptothecin [18] have been reported to inhibit *L. donovani* infection but never reached to the clinical stage. Therefore, there is still a crying need for new efficacious and safe drugs in the absence of an upcoming vaccine.

Chimanines, structurally simple 2-substituted quinolines, have been reported by Fournet et al. [19] in the early 90s and conducted ethno-pharmacological studies in South America and have discovered that alkaloids of the chemical family of quinolines had *in vitro* and *in vivo* antiparasitic properties. However, these molecules did not bear all the needed features of a drug-like entity, mainly because of their weak *in vitro* potency and metabolic instability (Tables 1 and 2). Two of these parent compounds identified by Alain Fournet [19] were selected as reference compounds: 2-n-propyl-quinoline (**2**) and 3-(quinolin-2-yl) prop-2-en-1-ol (**14e**). A library of substituted quinolines was thus prepared and tested *in vitro* with the aim of addressing these liabilities. The most potent compound (**26g**) was selected for further *in vivo* trials.

All compounds were tested *in vitro* against the intracellular form of *L. donovani* at CSIR-CDRI, and for druggability at Advinus Therapeutics.

2. Chemistry

The parent compounds 2-n-propyl-quinoline (**2**) and 3-(quinolin-2-yl) prop-2-en-1-ol (**14e**) were prepared by the method reported by Fakhfakh et al. [20]. Quinoline (**3**) was converted to quinoline *N*-oxide (**4**) using m-chloroperbenzoic acid, followed by bromination to 2-bromoquinoline (**5**) which on reaction with propargyl alcohol in presence of tetrakis (triphenylphosphine) palladium(0) catalyst and DIPEA as base affords 3-(quinolin-2-yl) prop-2-yn-1-ol (**6**) in good yield as shown in Scheme 1.

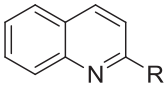
Different synthesis strategies were developed depending upon the position of the substitution on the quinoline ring which was targeted.

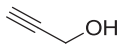
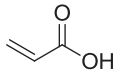
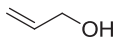
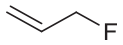
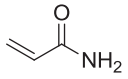
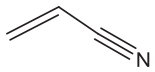
A series of di- and tri-substituted quinolines **14**(**a**–**e**) and **22** were synthesized starting from substituted anilines **7**(**a**–**e**) as depicted in Scheme 2. 4-hydroxy substituted quinolines [21] **8**(**a**–**e**) and **9**(**a**–**e**) were prepared starting from substituted anilines, ethylacetoacetate and polyphosphoric acid, which resulted in two regio isomers. The mixture of isomers, when treated with phosphorous tribromide gave 4-bromo quinoline derivatives **10**(**a**–**e**). The isomers were separated then by column chromatography and were used for next steps. Debromination of **10**(**a**–**e**) by using *n*-butyl lithium and quenching with water resulted in compounds **11**(**a**–**e**).

Appropriate aldehydes **12**(**a**–**e**) were obtained from **11**(**a**–**e**) by oxidation of methyl group with selenium dioxide in dioxane as shown in Scheme 2. Knoevenagal condensation followed by *in situ* decarboxylation of aldehydes with malonic acid gave propenoic acid derivatives **13**(**a**–**e**). The propenoic acids were reduced with ethylchloroformate and sodium borohydride gave **14**(**a**–**e**). 2-substituted quinoline acid (**13e**) was converted to corresponding amide (**16e**) and then to nitrile (**17e**) as per Scheme 2. Further 3-(quinolin-2-yl) prop-2-enoic acid (**13e**) was converted to 3-fluoroprop-1-en-1-yl] quinoline (**15e**) by the reaction of compound (**14e**) with diethylaminosulfur trifluoride (DAST). Similarly, compound (**22**) was obtained starting from compound (**9c**) as per Scheme 3.

Tetra-substituted quinoline derivatives were prepared from compound **10**(**a**,**c**). The substitution at C4 position on quinoline ring of compound **10**(**a**,**c**) was achieved by Suzuki coupling with appropriate aromatic boronic acids for aromatic substitution, whereas the cycloalkyl substitution was achieved by heating the appropriate amines with cesium carbonate using dimethylformamide as solvent.

Table 1
Activity and cytotoxicity data for 2-substituted quinoline derivatives.



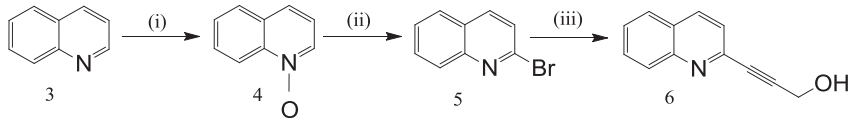
Compounds	R	IC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
2		>100	125.58 ± 10.2	<1.25
6		10.69 ± 1.4	26.21 ± 3.1	2.45
13e		50.2 ± 3.2	>400	>7.96
14e		10.04 ± 1.5	31.85 ± 5.2	3.17
15e		6.68 ± 1.1	38.56 ± 4.8	5.77
16e		20.41 ± 3.1	76.33 ± 5.4	3.74
17e		28.59 ± 3.5	154.61 ± 14.6	5.41
Miltefosine[d]		8.4 ± 0.7	52.74 ± 5.3	6.27

[a] IC_{50} (50% inhibitory concentration) and.
[b] CC_{50} (50% cytotoxic concentration) values (mean ± S.D.) are the average of two independent experiments.
[c] SI, selectivity index (ratio of CC_{50}/IC_{50}).
[d] Miltefosine was used as a reference drug.

Table 2
Metabolic stability[a] of 2-substituted quinoline derivatives.

Compounds	Solubility (mg/mL)	% Stability in HLM[b]	% Stability in MLM[c]
2	>100	98	100
6	>100	22	99
13e	ND	ND	ND
14e	>100	96	100
15e	>1	100	100
16e	>100	44	97
17e	>100	100	100

[a] Expressed as percentage of compound metabolized after 30 min of incubation.
[b] HLM, human liver microsome.
[c] MLM, mouse liver microsome.



Scheme 1. Synthesis of 3-(quinolin-2-yl) prop-2-yn-1-ol. **Reaction and conditions**: (i) *m*-Chloroperbenzoic acid, dichloromethane (ii) $POBr_3$/DCM (iii) Propargyl alcohol/Pd $[P(C_6H_5)_3]_4$/DIPEA, 70–75 °C.

The compounds were further converted to the final compounds **26**(**a**–**l**) as per Scheme 4.

Methoxy derivative **28**(**g**) was synthesized by reacting **25**(**g**) with sodium methoxide to obtain **27**(**g**) which on reaction with ethylchloroformate and sodium borohydride gave **28**(**g**) with a satisfactory yield (around 60%) as per Scheme 5.

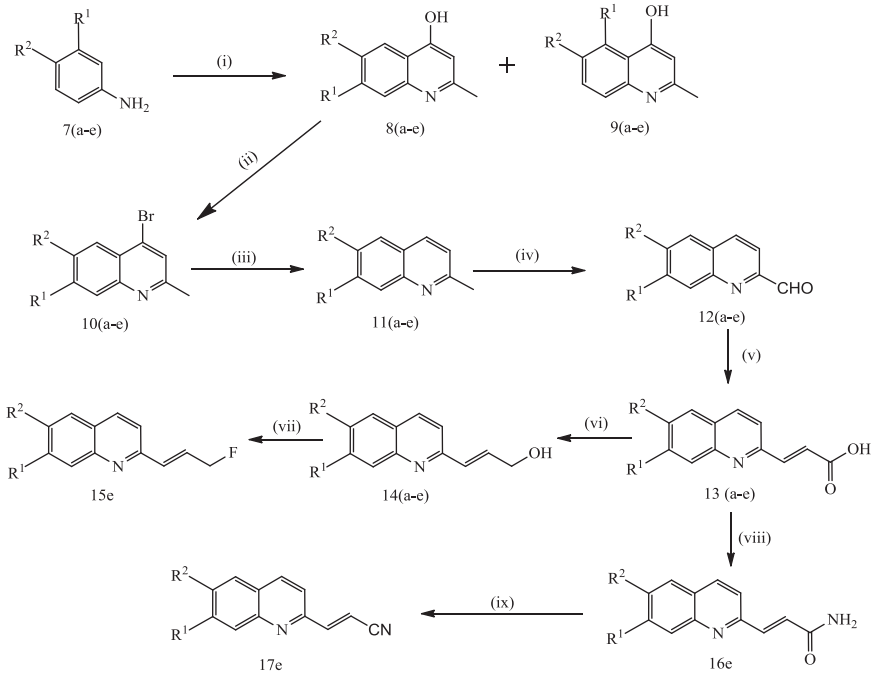
3. Results and discussion

3.1. In vitro activity profile and pharmacology

To identify new and potent antileishmanial agents, the simple quinoline derivatives were initially screened against the *L. donovani* intracellular amastigotes using the luciferase assay. The most active compounds, 2-*n*-propylquinoline (**2**) and 3-(quinolin-2-yl) prop-2-en-1-ol (**14e**) were resynthesized and tested in a murine model of visceral leishmaniasis.

The initial modifications on the quinoline ring at C2 position, as shown in Table 1 were aimed at finding the best side chain. In preliminary studies, prop-2-yn-1-ol (**6**), prop-2-enoic acid (**13e**), prop-2-enfluoride (**15e**), prop-2-enamide (**16e**), and prop-2-enenitrile (**17e**) derivatives were synthesized. Compound bearing an amide substituent (**16e**) had an IC_{50} value of 20.41 μM and was extensively metabolized in mouse liver microsomes (% metabolism in 30 min: Human Liver Microsome (HLM) = 44, Mouse Liver Microsome (MLM) = 97). The nitrile derivative (**17e**) had an IC_{50} value of 28.59 μM and was even more prone to extensive metabolism (% metabolism in 30 min: HLM = 100, MLM = 100). The Prop-2-yn-1-ol (**6**) compound with a propynyl alcohol side chain had an IC_{50} value of 10.69 μM (% metabolism in 30 min: HLM = 22, MLM = 99). The best active compound was (**15e**) with an IC_{50} value of 6.68 μM but it was quickly and completely metabolized (% metabolism in 30 min: HLM = 100, MLM = 100). Furthermore, it exhibited very poor solubility (>1 mg/mL). The next most active compound was the prop-2-en-1-ol (**14e**) which exhibited an IC_{50} value of 10.04 μM, but it showed poor metabolic stability (% metabolism in 30 min: HLM = 96, MLM = 100) (Tables 1 and 2). We further modified this latter compound to further increase potency and improve metabolic stability by introducing halogens, amines and aromatic rings at different positions on the quinoline ring.

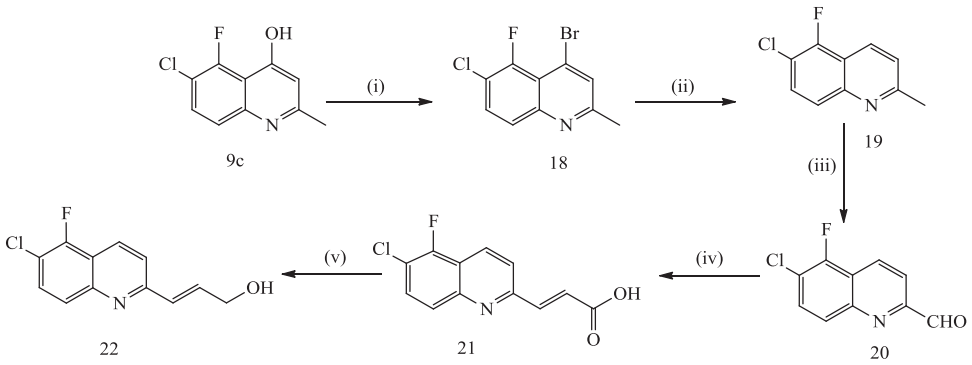
The second modification was aimed at introducing chloro, fluoro, and methoxy substitution on the ring (Tables 3 and 4). Introduction of methoxy group at C6 position of the compound (**14e**) resulted in compound (**14d**) which showed lower activity. A similar behavior was observed with 6-fluoro derivative compound (**14b**). However, substitution of chloro at C6-position of compound (**14e**) resulted in compound (**14a**) which showed improved activity and reduced cellular toxicity but it was completely metabolized in



7a, 8a, 9a, 10a, 11a, 12a, 13a, 14a: R_1=H, R_2=Cl;
7c, 8c, 9c, 10c, 11c, 12c, 13c, 14c: R_1=F, R_2=Cl;
7e, 8e, 9e, 10e, 11e, 12e, 13e, 15e, 14e, 15e, 16e, 17e: R_1=H, R_2=H.

7b, 8b, 9b, 10b, 11b, 12b, 13b, 14b:R_1=H, R_2=F;
7d, 8d, 9d, 10d, 11d, 12d, 13d, 14d: R_1=H, R_2=OCH_3;

Scheme 2. Synthesis of substituted quinoline derivatives. **Reagents and conditions**: (i) Polyphosphoric acid, Ethylacetoacetate, 120 °C, 16 h (ii) PBr_3, DMF, 3h; (iii) *n*-BuLi/THF, Water (iv) SeO_2, 1,4-dioxane, 70 °C (v) Malonic acid, pyridine, 1 h (vi) Ethylchloroformate, THF, TEA, $NaBH_4$ (vii) DAST/dichloromethane (viii) Ethylchloroformate/Aq. Ammonia (ix) $POCl_3$/110 °C/16 h.



Scheme 3. Synthesis of tri-substituted (quinolin-2-yl) prop-2-en-1-ols. **Reagents and conditions**: (i) Polyphosphoric acid, Ethylacetoacetate, 120 °C, 16 h (ii) PBr_3, DMF, 3h; (iii) *n*-BuLi/THF, Water (iv) SeO_2, 1,4-dioxane, 70 °C (iv) Malonic acid, pyridine, 1 h (v) Ethylchloroformate, THF, TEA, $NaBH_4$.

mouse liver microsomes (% metabolism in 30 min: HLM = 87, MLM = 100). Di-substituted compounds (**14c**) and (**22**) were synthesized by keeping chloro group intact at C6. Between compound (**14c**) having fluoro at C7 and compound (**22**) having fluoro C5, it was observed that the latter fluoro group at C7 position has lower cytotoxicity and significantly improved metabolic stability (% metabolism in 30 min: HLM = 50, MLM = 60). On these bases (chloro at C6 position and fluoro at C7 position) further optimization was performed by adding substituents at C4 position on quinoline ring.

Starting from compound (**14c**), several aryl groups were introduced at C4 position in the quinoline ring. The most active from this series were (**26a**–**d**), (**26f**), (**26k**) and (**26l**). The compound with an *O*-aryl group at C4 position (**26h**) gave better efficacy (1.96 μM) than (**14c**). This indicated that substitution at C4 position was a key feature to obtain high *in vitro* potency.

Quinoline propenols provided improvement in metabolic stability compared to the initial compound (**14e**). Insertion of morpholine at C4 resulted in improved potency as well as selectivity as shown by compound (**26a**). Insertion of a fluoro substituent at C7 of compound (**26a**) resulted in dramatic improvement in potency and metabolic stability (compound **26g**) (Table 5 and Table 6).

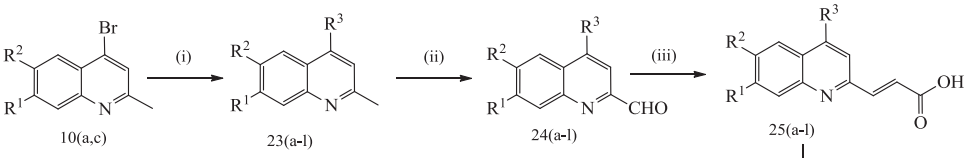
In general, these set of modifications resulted in improved metabolic stability in human and mouse liver microsomes but not in hamster liver microsomes (HamLM). Compound (**26g**) with **3-(6-Chloro-7-fluoro-4-morpholino) quinoline prop-2-en-1-ol** was the most potent with an IC_{50} value of 0.22 μM and 187 fold selectivity (% metabolism in 30 min: HLM = 35, MLM = 45) (Table 6). Hence this compound was selected for detailed *in vivo* evaluation and pharmacokinetics studies.

3.2. In vivo activity profile

Results are presented in Table 7. Based on the *in vitro* screening profile of >100 compounds, three compounds (**14e**, **26g**, **26k**) were identified for determining the *in vivo* response in the golden hamster model. These compounds were tested at 50 mg/kg × 5 days dose by the IP route. One compound, (**26g**) has shown some anti leishmanial activity (percent inhibition of parasite growth = 25.37 ± 19.14). To improve solubility of this compound, the hydrochloride salt was prepared and evaluated both by IP and PO routes. This hydrochloride salt of compound (**26g**) showed significant activity at 50 mg/kg (twice daily) dose after oral administration (percentage inhibition (PI) recorded at day 7 post-treatment was 84.26 ± 4.44). When animals were treated at the same dose regimen via IP route, a slightly lower activity was observed (77.16 ± 5.23 PI). Three more analogs of (**26g**) were tested (data not shown) at 50 mg/kg (twice daily) × 5 days, following PO administration, but none of them showed any promising activity.

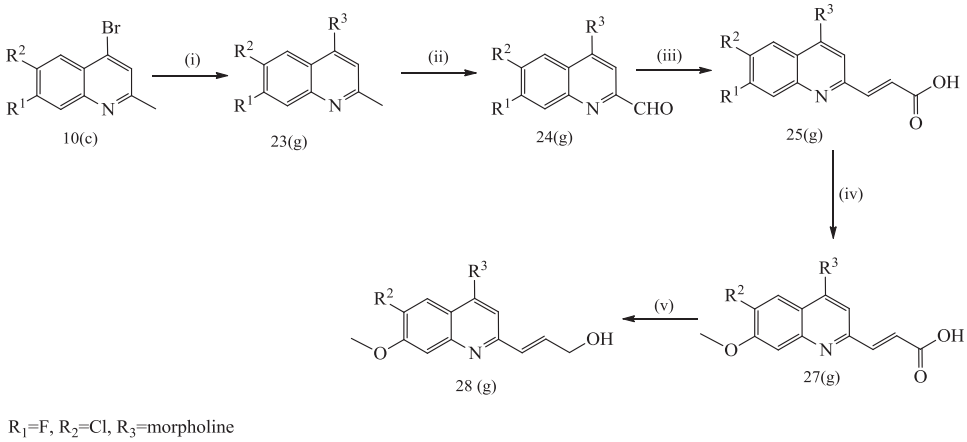
3.3. Pharmacokinetic study profile of compound (*26g*)

The pharmacokinetics results are presented in Table 8. Compound (**26g**) showed an extremely high plasma clearance (865 mL/min/kg, which is 10-fold higher than liver blood flow of 90 mL/min/kg). Its volume of distribution was high (7.5 L/kg, which was 11-fold



10a: R_1=H, R_2=Cl; 10c: R_1=F, R_2=Cl;
23a, 24a, 25a, 26a: R_1=H, R_2=Cl, R_3=morpholine;
23b, 24b, 25b, 26b:R_1=F, R_2=Cl, R_3=4-fluorophenyl;
23c, 24c, 25c, 26c: R_1=H, R_2=Cl, R_3=4-methoxyphenyl;
23d, 24d, 25d, 26d: R_1=H, R_2=Cl, R_3=4-fluorophenyl;
23e, 24e, 25e, 26e: R_1=F, R_2=Cl, R_3=*N*-methylpiperazine;
23f, 24f, 25f, 26f: R_1=F, R_2=Cl, R_3=2,6-dimethyl morpholine;
23g, 24g, 25g, 26g: R_1=F, R_2=Cl, R_3=morpholine;
23h, 24h, 25h, 26h:R_1=H, R_2=Cl, R_3=4-fluorophenoxy;
23i, 24i, 25i, 26i:R_1=F, R_2=Cl, R_3=*N*,*N*-dimethylamine;
23j, 24j, 25j, 26j:R_1=F, R_2=Cl, R_3=piperidine;
23k, 24k, 25k, 26k: R_1=F, R_2=Cl, R_3=4-methoxyphenyl;
23l, 24l, 25l, 26l:R_1=F, R_2=Cl, R_3=3-fluorophenyl.

Scheme 4. Synthesis of tetra substituted (quinolin-2-yl) prop-2-en-1-ols. **Reagents and conditions**: (i) Pd $(PPh_3)_4$, Aryl boronic acid, THF/water, K_2CO_3. 70 °C, 16 h or R_2NH/Cs_2CO_3/DMF, 4–16 h. (ii) SeO_2, 1,4-dioxane, 70 °C (iii) Malonic acid, pyridine, 1 h (iv) Ethylchloroformate, THF, TEA, $NaBH_4$, 0 °C.



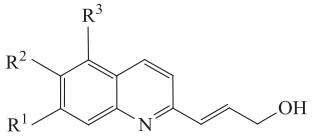
Scheme 5. Synthesis of tetra substituted methoxy (quinolin-2-yl) prop-2-en-1-ols. **Reagents and conditions**: (i) $Pd(PPh_3)_4$, Aryl boronic acid, THF/water, K_2CO_3. 70 °C, 16 h or R_3-NH/Cs_2CO_3/DMF, 4–16 h. (ii) SeO_2, 1,4-dioxane, 70 °C (iii) Malonic acid, pyridine, 1 h (iv) NaOMe, DMSO, 0 °C (v) Ethylchloroformate, THF, TEA, $NaBH_4$, 0 °C.

higher than total body water of 0.7 L/kg) indicating that the compound is well distributed. Its short intravenous elimination half-life of about 0.5 h was driven primarily by the abnormally high clearance. The high clearance value, in relation to liver plasma flow, suggested that the liver was not the only site of elimination. This was corroborated by direct *in vitro* experiments showing that the compound was largely stable in mouse liver microsomes (Table 6). *In vitro* whole blood and plasma stability experiments showed that compound (**26g**) was stable in plasma but unstable in whole blood. This could be due to metabolism with some cellular component of the whole blood. The oral solution bioavailability was found to be very low (~1%) (Fig. 2).

4. Conclusion

Overall, a library of new compounds were synthesized and assessed for *in vitro* antileishmanial activity and most of them were also evaluated for their metabolic stability. A significant number of compounds exhibited potent *in vitro* activity against *L. donovani*, as well as a high selectivity index (i.e., activity with low cytotoxicity). As a reminder, the parent compound (*n*-propyl-quinoline) had an IC_{50} value of about 40 μM whereas several new compounds were in the range of 0.2–2 μM with improved metabolic stability in liver microsomes. The compound (**26g**) was found most promising one, when evaluated *in vitro* in mouse macrophage cell line and *in vivo* in golden hamster/*L. donovani* model. This compound (**26g**) was further evaluated in a range of early DMPK and pharmacology assays. These results clearly state that the lead compound (**26g**) with morpholine at C4 position, chloro at C6 position and fluoro at C7 position showed very promising activity and metabolic stability compared to parent compound (**14e**), thus provide a new structural lead to develop an orally active antileishmanial agent.

Table 3
Activity and cytotoxicity data for di and tri substituted quinoline propenols.



Compounds	R^1	R^2	R^3	IC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
14a	H	Cl	H	0.86 ± 0.1	28.89 ± 2.1	33.59
14b	H	F	H	17.9 ± 1.4	51.21 ± 4.2	2.86
14c	F	Cl	H	14.35 ± 2.1	79.56 ± 9.4	5.54
14d	H	OMe	H	32.38 ± 2.3	45.85 ± 4.2	1.42
14e	H	H	H	10.04 ± 1.5	31.85 ± 5.2	3.17
22	H	Cl	F	4.1 ± 1.0	19.54 ± 2.9	4.76
Miltefosine[d]				8.4 ± 0.7	52.74 ± 5.3	6.27

[a] IC_{50} (50% inhibitory concentration) and.
[b] CC_{50} (50% cytotoxic concentration) values (mean ± S.D.) are the average of two independent experiments.
[c] SI, Selectivity index (ratio of CC_{50}/IC_{50}).
[d] Miltefosine was used as a reference drug.

5. Experimental section

5.1. Chemistry

5.1.1. General

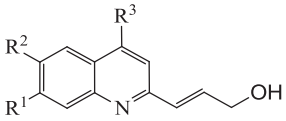
All the reagents and solvents were used as received. Starting materials were commercially available. Reactions were performed under anhydrous conditions unless noted otherwise. Reactions were followed by TLC analysis on Merck TLC aluminum sheets with silica gel 60 F254. The purities of compounds for biological testing were assessed by analytical HPLC. LC/MS analysis was performed on an Agilent 1200 MSD system with an Inertsil ODS-3V (250 × 4.6 mm, 5 μm particle size). Chromatograms for electrospray ionization (ESI) positive and negative base peak intensity and a UV total absorption chromatogram from 220 to 300 nm were generated, and values for *m*/*z* are given; generally, only ions that indicate the parent mass are reported, and unless otherwise stated, the value quoted is $(M + H)^+$ for positive-ion mode and $(M - H)^-$ for negative-ion mode. Melting points were measured on a Buchi-546 B and were uncorrected. Preparative HPLC (Agilent 1200 series) was performed on an Inertsil ODS-3V column (250 × 21 mm, 5 μm particle size) using a gradient

Table 4
Metabolic stability[a] data for di and tri substituted quinoline propenols.

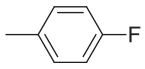
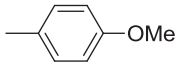
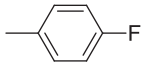
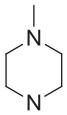
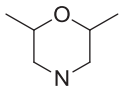
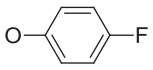
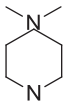
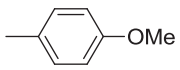
Compounds	% Stability in HLM[b]	% Stability in MLM[c]
14a	87	100
14b	76	100
14c	50	60
14d	ND	ND
14e	96	100
22	ND	ND

[a] Expressed as percentage of compound metabolized after 30 min of incubation.
[b] HLM, human liver microsome.
[c] MLM, mouse liver microsome.

Table 5
Activity and cytotoxicity data for tetra substituted quinoline propenols.



26(a-l), 28g

Compounds	R^1	R^2	R^3	IC_{50}[a] (μM)	CC_{50}[b] (μM)	SI[c]
26a	H	Cl		4.4 ± 0.9	46.89 ± 9.5	10.65
26b	F	Cl		1.43 ± 0.4	24.58 ± 1.2	7.92
26c	H	Cl		0.22 ± 0.7	37.12 ± 3.9	168.72
26d	H	Cl		1.57 ± 0.9	32.52 ± 2.8	20.71
26e	F	Cl		5.88 ± 1.2	44.57 ± 6.4	7.58
26f	F	Cl		4.75 ± 1.0	41.29 ± 4.8	8.97
26g	F	Cl		0.22 ± 0.06	41.25 ± 4.2	187.5
26h	H	Cl		1.96 ± 0.7	35.42 ± 4.7	18.07
26i	F	Cl		23.04 ± 1.7	41.58 ± 4.9	1.8
26j	F	Cl		2.84 ± 0.8	22.34 ± 3.5	7.86
26k	F	Cl		1.20 ± 0.2	26.75 ± 2.3	22.26
26l	F	Cl		1.79 ± 0.9	21.63 ± 4.6	12.08
28g	OCH_3	Cl		3.72 ± 0.9	51.78 ± 3.2	13.92
Miltefosine[d]				8.4 ± 0.7	52.74 ± 5.3	6.27

[a] IC_{50} (50% inhibitory concentration) and.
[b] CC_{50} (50% cytotoxic concentration) values (mean ± S.D.) are the average of two independent experiments.
[c] SI, selectivity index (ratio of CC_{50}/IC_{50}).
[d] Miltefosine was used as a reference drug.

of 10–90% acetonitrile/10 mm ammonium acetate or 0.1% TFA over 20 min at a flow rate 20 mL/min. Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a 400 MHz (VARIAN AVANCE 400 AS) spectrometer. Chemical shift data for the proton and carbon resonances were reported in parts per million (δ) relative to internal standards $(CH_3)_4$ Si (δ 0.0). All coupling constants (J) are given in Hz. The purities of the final compounds were determined with Agilent 1200 HPLC system.

Table 6
Metabolic stability[a] of tetra substituted quinoline propenols.

Compounds	Solubility (mg/mL)	% Stability in HLM[b]	% Stability in MLM[c]	% Stability in HamLM[d]
26a	>100	43	56	88
26b	<10	25	51	53
26c	<10	8	34	ND
26d	10	25	83	ND
26e	>100	27	27	89
26f	60	17	50	99
26g	>100	35	45	66
26h	<10	27	33	94
26i	ND	ND	ND	ND
26j	<10	39	81	100
26k	<10	14	30	23
26l	<10	25	40	59
28g	60	31	44	86

[a] Expressed as percentage of compound metabolized after 30 min of incubation.
[b] HLM, human liver microsome.
[c] MLM, mouse liver microsome.
[d] HamML, hamster liver microsome.

5.1.2. General procedure for synthesis of substituted quinoline hydroxy ***8***(***a***–***e***) and ***9***(***a***–***e***)

Substituted anilines **7**(**a**–**e**) (1 mmol) and PPA (6 mL) was heated to 80 °C, ethylacetoacetate (1.2 mmol) was added drop wise for 45 min. and heated to 120 °C for 16 h. Completion of the reaction was monitored by TLC. The RM was poured over crushed ice and neutralized with aqueous ammonia (25%); precipitated solid was filtered, washed with diethyl ether and dried. Product obtained was taken directly to next step without purification.

5.1.3. General procedure for synthesis of substituted quinoline bromo ***10***(***a***–***e***)

Phosphorous tribromide (1.5 mmol) was added drop wise to a solution of compounds **8**(**a**–**e**) (1 mmol) in DMF (10 mL) at 0 °C. After complete addition, the reaction mass to allow to room temperature and stirred for 3 h. After completion of the reaction, transferred the reaction mass to crushed ice and neutralized with aqueous ammonia. The precipitated solid obtained was filtered, and dried. The pure compounds were isolated by column chromatography using silica gel 230–400, eluting with 10% ethyl acetate in hexane.

5.1.4. General procedure for synthesis of substituted quinoline ***11***(***a***–***e***)

n-Butyl lithium (2.3 M, 1.2 mmol) was added to the solution of compounds **10**(**a**–**e**) (1 mmol) in dry THF at −78 °C and stirred for 30 min. Reaction was quenched with aqueous NH_4Cl solution (5 mL) and warmed to 0 °C. Ice cold water was added to the reaction mixture and extracted with ethyl acetate. The organic layer was washed with water, brine solution and dried over sodium sulfate, concentrated under reduced pressure. The crude product was triturated with 20% diethyl ether in hexane to afford the title compounds.

Table 7
In vivo efficacy of 2-substituted quinoline derivatives.

Compounds	Dose (mg/kg × days); route	Average percent inhibition ± SD[c] (n = No. of animals)	Remarks
14e	50 × 5; Once IP[a]	NI[d] ($n = 6$)	Inactive
26g	50 × 5; Once IP	25.37 ± 19.14 ($n = 6$)	Marginal activity
26g	50 × 5; Twice IP	77.16 ± 5.23 ($n = 5$)	Active
Hydrochloride salt	50 × 5; Twice PO[b]	84.26 ± 4.44 ($n = 5$)	Active
26k	50 × 5; Twice PO	NI ($n = 5$)	Inactive
Miltefosine[e]	30 × 5, Once PO	96.55 ± 1.05 ($n = 5$)	Standard antileishmanial

[a] SD = standard deviation.
[b] IP, intra peritoneal.
[c] PO, per oral.
[d] NI = no inhibition.
[e] Miltefosine was used as a reference drug.

5.1.5. General procedure for the synthesis of 4-(substituted) -2-methyl-substituted quinoline derivatives ***23***(***b***,***c***,***d***,***k***,***l***)

To a solution of **10**(**a**,**c**) (1 mmol) in THF and water (3:1), aryl boronic acid (1.2 mmol), and K_2CO_3 (1.5 mmol) was added under nitrogen atmosphere. Tetrakis triphenyl phosphine palladium (0) (0.05 mmol) was added and heated to 65 °C for 16 h. After completion of the reaction, organic layer separated from the aqueous layer. The aqueous layer was extracted with EtOAc. The combined organic layer was dried over sodium sulfate, evaporated and purified by column chromatography, eluting with ethyl acetate in hexane.

5.1.6. General procedure for the synthesis of 4-(substituted)-2-methyl-substituted quinoline derivatives ***23***(***a***,***e***,***f***,***g***,***h***,***i***,***j***)

To the solution of **10**(**a**,**c**) (1 mmol) in DMF (10 mL), was added cesium carbonate (1.5 mmol) and sec-amine (1.5 mmol). The reaction mass was heated to 100 °C for 4 h. After completion of reaction, transferred to cold water (100 mL) and extracted with ethyl acetate. The organic layer was dried over sodium sulfate, evaporated and purified by column chromatography, eluting with ethyl acetate: hexane.

5.1.7. General procedure for synthesis of substituted quinoline aldehydes ***12***(***a***–***e***) and ***24***(***a***–***l***)

A suspension of compound **11**(**a**–**e**) and **23**(**a**–**l**) (1 mmol) in 1,4-dioxane (10 mL) and SeO_2 (2 mmol) was heated to 80 °C for 2 h. After completion of the reaction, the inorganic compounds were filtered and poured in to ice water. The precipitated solid was then filtered and dried. The crude product was purified by column chromatography eluting with ethyl acetate: hexane mixture. % Yield = 55–80%.

5.1.8. General procedure for synthesis of substituted quinoline acids ***13***(***a***–***e***) and ***25***(***a***–***l***)

To a solution of compound **12**(**a**–**e**) and **24**(**a**–**l**) (1 mmol) in pyridine (10 mL), malonic acid (1.5 mmol) was added and heated to 70 °C for 2 h. After completion of the reaction, the solution was poured into ice water and acidified with hydrochloric acid to pH ~ 4. The precipitated solid was filtered and dried to get the compounds **13**(**a**–**e**) and **25**(**a**–**l**).

5.1.9. General procedure for synthesis of quinoline propenols ***14***(***a***–***e***) and ***26***(***a***–***l***)

To the solution of compound **13**(**a**–**e**) and **25**(**a**–**l**) (1 mmol) in THF (10 mL), triethyl amine (3 mmol), and ethylchloroformate (2 mmol) was added at 0 °C and stirred for 15 min. After completion of the reaction, the solid was filtered and washed with THF (20 mL). The filtrate was cooled to 5 °C and sodium borohydride solution (3 mmol) was added drop wise. After completion of the reaction, the organic layer was separated. The aqueous layer was extracted with ethyl acetate (10 mL × 2). All the organic layers were combined and dried over sodium sulfate. The crude product was obtained after evaporation and then purified by column chromatography using ethyl acetate: hexane (10–20%) as eluent.

5.1.10. General procedure for the synthesis of quinoline chalcone derivatives ***29***(***a***–***g***)

To a solution of compound **24**(**a**–**d**) (1 mmol) in acetic acid (2 mL), ketone (1 mmol) and sulfuric acid (0.2 mL) was added and

Table 8
Pharmacokinetics parameters of compound (**26g**) in male Swiss Albino mice following intravenous and per oral administration.

Compound	Dose/route	T_{max} (h)	C_{max}[a] (ng/mL)	AUC_{last}[b] (ng h/mL)	AUC_{inf} (ng h/mL)	$T_{1/2}$ (h)	Plasma clearance (mL/min/kg)	Vss[c] (L/kg)	%F[d]
26g	10 mg/kg (IV)	0.08	2225.16	192.59	192.64	0.47	865.18	7.54	–
	50 mg/kg (PO)	0.25	19.67	6.88	–	–	–	–	0.7

[a] Back-extrapolated concentration.
[b] AUC, area under the concentration time curve.
[c] Vss, volume of distribution at steady state.
[d] Bioavailability calculated using AUC_{last}.

heated to 60 °C for 2 h. After completion of the reaction, the mixture was cooled to RT, neutralized with $NaHCO_3$ solution and extracted with ethyl acetate (25 mL × 3). The organic layer was washed with water (25 mL) and saturated brine solution (25 mL), and then dried over sodium sulfate and evaporated. The crude product was purified by column chromatography using basic alumina as stationary phase and eluting with EtOAc: hexane as eluent.

5.2. Biology

5.2.1. Parasite

The WHO reference strain of *L. donovani* (MHOM/IN/80/Dd8) obtained from Imperial College, London (UK), was maintained as promastigotes *in vitro* and as amastigotes in golden hamsters. Promastigotes were cultivated in medium 199 (Sigma–Aldrich) supplemented with 0.1% gentamycin (Biovaccines Private Ltd., Chevella, India) and 10% Fetal Calf Serum [22].

5.2.2. Animals

For *in vivo* antileishmanial study (at CSIR-CDRI, Lucknow, India), healthy inbred hamsters weighing 40–45 g (8–10 week-old) of both sexes were used. Throughout the study, animals were housed in controlled animal quarters ((23 ± 2 °C; Relative humidity: 60%) with 12 h light–dark cycles). For Pharmacokinetic Studies (in Advinus Therapeutics, Bangalore, India), Male Swiss Albino mice, 8–12 weeks old, 30–40 g weight range employing a sparse sampling design were used. Animals were fasted 4 h before dose administration and feed was provided 4 h post dose. Animals were fed standard rodent pellet and had free access to drinking water. These studies were performed with the approval from the Institutional Animal Ethics Committee (IAEC) in respective places accordance with the requirement of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

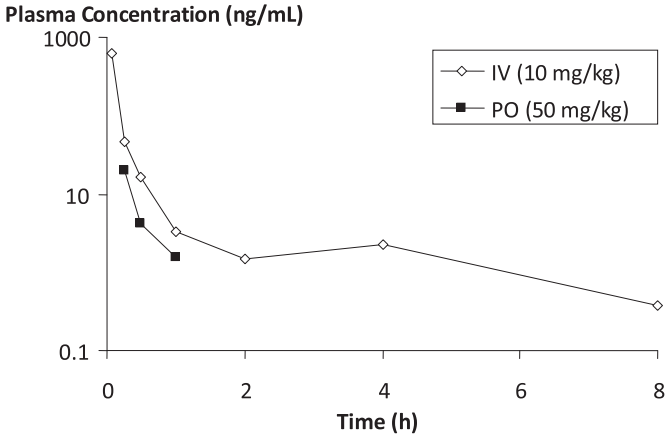


Fig. 2. Mean plasma concentration-time profile of compound (**26g**) in male Swiss Albino mouse after intravenous bolus (10 mg/kg) and oral gavage (50 mg/kg) administration.

5.2.3. Compound/chemicals

Analytical standards of compounds were supplied by the Department of Process Chemistry, Advinus Therapeutics Limited, Bangalore, India. Cremophor® EL (BASF, Germany), *N-N*-Dimethyl acetamide (Sigma–Aldrich, USA), Methyl Cellulose (Sigma–Aldrich, USA), acetonitrile (Rankem, India), methanol (Rankem, India), formic acid (Fluka, Germany), ammonium formate (Fluka, Germany) and other general laboratory chemicals and solvents were of analytical grade.

5.2.4. Antiamastigote activity

For assessing the activity of compounds against the amastigote stage of the parasite, mouse macrophage cell line (J-774A.1) infected with promastigotes expressing luciferase firefly reporter gene was used. Cells were seeded in a 96-well plate (4×10^3 cell/100 μL/well) in RPMI-1640 medium containing 10% fetal calf serum and the plates were incubated at 37 °C in a CO_2 incubator. After 24 h, the medium was replaced with fresh medium containing stationary phase promastigotes (4×10^4/100 μL/well). Promastigotes invade the macrophage and then 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 diluted serially up to 7 points in complete medium starting from 100 μM and the plates were incubated at 37 °C 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 3 min, the reading was taken in a luminometer [23]. The values are expressed as relative luminescence units (RLU). Data were plotted using Excel software. IC_{50} values of antileishmanial activity were calculated by nonlinear regression analysis of the concentration response curve using the four parameters Hill equations.

5.2.5. Cytotoxicity assay

The cell viability was determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole) assay [24]. Exponentially growing mammalian kidney epithelial cells (Vero Cell line) (1×10^5 cells/100 μl/well) were incubated with test compounds for 72 h. The test compounds were added at three fold dilutions up to 7 points in complete medium starting from 400 μM concentration, and were incubated at 37 °C in a humidified mixture of 5% CO_2 in an incubator. Podophyllotoxin was used as a reference drug and control wells containing dimethyl sulfoxide (DMSO) without compounds were also included in the experiment. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. After incubation, 25 μL of MTT reagent (5 mg/mL) in PBS medium was added to each well and incubated at 37 °C for 2 h. At the end of the

incubation period, the supernatant was removed by inverting the plate completely without disturbing the cell layer and 150 μL of pure DMSO were added to each well. After 15 min of shaking the readings were recorded as absorbance at 544 nm on a micro plate reader. The cytotoxic effect were expressed as 50% lethal dose (CC_{50}), i.e., as the concentration of a compound which reduced 50% of cell viability compared to cell in culture medium alone. CC_{50} values were estimated as described by Huber and Koella [25]. The selectivity index (SI) for each compound was calculated as ratio between, cytotoxicity (CC_{50}) and activity (IC_{50}) against Leishmania amastigotes.

5.2.6. In vivo assay

The method of Beveridge [26], as modified by Bhatnagar et al. [27] and Gupta et al. [28] was used for *in vivo* screening. Golden hamsters (Inbred strain) of either sex weighing 40–45 g were infected intracardiacally with 1×10^7 amastigotes per animal. The infection is well adapted to the hamster model and establishes itself in 15–20 days. Meanwhile, hamsters gain weight (85–95 g) and can be subjected to repeated spleen biopsies. Pre-treatment spleen biopsy in all the animals was carried out to assess the degree of infection. The animals with +1 grade of infection (5–15 amastigotes/100 spleen cell nuclei) were included in the chemotherapeutic trials. The infected animals were randomized into several groups on the basis of their parasitic burdens. Five to six animals were used for each test sample. Drug treatment at 50 mg/kg dose via IP or PO route was initiated two days after biopsy (48 h recovery) and continued for 5 consecutive days. Miltefosine (pure) at dose of 30 mg/kg × 5 days was used as reference drug. Post-treatment biopsies were done on day 7 of the last drug administration and amastigote counts were assessed by Giemsa staining. Intensity of infection in both, treated and untreated animals, as also the initial count in treated animals was compared and the efficacy was expressed in terms of percentage inhibition (PI) using the following formula:-

$$PI = 100 - [ANAT \times 100/(INAT \times TIUC)]$$

Where PI is Percent 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 Times Increase of parasites in Untreated Control animals.

5.2.7. Administration of test samples

Aqueous solution of test compounds was prepared by dissolving the accurately weighed sample in distilled water/PBS. Required quantity of the compounds were dissolved once and aliquoted in five tubes, one for each day and kept at 4 °C till used.

5.2.8. Metabolic stability assay

Screening studies for metabolic stability were performed with hamster, mouse and human liver microsomes, and degradation at 30 min in the presence and absence of cofactor was measured by LC/MS/MS analysis. Liver microsomes (Xenotech), NADPH (Sigma), sodium dihydrogen orthophosphate (Merck), disodium hydrogen orthophosphate (Merck), acetonitrile (Merck) and DMSO (Sigma) were procured for the study. Stock solution (200 μM) of test compound was prepared in Acetonitrile:DMSO mixture (96:4). NADPH solution (cofactor) was prepared by dissolving 100 mg of NADPH in phosphate buffer (24 mL) to produce final NADPH concentration of 5 mM. The reaction mixture containing sodium phosphate buffer (100 μM, pH 7.4), NADPH solution (5 mM), and liver microsomes (final protein concentration 1 mg/mL) was incubated at 37 °C for 10 min. The reaction was initiated by addition of 5 μL of compound stock solution (1 μM). Aliquots (50 μL) from the reaction mixture were withdrawn at 0 and 30 min and quenched by adding to 50 μL of stop solution comprising internal standard in a 79:20:1 mixture of acetonitrile, ethanol and acetic acid. The analyte/internal standard peak area ratio was determined in each sample by LC/MS/MS. The results are expressed as percent drug metabolized based on the levels at the start of the incubation (0 min sample). NADPH free reaction was performed similarly to verify non-microsomal degradation/solubility/stability issues. Percentage of compound metabolized after 30 min of incubation was calculated.

5.2.9. Pharmacokinetic studies

5.2.9.1. Study design. Tested compound was administered at 10 mg/kg (IV) and 50 mg/kg (PO). The study used a parallel design with two groups typically comprising 9 mice each for the IV and PO dose. Animals in IV group were administered a bolus in the tail vein. Animals in PO group were dose via oral gavages. Blood samples were collected from the orbital plexus from 3 mice at the following time points 0.083 (only for IV), 0.25, 0.50, 1, 2, 4, 8, 12 and 24 h into microfuge tubes containing K_2EDTA (20 μL of 200 mM per mL of blood). Plasma was harvested from blood samples by centrifugation at 8000 rpm for 5 min immediately after collection and stored below −70 °C until analyzed.

5.2.9.2. Bio-analysis. All *in vivo* plasma samples were analyzed using fit-for-purpose liquid chromatography tandem mass spectrometric (LC-MS/MS) method with suitable internal standard. The calibration curve (CC) range was 1–1000 ng/mL in all cases. Quality control (QC) samples were included at low (3X of lower limit of quantitation), medium (close to middle of CC) and high levels (85% of upper limit of quantitation). The CC and QC samples were prepared by spiking 2 μL of the test item spiking solution into 98 μL of mouse blood or plasma. After mixing, a 25 μL aliquot was mixed with 125 μL of internal standard solution. Study samples were similarly processed. After vortex mixing (5 min), the processed samples were centrifuged (14000 rpm, 10 min) and supernatant transferred to auto sampler vials and 5 μL of supernatant was injected in to the LC-MS/MS for analysis [29].

An Applied Biosystems, Sciex API 4000 triple quadrupole mass spectrometer (LC-MS/MS) equipped with a Turbo Ion spray source interfaced with mass spectrometer was used for analysis of all the compounds. Shimadzu Prominence LC-20 AD HPLC was system interfaced with the mass spectrometer. All compounds were analyzed in positive ion mode using an electrospray ion source and MRM (Multi reaction monitoring). Chromatographic separation was achieved by using Kromasil C8 analytical column (100 mm × 4.6 mm, 5 μ, Akzo Nobel, Sweden) maintained at 40 °C using a mobile phase comprising methanol (60%), 5 mM ammonium formate solution (40%) and formic acid (0.05%) with a flow rate of 0.5 mL/min. Chromatograms were acquired using Analyst® software version 1.4.1 and the data were processed using peak area ratio method. Calibration curves were obtained by plotting the peak area ratio against the analyte concentration. A weighted ($w = 1/X^2$, where X is concentration) least squares regression analysis was used to obtain a linear equation over the range of the calibration [30].

Pharmacokinetic parameters were calculated using non compartmental analysis tool of validated WinNonlin (version 5.2, Pharsight Co., Mountain View, CA) software. Estimated parameters included apparent clearance, volume of distribution at steady state (Vss), terminal half-life ($T_{1/2}$), area under the concentration-time curve from time zero to the last measurable concentration ($AUC_{0\text{-last}}$) and the area under the concentration-time curve extrapolated to infinity ($AUC_{0\text{-inf}}$). The maximum observed concentration of drug in plasma/blood (C_{max}) and the time of maximum observed

concentration in plasma/blood (T_{max}) were also reported., Bioavailability was calculated as ($AUC_{oral\ or\ IP}$/$Dose_{oral\ or\ IP}$)/(AUC_{IV}/$Dose_{IV}$) × 100 [31].

6. Funding

This work was supported by the Drug for Neglected Disease Initiative (DNDi). Specific funding from Bill & Melinda Gates Foundation, USA, with complementary core funding from Department for International Development (DFID), UK; Dutch Ministry of Foreign Affairs (DGIS), Nederland; Federal Ministry of Education and Research (BMBF), Germany; Spanish Agency for International Development Cooperation (AECID), Spain; Swiss Agency for Development and Cooperation (SDC), Switzerland and Médecins Sans Frontières (Doctors without Borders), International.

Acknowledgments

The authors thank Dr. T. K. Chakraborty, Director, CSIR-CDRI, Lucknow, for his encouragement and facilities. The transgenic *L. donovani* promastigotes were originally procured from Dr. Neena Goyal, Division of Biochemistry, CSIR-Central Drug Research Institute, Lucknow, India. This paper has CSIR-CDRI communication number 8544.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.08.028.

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