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Original article

Emergence of pyrido quinoxalines as new family of antimalarial agents

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ABSTRACT

A series of novel *N*-alkyl dihydro pyrido quinoxaline derivatives were synthesized using Gould–Jacobs reaction and evaluated their antimalarial activity *in vitro* against chloroquine sensitive (3D7) and drug resistant (Dd2) strains of *Plasmodium falciparum*. Among the compounds tested, 10 compounds were more potent than their structural standard analog ciprofloxacin, including 2 derivatives **5e** and **5h,** which showed 3.3–7.4 times more potency than ciprofloxacin against both the parasite strains. The results are encouraging and a lead molecule may emerge which is useful alone or in combination therapy.

1. Introduction

Malaria is a calamitous tropical disease causing millions of deaths annually throughout the world [1]. Since there is no vaccine available for malaria, chemotherapy is the mainstay in controlling the disease. It is known in prior art that certain antibiotics such as tetracycline [2], rifampin [3], clindamycin [4], erythromycin [5], chloramphenicol [6] show antimalarial activity *in vivo* either alone or in combination with other more commonly used antimalarial drugs [7]. The potential drug candidates for malaria treatment belongs to the family of amino quinolines, chloroquine has long been a mainstay in combating malaria [8]. However extensive usage of chloroquine has led to the emergence of resistant plasmodium strains, primarily due to acquisition of mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*PfCRT*) gene [9,10]. Several amino quinolines lost their efficacy and thus their usage has been staunched [11–13]. Due to the ineffectiveness of chloroquine family of drugs towards resistant strains of *Plasmodium*, there is an urgent need to develop new family of drugs, which can act equally act on chloroquine sensitive and resistant strains.

Quinolones are not only broad spectrum antibacterial agents [14–17] but also effective against malaria parasites and the drugs like Enoxacin, Ciprofloxacin and Grepafloxacin, Clinafloxacin kill malaria parasites with IC_{50} values in the range of 20–40 μM [18,19]. The exact mode of action of these drugs is still uncertain but recent articles propose that the apicoplast of parasite is the main target [20–23]. The apicoplast is a chloroplast-like organelle in malaria parasites and is essential for the viability of the parasite due to presence of multiple prokaryotic biosynthetic machinery in it. Quinolones are effective against both erythrocytic and hepatic stages of the parasite. The rational thinking behind the synthesis of pyrazine fused quinolones is based on the antimalarial activity of pyrazine and quinolones. Quinolone ethers [24,25] and apicidine tagged quinolones [26] are effective at nanomolar concentrations against *P. falciparum*. Charris et al. have synthesized several thieno fused quinolones and evaluated their antimalarial activity [27]. In fact, quinolones as part of combination therapy could be used when administered in conjunction with a rapidly acting antimalarial drug [28]. Several pyrazine containing drugs like sulfameto pyrazine, bispyrrolo[1,2-*a*]thieno[3,2-*e*]pyrazine are well known antimalarial agents [29]. Recently, Satish K. Awasthi and his co-workers were synthesized a novel series of fluoroquinolone analogs and evaluated their *in vitro* antimalarial activity against chloroquine sensitive strain of *P. falciparum* while Ciprofloxacin was used as standard [30]. Their results showed that

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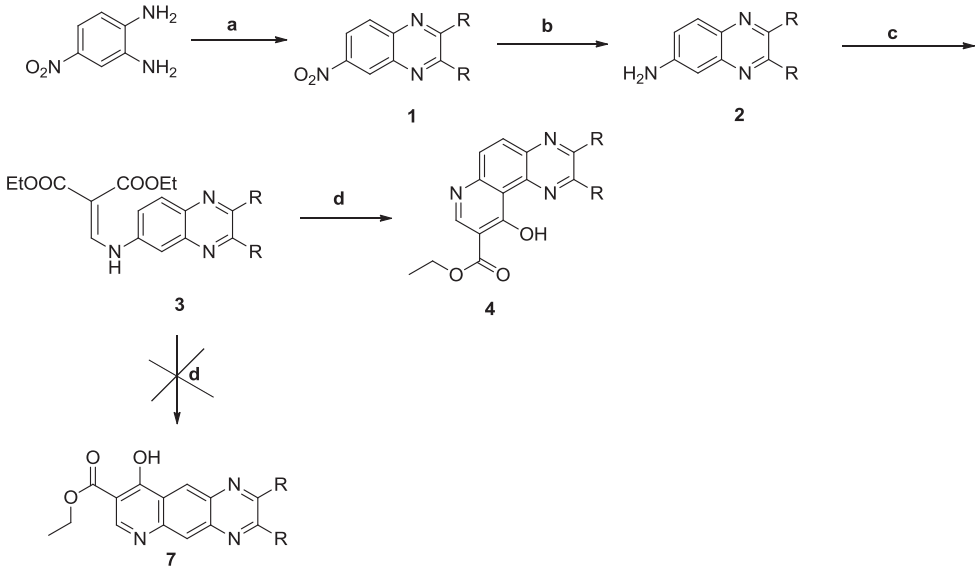
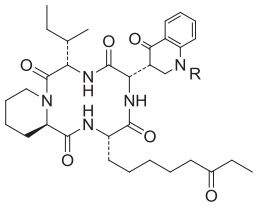


R= phenyl/4-fluorophenyl

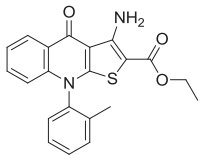
Reaction conditions: (**a**) benzil or 1,2-bis(4-fluorophenyl)ethane-1,2-dione/40% aq.HF/r.t./2 h (or) $GdCl_3.6H_2O$/EtOH/ reflux/ 6-8 h (**b**) 10%Pd-C/ EtOH/hydrazine hydrate/reflux/ 6h (**c**) EMME/130 °C/ 2-3 h/solvent free (**d**) diphenylether/260 °C/1-2 h.

Scheme 1. Synthesis of ethyl 10-hydroxy-2,3-bis(4-fluorophenyl)/diphenyl pyrido[3,2-*f*] quinoxaline-9-carboxylate.

one of the compounds was found most active when compared to Ciprofloxacin. Due to the biological significance of fluoroquinolones and pyrazines as antimalarial agents, we have designed novel pyrazine fused quinolones and antiplasmodial activity studies were performed and found promising candidates as lead molecules.



Apicidine containing quinolone
(Natural product)



Thieno fused quinolone (Charris *et al.*)
(Hetero ring fused quinolone)

2. Results and discussion

2.1. Chemistry

The synthetic methodology for the target compounds starts with the condensation of 4-nitro-*o*-phenylene diamine and benzil/ 1,2-bis(4-fluorophenyl)ethane-1,2-dione in the presence of aq.HF as a catalyst medium at room temperature yielded 6-nitro-2,3-diphenyl/bis[4-fluorophenyl] quinoxaline derivative **1** in good yields [31]. The nitro quinoxaline **1** was reduced in the presence of 10% Pd/C in refluxing ethanol and hydrazine hydrate as a promoter furnished 2,3-diphenyl/bis[4-fluorophenyl]quinoxaline-6-amine **2**. The same reaction was carried out in the presence of stannous chloride, which produced amine in low yields. Quinoxaline amine was reacted with ethoxy methylene diethyl malonate, produced an uncyclised product **3** in neat conditions. The uncyclised compound **3** underwent cyclization in hot diphenyl ether at 250 °C for 1–2 h afforded ethyl 10-hydroxy-2,3-diphenyl/bis-[4-fluorophenyl]pyrido[3,2-*f*]quinoxaline-9-carboxylate **4**. The NMR spectrum of **4b** showed two doublets at δ 8.1 and δ 8.6 with ortho coupling constant $J = 9.8$ Hz in aromatic region, which confirmed the formation of angular isomer but not the linear product **7** (Scheme 1).

Ethyl 10-hydroxy-2,3-diphenyl/bis-[4-fluorophenyl]pyrido[3,2-*f*]quinoxaline-9-carboxylate **4b** was alkylated using alkyl halides/ substituted benzyl halides in the presence of potassium carbonate in dimethyl formamide at 60 °C produced corresponding *N*-alkyl derivatives i.e., alkyl/benzyl 7-ethyl-2,3-diphenyl/bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-*f*] quinoxaline-9-carboxylate **5** in good yields. The *N*-alkylated dihydro pyrido-9-carboxylates **5** were saponificated in 10% aq. NaOH at 100 °C to obtain alkyl/benzyl 7-ethyl-2,3-diphenyl/bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-*f*]quinoxaline-9-carboxylicacid derivatives **6** (Table 1) (Scheme 2).

Majority of the acid derivatives **6** have poor solubility in dimethyl sulfoxide, hence recording their NMR spectra could not be recorded.

2.2. Antimalarial activity

Antimalarial activity of the compounds **3**, **4**, **5** and **6** were tested against the chloroquine sensitive (3D7) and drug resistant (Dd2) strains of *P. falciparum* using ciprofloxacin as standard [30].

All the synthesized 28 derivatives were initially tested against sensitive 3D7 strain; the potent hits were identified and the active leads were then evaluated against the Dd2 *P. falciparum* strain. Majority of the compounds were active against both sensitive and

Table 1
Physiochemical properties of N-alkyl dihydropyrido[3,2-*f*]quinoxaline derivatives.

Compound	R	R_1	M.P.(°C)	Yield[a]
5a	Ph	$-CH_2CH_3$	248–250	79
5b	Ph	$-CH_2CF_3$	>250	70
5c	Ph	$-CH_2CH_2CH_2CH_3$	236–238	80
5d	Ph	$-CH_2CCH$	>250	72
5e	Ph	$-4F-CH_2C_6H_4$	>250	70
5f	Ph	$-4CF_3-CH_2C_6H_4$	>250	70
5g	4F-Ph	$-CH_2CH_3$	212–214	85
5h	4F-Ph	$-CH_2CF_3$	>250	72
5i	4F-Ph	$-CH_2CH_2CH_2CH_3$	179–180	90
5j	4F-Ph	$-CH_2CCH$	175–177	80
5k	4F-Ph	$-4F-CH_2C_6H_4$	239–240	87
5l	4F-Ph	$-4CF_3-CH_2C_6H_4$	242	85
6a	Ph	$-CH_2CH_3$	248–250	70
6b	Ph	$-CH_2CF_3$	>250	70
6c	Ph	$-CH_2CH_2CH_2CH_3$	>250	75
6d	Ph	$-CH_2CCH$	>250	70
6e	Ph	$-4F-CH_2C_6H_4$	>250	70
6f	Ph	$-4CF_3-CH_2C_6H_4$	>250	65
6g	4F-Ph	$-CH_2CH_3$	240–242	75
6h	4F-Ph	$-CH_2CF_3$	>250	77
6i	4F-Ph	$-CH_2CH_2CH_2CH_3$	234–236	80
6j	4F-Ph	$-CH_2CCH$	>250	70
6k	4F-Ph	$-4F-CH_2C_6H_4$	>250	70
6l	4F-Ph	$-4CF_3-CH_2C_6H_4$	>250	70

[a] represents isolated yields.

Table 2
Antimalarial activity of pyrido quinoxaline derivatives against *P. falciparum* 3D7 and Dd2 strains.

S.No.	Compound	IC_{50} (μM±S.D.)	
		3D7	Dd2
1	**3a**	16.0(±0.42)	15.5(±1.55)
2	**3b**	21.3(±4.10)	–
3	**4a**	29.8(±4.54)	–
4	**4b**	15.7(±0.28)	26.9(±3.11)
5	**5a**	15.4(±1.27)	11.33(±2.07)
6	**5b**	13.1(±1.30)	–
7	**5c**	9.17(±0.99)	–
8	**5d**	ND[a]	–
9	**5e**	6.94(±0.91)	6.27(±0.84)
10	**5f**	ND[a]	–
11	**5g**	32.2(±7.3)	–
12	**5h**	3.92(±0.254)	4.60(±1.09)
13	**5i**	>50	–
14	**5j**	18.7(±1.89)	–
15	**5k**	7.59(±1.20)	7.11(±0.08)
16	**5l**	>50	–
17	**6a**	ND[a]	–
18	**6b**	ND[a]	–
19	**6c**	ND[a]	–
20	**6d**	ND[a]	–
21	**6e**	ND[a]	–
22	**6f**	ND[a]	–
23	**6g**	>50	–
24	**6h**	>50	–
25	**6i**	40.4(±9.60)	–
26	**6j**	>50	–
27	**6k**	>50	–
28	**6l**	>50	–
std	**Ciprofloxacin**	23.1(±3.78)	33.9(±0.96)

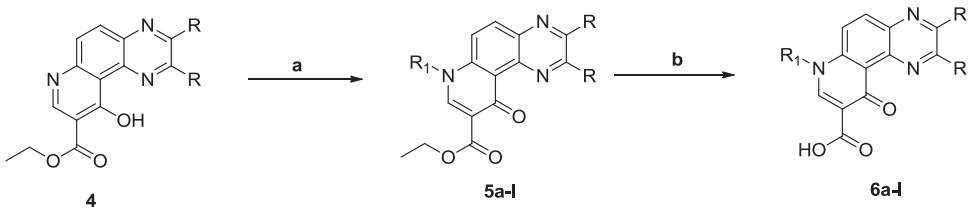
(The IC_{50} concentrations are average of two experiments, with two replicates for each experiment. SD is standard deviation mentioned in the bracket) std = standard.
[a] Could not be determined due to insolubility at higher concentrations (93 μM).

resistant strains of *P. falciparum*. The pharmacophore hydroxy pyrido quinoxaline **4** was itself more potent than ciprofloxacin and its subsequent *N*-alkylation improved its activity. *N*-alkylated dihydro pyrido quinoxaline-9-carboxylate derivatives **5** are 2–3 times more active compared to ciprofloxacin and potency was improved due to the enhanced solubility. *N*-alkylated dihydro pyrido quinoxaline-9-carboxylic acid derivatives **6** are very poor pharmacophores did not inhibit or weakly inhibited *P. falciparum* growth.

Amongst the derivatives synthesized, enamine and hydroxy pyrido quinoxaline derivatives **3** & **4** showed more potency than ciprofloxacin. In *N*-alkylated dihydro pyrido quinoxaline-9-carboxylate derivatives **5**, ethyl 7-butyl-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-*f*]quinoxaline-9-carboxylate **5c**, ethyl 7-(4-fluorobenzyl)-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-*f*]quinoxaline-9-carboxylate **5e** and ethyl 7-(4-fluorobenzyl)-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-*f*]quinoxaline-9-carboxylate **5k** are promising and ethyl 2,3-bis(4-fluorophenyl)-10-oxo-7-(2,2,2-trifluoroethyl)-7,10-dihydropyrido[3,2-*f*]quinoxaline-9-carboxylate **5h** was the most potent among all the compounds tested, and it was five times more active than ciprofloxacin (IC_{50}: **5h**, 3.92 μM for 3D7 and 4.6 μM for Dd2; ciprofloxacin, 23.1 μM for 3D7 and 33.9 μM for Dd2). The above results indicated that the presence of trifluoroethyl, butyl and fluorobenzyl moieties on *N*1-position offer optimum antimalarial activity.

Of the 12 *N*-alkylated dihydro pyrido quinoxaline-9-carboxylic acid derivatives **6**, six compounds did not show any effect on parasite growth, five compounds showed marginal antiparasite activity at concentrations above 50 μM and the compound 7-butyl-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-*f*]quinoxaline-9-carboxylic acid **6i** inhibited parasite growth with IC_{50} value of 40.4 μM (Table 2).

It is necessary to find cytotoxicity of a compound to have selective antiplasmodial activity. We have selected high active compounds (**5e**, **5h**, **5k**) calculated their IC_{50} values on two human cell lines HEP-G2, THP-1 and calculated their selective indices (Table 3). All three compounds have registered good selective indices in



R= phenyl/4-fluorophenyl

Reaction conditions: (**a**) R-X/DMF/60 °C/16-24 h (**b**) 10% aq.NaOH/ reflux/ 6 h

Scheme 2. Synthesis of 2,3-bis(4-fluorophenyl)/diphenyl-10-oxo-7-alkyl-7,10-dihydropyrido [3,2-*f*]quinoxaline-9-carboxylic acid.

Table 3
Cytotoxicity of pyrido quinoxaline derivatives.

S.No.	Compound	Antiplasmodial activity (W2)	Human cell toxicity (IC_{50}) (μM)		Selectivity index	
		IC_{50} (μM) (±S.D.)	**HEP G2**	**THP1**	**HEP G2**	**THP1**
1	**5e**	6.94(±0.91)	150	200	21.61	28.81
2	**5h**	3.92(±0.254)	250	275	63.77	70.15
3	**5k**	7.59(±1.20)	200	140	26.35	18.44
std	**Ciprofloxacin**	23.1(±3.78)	–	60.5	–	2.61
std	**Doxorubicin**	–	0.2	0.05	–	–

std = standard.

which **5h** showed better values 63.77, 70.15 makes it as further effective.

Importantly, all the compounds that inhibited parasite growth showed similar IC_{50} concentrations for both chloroquine sensitive and resistant strains. Ciprofloxacin inhibits prokaryotic DNA gyrase. Although the molecular target of ciprofloxacin is not clearly known, but the apicoplast DNA gyrase appears to be a likely target. The pyrido quinoxaline may target the enzyme as ciprofloxacin and the identification of ciprofloxacin target in malaria parasite will benefit structure-based drug design of ciprofloxacin–based compounds. This result supports the pyrido quinoxaline pharmacophore as a tool to combat resistant *P. falciparum* malaria and further structural modification to this pharmacophore may provide ore potent leads.

3. Conclusions

We have successfully synthesized a library of *N*-alkylated dihydro pyrido quinoxaline derivatives and screened against sensitive and resistant strains of the human malaria parasite *P. falciparum*. Out of evaluated 28 compounds, 10 compounds were identified as more potent than ciprofloxacin. Compound **5h** was the most potent among all screened compounds with IC_{50} of 3.92 μM. These results are encouraging and could be used as a starting point to design more potent antimalarial compounds. This study has revealed pyrido quinoxalines as a new family of antimalarial agents, and provides a new platform to design novel antimalarial agents other than traditional chloroquine family. More research is going in the direction to evaluate the mode of action, docking studies and the results will be published in future.

4. Experimental protocols

4.1. Synthetic chemistry

Melting points of compounds were recorded on Casia-Siamia (VMP-AM) melting point apparatus and are uncorrected. IR spectra were recorded on a Perkin–Elmer FT-IR 240-C spectrophotometer using KBr optics. ^{1}H NMR spectra were recorded on Bruker AV 300 MHz in $CDCl_3$ or DMSO-d_6 expressed as δ (ppm) values using TMS as internal standard. Electron impact (EI) and chemical ionization mass spectra were recorded on a VG 7070 H instrument at 70 eV. All high-resolution spectra were recorded on QSTARXL hybrid MS/MS system (Applied Biosystems, USA) under electrosprayionization. CHN analyses were recorded on a vario EL analyzer.

4.1.1. Diethyl 2-(2,3-bis(4-fluorophenyl)/diphenyl quinoxalin-6-ylamino)methylene)malonate (**3**)

4.1.1.1. General procedure.
2,3-Di(4-fluorophenyl)/diphenylquinoxaline-6-amine (1.0 mmol) and ethoxy methylene diethyl malonate (EMME) (1.1 mmol) were heated at 130 °C for 5–6 h. Progress of the reaction was monitored by thin layer chromatography. The low volatiles of the reaction mixture were removed under reduced pressure and the residue was diluted with hexane, the separated solid was filtered, thoroughly washed with hexane and further purified through column chromatography.

4.1.2. Diethyl 2-(((2,3-bis(4-fluorophenyl)quinoxalin-6-yl)amino) methylene)malonate (**3b**)

Yellow colored solid; IR (KBr, cm^{-1}): 3421.6 (-NH stretch), 1727.27 (ester CO) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 11.28 (d, 1H, NH, J = 13.2 Hz); 8.70 (d, 1H, CH adjacent to NH, J = 13.2 Hz); 8.14 (d, 1H, Ar–H, J = 8.8 Hz); 7.85 (d, 1H, ArH, J = 2.26 Hz); 7.46–7.57 (m, 5H, ArH); 7.01–7.11 (m, 4H, ArH); 4.25–4.41 (m, 4H, O–$\underline{CH_2}$); 1.32–1.47 (m, 6H, O–CH_2–$\underline{CH_3}$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 168.7, 165.0, 163.2 (t, J = 250.3 Hz), 163.1 (d, J = 249.7 Hz), 153.1, 151.1, 150.5, 141.9, 140.5, 138.7, 134.7, 131.6 (d, J = 8.2 Hz), 130.9, 121.9, 115.4 (d, J = 21.4 Hz), 112.5, 96.0, 60.7, 60.3, 14.4, 14.2. ESI–MS: 504 (M + H), 526 (M + Na); Anal. Calcd. for $C_{28}H_{23}F_2N_3O_4$:C, 66.79; H, 4.60; N, 8.35%. Found: C, 66.78; H, 4.59; N, 8.34%.

4.1.3. Ethyl 2,3-bis(4-fluorophenyl/phenyl)-10-hydroxypyrido[3,2-f] quinoxaline-9-carboxylate (**4**)

4.1.3.1. General procedure. Diethyl 2-(((2,3-bis(4-fluorophenyl)/diphenyl)quinoxalin-6-ylamino)methylene)malonate **3b** (4.20 g) was taken in diphenyl ether (20 ml) and heated at 260 °C for 1.5–2.0 h. Progress of the reaction was monitored by thin layer chromatography. After completion of the reaction, low volatiles formed during the reaction were removed under reduced pressure. The residue was diluted with hexane (100 ml) and the separated solid was filtered, thoroughly washed with hexane and dried. The crude solid was purified through column chromatography.

4.1.4. Ethyl 2,3-bis(4-fluorophenyl)-10-hydroxypyrido[3,2-f] quinoxaline-9-carboxylate (**4b**)

Pale yellow colored solid; IR (KBr, cm^{-1}): 3431.6 (OH stretch), 1729.0 (ester CO) cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 15.25 (bs, 1H, OH); 9.40 (s, 1H, CH adjacent to N); 8.60 (d, 1H, ArH, J = 9.8 Hz); 8.10 (d, 1H, ArH, J = 9.8 Hz); 7.54–7.61 (m, 4H, ArH); 7.07–7.16 (m, 4H, ArH); 4.51 (q, 2H, O–$\underline{CH_2}$, J = 7.1 Hz); 1.47 (t, 3H, O–CH_2–$\underline{CH_3}$, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 166.6, 164.2, 163.6 (d, J = 251.9 Hz), 163.4 (d, J = 250.8 Hz) 154.6, 152.2, 152.1, 148.1, 140.3, 139.7, 134.0, 133.7 (d, J = 2.7 Hz), 132.8 (d, J = 2.7 Hz), 131.9 (d, J = 8.2 Hz), 131.6 (d, J = 8.2 Hz), 131.4, 116.1 (d, J = 22.0 Hz), 115.7 (d, J = 22.0 Hz), 111.8, 111.5, 61.2, 14.3. ESI–MS (M + H): 458.18; Anal. Calcd. for $C_{26}H_{17}F_2N_3O_3$: C, 68.27; H, 3.75; N, 9.19%. Found: C, 68.28; H, 3.72; N, 9.20%.

4.1.5. Ethyl-7-alkyl-2,3-(bis(4-fluorophenyl))/diphenyl-10-oxo-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylate (**5**)

4.1.5.1. General procedure. Ethyl 2,3-(bis(4-fluorophenyl)/diphenyl)-10-hydroxypyrido[3,2-*f*]quinoxaline-9-carboxylate (1.2 mmol), alkyl/substituted benzyl halide (3.6 mmol) were taken in 5 ml of dimethyl formamide. Freshly fused potassium carbonate (10 mmol) was added to the above mixture and contents of the

reaction mixture was stirred at 60 °C for 16–18 h. Progress of the reaction mixture was monitored by TLC and after completion of the reaction, the mixture was poured into ice cold water to furnish pale yellow solid. The solid was filtered, washed with hexane and further purified through column chromatography.

*4.1.6. Ethyl 7-ethyl-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylate (**5a**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 1725.25 (ester CO) cm^{-1}; ^1H NMR (TFA + $CDCl_3$, 300 MHz): δ 9.42 (s, 1H, ArH), 9.00 (d, 1H, ArH, $J = 9.8$ Hz), 8.55 (d, 1H, ArH, $J = 9.8$ Hz), 7.42–7.90 (m, 10H, ArH), 4.98 (q, 2H, OCH_2, $J = 7.3$ Hz), 4.60 (q, 2H, NCH_2, $J = 6.7$ Hz), 1.80 (t, 3H, $J = 7.3$ Hz), 1.50 (t, 3H, $J = 6.7$ Hz). ESI–MS (M + H): 450; Anal. Calcd. for $C_{28}H_{23}N_3O_3$: C, 74.82; H, 5.16; N, 9.35%. Found: C, 74.80; H, 5.15; N, 9.34%.

*4.1.7. Ethyl 10-oxo-2,3-diphenyl-7-(2,2,2-trifluoroethyl)-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5b**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 1725.25 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.39 (s, 1H, CH attached to N), 8.34 (d, 1H, Ar–H, $J = 6.0$ Hz), 8.19 (d, 1H, Ar–H, $J = 6.0$ Hz),7.54–7.64 (m, 4H, Ar–H), 7.35–7.52 (m, 6H, ArH), 6.75–6.87 (m, 2H, $\underline{CH_2}CF_3$), 4.51 (q, 2H, O–$\underline{CH_2}$, $J = 6.8$ Hz), 1.47 (t, 3H, O–$CH_2\underline{CH_3}$, $J = 6.8$ Hz). ESI–MS (M + H): 504; Anal. Calcd. for $C_{28}H_{20}F_3N_3O_3$: C, 66.80; H, 4.00; N, 8.35%. Found: C, 66.79; H, 4.10; N, 8.34%.

*4.1.8. Ethyl 7-butyl-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylate (**5c**)*

Pale yellow colored solid; IR: (KBr, cm^{-1}): 1702.27 (ester CO) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 8.48 (s, 1H, CH attached to N); 8.25–8.37 (m, 1H, ArH); 7.82 (d, 1H, Ar–H, $J = 7.7$ Hz); 7.57–7.68 (m, 3H, ArH); 7.27–7.42 (m, 7H, ArH); 4.45–4.49 (m, 1H, CH); 4.39 (q, 2H, O/N–$\underline{CH_2}$, $J = 6.9$ Hz); 4.26 (t, 1H, CH, $J = 7.3$ Hz); 1.79–2.02 (m, 2H, $\underline{CH_2}$); 1.37–1.57 (m, 5H, $\underline{CH_2}$); 1.03 (t, 2H, CH, $J = 7.3$ Hz); 0.77 (t, 1H, –CH, $J = 7.3$ Hz). ESI–MS (M + H): 478; Anal. Calcd. for for $C_{30}H_{27}N_3O_3$: C, 75.45; H, 5.70; N, 8.80%. Found: C, 75.47; H, 5.69; N, 8.82%.

*4.1.9. Ethyl 10-oxo-2,3-diphenyl-7-(prop-2-ynyl)-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5d**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 3040.20 (CCH), 1699.02 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.63 (s, 1H, CH attached to N); 8.20 (d, 1H, ArH, $J = 8.9$ Hz); 7.80–7.90 (m, 2H, ArH); 7.50–7.65 (m, 5H, ArH); 7.00–7.14 (m, 5H, ArH); 4.91 (d, 2H, N–$\underline{CH_2}$, $J = 2.4$ Hz), 4.42 (q, 4H, N–$\underline{CH_2}$, $J = 7.0$ Hz); 2.95 (s, 1H, $C\underline{CH}$); 1.40 (t, 3H, $\underline{CH_3}$, $J = 7.0$ Hz). ESI–MS (M + H): 460; Anal. Calcd. for $C_{29}H_{21}N_3O_3$: C, 75.80; H, 4.61; N, 9.14%. Found: C, 75.79; H, 4.66; N, 9.17%.

*4.1.10. Ethyl 7-(4-fluorobenzyl)-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5e**)*

Yellow colored solid; IR (KBr, cm^{-1}): 1678.27 (ester CO) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 8.65 (s, 1H, CH attached to N); 8.18 (d, 1H, Ar–H, $J = 9.5$ Hz); 7.75 (d, 1H, ArH, $J = 9.5$ Hz); 7.54–7.63 (m, 5H, ArH); 7.29–7.43 (m, 5H, ArH); 7.20–7.25 (m, 2H, ArH); 7.05–7.10 (m, 2H, ArH); 5.63 (s, 2H, $\underline{CH_2}$Ph); 4.39 (q, 2H, O–$\underline{CH_2}$, $J = 6.3$ Hz); 1.40 (t, 3H, O–CH_2–$\underline{CH_3}$, $J = 6.3$ Hz). ESI–MS (M + H): 530; Anal. Calcd. for $C_{33}H_{24}FN_3O_3$: C, 74.85; H, 4.57; N, 7.93%. Found: C, 74.89; H, 4.55; N, 7.95%.

*4.1.11. Ethyl 7-(4-(trifluoromethyl)benzyl)-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5f**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 1698.24 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.51 (s, 1H, CH attached to N); 8.13 (d, 1H, Ar–H, $J = 9.8$ Hz); 7.85–7.92 (m, 2H, ArH); 7.60–7.69 (m, 5H, ArH); 7.44–7.51 (m, 2H, Ar–H); 7.32–7.42 (m, 7H, ArH); 5.57 (s, 2H, $\underline{CH_2}$Ph); 4.45 (q, 2H, O–$\underline{CH_2}$, $J = 7.5$ Hz); 1.42 (t, 3H, $OCH_2\underline{CH_3}$, $J = 7.5$ Hz). ESI–MS (M + H): 580; Anal. Calcd. for $C_{34}H_{24}F_3N_3O_3$: C, 70.46; H, 4.17; N, 7.25%. Found: C, 70.43; H, 4.19; N, 7.27%.

*4.1.12. Ethyl 7-ethyl-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5g**)*

Yellow colored solid; IR (KBr, cm^{-1}): 1731.51 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.45 (s, 1H, CH attached to N); 8.30 (d, 1H, Ar–H, $J = 9.8$ Hz); 7.84–7.90 (m, 2H, ArH); 7.80 (d, 1H, Ar–H, $J = 9.8$ Hz); 7.60–7.67 (m, 2H, ArH); 7.00–7.13 (m, 4H, ArH); 4.33–4.55 (m, 4H, O–$\underline{CH_2}$); 1.63 (t, 3H, O–CH_2–$\underline{CH_3}$, $J = 7.5$ Hz); 1.42 (t, 3H, O–CH_2–$\underline{CH_3}$, $J = 6.7$ Hz). ESI–MS (M + H): 486; Anal. Calcd. for $C_{28}H_{21}F_2N_3O_3$: C, 69.27; H, 4.36; N, 8.66%. Found: C, 69.28; H, 4.37; N, 8.67%.

*4.1.13. Ethyl 2,3-bis(4-fluorophenyl)-10-oxo-7-(2,2,2-trifluoroethyl)-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylate (**5h**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 1687.92 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.50 (s, 1H, CH attached to N); 8.08 (d, 1H, Ar–H, $J = 9.06$ Hz); 7.82–7.91 (m, 2H, ArH); 7.58–7.66 (m, 2H, ArH); 7.55 (d, 1H, Ar–H, $J = 9.0$ Hz); 7.31–7.38 (m, 1H, ArH); 6.93–7.08 (m, 3H, ArH); 5.48 (s, 2H, $\underline{CH_2}$–CF_3); 4.33–4.55 (m, 2H, O–$\underline{CH_2}$); 1.42 (t, 3H, $OCH_2\underline{CH_3}$, $J = 7.5$ Hz). ESI–MS (M + H): 540; Anal. Calcd. for $C_{28}H_{18}F_5N_3O_3$: C, 62.34; H, 3.36; N, 7.79%. Found: C, 62.28; H, 3.37; N, 7.77%.

*4.1.14. Ethyl 7-butyl-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5i**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 1712.45 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 9.28 (s, 1H, CH attached to N); 8.45 (s, 1H, Ar–H); 8.31 (d, 1H, Ar–H, $J = 7.1$ Hz); 7.78–7.87 (m, 2H, ArH); 7.57–7.67 (m, 2H, ArH); 6.95–7.14 (m, 4H, ArH); 4.21–4.54 (m, 4H, O/N–$\underline{CH_2}$); 1.79–2.04 (m, 2H, $\underline{CH_2}$); 1.37–1.56 (m, 4H, $\underline{CH_2}$); 1.20–1.29 (m, 1H, CH); 0.99–1.08 (m, 2H, CH); 0.78 (t, 1H, CH, $J = 7.3$ Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 174.2, 165.0, 153.1, 151.5, 150.4, 147.0, 141.5, 140.3, 136.7, 134.5, 133.9, 133.4, 132.7 (d, $J = 8.7$ Hz), 131.6 (d, $J = 8.7$ Hz), 118.9, 115.4 (d, $J = 21.9$ Hz), 115.2 (d, $J = 21.9$ Hz), 61.8, 54.9, 19.8, 18.7, 14.3, 13.6. ESI–MS (M + H): 514; Anal. Calcd. for $C_{30}H_{25}F_2N_3O_3$: C, 70.16; H, 4.91; N, 8.18%. Found: C, 70.17; H, 4.95; N, 8.17%.

*4.1.15. Ethyl 2,3-bis(4-fluorophenyl)-10-oxo-7-(prop-2-ynyl)-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5j**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 3010.20 (CCH), 1717.84 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.46 (s, 1H, CH); 8.30 (d, 1H, Ar–H, $J = 8.8$ Hz); 7.80–7.90 (m, 2H, ArH); 7.60–7.67 (m, 2H, ArH); 6.99–7.13 (m, 5H, ArH); 5.01 (partially split doublet, 2H, N–$\underline{CH_2}$, $J = 2.4$ Hz); 4.41 (q, 4H, N–$\underline{CH_2}$, $J = 7.2$ Hz); 2.95 (s, 1H, $C\underline{CH}$); 1.40 (t, 3H, $\underline{CH_3}$, $J = 7.2$ Hz). ESI–MS (M + H): 496; Anal. Calcd. for $C_{29}H_{19}F_2N_3O_3$: C, 70.30; H, 3.87; N, 8.48%. Found: C, 70.29; H, 3.86; N, 8.47%.

*4.1.16. Ethyl 7-(4-fluorobenzyl)-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylate (**5k**)*

White colored solid; IR (KBr, cm^{-1}): 1678.48 (ester CO) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 8.65 (s, 1H, CH attached to N); 8.15 (d, 1H, Ar–H, $J = 9.5$ Hz); 7.81–7.86 (m, 2H, ArH); 7.75 (d, 1H, Ar–H, $J = 9.5$ Hz); 7.59–7.64 (m, 2H, ArH); 7.35–7.39 (m, 1H, ArH); 7.20–7.25 (m, 2H, ArH); 7.01–7.11 (m, 4H, ArH); 6.98 (t, 1H, ArH, $J = 8.7$ Hz); 5.62 (s, 2H, $\underline{CH_2}$Ph); 4.40 (q, 2H, O–$\underline{CH_2}$, $J = 6.9$ Hz); 1.40 (t, 3H, O–CH_2–$\underline{CH_3}$, $J = 6.9$ Hz). ESI–MS (M + H): 566.4; Anal. Calcd. for $C_{33}H_{22}F_3N_3O_3$: C, 70.08; H, 3.92; N, 7.43%. Found: C, 70.09; H, 3.95; N, 7.45%.

*4.1.17. Ethyl 7-(4-(trifluoromethyl)benzyl)-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylate (**5l**)*

Yellow colored solid; IR (KBr, cm^{-1}): 1694.60 (ester CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 8.53 (s, 1H, CH attached to N); 8.13 (d, 1H, Ar–H, J = 9.0 Hz); 7.82–7.94 (m, 2H, ArH); 7.58–7.72 (m, 5H, ArH); 7.51 (d, 1H, Ar–H, J = 9.8 Hz); 7.31 (s, 1H, ArH); 7.01–7.15 (m, 4H, ArH); 5.58 (s, 2H, $\underline{CH_2}$Ph); 4.45 (q, 2H, O–$\underline{CH_2}$, J = 7.5 Hz); 1.42 (t, 3H, O–CH_2–$\underline{CH_3}$, J = 7.5 Hz). ESI–MS (M + H): 616; Anal. Calcd. for $C_{29}H_{19}F_2N_3O_3$: C, 70.08; H, 3.92; N, 7.43%. Found: C, 70.09; H, 3.95; N, 7.45%.

*4.1.18. 7-Alkyl-2,3-(bis(4-fluorophenyl))/diphenyl-10-oxo-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylic acid (**6**)*

4.1.18.1. General procedure. Ethyl 7-alkyl-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-*f*]quinoxaline-9-carboxylate (1.0 mmol) was taken in 10% aq. NaOH solution and heated at 100 °C for 6–10 h. Progress of the reaction was monitored by TLC. After completion of the reaction, the contents of the reaction mixture was poured in to ice cold water. It was acidified with 10% aq.HCl till the pH attains 2. The solid was filtered, washed with water and oven dried. The crude compound was purified using column chromatography.

*4.1.19. 7-Ethyl-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylic acid (**6a**)*

White colored solid; IR (KBr, cm^{-1}): 3350.17 (OH stretch), 1725.40 (acid CO) cm^{-1}; ESI–MS (M + H): 422; Anal. Calcd. for $C_{26}H_{19}N_3O_3$: C, 74.10; H, 4.54; N, 9.97%. Found: C, 74.09; H, 4.57; N, 9.95%.

*4.1.20. 10-Oxo-2,3-diphenyl-7-(2,2,2-trifluoroethyl)-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylic acid (**6b**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 3450 (OH stretch); 1720 (acid CO) cm^{-1}; ESI–MS (M + H): 476; Anal. Calcd. for $C_{26}H_{16}F_3N_3O_3$: C, 65.68; H, 3.39; N, 8.84%. Found: C, 65.69; H, 3.37; N, 8.85%.

*4.1.21. 7-Butyl-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylic acid (**6c**)*

White colored solid; IR (KBr, cm^{-1}): 3450 (OH stretch), 1780 (acid CO) cm^{-1}; ESI–MS (M + H): 450; Anal. Calcd. for $C_{28}H_{23}N_3O_3$: C, 74.82; H, 5.16; N, 9.35%. Found: C, 74.82; H, 5.17; N, 9.37%.

*4.1.22. 10-Oxo-2,3-diphenyl-7-(prop-2-ynyl)-7,10-dihydropyrido [3,2-f]quinoxaline-9-carboxylic acid (**6d**)*

IR (KBr, cm^{-1}): 3420.80 (OH stretch), 1726.27 (acid CO) cm^{-1}; ESI–MS (M + H): 431; Anal. Calcd. for $C_{27}H_{17}N_3O_3$: C, 75.16; H, 3.97; N, 9.74%. Found: C, 75.12; H, 3.93; N, 9.73%.

*4.1.23. 7-(4-fluorobenzyl)-10-oxo-2,3-diphenyl-7,10-dihydropyrido [3,2-f]quinoxaline-9-carboxylic acid (**6e**)*

White colored solid; IR (KBr, cm^{-1}): 3650 (OH stretch), 1780 (acid CO) cm^{-1}; ESI–MS (M + H): 502; Anal. Calcd. for $C_{31}H_{20}FN_3O_3$: C, 74.24; H, 4.02; N, 8.38%. Found: C, 74.22; H, 4.03; N, 8.37%.

*4.1.24. 7-(4-(Trifluoromethyl)benzyl)-10-oxo-2,3-diphenyl-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylic acid (**6f**)*

White colored solid; IR (KBr, cm^{-1}): 3650 (OH stretch), 1780 (acid CO) cm^{-1}; ESI–MS (M + H): 552; Anal. Calcd. for $C_{32}H_{20}F_3N_3O_3$: C, 69.69; H, 3.66; N, 7.62%. Found: C, 69.70; H, 3.63; N, 7.64%.

*4.1.25. 7-Ethyl-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido [3,2-f]quinoxaline-9-carboxylic acid (**6g**)*

White colored solid; IR (KBr, cm^{-1}): 3441.48 (OH stretch), 1717.84 (acid CO) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 15.66 (bs, 1H, OH); 8.88 (s, 1H, CH attached to N); 8.46 (d, 1H, Ar–H, J = 9.9 Hz); 7.94 (d, 1H, Ar–H, J = 9.9 Hz); 7.79–7.84 (m, 2H, ArH); 7.63–7.68 (m, 2H, ArH); 7.05–7.13 (m, 4H, ArH); 4.53 (q, 2H, O–$\underline{CH_2}$, J = 6.6 Hz); 1.70 (t, 3H, O–CH_2–$\underline{CH_3}$, J = 6.6 Hz). ESI–MS (M + H): 458; Anal. Calcd. for $C_{26}H_{17}F_2N_3O_3$: C, 68.27; H, 3.75; N, 9.19%. Found: C, 68.28; H, 3.77; N, 9.20%.

*4.1.26. 2,3-Bis(4-fluorophenyl)-10-oxo-7-(2,2,2-trifluoroethyl)-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylic acid (**6h**)*

Pale yellow colored solid; IR (KBr, cm^{-1}): 3449.08 (OH stretch), 1719.27 (acid CO), 1150 (CF_3strech) cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 15.72 (bs, 1H, OH); 8.68 (s, 1H, CH attached to N); 8.27 (d, 1H, Ar–H, J = 9.0 Hz); 8.00–8.06 (m, 2H, ArH); 7.67–7.75 (m, 3H, ArH); 7.10–7.20 (m, 4H, ArH); 5.62 (s, 2H, $\underline{CH_2}CF_3$). ESI–MS (M + H): 512. Anal. Calcd. for $C_{26}H_{14}F_5N_3O_3$: C, 61.06; H, 2.76; N, 8.22%. Found: C, 61.08; H, 2.77; N, 8.23%.

*4.1.27. 7-Butyl-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido [3,2-f]quinoxaline-9-carboxylic acid (**6i**)*

White colored solid; IR (KBr, cm^{-1}): 3460.20 (OH stretch), 1717.20 (acid CO) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 15.98 (bs, 1H, OH); 9.01 (s, 1H, CH attached to N); 8.49 (d, 1H, Ar–H, J = 9.6 Hz); 8.13 (d, 1H, Ar–H, J = 9.6 Hz); 7.76–7.86 (m, 2H, ArH); 7.61–7.71 (m, 2H, ArH); 7.0–7.17 (m, 4H, ArH); 4.62 (t, 2H, N–$\underline{CH_2}$, J = 7.55 Hz); 1.92–2.07 (m, 2H, CH); 1.37–1.57 (m, 2H, CH); 1.03 (t, 3H, CH, J = 7.36 Hz). ESI–MS (M + H): 486; Anal. Calcd. for $C_{28}H_{21}F_2N_3O_3$: C, 69.27; H, 4.36; N, 8.66%. Found: C, 69.28; H, 4.37; N, 8.64%.

*4.1.28. 2,3-Bis(4-fluorophenyl)-10-oxo-7-(prop-2-ynyl)-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylic acid (**6j**)*

White colored solid; IR (KBr, cm^{-1}): 3449.21 (OH stretch), 1717.26 (acid CO) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 15.66 (bs, 1H, OH); 8.50 (s, 1H, Ar–H); 8.34 (d, 1H, Ar–H, J = 8.8 Hz); 7.84–7.94 (m, 2H, ArH); 7.64–7.72 (m, 2H, ArH); 7.03–7.17 (m, 4H, ArH); 5.05 (partially splitted doublet, 2H, N–$\underline{CH_2}$, J = 2.4 Hz); 4.40–4.48 (m, 2H, N–$\underline{CH_2}$, J = 7.2 Hz); 2.99 (s, 1H, C$\underline{CH}$). ESI–MS (M + H): 468. Anal. Calcd. for $C_{27}H_{15}F_2N_3O_3$: C, 69.38; H, 3.23; N, 8.99%. Found: C, 69.39; H, 3.24; N, 9.01%.

*4.1.29. 7-(4-Fluorobenzyl)-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-f]quinoxaline-9-carboxylic acid (**6k**)*

White colored solid; IR (KBr, cm^{-1}): 3449.15 (OH stretch), 1719.28 (acid CO) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 9.41 (bs, 1H, CH attached to N); 8.39 (d, 1H, Ar–H, J = 9.6 Hz); 8.24 (d, 1H, ArH, J = 9.6 Hz); 7.73–7.80 (m, 2H, ArH); 7.59–7.68 (m, 2H, ArH); 7.33–7.41 (m, 2H, ArH); 7.10–7.25 (m, 6H, ArH); 6.04 (s, 2H, $\underline{CH_2}$Ph). ESI–MS (M + H): 538; Anal. Calcd. for $C_{31}H_{18}F_3N_3O_3$: C, 69.27; H, 3.38; N, 7.82%. Found: C, 69.29; H, 3.37; N, 7.84%.

*4.1.30. 7-(4-(Trifluoromethyl)benzyl)-2,3-bis(4-fluorophenyl)-10-oxo-7,10-dihydropyrido[3,2-f] quinoxaline-9-carboxylic acid (**6l**)*

White colored solid; IR (KBr, cm^{-1}): 3449.50 (OH stretch), 1718.20 (acid CO) cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz): δ 15.70 (bs, 1H, OH); 8.98 (s, 1H, CH attached to N); 8.29–8.34 (m, 2H, ArH); 7.90–8.10 (m, 7H, ArH); 7.42–7.54 (m, 5H, ArH); 6.02 (s, 2H, $\underline{CH_2}$Ph). ESI–MS (M + H): 588; Anal. Calcd. for $C_{32}H_{18}F_5N_3O_3$: C, 65.42; H, 3.09; N, 7.15%. Found: C, 65.45; H, 3.11; N, 7.20%.

4.2. Biological experiments

4.2.1. Parasite culture and assessment of antimalarial activity

The *P. falciparum* 3D7 and Dd2 strains were cultured in human erythrocytes (at 2% haematocrit) in RPMI 1640 medium (supplemented with 41.1 mg/lit hypoxanthine, 300 mg/lit glutamine, 2.5% human serum, and 0.5% Albumax II) in the presence of a gas mixture (5% CO_2, 5% O_2, and 90% N_2) [32]. Compound stocks were made in DMSO; chloroquine stock was made in sterile water; and ciprofloxacin stock was made in absolute ethanol. The stock (1.5 μl) was added to 98.5 μl culture medium in the wells of the first row of a 96-well plate; 50 μl of this medium was used to make serial two-fold dilutions across the remaining rows; and 50 μl of the culture medium was discarded from the last row, which left all wells with 50 μl culture medium. Control wells contained DMSO (0.75%) or 500 nM chloroquine. 50 μl of parasite suspension (2% ring-infected erythrocytes at 4% haematocrit) was added to each well, and plates were incubated in a *modular incubator chamber* (*Billups-Rothenberg, Inc.)* with the gas mixture at 37 °C for 50 h. 100 μl lysis buffer (20 mM Tris-Cl, 5 mM EDTA, 0.008% saponin, 0.08% Triton X-100, pH 7.5) with SYBR Green 1 (at the manufacturer's recommended dilution) was added to each well at the end of incubation, the plate was incubated at 37 °C for 30 min to allow cell lysis, and fluorescence was measured (Ex: 485 nm, Em: 530 nm, gain setting: 50) using an Infinite M200 multimode microplate reader (TECAN) as described previously [33]. 500 nM chloroquine completely inhibits the development of 3D7 strain; hence, fluorescence value of the wells containing chloroquine was subtracted from inhibitor-treated and DMSO-containing cultures to account for background fluorescence. Fluorescence values of cultures treated with different concentrations of compounds or ciprofloxacin were normalized as percentage of DMSO-treated cultures, plotted against the concentrations, and analyzed using nonlinear regression analysis to determine IC_{50} concentrations as described earlier [34].

4.2.1.1. Cytotoxicity MTT assay procedure. Cytotoxicity was measured using the MTT assay, according to the method of Mossman (1983). Briefly, the cells (5×10^3) were seeded in each well containing 100 μl of medium in 96 well plates. After overnight incubation at 37 °C in 5% CO_2, the cells were treated with 100 μl of medium with various product concentrations at identical conditions and incubated for 72 h. The cell viability was assessed after 24 h, by adding 10 μl of MTT (5 mg/ml) per well. The plates were incubated at 37 °C for additional 3 h. The medium was discarded and the formazan blue, which formed in the cells, was dissolved with 100 μl of DMSO. The rate of color formation was measured at 570 nm in a spectrophotometer (Spectra MAX Plus; Molecular Devices; supported by SOFTmax PRO-5.4). The percent inhibition of cell viability was determined with reference to the control values (without test compound). The data were subjected to linear regression analysis and the regression lines were plotted for the best straight-line fit. The IC_{50} (inhibition of cell viability) concentrations were calculated using the respective regression equation.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.03.010.

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