Pyrrolidine-Acridine hybrid in Artemisinin-based combination: a pharmacodynamic study

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SUMMARY

Aiming to develop new artemisinin-based combination therapy (ACT) for malaria, antimalarial effect of a new series of pyrrolidine-acridine hybrid in combination with artemisinin derivatives was investigated. Synthesis, antimalarial and cytotoxic evaluation of a series of hybrid of 2-(3-(substitutedbenzyl)pyrrolidin-1-yl)alkanamines and acridine were performed and mode of action of the lead compound was investigated. *In vivo* pharmacodynamic properties (parasite clearance time, parasite reduction ratio, dose and regimen determination) against multidrug resistant (MDR) rodent malaria parasite and toxicological parameters (median lethal dose, liver function test, kidney function test) were also investigated. 6-Chloro-*N*-(4-(3-(3,4-dimethoxybenzyl)pyrrolidin-1-yl)butyl)-2-methoxyacridin-9-amine (*15c*) has shown a dose dependent haem bio-mineralization inhibition and was found to be the most effective and safe compound against MDR malaria parasite in Swiss mice model. It displayed best antimalarial potential with artemether (AM) *in vitro* as well as *in vivo*. The combination also showed favourable pharmacodynamic properties and therapeutic response in mice with established MDR malaria infection and all mice were cured at the determined doses. The combination did not show toxicity at the doses administered to the Swiss mice. Taken together, our findings suggest that compound *15c* is a potential partner with AM for the ACT and could be explored for further development.

Key words: Malaria, multidrug resistant, pyrrolidine-acridine hybrid, ACT, combination therapy, pharmacodynamics.

INTRODUCTION

Malaria continues to be the most prevalent parasitological diseases across the world, representing a global public health problem with approximately 300 million cases and 0·6 million deaths annually (World Health Organization, 2014). Although efforts to develop vaccine against the disease are yet to be translated to clinics, malaria can be effectively cured by chemotherapy. Unfortunately there was extensive resistance of malaria parasite to most of the available antimalarials including chloroquine, mefloquine, amodiaquine, pyrimethamine, sulfadoxine, atovaquone or their combinations thereof in specific areas. As a result, new antimalarials representing artemisinin or its derivatives, which kill the parasite rapidly became the drug of choice. However, artemisinin monotherapy has been banned to prevent the origin of resistance and only combination therapy is recommended, which also reduce transmission (White, 2008*a*, *b*). However, there are now evidences that parasite has developed resistance against artemisinin in Cambodia, the Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam (Noedl *et al*. 2008; Okombo *et al*. 2014; Wells *et al*. 2015; WHO, 2016). Continued use of antimalarials with high level of resistance as the partner drug in artemisinin-based combination therapy (ACT) may enhance the chances of further drug resistance thereby increasing the malaria mortality rate (Bloland, 2001; White, 2008*a*, *b*). Therefore, development of new antimalarial agents directed towards potential and safe targets and their effectiveness in combination therapy could be a successful way forward in curing the malaria infection (Fong *et al*. 2015).

The acridine core is a privileged unit in medicinal chemistry as it is represented in several analogues endowed with diverse biological activities including antileishmanial, antitrypanosomal (Gamage *et al*. 1994), antitumour, (Baguley *et al*. 1981), antiprion (May *et al*. 2006) and anti-Alzheimer (Fang *et al*. 2008). In context of their antimalarial activity, different modes of action of acridine derivatives

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have been proposed *viz*. DNA intercalation (Chen *et al*. 1978), haem-binding (Dorn *et al*. 1998) and inhibition of topoisomerase II (Gamage *et al*. 1994). An acridine-based antimalarial pyronaridine, was demonstrated to be effective against chloroquine resistant strain of *Plasmodium falciparum* (*P. falciparum*) (Chang *et al*. 1992). Later, we and other independent groups also reported the antimalarial effect of pyronaridine against multidrug resistant (MDR) malaria parasite (Looareesuwan *et al*. 1996; Ringwald *et al*. 1996; Dutta *et al*. 2000; Tripathi *et al*. 2000). Recently pyronaridine-artesunate combination had been shown to be effective and safe for uncomplicated malaria in different clinical trials (Duparc *et al*. 2013) and this has been commercialized as Pyramax®. In a separate study it was reported that pyrrolidinoaminoalkane class of compounds show effectiveness as CQ-resistance reversers (De *et al*. 1993; Walter *et al*. 1993; Batra and Bhaduri, 1997). We anticipated that covalently linking the pyrrolidinoaminoalkane to acridine would offer a new series of hybrid compounds, which may display antimalarial effect. It may be noted that literature include reports of antimalarial effect of hybrid compounds wherein the 9-aminoacridine is covalently linked to polyarylmethyl group *III* & *IV* (Gemma *et al*. 2009), imidazole group *V* (Fattorusso *et al*. 2008), artemisinin *VI* & *VII* (Jones, 2009) or quinoline *VIII* (Kumar *et al*. 2010) (Fig. 1A). In this context we prepared a series of hybrid compounds belonging to prototype *IX* in which the pyrrolidinoaminoalkane subunit is tethered to 6-chloro-2-methoxyacridine at 9-position and investigated their antimalarial activity (Fig. 1B). The most active compound of the series was further examined for its potential as partner to artemisinin derivatives for the ACT. The drug interaction, pharmacodynamics and toxicological parameters of the hybrid compound and artemether (AM) were studied to assess the dose and regimen.

MATERIALS AND METHODS

Chemicals

AlbumaxII, Sybr Green-I, were purchased from Invitrogen. RPMI-1640, hypoxanthine and saponin were bought from Sigma Aldrich. Tween-20 and Giemsa's stain were procured from Merck, India. The chemicals used in the synthesis were either procured from Sigma-Aldrich or Spectrochem, India whereas the solvents were procured from Spectrochem, India, Central Drug House, India or Merck, India.

Animals

Outbred Swiss mice of 6–8 weeks (20–22 g and either sex) were procured from National Laboratory Animal Centre, CSIR-Central Drug Research Institute, Lucknow, India. Animals were housed in polypropylene cages (~100 cm^2 areas per mouse) on paddy husk saw dust with proper ventilation, 12 h light/dark cycle and food water *ad libitum*. Housing temperature was maintained at 22–24 °C and humidity was controlled to 45–60%. All studies were conducted under approved protocol by the Institutional Animal Ethics Committee of CSIR-Central Drug Research Institute, Lucknow, India.

Chemistry

General Procedure for the synthesis of N-(2-(3-benzylpyrrolidin-1-yl)alkyl)-6-chloro-2-methoxyacridin-9-amines 13a-e-15a-e *as exemplified for 6-chloro-N-(4-(3-(3,4-dimethoxybenzyl)pyrrolidin-1-yl) butyl)-2-methoxyacridin-9-amine* 15c. To a solution of *12c* (1·0 g, 3·42 mmol) in acetonitrile (20 mL) were added Et_3N (1·75 mL, 13·7 mmol) and 6,9-dichloro-2-methoxyacridine (0·95 g, 3·42 mmol). The reaction mixture was heated at reflux and after completion as monitored by thin layer chromatography (TLC), the solvent was removed under vacuum. The residue was extracted with $CHCl_3$ (3 mL × 50 mL) and water (50 mL) and the organic phases were combined, dried over Na_2SO_4 and concentrated in vacuum to obtain the crude product. Purification of the crude product by column chromatography over basic alumina using MeOH/$CHCl_3$ (1:99, v/v) as eluent afforded 1·26 g (69%) of *15c* as yellow oil.

6-Chloro-N-(4-(3-(3,4-dimethoxybenzyl)pyrrolidin-1-yl) butyl)-2-methoxyacridin-9-amine (15c). $R_f = 0·53$ (MeOH/$CHCl_3$, 10:90, v/v); IR (Neat) $\nu_{max} = 3321$ (NH) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) $\delta =$ 1·65–1·72 (m, 2H, CH_2), 1·80–1·84 (m, 5H, 2 × CH_2 and C*H*H), 2·13–2·18 (m, 1H, CH*H*), 2·45–2·56 (m, 4H, 2 × CH_2), 2·59–2·76 (m, 3H, CH_2 and CH), 3·72–3·77 (m, 2H, CH_2), 3·84 (s, 3H, OCH_3), 3·85 (s, 3H, OCH_3), 3·95 (s, 3H, OCH_3), 6·67–6·78 (m, 3H, ArH), 7·26–7·28 (m, 2H, ArH), 7·41 (dd, 1H, $J_1 = 2·6$ Hz, $J_2 = 9·4$ Hz, ArH), 7·98–8·05 (m, 3H, ArH); ^{13}C NMR (75 MHz, $CDCl_3$) $\delta =$ 26·6, 29·7, 30·5, 39·2, 41·2, 50·8, 53·5, 54·2, 55·7, 56·0, 56·1, 60·2, 100·2, 111·3, 112·1, 115·7, 117·8, 120·5, 124·3, 128·1, 131·3, 133·8, 134·9, 146·8, 147·4, 148·4, 148·9, 150·3, 155·8; ESI-MS $m/z = 534·3$ $[M + H]^+$; ESI-HR-MS $m/z =$ 534·2518, calcd. for $C_{31}H_{36}ClN_3O_3$ $[MH]^+$ 534·2523.

In vitro *antimalarial evaluation*. The compounds were evaluated for antimalarial activity against 3D7 (CQ-sensitive) as well as K1 (CQ-resistant) strains of *P. falciparum* using Malaria SYBR Green I nucleic acid staining dye based fluorescence (MSF) assay as mentioned by Singh *et al*. (2011).

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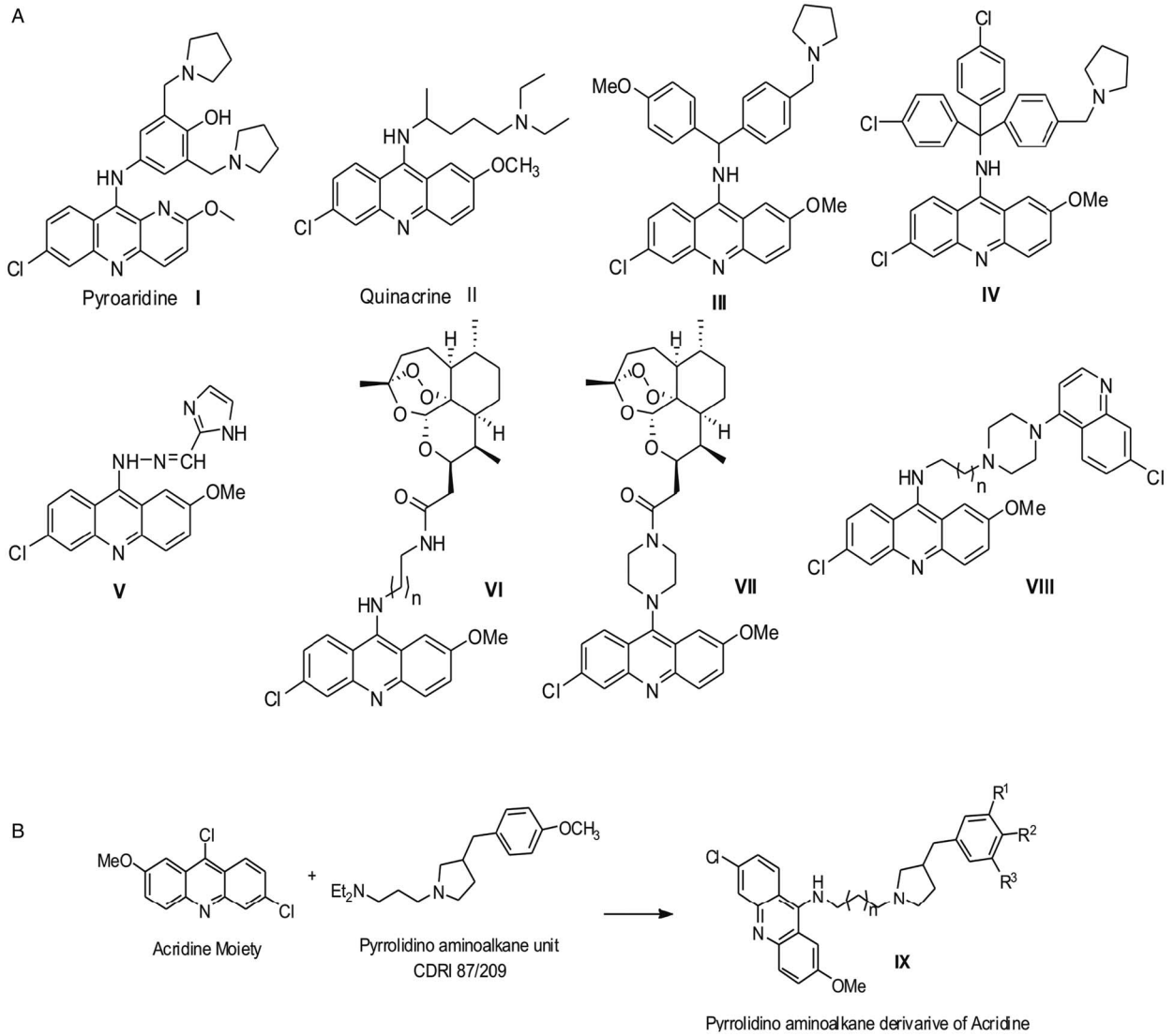

Fig. 1. (A) Structure of acridine-based hybrid compounds, (B) proposed structure of acridine tethered pyrrolidino aminoalkane.

Experiments were performed in triplicates and repeated at least three times.

Cytotoxicity assay. Cytotoxicity of the compounds was carried out using Vero cell line (C1008; Monkey kidney fibroblast) following the method as mentioned in Sharma *et al*. (2014). The cells were incubated with compound-dilutions for 72 h and MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used as reagent for detection of cytotoxicity. Fifty per cent cytotoxic concentration (CC_{50}) was determined using non-linear regression analysis of dose response curves using pre-programmed Excel spreadsheet. Selectivity index (SI) was calculated as

$$SI = CC_{50}/IC_{50}$$

In vitro *combinations assay*. *In vitro* interaction of newly synthesised compound (15c) and antimalarials arteether (AE), AM and artesunate (AS) were determined according to Pandey *et al*. (2013). Experiments were performed in triplicates and repeated at least three times. Fractional inhibitory concentration (FIC) was interpreted by the following formula and subsequent isobologram were plotted.

FIC = Conc. of drug in combination to produce IC_{50}/Conc. of drug alone require to produce IC_{50}.

The sum FIC (ΣFIC) value for each of the preparations determined by the following formula was used to classify the drug–drug interaction.

$$\Sigma FIC = \frac{IC_{50}\text{ of drug A in combination}}{IC_{50}\text{ of drug A alone}} + \frac{IC_{50}\text{ of drug B in combination}}{IC_{50}\text{ of drug B alone}}$$

ΣFIC < 1 represents synergism, ΣFIC = 1 represents additive interaction while >1 was considered as antagonistic (Bell, 2005; Kelly *et al*. 2009). Mean

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ΣFIC for all the tested ratios were used to classify the overall trend of the interaction.

In vivo -*day 4 parasitaemia suppression test*. For *in vivo* evaluation, drugs were administered orally as Tween 80-water formulation. Different groups of Swiss mice (5 mice/group) were inoculated with 5×10^5 *Plasmodium yoelii nigeriensis* MDR infected erythrocytes obtained in anticoagulant through cardiac puncture from infected mice. After 2–4 h of infection, these groups were treated with daily oral doses for 4 days (0, 1, 2, and 3) at 100 mg kg^{-1} day^{-1}. Mice in the control group were injected with parasites only and treated with vehicle. Parasitaemia was monitored on day 4 by Giemsa stained thin blood smears and suppression was determined relative to the control group.

Determination of antimalarial response of the lead combinations against P. yoelii nigeriensis *MDR*. Different groups of Swiss mice (10 mice per group) were inoculated with 5×10^5 *P. yoelli nigeriensis* MDR -infected erythrocytes. For simultaneous drug response assessment, these groups were treated with daily oral doses of alone partners (AM or 15c) and their combinations at 2–4 h after infection whereas treatment was done after observation of parasitaemia in the blood of infected mice (5 mice per group) for the evaluation of therapeutic antimalarial response. Mice in the control group were injected with parasites only and treated with vehicle.

Parasitaemia was monitored by Giemsa stained thin blood smears on pre-determined days and survival of the mice was duly recorded (Tripathi *et al*. 2011).

Determination of pharmacodynamic properties. The pharmacodynamic properties (parasite reduction ratio (PRR), parasite clearance time (PCT), recrudescence studies etc.) were explored according to the protocol defined by White (1997) with some modifications. Briefly, a calculated amount of parasites was administered intraperitonially to a group of 5 Swiss mice and parasitaemia was determined on the successive days. Upon appearance of parasitaemia in the blood, single dose of the different combinations as well as individual drugs were given. Giemsa's stained thin blood smears were examined under 100X objective (oil immersion). The PRR was calculated as per the formula

$$\tau = P_0/P_2$$

P_0 is parasite burden per micro litre (μL) at the time of drug treatment.

P_2 is parasite burden μL after 24 h of drug treatment.

The parasite burden was calculated as follows:

Parasite burden (P) per $\mu L = 4 \times 10^6 \times$ parasitaemia 100^{-1} (The Swiss mice have 4×10^6 RBCs per μL of blood).

The PCT (T) was determined with the following assumption: if the blood volume of an average mouse of 20–22 g is assumed to be 2·0 mL and P is the parasite count per μL, then the total parasite burden (B) is given by:

$$B = 2 \times 10^3 \times P = 2P \times 10^3$$

Therefore,

$$\log_e B = \log_e P + 7 \cdot 6$$

If $\tau = P0/P_2$, then the time (T) in days for which parasites are present in the body (with an asexual cycle of ~1 day) is given by:

$$T = (\log_e P + 7 \cdot 6)/\log_e \tau$$

T is the time for which therapeutic concentration of an antimalarial drug must be present in the host plasma, P is the parasite burden per μL and τ is parasite reduction ratio.

Determination of median lethal dose. Median lethal dose (LD_{50}) was determined according to the method of Srivastava *et al*. (2005) with some modifications. Briefly, higher oral doses of test compound was given to four groups of 5 Swiss mice (20–22 g) and housed according to ethical guidelines. Mice were observed for 2 days and mortality was duly recorded. On the basis of mortality in the different groups, LD_{50} was calculated with the help of Prism software.

Biochemical evaluation of liver, kidney and blood parameters. Four groups of Swiss mice (5 mice per group) were treated with alone partners (200 mg kg^{-1} day^{-1} 15c or 60 mg kg^{-1} day^{-1} AM) or their combinations for 4 days. Blood was withdrawn by cardiac puncture and allowed to stand undisturbed for 30 min. Serum was separated and levels of urea, blood urea nitrogen, total bilirubin, creatinine, alanine transaminase, aspartate aminotransferase and alkaline phosphatase were estimated using fully automated biochemical analyser (Merck-selectra junior).

Assessment of haem bio-mineralization *inhibition*. Haem biomineralization inhibition in the extract of *P. yoelii*-infected erythrocytes was assayed by a method described by Agrawal *et al*. (2002) and the quantification of hemozoin was done using an extinction coefficient of 91 mM^{-1} cm^{-1} at 400 nm.

Statistical analysis

Logit regression analysis of dose response curves using pre-programmed Excel spreadsheet (a kind

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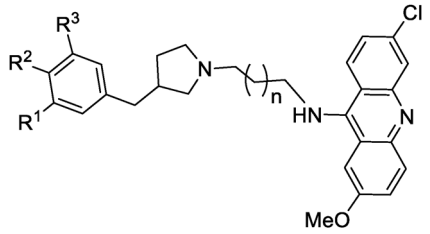
Table 1. *In vitro* antimalarial activity of pyrrolidinoaminoalkane derivatives of acridine against CQsensitive (3D7) strain and CQ resistant (K1) strain of *P. falciparum*. Experiments were performed in triplicates and repeated 3 times.



Compound no.	R^1	R^2	R^3	n	IC_{50} against *Pf*3D7 (nM)	SI for *Pf* 3D7	IC_{50} against *Pf* K1 (nM)	SI for *Pf* K1	LogP	CLogP
13a	H	H	H	0	258	43	351	32	5·79	7·78
13b	H	OMe	H	0	311	27	342	25	5·66	7·57
13c	H	OMe	OMe	0	190	41	109	37	5·53	7·44
13d	OMe	OMe	OMe	0	39	377	3492	1·6	5·41	7·08
13e	OCH_2O		H	0	54·0	246	3267	11	5·57	7·75
14a	H	H	H	1	279	532	1117	3·6	5·89	8·05
14b	H	OMe	H	1	360	30	1361	13	5·77	7·82
14c	H	OMe	OMe	1	28	120	78·4	23	5·64	7·71
14d	OMe	OMe	OMe	1	3·7	2829	9·5	609	5·51	7·35
14e	OCH_2O		H	1	432	1·5	1558	10	5·67	8·01
15a	H	H	H	2	80	2246	2998	18	6·35	8·10
15b	H	OMe	H	2	17	628	10·9	495	6·22	7·85
15c	H	OMe	OMe	2	30·3	140	9·6	236	6·09	7·75
15d	OMe	OMe	OMe	2	9·3	19 011	5·2	3400	5·97	7·40
15e	OCH_2O		H	2	1·7	1767	109.	14	6·12	8·06
CQ	–		–	–	6·3	23 305	253	495	3·73	5·06

gift from Drugs for Neglected Diseases Initiative-Geneva) was done for IC_{50} assessment. Mean, S.D. and S.E.M. were also calculated using Excel spreadsheet. Two tailed student *t*-test was performed for significance.

RESULTS

Chemistry

The synthesis of the title compounds is outlined in the Supplementary Material (Scheme 1). The dimesylates *6a-e* were prepared from substituted benzaldehyde and γ-butyrolactone via the reported procedure (Batra *et al*. 2000). Reaction of *6a-e* with several mono-Boc protected diamines afforded the Boc-protected pyrrolidinoaminoalkanes *7a-e-9a-e*. Deprotecting the Boc-group in *7–9* with 4 N aq. HCl gave the free amines *10a-e-12a-e*. Treating these free amines with 6,9-dichloro acridine in the presence of Et_3N in MeCN as medium produced the final products *13a-e-15a-e* in moderate to good yields. All prepared compounds were investigated for their *in vitro* and *in vivo* antimalarial efficacy.

In vitro *antimalarial activity and cytotoxicity*

The compounds *13a-e-15a-e* were subjected to *in vitro* antimalarial assay against CQ-sensitive (3D7) as well as CQ resistant (K1) strains of *P. falciparum*. The antimalarial activity (IC_{50}, nM) was quantified as 50% inhibition of parasite growth and the results are reported in Table 1. The two points of variations were the phenyl ring and the alkane spacer. The five substitutions on the phenyl ring in compounds of *13a-e-15a-e*, included hydrogen, 4-methoxy, 3,4-dimethoxy, 3,4,5-trimethoxy and 3,4-methylenedioxy, whereas the three variations in the alkane spacer included $n = 0$, 1, 2. As illustrated in Table 1, out of 15 compounds from the series, 8 compounds displayed antimalarial efficacy between 1·7 and 54 nM against 3D7 strain of *P. falciparum*. In general compounds (*15*) having butyl spacer displayed relatively potent activity as compared with the compounds bearing propyl (*14*) or ethyl spacer (*13*). On the other hand compounds bearing substitutions on the phenyl ring displayed better antimalarial effect as compared to the compounds with unsubstituted phenyl ring (*13a, 14a, 15a*). All compounds from *13a-e-15a-e* besides having potent antimalarial efficacy displayed good SI. Compound *14d*, which displayed the activity with $IC_{50} = 3·7$ nM showed SI of 2829, which was considered to be safe. Similarly *15d* was also found to be safe having also good SI value of 19 011.

Next the antimalarial effect of these compounds against CQ-resistant (K1) strain of *P. falciparum* was investigated. The IC_{50} of CQ for this strain was

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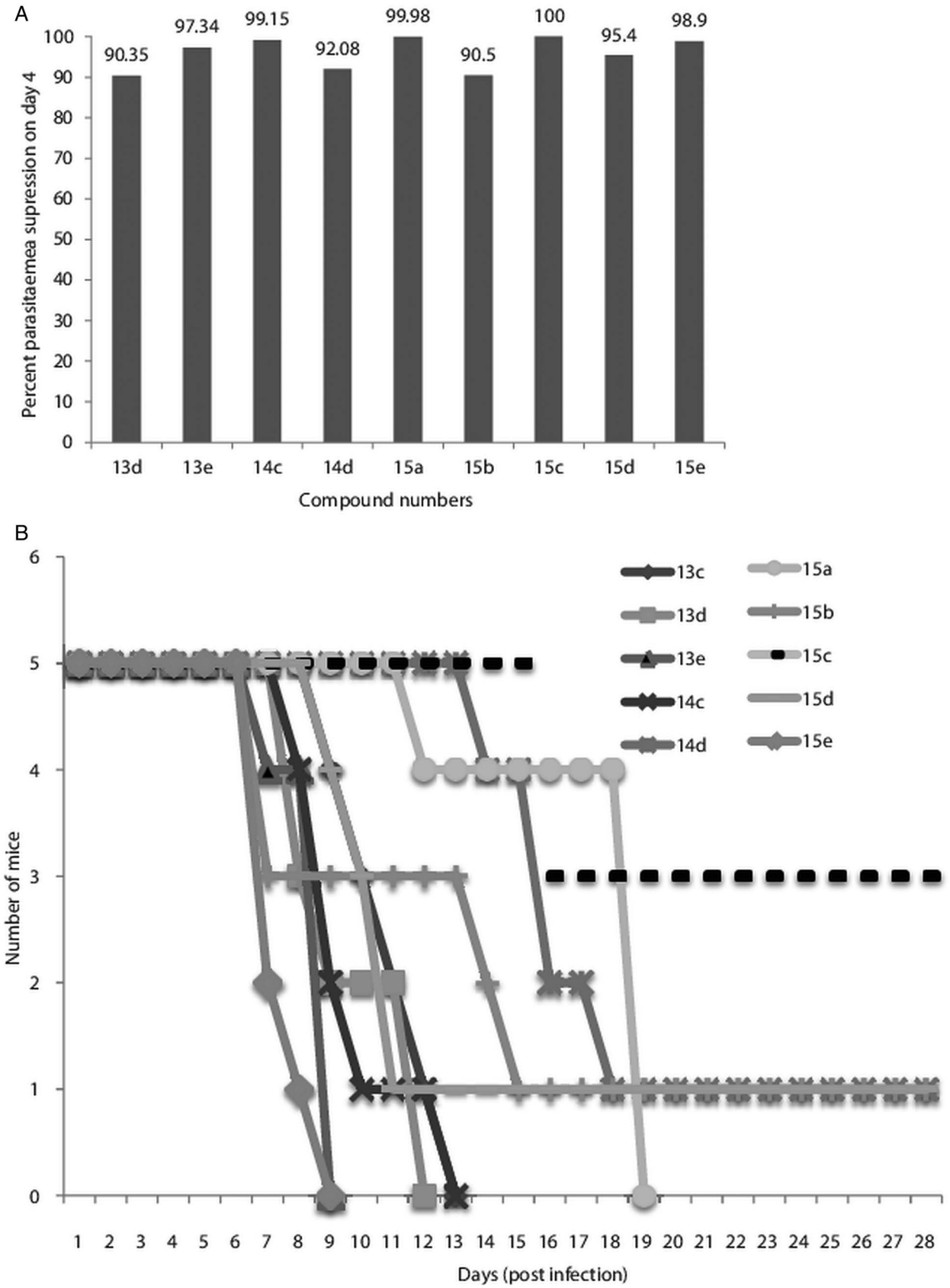

Fig. 2. Antimalarial potential of selected 3-(substituted benzyl)-pyrrolidino-aminoalkanes (A) Parasitaemia suppression on day4 at 100 mg $kg^{-1} \times 4$ doses against *P.yoelii nigeriensis* multidrug resistant (MDR) infected Swiss mice. (B) Mice survival graph.

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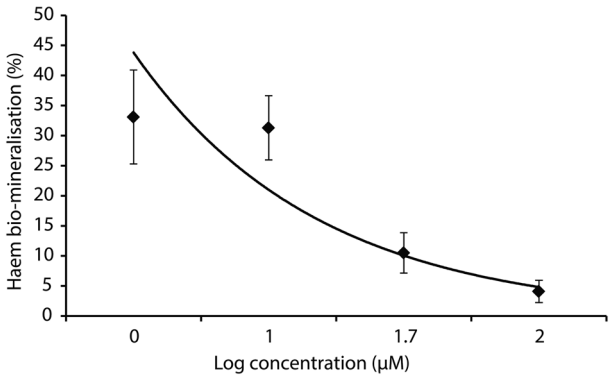

Fig. 3. Dose dependent inhibitory activity of *15c* on haem bio-mineralization. Error bars represent mean ± standard deviation.

observed to be 253 nM. Compounds *14c-d and 15b-e* displayed IC_{50}, which was several folds better as compared with CQ. Interestingly compound *15e*, which had better efficacy than CQ against CQS strain was found to be relatively less potent as compared with other analogues. Nevertheless compounds *14d* and *15d* bearing trimethoxy substitution on the phenyl ring in the pyrrolidine moiety elicited potent antimalarial effect with IC_{50} of 9·53 and 5·2 nM, respectively. Compound *15d* also had a good SI of 3400 as compared with 495 of CQ. However, several of these compounds (*13d-e, 14a-b, 14b, 15a* and *15e*) were less efficacious in K1 when compared with their activity in 3D7. Interestingly, *15c* was found to have more potent antimalarial activity in CQ-resistant K1 strain.

Day 4 Parasitaemia suppression

Based on the results of the *in vitro* assays of these compounds, a few of them were examined for their *in vivo* antimalarial activity against MDR strain *P. yoelii nigeriensis* in outbred Swiss mice at a dose of 100 mg kg^{-1} day^{-1} × 4 days *via* oral route and the results are presented in Fig. 2. It was observed that all compounds at a dose of 100 mg kg^{-1} × 4 days dose showed >90% suppression of parasitaemia on day 4. However, compound *15c* having dimethoxy substitution on the phenyl ring and 4-carbon spacer could make survival of 3 out of 5 mice beyond 28 days of observation period.

Mode of action and in vitro *antimalarial interaction of 15c with artemisinin derivatives*

In an attempt to investigate the possible mode of action of *15c*, the haem bio-mineralization inhibition assay was performed wherein *15c* showed a dose dependent haem bio-mineralisation inhibitory activity against *P. yoelii nigeriensis* (Fig. 3). Antimalarial activity of *15c* in combination with artemisinin derivatives AM, AE and AS was investigated

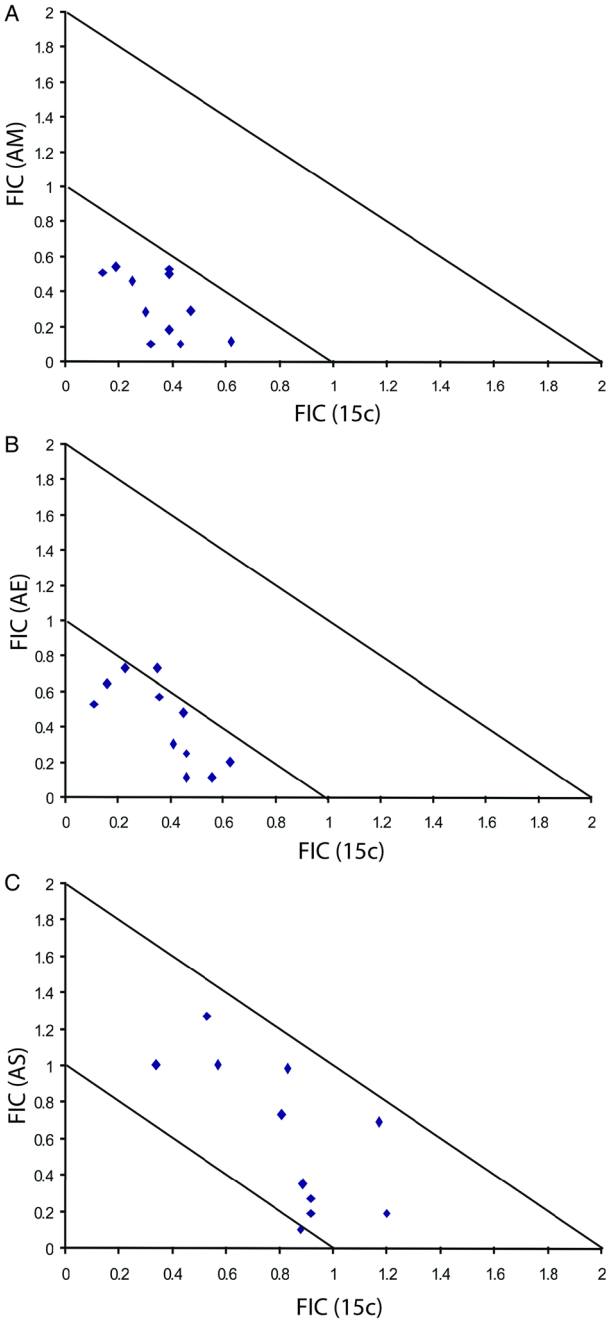

Fig. 4. Isobologram representing antimalarial interaction of 15c with artemisinin derivatives with (A) artemether, (B) arteether, (C) artesunate. Diagonal lines presents the boundaries of synergy (<1), additivity (1) and antagonism (>1).

against 3D7 strain of *P. falciparum*. Interaction of *15c* with AM and AE were found to be slightly synergistic (mean ΣFIC 0·68 ± 0·16 and 0·80 ± 0·15, respectively) whereas it was slightly antagonistic with AS (mean ΣFIC 1·43 ± 0·31). From the results it was concluded that the differences between the interactions of *15c* with the 3 artemisinins were minor. These interactions were supported by the isobolograms representing the interactions between the partner drugs (Fig. 4).

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Table 2. Antimalarial potential (prophylactic) of 15c with artemether against *P. yoelii nigeriensis* multidrug resistant in Swiss mice.

Drug/ combination	Dose (Mg kg^{-1} × 4days)	Parasitaemia (Mean ± S.D.) at different days (number of mice without parasitaemia/total survived mice)							Mean survival time (Days)	% cure
		Day4	Day7	Day10	Day14	Day18	Day21	Day24	Day28		
15c + AM	100 + 25	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	>28	100
,,	50 + 25	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	>28	100
,,	100 + 12·5	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	>28	100
,,	50 + 12·5	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	>28	100
,,	100 + 6·25	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	>28	100
,,	50 + 6·25	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	>28	100
,,	– + 25	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	0 ± 0 (10/10)	>28	100
,,	– + 12·5	0 ± 0 (10/10)	0·002 ± .006 (9/10)	9·4 ± 26·89 (8/10)	0 ± 0 (8/8)	7·06 ± 7·95 (4/8)	0 ± 0 (4/4)	0·58 ± 0·63 (2/4)	5 ± 7·07 (2/3)	21·6	20
,,	– + 6·25	0 ± 0 (10/10)	0·42 ± 1·04 (6/10)	6·5 ± 12·31 (6/8)	5·71 ± 13·99 (6/7)	3·67 ± 5·67 (3/6)	21·25 ± 38·6 (3/4)	0·8 ± 1·13 (2/3)	0 ± 0 (2/2)	19·6	20
,,	100 + –	0·05 ± 0·12 (7/10)	0·9 ± 1·58 (6/10)	10·25 ± 23·5 (4/10)	10·33 ± 15·76 (2/6)	9·9 ± 12·97 (1/5)	0·3 ± 0·4 (1/4)	0·01 ± .01 (2/4)	0·002 ± .004 (3/4)	20·2	10
,,	50 + –	0·2 ± 0·25 (3/10)	26·4 ± 27·2 (0/10)	41·2 ± 37·08 (0/6)	15·9 ± 14·1 (0/2)	0·8 ± 0·0 (0/1)	0 ± 0 (1/1)	0·02 ± 0 (0/1)	0 ± 0 (1/1)	13·4	0
Control		39·1 ± 18·6 (0/10)								5·7	0

In vivo *antimalarial potential of 15c with AM against MDR rodent malaria parasite*

Antimalarial potential of oral *15c* with AM, was evaluated against MDR *P. yoelii nigeriensis* in random-bred Swiss mice. Compound *15c* individually and its different combinations with AM were administered orally to different groups of mice. *15c* at 100 mg kg^{-1} day^{-1} and AM at 12·5 mg kg^{-1} day^{-1} separately for 4 days could produce only 10, and 20% cure, respectively, while the combination of lower doses of *15c* (50 mg kg^{-1} day^{-1}) and AM (6·25 mg kg^{-1} day^{-1}) for 4 days, produced 100% cure. Additionally, the mean survival time for alone drugs at higher concentrations (100 mg kg^{-1} *15c* or 12·5 mg kg^{-1} AM) were also short (~20 days) in comparison with the combination at lower doses (50 mg kg^{-1} *15c* with 6·25 mg kg^{-1} AM; >28 days) (Table 2).

In vivo *Pharmacodynamics of 15c with AM*

Pharmacodynamic study showed a gradual decrease in parasitaemia with the single dose of 30 and 60 mg kg^{-1} AM alone and its combination with 200 mg kg^{-1} *15c* while a gradual increase was observed in *15c* group after 12 h of dosing, After 24 h, there was negligible parasitaemia in the AM alone and combination group whereas in *15c* alone group, all the mice possess significant parasitaemia in their blood (Table 3). Divided doses of *15c* 100 mg kg^{-1} at every 12 h could not produce any significant improvements in the results (data not shown).

On the basis of above observations, pharmacodynamic properties, i.e. PRR and PCT were calculated (Table 3). The PCT was much lower and PRR was higher in the combination groups (200 + 60) compared with alone groups. PRR for 30 and 60 mg kg^{-1} AM alone were 12 and 17·3, respectively, while *15c* could not reduce the parasite load. Combination of 30 mg kg^{-1} of AM with 200 mg kg^{-1} of *15c* was not found as much pharmacodynamically favourable over alone treatment with a PRR value of 14. On the other hand, combination of 200 mg kg^{-1} *15c* with 60 mg kg^{-1} AM had reduced the parasite load significantly having the PRR value 880. The calculated PCT in the most potent combination 60 mg kg^{-1} AM + 200 mg kg^{-1} *15c* is found to be much lower than AM alone (2·8 *vs* 6·5 days).

Therapeutic antimalarial potential of 15c with AM against MDR rodent malaria parasite

15c and AM combinations were also evaluated and optimized against established infection of *P. yoelii nigeriensis* in Swiss mice. The combination (200 mg kg^{-1} *15c* + 60 mg kg^{-1} AM) was having the lowest PCT (2·8 days) and highest PRR (880) was found as most appropriate combination.

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Table 3. *In vivo* pharmacodynamics of 15c with artemether.

Drug/combination (Mg $kg^{-1} \times 1$ day) *15c* + AM	Mean % parasitaemia at different time intervals ± S.D.					PRR	PCT (Days)
	0 h	6th hour	12th hour	18th hour	24th hour		
200 + 30	1·26 ± 0·46	1·38 ± 0·38	1·02 ± 0·52	0·42 ± 0·25	0·09 ± 0·1	14	7·05
200 + 60	1·76 ± 0·38	1·52 ± 0·71	0·82 ± 0·41	0·16 ± 0·16	0·002 ± 0·004	880	2·8
– + 30	0·86 ± 0·44	1·26 ± 0·69	0·72 ± 0·56	0·31 ± 0·21	0·07 ± 0·12	12	7·2
– + 60	1·04 ± 0·49	1·24 ± 0·55	0·48 ± 0·39	0·10 ± 0·12	0·06 ± 0·005	17·3	6·5
200 + –	0·98 ± 0·61	2·07 ± 1·2	2·78 ± 1·7	3·92 ± 2·2	12·2 ± 6·34	NA[a]	NA[a]
Control	0·72 ± 0·43	3·16 ± 2·3	3·9 ± 2·8	6·42 ± 4·09	25·6 ± 18·6	–	–

PCT, parasite clearance time; PRR, parasite reduction ratio.
[a] Parasitaemia not reduced.

When 200 mg kg^{-1} *15c* + 60 mg kg^{-1} AM was given to infected Swiss mice for 4 days, no parasitaemia was observed throughout the study and all the mice in the group survived beyond the observation period of 28 days. While the combination having higher PCT of 7·05 (200 mg kg^{-1} *15c* + 30 mg kg^{-1} AM) when given for 4 days, recrudescence occurred on day 24 and cure rate was also reduced to 80%. In alone groups maximum 20% cure was observed and mean survival time was also lower than the combination groups (>28 *vs* <20 days), which further indicated the strong antimalarial potential of *15c* with AM (Table 4).

In vivo *toxicity of 15c in combination with AM*

On the basis of mortality in the different groups of treated mice, LD_{50} of newly synthesized compound 15c was calculated and it was 10 times higher than its curative dose in combination (data not shown).

The potent antimalarial combination (200 mg kg^{-1} *15c* + 60 mg kg^{-1} AM) had no significant effect on key parameters of liver and kidney. All the liver and kidney parameters were found to be normal for both the combinations (data not shown).

DISCUSSION

The increase of resistance by the parasite to common antimalarial chemotherapy was the major reason for WHO advocating the use of ACT as the first-line of drugs in endemic areas. Unfortunately, there are now reports that the malaria parasite has developed resistance to artemisinin derivatives too (Noedl *et al*. 2008; Wells *et al*. 2015). Furthermore, the continued application of ineffective antimalarial as a partner drug in ACT may increase the chances of drug resistance thereby leading to rise in malaria mortality. Therefore, ACT with a new partner drug directed towards safer target might be effective and well tolerated (Bloland, 2001; White, 2008*a*, *b*). The present study concerns with exploration of pyrrolidinoaminoalkane-acridine hybrids as a partner in ACT and its pharmacodynamic studies.

The antimalarial potential of mepacrine (quinacrine), an acridine derivative was reported 80 years ago. It had been demonstrated to not only inhibit the asexual stages of malaria parasite but also display inhibitory effect on gametocytes. Notably, it is found to be effective against chloroquine and pyrimethamine resistant malaria parasite (Chavalitshewinkoon-Petmitr *et al*. 2001; Schlitzer, 2007). More recently, pyronaridine another acridine derivative incorporating the pyrrolidine unit in combination with AS is being used in clinics as Pyramax® and is considered to be a promising ACT for uncomplicated *falciparum* malaria (Croft *et al*. 2012). Acridine derivatives show wide spectrum effect on malaria parasite metabolic pathways such as inhibition of hemozoin (β-hematin) formation, mitochondrial bc_1 complex and DNA topoisomerase II and interaction with DNA (Ramharter *et al*. 2008; Valdés, 2011; Padmanaban *et al*. 2012). Therefore we envisaged to prepare a new series of acridine hybrid, which carried the pyrrolidine unit, which in itself had shown the potential as CQ-resistance reverser. We found that all the compounds displayed potent antimalarial activity *in vitro*. Among the series, compound *15c* displayed the best *in vivo* antimalarial potential. As a consequence compound *15c* was selected to examine the *in vitro* as well as *in vivo* antimalarial efficacy in combination with the three most active and widely used artemisinine derivatives *viz*. AM, AE and AS. It is known that if the combination of two drugs has ΣFIC of <1, then the combination is termed as synergistic whereas if it is 1 it is considered to be additive. During the assessment of the combinations it was found that mean ΣFIC value for the *15c* + AM was 0·68 whereas it was 0·80 for *15c* + AE thereby indicating the overall slight synergistic antimalarial potential of *15c* in combination with AM or AE *in vitro*. The ΣFIC value for *15c* + AS combination was found to be >1 suggesting a marginal trend towards antagonism. This might be due to higher *in vitro* antimalarial activity of AS than AM or AE (~2 times; our unpublished data) and *15c* failed to enhance the activity of AS above that level.

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Table 4. Therapeutic antimalarial potential of 15c with artemether against *P. yoelii nigeriensis* multidrug resistant in Swiss mice.

Drug/ combination	Dose (Mg $kg^{-1} \times 4$ days)	Parasitaemia (Mean% ± S.D.) at different days (number of mice without parasitaemia/total survived mice)								Mean survival time (Days)	% cure
		0 h	Day4	Day7	Day10	Day14	Day18	Day21	Day24	Day28		
15c + AM	200 + 60	0·5 ± 0·32 (0/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	>28	100
,,	200 + 30	1·02 ± 0·32 (0/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0 ± 0 (5/5)	0·56 ± 0·0 (4/5)	0 ± 0 (5/5)	>28	80
,,	– + 60	0·81 ± 0·38 (0/5)	0 ± 0 (5/5)	0·54 ± 0·75 (3/5)	14·28 ± 17·33 (1/5)	25·25 ± 16·75 (0/2)	0·1 ± 0·0 (0/1)	0 ± 0 (1/1)	0 ± 0 (1/1)	0 ± 0 (1/1)	16·6	20
,,	– + 30	0·76 ± 0·34 (0/5)	0 ± 0 (5/5)	13·14 ± 25·53 (3/5)	19·16 ± 18·36 (0/3)	Died					12·6	0
,,	200 + –	0·38 ± 0·28 (0/5)	0·04 ± 0·08 (4/5)	5·18 ± 3·52 (0/5)	32·3 ± 17·68 (0/5)	38·3 ± 29·52 (0/4)	32·33 ± 32·04 (1/2)	0 ± 0 (1/1)	0 ± 0 (1/1)	0 ± 0 (1/1)	19·2	20
Control	–	0·4 ± 0·38 (0/5)	90 ± 0 (0/1)	Died							5·2	0

The synergistic *in vitro* interaction of *15c* with AM invoked us to combine *15c* with AM for its antimalarial response *in vivo* against MDR malaria parasite. It was encouraging to discover that the combination of *15c* with AM enhanced the antimalarial response of both the partners. Compound *15c* could cure only 10% of the Swiss mice infected with *P. yoelii nigeriensis* MDR at 100 mg $kg^{-1} \times 4$ days and AM at its 12·5 mg $kg^{-1} \times 4$ days dose produced only 20% cure. Interestingly, a combination with lower doses of same partners (50 mg $kg^{-1} \times 4$ days *15c* with 6·25 mg $kg^{-1} \times 4$ days AM) potentiate the curative effect up to 100% and also enhanced the mean survival time.

The inappropriate treatment strategies with antimalarials induce the problems of recrudescence, toxicity of a particular drug and its improper therapeutic response. Therefore the pharmacokinetic-pharmacodynamic (PK-PD) properties (curative doses, maximum effect produced by the drug, parasite multiplication rate, parasite reduction ratio, PCT and recrudescence study) of the drug should be explored appropriately before its application in the patients. As a consequence, to determine a proper dose and regimen, the pharmacodynamic studies of the combination of 15c + AM were carried out and the pharmacodynamic parameters were found to be favourable in the group treated with 200 mg kg^{-1} 15c in combination with 60 mg kg^{-1} AM in comparison with alone treatment. The PCT was lower in the combination group (2·8 days in combination *vs* 6·5 days in alone group) while PRR was higher (880 in combination *vs* 17·3 in alone group). However, combination of 200 mg kg^{-1} *15c* with 30 mg kg^{-1} AM could not show any improvement in pharmacodynamics properties over alone treatment. Low PCT and high PRR values indicate the combination to be pharmacological as well as pharmacodynemic worth pursuing (White, 1997).

Our *in vivo* screening data for established infection also validated the pharmacodynamic studies. When 200 mg kg^{-1} *15c* + 60 mg kg^{-1} AM was given for 4 consecutive days all the infected mice were cured and no parasitaemia was observed till the end of the experiment, while 200 mg kg^{-1} *15c* + 30 mg kg^{-1} AM (required PCT 7·05 days) when given for 4 days there was only 80% cure. However, both the combinations were better than their individual effects. Moreover, low PCT and high PRR value may be considered helpful in reducing the chances of drug resistance (White, 1997).

Most of the acridine derivatives are associated with toxicity. For example, Loiseau and Nguyen (1996) reported that a new series of acridine derivatives, which possess potent antimalarial activity against murine malaria model showed a high level of toxicity. Moreover, single oral dose toxicology studies with Pyramax in the rat displayed toxicity characteristically reflected by decreased body weight gain, diarrhoea

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and soft stools at ⩾1000 mg kg^{-1} and chromaturia at 2000 mg kg^{-1}. These observations were in line with the inherent acute toxicity expressed by pyronaridine alone (Croft *et al*. 2012). Interestingly, compound *15c* has not shown hepatotoxicity or nephrotoxicity and displayed acceptable safety index, both *in vitro* and *in vivo* (LD_{50} ~ 2000 mg kg^{-1} alone and safety index of ~10 in combination).

In summary, we have synthesized and investigated the antimalarial efficacy of a new series of acridine-based hybrids. The compounds displayed oral efficacy against MDR *P. yoelii nigeriensis* in mice. Further it was shown that one of the derivatives (*15c*) serves as good partner in ACT in particular with AM. However, detailed absorption-distribution-metabolism-excretion and clinical safety studies are required to utilize this combination for further development.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at http://dx.doi.org/10.1017/S0031182016000937.

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