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Bioorganic & Medicinal Chemistry 16 (2008) 6027–6033
Bioorganic & Medicinal Chemistry

Pyridinium cationic-dimer antimalarials, unlike chloroquine, act selectively between the schizont stage and the ring stage of *Plasmodium falciparum*

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Received 24 March 2008; revised 19 April 2008; accepted 22 April 2008
Available online 25 April 2008

Abstract—Malaria is a leading cause of death in developing countries, and the emergence of strains resistant to the main therapeutic agent, chloroquine, has become a serious problem. We have developed cationic-dimer type antimalarials, MAP-610 and PMAP-H10, which are structurally different from chloroquine. In this study, we introduced several substituents on the terminal phenyl rings of PMAP-H10. The electronic and hydrophobic properties of the substituents were correlated with the antimalarial activity and cytotoxicity of the compounds, respectively. Studies with synchronized cultures of malarial plasmodia showed that our cationic-dimers act selectively between the schizont stage and the ring stage of the parasitic cycle, unlike chloroquine, which has a stage-independent action. Thus, the mechanism of action of our antimalarials appears to be different from that of chloroquine, and our compounds may be effective against chloroquine-resistant strains.

1. Introduction

Malaria kills 1–1.5 million people and affects 300–500 million people every year, and the total number of malaria-affected individuals is estimated to be up to about 800 million.[1,2] The problem is most serious in sub-Saharan African countries. Although the number of countries where malaria is prevalent has been decreasing,[2] the movement of people to infected areas has caused new social problems.[3] Pathogenic malaria parasites include *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*.[4] Among them, *Plasmodium falciparum* (*P. falciparum*) causes severe infection.[1] Chloroquine (**1**) (Fig. 1) is widely used for prevention or for initial treatment of malaria, as it has few side effects. However, the emergence of chloroquine-resistant strains remains a problem.[5,6]

Among antimalarials active against drug-resistant strains, bis-cation type antimalarial compounds G25 (**2**) were developed by Vial et al.[7–9] Furamidine (**3**), a derivative of the antiparasitic pentamidine, can be cationized under appropriate conditions, and exerts antimalarial activity (Fig. 1) through the inhibition of hemozoin formation.[10–12]

Considering that malaria is mainly prevalent in developing countries and that chloroquine-resistance is becoming a problem, we have been seeking new antimalarials that can be synthesized inexpensively and whose chemical structures are different from that of chloroquine (**1**). We have reported that cationic-dimer type antimalarials of the MAP series can be synthesized in just two steps from an inexpensive starting material, isonicotinic acid (Fig. 1).[13] A structure–activity relationship study led to 1,1′-(1,10-decanediyl)bis[4-[(hexylamino)carbonyl] pyridinium bromide] (**4**: MAP-610) and 1,1′-(1,12-dodecanediyl)bis[4-[(butylamino)-carbonyl]pyridinium bromide] (**5**: MAP-412) as potent antimalarials. 1,1′-(1,10-Decanediyl)bis[4-[(phenylamino)carbonyl]pyridinium bromide] (**6**: PMAP-H10), which possesses phenyl rings at both ends, also showed potent antimalarial activity, not only in vitro, but also in vivo. It is possible to introduce various substituents onto the phenyl rings of PMAP-H10 (**6**), so we examined the effects of introducing several

Keywords: Antimalarials; Cationic-dimer; Hammett's σ; Hansch–Fujita π; Synchronized culture.
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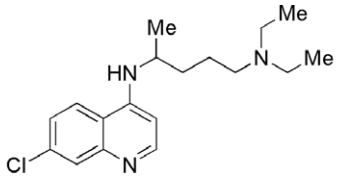
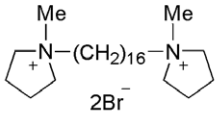
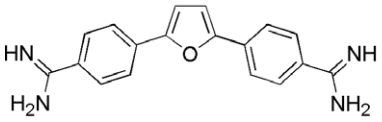
0968-0896/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.bmc.2008.04.051



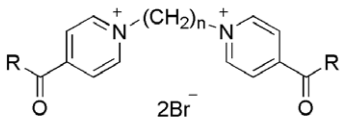
Chloroquine (**1**)



G25 (**2**)



Furamidine (**3**)



	R	n
MAP-610 (**4**)	$C_6H_{13}NH$	10
MAP-412 (**5**)	C_4H_9NH	12
PMAP-H10 (**6**)	PhNH	10

Figure 1. Chemical structures of well-known antimalarials (**1**–**3**) and MAPs (**4**–**6**).

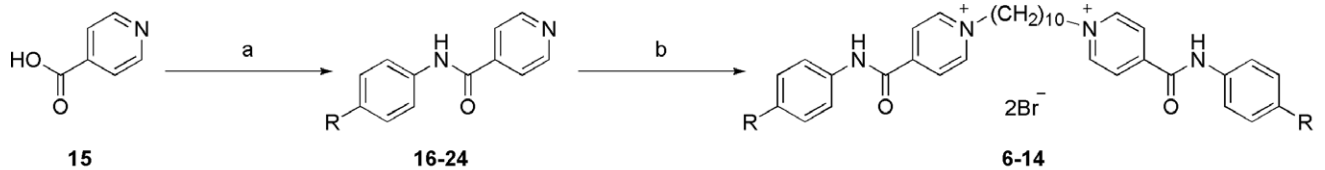
substituents onto the phenyl rings of the MAP series compounds in order to understand the influence of the electronic and hydrophobic properties of the substituents on the antimalarial activity and cytotoxicity. We were also interested to know whether there is a difference in the mechanism of action between our cation-dimers and chloroquine (**1**), so we examined the site of action of our compounds in *P. falciparum*. Here, we describe the results of a structure–activity relationship study and discuss the site of action of our cation-dimer type antimalarials.

2. Chemistry

Introduction of substituents into the phenyl rings at both ends of PMAP-H10 (**6**) was performed with anilino derivatives possessing substituents with various electronic or hydrophilic properties. These compounds were synthesized from isonicotinic acid (**15**) as a starting material, according to Scheme 1. Substituted anilines were reacted with isonicotinic acid (**15**), HOBt and EDC in DMF to afford the intermediates **16**–**24**. The intermediates were reacted with 1,10-dibromodecane to afford the target molecules **6**–**14**.

3. Results and discussion

We examined the antimalarial activity against FCR-3 strain (*P. falciparum*) and the cytotoxicity against FM3A F28-7 cells (derived from breast cancer of mice) of new compounds in the MAP series bearing various substituents on the phenyl rings at both ends (PMAP series). Chloroquine (**1**) was used as a positive control. As shown in Table 1, most of the tested compounds showed antimalarial activities comparable with that of chloroquine (**1**, EC_{50}: 18 nM), indicating that the introduction of substituents into the phenyl rings did not significantly decrease the antimalarial activity. In contrast, IC_{50} values of cytotoxicity were at the micromolar level. PMAP-H10 (**6**) and PMAP-CN10 (**13**) showed less cytotoxicity than chloroquine (**1**). The nature of the substituents on the phenyl rings at both sides appeared to influence both antimalarial and cytotoxic activities.



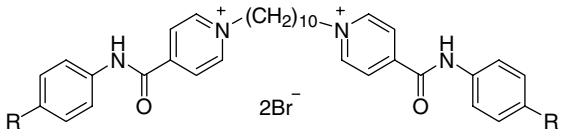
15 **16-24** **6-14**

		R
6	PMAP-H10	H
7	PMAP-M10	CH_3
8	PMAP-F10	F
9	PMAP-I10	I
10	PMAP-Cl10	Cl

		R
11	PMAP-Br10	Br
12	PMAP-TF10	CF_3
13	PMAP-CN10	CN
14	PMAP-NO10	NO_2

Scheme 1. Reagents and yields: (a) corresponding aniline, HOBt, EDC, DMF, 17–65%; (b) 1,10-dibromodecane, appropriate solvent, 36–82%.

Table 1. In vitro antimalarial activities and cytotoxicities of chloroquine (**1**) and MAPs (**4**–**14**)[a]



Compounds	R	EC_{50} (nM)		σ[d]	π[e]
		Antimalarial activity[b]	Cytotoxicity[c]		
Chloroquine (**1**)	—	18	32,000	—	—
MAP-610 (**4**)	—	5	10,500	—	—
MAP-412 (**5**)	—	100	52,900	—	—
PMAP-M10 (**7**)	Me	39	25,600	−0.17	0.56
PMAP-H10 (**6**)	H	29	42,800	0.00	0.00
PMAP-F10 (**8**)	F	21	27,800	0.06	0.14
PMAP-I10 (**9**)	I	12	1420	0.18	1.12
PMAP-Cl10 (**10**)	Cl	8	1890	0.23	0.71
PMAP-Br10 (**11**)	Br	15	1390	0.23	0.86
PMAP-TF10 (**12**)	CF_3	33	5180	0.54	0.88
PMAP-CN10 (**13**)	CN	35	42,400	0.66	−0.57
PMAP-NO10 (**14**)	NO_2	74	5990	0.78	−0.28

[a] In vitro antimalarial activities and cytotoxicities were determined as described in Section 5.
[b] Antimalarial activities were examined against chloroquine-sensitive *P. falciparum* (FCR-3 strain).
[c] Cytotoxicity was examined against mouse mammary tumor FM3A cells.
[d] Electronic parameters are quoted from Ref. 15.
[e] Hydrophobic parameters are quoted from Ref. 15.

In considering the relationship between substituents on phenyl rings and bioactivities, Hammett's σ value[14,15] and Hansch–Fujita's π value[16–18] are commonly used as electronic property parameters and hydrophobic parameters, respectively. Figure 2 shows the relationship of antimalarial activity with Hammett's σ or Hansch–Fujita's π. The results suggest that there is an optimal electronic character of the substituent on the phenyl groups for antimalarial activity in the PMAP series compounds. Among them, the non-substituted compound **6** and halogen-substituted derivatives **8**–**11** exhibited potent antimalarial activities. On the other hand, there appeared to be no correlation between antimalarial activity and Hansch–Fujita's π.

Figure 3 shows the relationship of cytotoxicity with Hammett's σ or Hansch–Fujita's π. The cytotoxicity appeared to be correlated with Hansch–Fujita's π, but not with Hammett's σ. Among the potent antimalarial compounds **6** and **8**–**11**, the more hydrophilic compound **7** showed lower cytotoxicity and the more hydrophobic compounds **9**–**11** showed higher cytotoxicity. From the viewpoints of both antimalarial activity and cytotoxicity, compound **6** seems to be the best candidate in the PMAP series.

P. falciparum invades the human body as sporozoites through the salivary glands of *Anopheles* mosquitoes. Sporozoites first invade liver cells, and then transform into several thousand merozoites after several cycles of division in hepatocytes over a period of a few days. After being released from liver cells, merozoites invade blood cells.[19] Infectious plasmodia in blood cells proliferate vigorously through a cycle involving merozoites, rings, trophozoites, and schizontes.[4] Plasmodia can be cultured at a single stage by means of synchronized culture methods.[20] In order to elucidate the site of action of

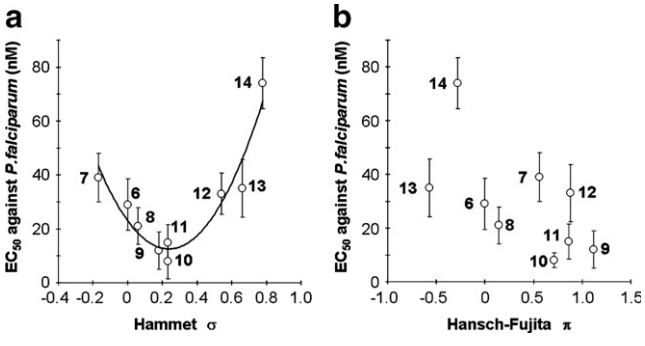


Figure 2. Correlations of antimalarial activities of PMAPs (**6**–**14**) with (a) Hammett substituent constants (σ) and (b) Hansch–Fujita π constants.

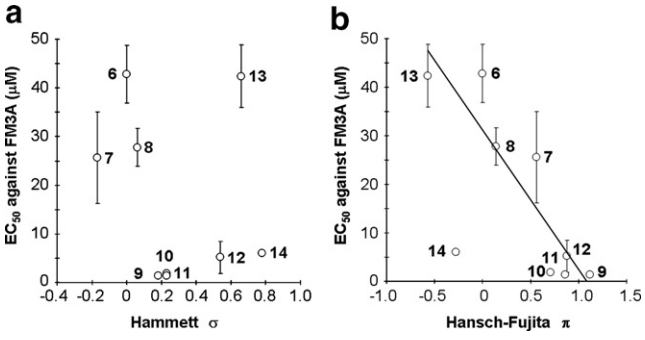


Figure 3. Correlations of cytotoxicity of PMAPs (**6**–**14**) with (a) Hammett substituent constants (σ) and (b) Hansch–Fujita π constants.

our cationic-dimer type antimalarials, we treated plasmodial cultures synchronized at various stages with MAP-610 (**4**) or PMAP-H10 (**6**), which showed antimalarial activities in vivo.[13] At time zero, plasmodia were synchronized in the ring, middle trophozoite, late trophozoite, or schizont stage, and then MAP-610 (**4**) or PMAP-H10 (**6**) was added at 100 times the corresponding IC_{50}. The infectious ratio was determined hourly thereafter. In cultures synchronized at the ring, middle trophozoite or late trophozoite stage, no significant difference between the effects of vehicle and drug was found immediately after drug treatment, indicating that our compounds did not affect malaria at those stages (Fig. 4a–c). But, in cultures synchronized at the schizont stage, the infection ratio was reduced immediately after drug treatment (Fig. 4d). These results indicate that our compounds act selectively on malaria parasites between the schizont stage and the ring stage. In contrast, it was reported that chloroquine acts at the ring and trophozoite stages, as well as the schizont stage, that is, its activity is stage-independent.[21,22] Therefore, our cationic-dimer type compounds appear to have a different mechanism of antimalarial activity from chloroquine, and might be effective against chloroquine-resistant malaria.

4. Conclusion

For the structural development of our cationic-dimer type antimalarials, we synthesized several compounds bearing various substituents on the phenyl rings of PMAP-H10 (**6**) and performed a structure–activity relationship study using Hammett's σ and Hansch–Fujita's π values. The results indicate that there is an optimal Hammett's σ value for potent antimalarial activity, while cytotoxicity is correlated with Hansch–Fujita's π values. In order to elucidate the site of action of our cationic-dimer type antimalarials, synchronized culture methods were employed. The results indicated that MAP-610 (**4**) and PMAP-H10 (**6**) act between the schizont stage and the ring stage, unlike chloroquine (**1**), whose action is stage-independent. Therefore, our cation-dimers appear to have a different mechanism of action from chloroquine, and might be effective against chloroquine-resistant malaria.

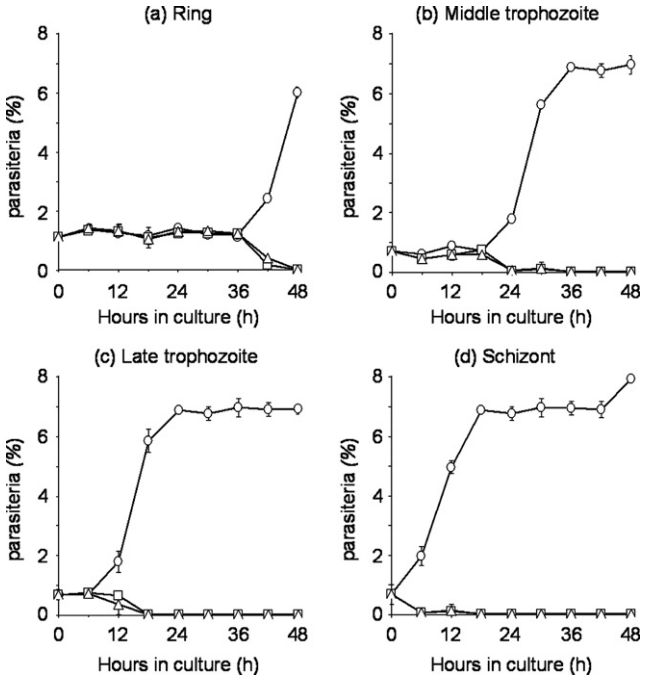


Figure 4. Synchronous growth of *P. falciparum* in the absence (open circles) of compounds and in the presence (open triangles) of MAP-610 (**4**) and in the presence (open squares) of PMAP-H10 (**6**). Cultures were initially synchronized at 0 h in (a) ring stage, (b) middle trophozoite stage, (c) late trophozoite stage and (d) schizont stage.

5. Experimental

5.1. Chemistry

Melting points were determined with a Yanagimoto hot-stage melting point apparatus and are uncorrected. IR spectra were recorded on a JASCO FT/IR350 (KBr). NMR spectra were recorded on a VarianVXR-300 (^{1}H 300 MHz) or a VarianVXR-500 (^{1}H 500 MHz) spectrometer. Proton chemical shifts were referenced to the TMS internal standard. Elemental analysis was carried out with a Yanagimoto MT-5 CHN recorder elemental analyzer and results were within ±0.4% of the theoretical values. FAB-MS was carried out with a VG70-SE.

5.2. General procedure for synthesis of substituted *N*-phenyl-4-pyridinecarboxamides (GP-A)

To a solution of isonicotinic acid (**14**) (3.0 mmol) in DMF (6.0 mL) were added isonicotinic acid (3.0 mmol), HOBt (3.5 mmol) and EDC (3.6 mmol). The mixture was stirred overnight at 100 °C, then poured into satd $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with H_2O and brine, and then dried over $MgSO_4$. The solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography or recrystallization to give the corresponding amide intermediates **16**–**24**.

5.2.1. *N*-Phenyl-4-pyridinecarboxamide (16). According to the general procedure (GP-A), **16** was obtained in 17% yield as colorless plates after recrystallization from EtOAc/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.49 (br s, 1H), 8.79 (dd, 2H, J = 4.3, 1.7 Hz), 7.86 (dd, 2H, J = 4.3, 1.7 Hz), 7.77 (d, 2H, J = 7.6 Hz), 7.38 (td, 2H, J = 7.6, 1.5 Hz), 7.14 (tt, 1H, J = 7.6, 1.5 Hz).

5.2.2. *N*-(4-Methylphenyl)-4-pyridinecarboxamide (17). According to the general procedure (GP-A), **17** was obtained in 41% yield as colorless plates after recrystallization from EtOH/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.41 (br s, 1H), 8.78 (dd, 2H, J = 4.6, 1.6 Hz), 7.85 (dd, 2H, J = 4.6, 1.6 Hz), 7.65 (d, 2H, J = 8.0 Hz), 7.18 (d, 2H, J = 8.0 Hz), 2.29 (s, 3H).

5.2.3. *N*-(4-Fluorophenyl)-4-pyridinecarboxamide (18). According to the general procedure (GP-A), **18** was obtained in 42% yield as a colorless powder after recrystallization from EtOH/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.55 (br s, 1H), 8.79 (dd, 2H, J = 4.7,

1.7 Hz), 7.86 (dd, 2H, J = 4.7, 1.7 Hz), 7.79 (m, 2H), 7.22 (m, 2H).

5.2.4. *N*-(4-Iodophenyl)-4-pyridinecarboxamide (19). According to the general procedure (GP-A), **19** was obtained in 26% yield as a colorless powder after recrystallization from EtOH/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.57 (br s, 1H), 8.79 (dd, 2H, J = 4.5, 1.7 Hz), 7.85 (dd, 2H, J = 4.5, 1.7 Hz), 7.72 (dd, 2H, J = 6.8, 2.1 Hz), 7.62 (dd, 2H, J = 6.8, 2.1 Hz).

5.2.5. *N*-(4-Chlorophenyl)-4-pyridinecarboxamide (20). According to the general procedure (GP-A), **20** was obtained in 52% yield as a colorless powder after recrystallization from EtOAc/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.61 (br s, 1H), 8.79 (dd, 2H, J = 4.7, 1.4 Hz), 7.85 (dd, 2H, J = 4.7, 1.4 Hz), 7.81 (dd, 2H, J = 7.0, 1.9 Hz), 7.44 (dd, 2H, J = 7.0, 1.9 Hz).

5.2.6. *N*-(4-Bromophenyl)-4-pyridinecarboxamide (21). According to the general procedure (GP-A), **21** was obtained in 65% yield as colorless plates after recrystallization from EtOAc/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.62 (br s, 1H), 8.79 (dd, 2H, J = 4.5, 1.7 Hz), 7.85 (dd, 2H, J = 4.5, 1.7 Hz), 7.76 (d, 2H, J = 9.0 Hz), 7.57 (d, 2H, J = 9.0 Hz).

5.2.7. *N*-[4-(Trifluoromethyl)phenyl]-4-pyridinecarboxamide (22). According to the general procedure (GP-A), **22** was obtained in 35% yield as colorless needles after recrystallization from EtOAc/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.82 (br s, 1H), 8.81 (dd, 2H, J = 4.4, 1.7 Hz), 8.01 (d, 2H, J = 8.8 Hz), 7.88 (dd, 2H, J = 4.4, 1.7 Hz), 7.76 (dd, 2H, J = 8.8 Hz).

5.2.8. *N*-(4-Cyanophenyl)-4-pyridinecarboxamide (23). According to the general procedure (GP-A), **23** was obtained in 22% yield as colorless plates after recrystallization from EtOAc/*n*-hexane; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.90 (br s, 1H), 8.81 (dd, 2H, J = 4.3, 1.7 Hz), 7.98 (d, 2H, J = 9.0 Hz), 7.87 (dd, 2H, J = 4.3, 1.7 Hz), 7.84 (d, 2H, J = 9.0 Hz).

5.2.9. *N*-(4-Nitrophenyl)-4-pyridinecarboxamide (24). According to the general procedure (GP-A), **24** was obtained in 30% yield as yellow needles after recrystallization from EtOH/*n*-hexane; ^{1}H NMR (500 MHz, DMSO-d_6) δ: 11.02 (br s, 1H), 8.81 (dd, 2H, J = 4.9, 1.5 Hz), 8.29 (d, 2H, J = 9.2 Hz), 8.06 (d, 2H, J = 9.2 Hz), 7.88 (dd, 2H, J = 4.9, 1.5 Hz).

5.3. General procedure for synthesis of PMAPs (GP-B)

To a solution of the appropriate *N*-phenyl-4-pyridinecarboxamide (0.5 mmol) in an appropriate solvent was added 1,10-dibromodecane (0.2 mmol). The mixture was stirred overnight at 100 °C and concentrated under reduced pressure. The residue was recrystallized or triturated using an appropriate solvent to give the target compounds **6**–**14**.

5.3.1. 1,1′-(1,10-Decanediyl)bis[4-[(phenylamino)carbonyl]pyridinium bromide] (6): PMAP-H10. According to the general procedure (GP-B), **6** was obtained in 36% yield as a pale yellow powder after recrystallization from MeOH/EtOAc. This reaction was carried out in DMF. Mp 209.0–210.0 °C; IR (KBr) cm^{-1}: 3415 (NH), 1673 (CO); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.91 (s, 2H), 9.31 (d, 4H, J = 6.7 Hz), 8.58 (d, 4H, J = 6.7 Hz), 7.78 (d, 4H, J = 7.7 Hz), 7.42 (t, 4H, J = 7.7 Hz), 7.20 (t, 2H, J = 7.7 Hz), 4.67 (t, 4H, J = 7.5 Hz), 1.95 (m, 4H), 1.29 (m, 12H); FAB-MS *m*/*z*: 615, 617 [M+H–HBr]$^+$; Anal. Calcd for $C_{34}H_{40}Br_2N_4O_2\cdot 1/2H_2O$: C, 57.88; H, 5.86; N, 7.94. Found: C, 57.79; H, 5.72; N, 7.82.

5.3.2. 1,1′-(1,10-Decanediyl)bis[4-[[4-(methyl)phenylamino]carbonyl]pyridinium bromide] (7): PMAP-M10. According to the general procedure (GP-B), **7** was obtained in 79% yield as yellow needles after recrystallization from MeOH. This reaction was carried out in DMF. Mp 238.0–239.0 °C; IR (KBr) cm^{-1}: 3283 (NH), 1678 (CO); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.85 (s, 2H), 9.30 (d, 4H, J = 6.7 Hz), 8.56 (d, 4H, J = 6.7 Hz), 7.67 (d, 4H, J = 8.2 Hz), 7.22 (d, 4H, J = 8.2 Hz), 4.67 (t, 4H, J = 7.5 Hz), 2.31 (s, 6H), 1.96 (m, 4H), 1.29 (m, 12H); FAB-MS *m*/*z*: 643, 645 [M+H–HBr]$^+$; Anal. Calcd for $C_{36}H_{44}Br_2N_4O_2\cdot 1/2H_2O$: C, 58.94; H, 6.18; N, 7.64. Found: C, 59.08; H, 6.07; N, 7.82.

5.3.3. 1,1′-(1,10-Decanediyl)bis[4-[[4-(fluoro)phenylamino]carbonyl]pyridinium bromide] (8): PMAP-F10. According to the general procedure (GP-B), **8** was obtained in 72% yield as a yellow powder after recrystallization from MeOH/EtOAc. This reaction was carried out in dioxane. Mp 239.0–240.0 °C; IR (KBr) cm^{-1}: 3410 (NH), 1662 (CO); ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.98 (s, 2H), 9.32 (d, 4H, J = 6.7 Hz), 8.58 (d, 4H, J = 6.7 Hz), 7.82 (m, 4H), 7.24 (m, 4H), 4.67 (t, 4H, J = 7.4 Hz), 1.94 (m, 4H), 1.29 (m, 12H); FAB-MS *m*/*z*: 651, 653 [M+H–HBr]$^+$; Anal. Calcd for $C_{34}H_{38}Br_2F_2N_4O_2\cdot 1/2H_2O$: C, 55.07; H, 5.30; N, 7.56. Found: C, 55.15; H, 5.21; N, 7.78.

5.3.4. 1,1′-(1,10-Decanediyl)bis[4-[[4-(iodo)phenylamino]carbonyl]pyridinium bromide] (9): PMAP-I10. According to the general procedure (GP-B), **9** was obtained in 82% yield as a yellow powder after recrystallization from MeOH. This reaction was carried out in DMF. Mp > 300.0 °C; IR (KBr) cm^{-1}: 3347 (NH), 1681 (CO); ^{1}H NMR (500 MHz, DMSO-d_6) δ: 10.86 (br s, 2H), 9.30 (d, 4H, J = 6.9 Hz), 8.56 (d, 4H, J = 6.9 Hz), 7.66 (d, 4 H, J = 8.4 Hz), 7.22 (d, 4H, J = 8.4 Hz), 4.67 (t, 4H, J = 7.3 Hz), 1.94 (m, 4H), 1.29 (m, 12H); FAB-MS *m*/*z*: 867, 869 [M+H–HBr]$^+$; Anal. Calcd for $C_{34}H_{38}Br_2I_2N_4O_2\cdot 1/2H_2O$: C, 42.66; H, 4.11; N, 5.85. Found: C, 42.47; H, 4.10; N, 5.81.

5.3.5. 1,1′-(1,10-Decanediyl)bis[4-[[4-(chloro)phenylamino]carbonyl]pyridinium bromide] (10): PMAP-Cl10. According to the general procedure (GP-B), **10** was obtained in 48% yield as yellow plates after recrystallization from MeOH/EtOAc. This reaction was carried out in DMF. Mp 261.5–263.0 °C; IR (KBr) cm^{-1}: 3432 (NH), 1665 (CO); ^{1}H NMR (300 MHz, DMSO-

d_6) δ: 11.05 (s, 2H), 9.32 (d, 4H, J = 6.7 Hz), 8.57 (d, 4H, J = 6.7 Hz), 7.83 (dd, 4H, J = 6.8, 2.1 Hz), 7.49 (dd, 4H, J = 6.8, 2.1 Hz), 4.67 (t, 4H, J = 7.3 Hz), 1.94 (m, 4H), 1.28 (m, 12H); FAB-MS m/z: 683, 685 $[M+H-HBr]^+$; Anal. Calcd for $C_{34}H_{38}Br_2Cl_2N_4O_2 \cdot H_2O$: C, 52.13; H, 5.15; N, 7.15. Found: C, 52.10; H, 5.13; N, 7.09.

5.3.6. 1,1′-(1,10-Decanediyl)bis[4-[[4-(bromo)phenylamino]carbonyl]pyridinium bromide] (11): PMAP-Br10. According to the general procedure (GP-B), **11** was obtained in 47% yield as a yellow powder after recrystallization from MeOH/EtOAc. This reaction was carried out in DMF. Mp 280.5–282.0 °C; IR (KBr) cm^{-1}: 3402 (NH), 1664 (CO); ^1H NMR (300 MHz, DMSO-d_6) δ: 11.06 (s, 2H), 9.32 (d, 4H, J = 6.8 Hz), 8.57 (d, 4H, J = 6.8 Hz), 7.78 (dd, 4H, J = 6.9, 2.0 Hz), 7.63 (dd, 4H, J = 6.9, 2.0 Hz), 4.68 (t, 4H, J = 7.3 Hz), 1.94 (m, 4H), 1.28 (m, 12H); FAB-MS m/z: 771, 773, 775, 777 $[M+H-HBr]^+$; Anal. Calcd for $C_{34}H_{38}Br_4N_4O_2$: C, 47.80; H, 4.48; N, 6.56. Found: C, 47.66; H, 4.53; N, 6.48.

5.3.7. 1,1′-(1,10-Decanediyl)bis[4-[[4-(trifluoromethyl)phenylamino]carbonyl]pyridinium bromide] (12): PMAP-TF10. According to the general procedure (GP-B), **12** was obtained in 74% yield as a yellow powder after recrystallization from MeOH/EtOAc. This reaction was carried out in dioxane. Mp 215.0–217.0 °C; IR (KBr) cm^{-1}: 3400 (NH), 1681 (CO); ^1H NMR (300 MHz, DMSO-d_6) δ: 11.23 (s, 2H), 9.33 (d, 4H, J = 6.7 Hz), 8.59 (d, 4H, J = 6.7 Hz), 8.02 (d, 4H, J = 8.5 Hz), 7.81 (d, 4H, J = 8.5 Hz), 4.68 (t, 4H, J = 7.3 Hz), 1.95 (m, 4H), 1.27 (m, 12H); FAB-MS m/z: 751, 753 $[M+H-HBr]^+$; Anal. Calcd for $C_{36}H_{38}Br_2F_6N_4O_2 \cdot 2H_2O$: C, 49.78; H, 4.87; N, 6.45. Found: C, 49.92; H, 4.76; N, 6.43.

5.3.8. 1,1′-(1,10-Decanediyl)bis[4-[[4-(cyano)phenylamino]carbonyl]pyridinium bromide] (13): PMAP-CN10. According to the general procedure (GP-B), **13** was obtained in 66% yield as a colorless powder after recrystallization from MeOH. This reaction was carried out in DMF. Mp 284.5–285.5 °C; IR (KBr) cm^{-1}: 3414 (NH), 1679 (CO); ^1H NMR (300 MHz, DMSO-d_6) δ: 11.34 (s, 2H), 9.37 (d, 4H, J = 6.3 Hz), 8.61 (d, 4H, J = 6.3 Hz), 8.02 (d, 4H, J = 8.7 Hz), 7.91 (d, 4H, J = 8.7 Hz), 4.70 (t, 4H, J = 7.1 Hz), 1.95 (m, 4H), 1.28 (m, 12H); FAB-MS m/z: 665, 667 $[M+H-HBr]^+$; Anal. Calcd for $C_{36}H_{38}Br_2N_6O_2 \cdot H_2O$: C, 56.55; H, 5.27; N, 10.99. Found: C, 56.38; H, 5.11; N, 10.94.

5.3.9. 1,1′-(1,10-Decanediyl)bis[4-[[4-(nitro)phenylamino]carbonyl]pyridinium bromide] (14): PMAP-NO10. According to the general procedure (GP-B), **14** was obtained in 82% yield as a pale brown powder after recrystallization from MeOH. This reaction was carried out in DMF. Mp 240.0–241.0 °C; IR (KBr) cm^{-1}: 3382 (NH), 1687 (CO); ^1H NMR (500 MHz, DMSO-d_6) δ: 11.44 (s, 2H), 9.35 (d, 4H, J = 6.9 Hz), 8.60 (d, 4H, J = 6.9 Hz), 8.33 (d, 4H, J = 9.2 Hz), 8.07 (d, 4H, J = 9.2 Hz), 4.69 (t, 4H, J = 7.3 Hz), 1.95 (m, 4H), 1.28 (m, 12H); FAB-MS m/z: 705, 707 $[M+H-HBr]^+$; Anal. Calcd for $C_{34}H_{38}Br_2N_6O_6 \cdot H_2O$: C, 50.76; H, 5.01; N, 10.45. Found: C, 50.67; H, 4.97; N, 10.57.

5.4. Culture of *P. falciparum*

Antimalarial activities of synthesized compounds against *P. falciparum* (FCR-3 strain) were determined. *P. falciparum* was cultivated by a modification of the method of Trager and Jensen using a 5% hematocrit suspension of type A human red blood cells (RBCs) in RPMI 1640 medium supplemented with 44 mM HEPES, 24 mM $NaHCO_3$, 25 μg/mL gentamicin, and heat-inactivated 10% type A human serum. The plates were held under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 37 °C. The medium was changed daily until 5% parasitemia (five parasite-infected RBCs per 100 RBCs).

5.5. Culture of mammalian cells

Cytotoxicity of the test compounds toward mouse mammary tumor FM3A cells (wild-type, subclone F28-7) was determined. FM3A cells were maintained in suspension culture at 37 °C in a 5% CO_2 atmosphere in plastic bottles containing ES medium supplemented with 2% heat-inactivated fetal bovine serum. FM3A cells grew with a doubling time of about 12 h.

5.6. In vitro antimalarial activity assay[23]

Five microliters of solution of a test compound in DMSO at several concentrations was added to individual wells of a 24-well plate. RBCs with 0.3% parasitemia were added to each well, containing 995 μL of culture medium, to give a final hematocrit level of 3%. Plates were incubated at 37 °C for 72 h under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. To evaluate the antimalarial activities of the test compounds, thin blood films from each culture were prepared and stained with Diff-Quik stain. Test compounds were tested in duplicate at each concentration. Compound-free control cultures were run simultaneously. All data points represent the mean of three experiments (parasitemia in controls reached between 4% and 5% at 72 h). The 50% inhibitory concentration (IC_{50}), which is the concentration giving 50% inhibition of parasite proliferation compared with the control, was calculated from the growth inhibition curve for each compound.

5.7. Cytotoxicity against mammalian cell line[23]

Prior to exposure to the test compounds, cell density was adjusted to 5×10^4 cells/mL. Cell suspension (995 μL/well) was dispensed into the 24-well test plate, and each test compound at various concentrations suspended in DMSO (5 μL) was added to individual wells. The plate was incubated at 37 °C for 48 h under an atmosphere of 5% CO_2 in air. All the test compounds were assayed in duplicate at each concentration. Cell numbers were counted microscopically. All data points represent the mean of three experiments. The IC_{50} value refers to the concentration of the compound necessary to inhibit the increase in cell density at 48 h by 50% of the control.

5.8. Synchronization of parasites in culture[21,23,24]

The culture was synchronized by D-sorbitol treatment. It is known that *P. falciparum* at the trophozoite and schizont stages is sensitive to D-sorbitol, and ring-stage parasites were obtained in synchronous culture. The culture was centrifuged at 2000 rpm for 5 min and the medium was removed. The pellet was resuspended in 5% D-sorbitol in distilled water for 20 min at room temperature and washed twice with culture medium and RPMI. Then, the residue was added to freshly erythrocytes. The synchronous culture was maintained in wells containing 2 mL 5% erythrocytes (v/v) in RPMI under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. At 40 h after the first synchronization, the culture was treated again with D-sorbitol in the same manner, and culture in which the ratio of the ring stage was more than 0.8 (>80%) was used in this experiment. At the start of every assay, the ratio of each stage was checked under a microscope in the same manner as described for the ring stage.

5.9. Determination of stage at which compounds inhibit parasite growth[21,23,24]

The assay for determination of the stage at which parasite growth was inhibited was performed for four groups divided by the starting stage of the assay. (Group a: parasites exposed to compounds at the ring stage, Group b: at the middle trophozoite stage, Group c: at the late trophozoite stage, Group d: at the schizont stage). Twenty-four-well plastic plates were used in this experiment, and each well contained of 995 μL of 5.0% erythrocyte suspension having about 1.0% parasitemia and 5 μL of test compound in 0.1% DMSO diluted with water (MilliQ-purified). The assay plate was incubated at 37 °C in 90% N_2, 5.0% O_2 and 5.0% CO_2 for 48 h. The time of addition of the test compound solution was defined as 0 h for each group. After synchronization, parasites were cultured at each stage in the medium containing the test compound at a concentration of 100 times the IC_{50}. Thin blood films of the cultures were made at 0, 6, 12, 18, 24, 30, 36, 42, 48 h, and the infection ratio at each time was calculated. At 24 h, 800 μL of the medium was replaced with fresh medium containing 4 μL of test compound solution. The slides were stained with Diff-Quik stain. For each group, a compound-free control culture was run simultaneously. All experiments were carried out in duplicate. The number of parasite-infected erythrocytes per at least 2000 erythrocytes was counted under a microscope and the infection ratio was calculated. Graphs of infection ratio were independently drawn for groups A to D, and the graph for each group was compared with the control. The stage at the time point when differences were seen between compound-added groups and the control groups were judged as the stage at which the test compound inhibits the growth of the parasites.

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