# Dihydroethanoanthracene Derivatives Reverse *In Vitro* Quinoline Resistance in *Plasmodium falciparum* Malaria

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**Abstract:** The capacity of ten molecules for reversing resistance in *Plasmodium falciparum in vitro* to quinoline antimalarial drugs, such as chloroquine (CQ), quinine (QN), mefloquine (MQ) and monodesethylamodiaquine (MDAQ), was assessed against 27 *Plasmodium falciparum* isolates. Four of these compounds were 9,10-dihydroethanoanthracene derivatives (DEAs). These DEAs reversed 75 to 92% of the CQ resistant strains. These synthetic compounds were more effective in combination with CQ than verapamil, ketotifen, chlorpromazine, reserpine or nicardipine, which reversed less than 50% of the CQ resistant strains. DEAs significantly reversed 67 to 100% of MDAQ resistant parasites. These compounds were more effective in combination with MDAQ than ketotifen (60% of reversal), chlorpromazine (45%), verapamil (33%), reserpine (30%) or nicardipine (9%). The reversal activity of MQ resistance was less pronounced, regardless of the molecule tested, and was homogeneous with a rate ranging from 42% for ketotifen to 58% for reserpine, nicardipine, verapamil and cyproheptadine. The four DEAs significantly reversed 50 to 55% of the parasites resistant to MQ. Fifty-six to 78 % of the QN resistant parasites were reversed by the synthetic DEAs. There were few differences in the rate of reversal activity on QN resistant strains between the ten compounds, with rates ranging between 56 to 78% for the ten chemosensitizers. The use of DEAs in combination with quinoline seems to be thus a promising strategy for limiting the development of drug resistant strains and for treating patients in drug resistant areas.

**Key Words**: Malaria, quinoline resistance, antimalarial drug, chemosensitizer, reversal agent.

## INTRODUCTION

The current option for reducing the morbidity and mortality of malaria are chemoprophylaxis and chemotherapy. Until recently, chloroquine (CQ) has been a key weapon in the fight against this disease. During the past 20 years there has been an emergence of strains of *Plasmodium falciparum* that are resistant to CQ and other antimalarial drugs [1, 2]. Failure of antimalarial prophylaxis with CQ, a combination of CQ plus proguanil [3], and mefloquine [4, 5], and clinical failure with halofantrine [6] and quinine [7] have been observed in Africa. The spread of drug resistant strains has increased the prevalence of malaria [2], which as of now afflicts 40% of the world's population and accounts for over three million deaths each year. One strategy for reducing the prevalence of malaria is the combinatorial use of drugs. Since 2001, more than 56 countries have officially adopted artemisinin-based combination therapies (ACTs) for the treatment of falciparum malaria [8]. However, individual *P. falciparum* isolates resistant *in vitro* to artemisinin, whether associated or not associated with clinical failures, have been described in Cambodia [9, 10]. Another strategy for reducing the prevalence of malaria is to reverse quinoline resistance chemically.

Classical chemosensitizers, such as calcium channel blockers like verapamil, diltiazem, nifedipine, nicardipine or β-blockers like propanolol, are known to effectively overcome CQ resistance *in vitro* [11-13]. Verapamil also reverses CQ resistance of laboratory strains and in clinical isolates [14-16]. Antipsychotic agents, such as phenothiazine drugs, can enhance the *in vitro* potency of CQ against *P. falciparum* CQ-resistant strains [17, 18]. Desipramine and other tricyclic antidepressant drugs, such as imipramine, reverse *in vitro* CQ resistance in *P. falciparum* at concentrations observed in the plasma of human patients undergoing treatment for depression [19, 20]. However desipramine did not enhance the efficacy of CQ in clinical trials [21]. Tricyclic histamine (H-1) receptor antagonists, such as cyproheptadine, promethazine or ketotifen, also reverse *in vitro* CQ resistance in *P. falciparum* [22, 23]. The combination of CQ with chlorpheniramine enhances the efficacy of the former in *P. falciparum* malaria and is effective in CQ resistant malaria in Nigerian children [24, 25].

Quinine (QN) response in *P. falciparum* can be modulated by several molecules which also modulate chloroquine response in *P. falciparum*. Namely, these are calcium channel blockers such as verapamil, diltiazem or tiapamil [11, 12, 26, 27], calmodulin inhibitors such as chlorpromazine [11],

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1573-4064/08 $55.00+.00 

an uncharged polyethoxylated nonylphenol synthetic surfactant (NP30) [28], and natural alkaloids such as malagashanine isolated from the Madagascan plant *Strychnos myrtoides* [29].

Studies to reverse mefloquine (MQ) resistance of *P. falciparum* have been very limited. Mefloquine resistance can be partly reversed *in vitro* by penfluridol [30, 31]. Further studies have shown that resistance to MQ in *P. yoelii* can be reversed by penfluridol with MQ in infected mice [32]. A preliminary study has shown the reversal of MQ resistance of *P. falciparum in vitro* by two monoindole alkaloids, namely icajine and isoretuline isolated from the plant *Strychnos myrptoides* [33]. In another *in vitro* study, the synthetic surfactant NP30 had a reversal effect against MQ resistant *P. falciparum* [28]. More recently, ketoconazole, a cytochrome $P_{450}$ inhibitor, could reverse MQ resistance in *P. yoelii nigeriensis* [34] and *P. knowlesi* [35]. Fluoxetine, a P-glycoprotein inhibitor, potentiated the MQ response in multidrug-resistant *P. falciparum* at clinically achievable concentrations [36]. Furthermore, verapamil showed an ability *in vitro* to reverse amodiaquine resistance [37].

The aim of the present work was to determine the capacity of ten molecules for reversing the resistance of different *P. falciparum* strains towards four conventional drugs: CQ, QN, MQ and monodesethylamodiaquine (MDAQ), the active metabolite of amodiaquine. Four of these compounds were 9,10-dihydroethanoanthracenes (DEAs) (Fig. **1**), which were selected from about sixty compounds [38, 39]. BG958 and BG920 were able to reverse CQ resistance in W2 CQ resistant clones and to accumulate CQ; BG918 was able to accumulate CQ without reversing CQ resistance, and BG1001 reversed CQ resistance but did not accumulate CQ [38-40]. Nicardipine, a calcium channel blocker similar to verapamil, was shown previously to reverse CQ resistance in a malaria mouse model [41]. Chlorpromazine is a calmodulin inhibitor with known reversal activity for CQ and QN *P.*

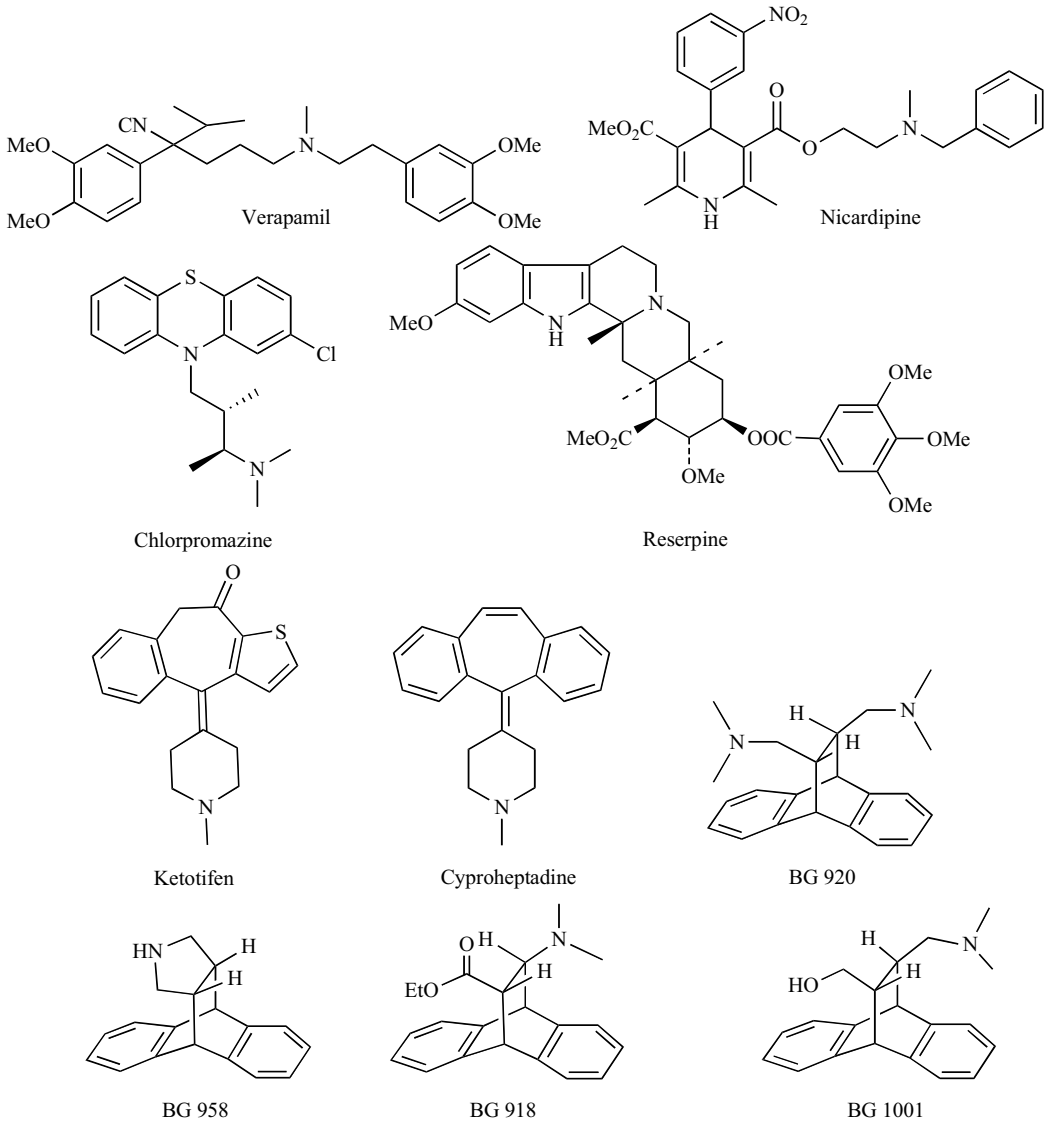


**Fig. (1).** Chemical structures of the chemosensitizers evaluated in combination with chloroquine, monodesethylamodiaquine, quinine and mefloquine against *P. falciparum* resistant parasites.

*falciparum* resistance [17, 18]. Ketotifen [13] and cyproheptadine [23], histamine (H-1) receptor antagonists, also reversed CQ *P. falciparum* resistance. Reserpine is an alkaloid with an ability to modulate multi-drug resistance in cancer cells [42, 43].

## RESULTS

### Chloroquine

The CQ $IC_{50}$ ranged between 12 and 94 nM for isolates susceptible to CQ and between 111 to 472 nM for parasites resistant to CQ (from resistant to susceptible) (Fig. **2**).

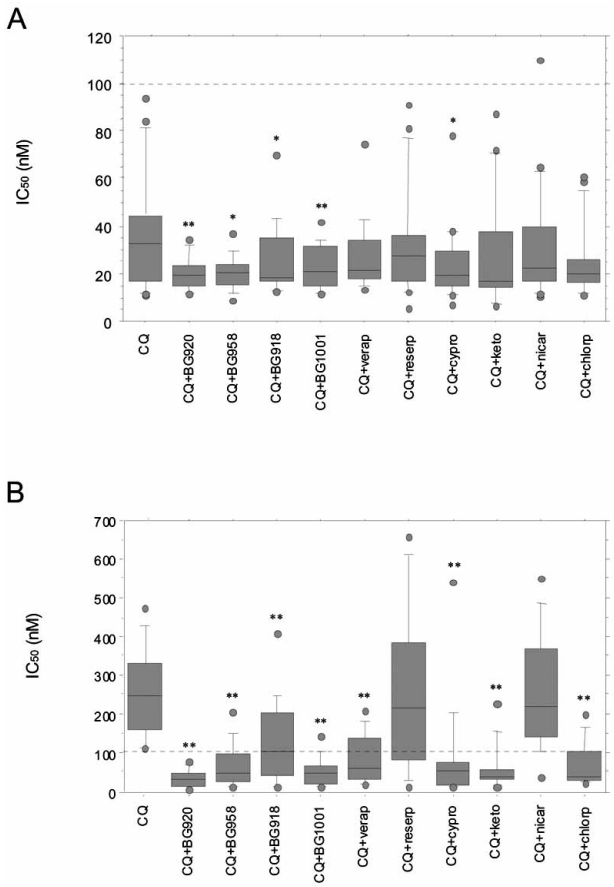


**Fig. (2).** Box-and-whisker plots showing activities of 0.5-µM modulator molecules on the CQ $IC_{50}$ in 15 CQ susceptible (A) and 12 CQ resistant strains (B). Lines in the boxes represent the 75$^{th}$ percentile, median and 25$^{th}$ percentile of the $IC_{50}$ values ( in nM); whiskers represent the lower and upper adjacent values, and dots represent outside values. Asterisks indicate a significant difference between $IC_{50}$ of CQ alone and $IC_{50}$ in the presence of modulator molecules (*: $p<0.05$; **: $p<0.01$) according to Wilcoxon signed-rank test. The resistance threshold is figured by the dotted line.

In the CQR strains, BG920, BG958, BG1001, verapamil, cyproheptadine, ketotifen and chlorpromazine showed a significant reversal effect (Fig. **2B**). However, synergy with the CQ effects was also observed in combination with the four DEAs and with cyproheptadine in CQ susceptible parasites (Fig. **2A**).

Among 25 tested strains, the CQ $IC_{50}$ for 21 strains decreased in the presence of BG958, BG1001 and chlorpromazine, 20 in the presence of BG920 and cyproheptadine, 19 with ketotifen, 18 with verapamil, 17 with BG918, 14 with reserpine and only 8 with nicardipine (Table **1**). On the contrary, the CQ $IC_{50}$ did not increase in the presence of BG1001, only one increased with BG858 and BG918, 2 with BG920, 3 with verapamil, ketotifen and cyproheptatide, 4 with chlorpromazine, 5 with reserpine and 10 with nicardipine.

The strains were classified according to their susceptibility to CQ: susceptible for CQ $IC_{50}<80$ nM, intermediate for CQ $IC_{50}$ between 80 and 100 nM and resistant for CQ $IC_{50}>100$ nM. The distribution of the rate of decreasing, stable and increasing CQ $IC_{50}$ values in the presence of the different chemosensitizers according to their CQ susceptibilities are summarized in Table **2**.

BG920, BG958 and BG1001 decreased the CQ $IC_{50}$ in 100% of CQ resistant *P. falciparum* parasites. These three DEAs reversed 75 to 92% of CQ resistant strains. Fifty-eight percent and only 17% of CQ resistant parasites had a decreased CQ $IC_{50}$ and reversed resistance, respectively, by BG918. Verapamil, ketotifen, chlorpromazine, reserpine or nicardipine reversed less than 50% of the CQ resistant strains.

### Monodesethylamodiaquine

The MDAQ $IC_{50}$ ranged between 10 and 74 nM for isolates susceptible to MDAQ and between 80 to 288 nM for parasites resistant to MDAQ (Fig. **3**).

The reversal effect of the chemosensitizers on MDAQ resistant strains was similar to those obtained for CQ on CQ resistant strains (Fig. **3B**). No synergistic effects between the ten molecules were found for MDAQ susceptible strains (Fig. **3A**). On the contrary, verapamil, reserpine, nicardipine and chlorpromazine increased the MDAQ $IC_{50}$ in MDAQ susceptible parasites, while the four DEAs had no effect on the MDAQ $IC_{50}$ in MDAQ susceptible strains.

Among 25 strains, 20 had a decreased MDAQ $IC_{50}$ in the presence of BG1001, 19 in the presence of BG958, 18 with BG920 and cyproheptadine, 15 with ketotifen, 14 with BG918, 12 with verapamil, 10 with reserpine, 9 with chlorpromazine and only 3 with nicardipine (Table **3**). In contrast, only one MDAQ $IC_{50}$ increased in the presence of BG920, 3 with BG858 and BG1001, 4 with BG918, 7 with cyproheptatide, 8 with verapamil and ketotifen, 10 with reserpine and 11 with chlorpromazine and nicardipine.

The strains were classified according to their susceptibility to MDAQ: susceptible for MDAQ $IC_{50}<60$ nM, intermediate for MDAQ $IC_{50}$ between 60 and 80 nM and resistant for MDAQ $IC_{50}>80$ nM. The distribution of the rates of decreasing, stable and increasing MDAQ $IC_{50}$ values in the presence of the different chemosensitizers according to their MDAQ susceptibilities are summarized in Table **4**.

BG920, BG958, BG918 and BG1001 decreased the MDAQ $IC_{50}$ in 75 to 100% of MDAQ resistant *P. falciparum* parasites. These four DEAs reversed 50 to 100% of MDAQ resistant strains. Verapamil, chlorpromazine, reserpine or nicardipine reversed less than 50% of the MDAQ resistant strains (9 to 45%).

**Table 1. Distribution of *P. falciparum* Strains According to the Effects on CQ $IC_{50}$ with the Combination of CQ with BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM**

| Compounds | Synergy | | | | No effect | | | | Antagonism | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | Median Ratio | Q1 Ratio | Q3 Ratio | no | Median Ratio | Q1 Ratio | Q3 Ratio | no | Median Ratio | Q1 Ratio | Q3 Ratio |
| BG920 | 20 | 0.21 | 0.16 | 0.49 | 5 | 0.94 | 0.87 | 0.98 | 2 | 1.30 | 1.21 | 1.40 |
| BG958 | 21 | 0.35 | 0.14 | 0.60 | 4 | 0.97 | 0.93 | 1.08 | 1 | 1.24 | - | - |
| BG918 | 17 | 0.65 | 0.43 | 0.74 | 9 | 0.99 | 0.93 | 1.09 | 1 | 1.22 | - | - |
| BG1001 | 21 | 0.43 | 0.28 | 0.54 | 6 | 0.97 | 0.89 | 1.08 | 0 | | | |
| Verapamil | 18 | 0.48 | 0.41 | 0.62 | 6 | 1.00 | 0.84 | 1.09 | 3 | 1.25 | 1.20 | 2.80 |
| Cyproheptadine | 20 | 0.29 | 0.21 | 0.46 | 4 | 0.98 | 0.88 | 1.04 | 3 | 1.22 | 1.20 | 1.39 |
| Ketotifen | 19 | 0.45 | 0.31 | 0.55 | 5 | 0.98 | 0.85 | 1.07 | 3 | 1.56 | 1.27 | 3.05 |
| Nicardipine | 8 | 0.68 | 0.45 | 0.73 | 9 | 0.97 | 0.94 | 1.06 | 10 | 1.36 | 1.23 | 1.46 |
| Chlorpromazine | 21 | 0.48 | 0.39 | 0.70 | 2 | 0.93 | 0.92 | 0.93 | 4 | 1.41 | 1.21 | 1.78 |
| Reserpine | 14 | 0.57 | 0.39 | 0.70 | 8 | 1.02 | 0.97 | 1.14 | 5 | 1.68 | 1.36 | 2.02 |

Synergy = decreased $IC_{50}$, No effect = stable $IC_{50}$, Antagonism = increased $IC_{50}$.

Ratio = CQ $IC_{50}$ with CQ in combination with a chemosensitizer / CQ $IC_{50}$ with CQ alone.

Q1 = 25%-interquartile, Q3 = 75%-interquartile.

**Table 2. Distribution of the CQ $IC_{50}$ According to the Effect (Decrease, Reversed, Stable and Increase) of BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM and According to the Basic Susceptibility of the Strains to CQ (Susceptible, Intermediate and Resistant Strains)**

| Compounds | Susceptible Strains $IC_{50} < 80$ nM | | | | Intermediate Strains $80 < IC_{50} < 100$ nM | | | | Resistant Strains $IC_{50} > 100$ nM | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | D | S | I | no | D | S | I | no | D | Rev | S | I |
| BG920 | 13 | 6 | 2 | 5 | 2 | 2 | 0 | 0 | 12 | 12 | 11 | 0 | 0 |
| BG958 | 13 | 8 | 4 | 1 | 2 | 2 | 0 | 0 | 11 | 11 | 9 | 0 | 0 |
| BG918 | 13 | 8 | 5 | 0 | 2 | 2 | 0 | 0 | 12 | 7 | 2 | 4 | 1 |
| BG1001 | 13 | 7 | 6 | 0 | 2 | 2 | 0 | 0 | 12 | 12 | 9 | 0 | 0 |
| Verapamil | 13 | 5 | 5 | 3 | 2 | 2 | 0 | 0 | 12 | 11 | 6 | 1 | 0 |
| Cyproheptadine | 13 | 7 | 6 | 0 | 2 | 2 | 0 | 0 | 12 | 12 | 11 | 0 | 0 |
| Ketotifen | 13 | 5 | 5 | 3 | 2 | 2 | 0 | 0 | 12 | 12 | 6 | 0 | 0 |
| Nicardipine | 13 | 3 | 4 | 6 | 2 | 1 | 0 | 1 | 12 | 4 | 1 | 5 | 3 |
| Chlorpromazine | 13 | 7 | 2 | 4 | 2 | 2 | 0 | 0 | 12 | 12 | 6 | 0 | 0 |
| Reserpine | 13 | 5 | 5 | 3 | 2 | 2 | 0 | 0 | 12 | 7 | 2 | 3 | 2 |

D = decrease, Rev = reversal, S = stable, I = increase.

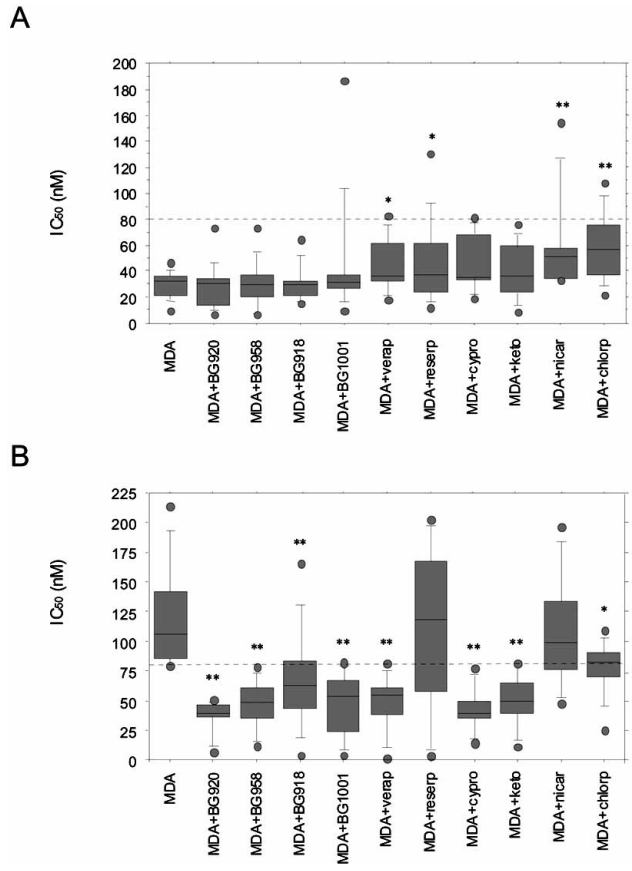


**Fig. (3).** Box-and-whisker plots showing the activities of 0.5-µM modulator molecules on the MDAQ $IC_{50}$ in MDAQ 15 susceptible (A) and 12 MDAQ resistant strains (B). Lines in the boxes represent the 75$^{th}$ percentile, median and 25$^{th}$ percentile of the $IC_{50}$ values (in nM); whiskers represent the lower and upper adjacent values, and dots represent outside values. Asterisks indicate a significant difference between $IC_{50}$ of MDAQ alone and $IC_{50}$ in presence of modulator molecules (*: $p<0.05$; **: $p<0.01$) according to Wilcoxon signed-rank test. The resistance threshold is figured by the dotted line.

## Quinine

The QN $IC_{50}$ ranged between 21 and 453 nM for isolates susceptible to QN and between 500 to 1310 nM for parasites resistant to QN (Fig. **4**).

All molecules, except reserpine and nicardipine, showed a significant decrease in the QN $IC_{50}$ in QN resistant strains (Fig. **4B**). No effect was observed in QN susceptible strains (Fig. **4A**).

Among 25 strains, the QN $IC_{50}$ of 19 strains decreased in the presence of verapamil and cyproheptadine, 17 in the presence of BG958 and BG920, 16 with BG918, BG1001, ketotifen and nicardipine, and 14 with reserpine and chlorpromazine (Table **5**). In contrast, the QN $IC_{50}$ for only 2 increased in the presence of cyproheptadine, 3 with BG1001, 4 with nicardipine, 5 with BG920, BG958, BG918, verapamil, reserpine and ketotifen, and 11 with chlorpromazine.

The strains were classified according to their susceptibility to QN: susceptible for QN $IC_{50}<400$ nM, intermediate for QN $IC_{50}$ between 400 and 500 nM and resistant for QN $IC_{50}>500$ nM. The distribution of the rate of decreasing, stable and increasing $IC_{50}$ values in the presence of the different chemosensitizers according to their QN susceptibilities are summarized in Table **6**.

BG920, BG958, BG918 and BG1001 decreased the QN $IC_{50}$ in 78% of the QN resistant *P. falciparum* parasites. These four DEAs reversed 56 to 78% of QN resistant strains. The other compounds reversed 56 to 89% of QN resistant strains.

## Mefloquine

The MQ $IC_{50}$ ranged between 6 and 25 nM for isolates susceptible to MQ and between 32 to 78 nM for parasites resistant to MQ (Fig. **5**).

Modulators added to MQ did not statistically modify the MQ $IC_{50}$ of susceptible strains compared with the MQ $IC_{50}$ when MQ was used alone (Fig. **5A**). Conversely, in the MQ resistant strain group, most of the modulators increased significantly the activity of MQ. Only ketotifen and chlorpromazine did not affect the MQ $IC_{50}$ in MQ resistant isolates (Fig. **5B**).

Among 25 strains, the MQ $IC_{50}$ of 15 strains decreased in the presence of BG918 and BG920, 14 in the presence of cyproheptadine and reserpine, 13 with verapamil, 12 with BG958, BG1001 and nicardipine, and 10 with ketotifen and chlorpromazine (Table **7**). Conversely, the MQ $IC_{50}$ for 3 strains increased in the presence of BG920, BG918 and nicardipine, 5 with BG1001, verapamil, cyproheptadine and reserpine, 6 with BG958 and ketotifen, and 10 with chlorpromazine.

The strains were classified according to their susceptibility to MQ: susceptible for MQ $IC_{50}<20$ nM, intermediate for MQ $IC_{50}$ between 20 and 30 nM and resistant for MQ $IC_{50}>30$ nM. The distribution of the rate of decreasing, stable and increasing MQ $IC_{50}$ values in the presence of the different chemosensitizers according to their MQ susceptibilities are summarized in Table **8**.

BG920, BG958, BG918 and BG1001 decreased the MQ $IC_{50}$ in 63 to 72% of MQ resistant parasites. These four DEAs reversed 50 to 55% of MQ resistant strains. The other compounds reversed 50 to 58% of MQ resistant strains.

## DISCUSSION

Quinoline resistance mechanisms in *P. falciparum* remain controversial. Quinoline resistance in the erythrocytic stages of *P. falciparum* is frequently compared to multidrug resistance in mammalian cells, in part because of the initial observation that chloroquine resistance can be reversed by verapamil [14]. BG920, BG958 and BG1001 decreased the CQ $IC_{50}$ in all CQ resistant *P. falciparum* parasites. The three DEAs reversed 75 to 92% of CQ resistant strains. These synthetic compounds are more effective in combination with CQ than verapamil, ketotifen, chlorpromazine, reserpine or nicardipine, which reverse less than 50% of CQ resistant strains. Nicardipine, which was shown to have reversal activity for CQ resistance in a mouse model of malaria [41, 44], reversed only 8% of *P. falciparum* CQ resistant

**Table 3. Distribution of *P. falciparum* Strains According to the Effects on MDAQ $IC_{50}$ with the Combination of MDAQ with BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM**

| Compounds | Synergy | | | | No effect | | | | Antagonism | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | Median Ratio | Q1 Ratio | Q3 Ratio | no | Median Ratio | Q1 Ratio | Q3 Ratio | no | Median Ratio | Q1 Ratio | Q3 Ratio |
| BG920 | 18 | 0.42 | 0.22 | 0.58 | 6 | 0.94 | 0.84 | 1.12 | 1 | 2.35 | - | - |
| BG958 | 19 | 0.48 | 0.28 | 0.71 | 5 | 0.83 | 0.83 | 1.04 | 3 | 1.50 | 1.23 | 2.73 |
| BG918 | 14 | 0.62 | 0.45 | 0.66 | 6 | 0.96 | 0.91 | 1.01 | 4 | 1.64 | 1.30 | 3.69 |
| BG1001 | 20 | 0.54 | 0.35 | 0.64 | 2 | 1.01 | 0.99 | 1.04 | 3 | 1.96 | 1.25 | 4.53 |
| Verapamil | 12 | 0.46 | 0.32 | 0.65 | 5 | 0.89 | 0.87 | 1.06 | 8 | 1.90 | 1.68 | 2.79 |
| Cyproheptadine | 18 | 0.52 | 0.39 | 0.59 | 2 | 0.90 | 0.86 | 0.93 | 7 | 1.84 | 1.49 | 2.56 |
| Ketotifen | 15 | 0.43 | 0.30 | 0.64 | 4 | 0.91 | 0.85 | 0.97 | 8 | 1.66 | 1.40 | 2.01 |
| Nicardipine | 3 | 0.57 | 0.22 | 0.70 | 12 | 0.99 | 0.93 | 1.06 | 11 | 1.86 | 1.49 | 3.27 |
| Chlorpromazine | 9 | 0.65 | 0.43 | 0.68 | 5 | 1.09 | 0.94 | 1.15 | 11 | 2.01 | 1.73 | 3.21 |
| Reserpine | 10 | 0.56 | 0.38 | 0.70 | 5 | 1.05 | 0.83 | 1.10 | 10 | 1.47 | 1.37 | 1.84 |

Synergy = decreased $IC_{50}$, No effect = stable $IC_{50}$, Antagonism = increased $IC_{50}$.

Ratio = MDAQ $IC_{50}$ with MDAQ in combination with a chemosensitizer / MDAQ $IC_{50}$ with MDAQ alone.

Q1 = 25%-interquartile, Q3 = 75%-interquartile.

strains *in vitro*. Only reserpine, which was shown to modulate multi-drug resistance in cancer cells [42, 43], had not been tested previously in *P. falciparum* strains. Its CQ resistance reversal activity is average with only 17% of CQ resistant parasites showing a reversal. BG918 decreased significantly the CQ $IC_{50}$ in 58% of CQ resistant strains but reversed only 17% of resistant parasites. This result confirms previous studies: BG918 can accumulate CQ in resistant parasites without reversing CQ resistance [38, 39].

**Table 4. Distribution of the MDAQ $IC_{50}$ According to the Effect (Decrease, Stable and Increase) of BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM and According to the Basic Susceptibility of the Strains to MDAQ (Susceptible, Intermediate and Resistant Strains)**

| Compounds | Susceptible Strains $IC_{50}$ < 60 nM | | | | Intermediate Strains 60 < $IC_{50}$ < 80 nM | | | | Resistant Strains $IC_{50}$ > 80 nM | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | D | S | I | no | D | S | I | no | D | Rev | S | I |
| BG920 | 12 | 5 | 6 | 1 | 2 | 2 | 0 | 0 | 11 | 11 | 11 | 0 | 0 |
| BG958 | 13 | 6 | 4 | 3 | 2 | 2 | 0 | 0 | 12 | 11 | 10 | 1 | 0 |
| BG918 | 10 | 4 | 3 | 3 | 2 | 1 | 0 | 1 | 12 | 9 | 6 | 3 | 0 |
| BG1001 | 11 | 6 | 2 | 3 | 2 | 2 | 0 | 0 | 12 | 12 | 8 | 0 | 0 |
| Verapamil | 13 | 3 | 2 | 8 | 2 | 2 | 0 | 0 | 12 | 7 | 4 | 3 | 0 |
| Cyproheptadine | 13 | 6 | 0 | 7 | 2 | 1 | 1 | 0 | 12 | 11 | 9 | 1 | 0 |
| Ketotifen | 13 | 4 | 1 | 8 | 2 | 1 | 1 | 0 | 10 | 8 | 6 | 2 | 0 |
| Nicardipine | 13 | 2 | 5 | 6 | 2 | 0 | 1 | 1 | 11 | 1 | 1 | 6 | 4 |
| Chlorpromazine | 12 | 1 | 2 | 9 | 2 | 0 | 2 | 0 | 11 | 8 | 5 | 1 | 2 |
| Reserpine | 13 | 4 | 2 | 7 | 2 | 1 | 0 | 1 | 10 | 5 | 3 | 3 | 2 |

D = decrease, Rev = reversal, S = stable, I = increase.

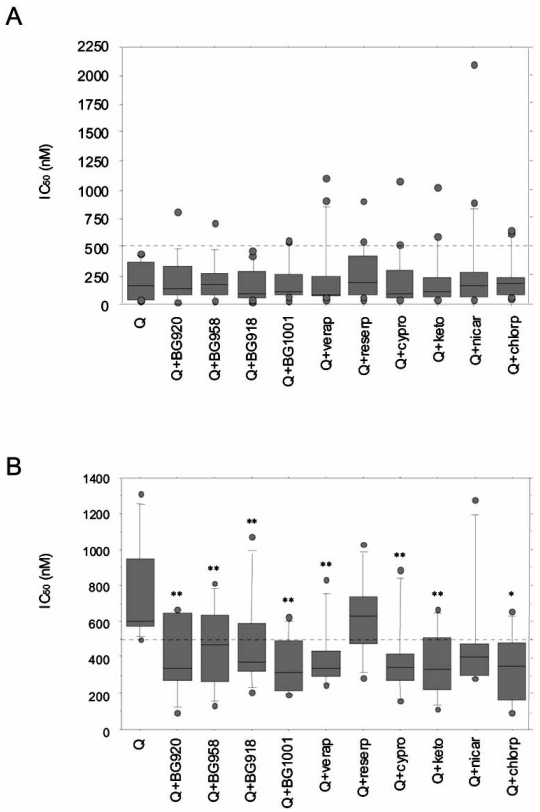


**Fig. (4).** Box-and-whisker plots showing the activities of 0.5-µM modulator molecules on the QN $IC_{50}$ in 16 QN susceptible (A) and 9 QN resistant strains (B). Lines in the boxes represent the 75$^{th}$ percentile, median and 25$^{th}$ percentile of the $IC_{50}$ values ( in nM); whiskers represent the lower and upper adjacent values, and dots represent outside values. Asterisks indicate a significant difference between $IC_{50}$ of QN alone and $IC_{50}$ in presence of modulator molecules (*: $p<0.05$; **: $p<0.01$) according to Wilcoxon signed-rank test. The resistance threshold is figured by the dotted line.

Studies to reverse MDAQ resistance in *P. falciparum* have been very limited. Only verapamil was shown to reverse MDAQ resistance *in vitro* [37]. BG958, BG920 and BG1001 significantly decreased the MDAQ $IC_{50}$ in 92 to 100% of the MDAQ resistant parasites and reversed 67 to 100% of these strains. BG918 was less active with a 75% decrease in the $IC_{50}$ and 50% of MDAQ resistant strains reversed. However, our compounds were more effective again in combination with MDAQ than ketotifen (60% of reversal), chlorpromazine (45%), verapamil (33%), reserpine (30%) or nicardipine (9%). Similar to CQ resistance reversal, BG920, BG958 and BG1001 were among the four best chemosensitizers.

The four DEAs decreased significantly the QN $IC_{50}$ in 78% of strains with a reduced susceptibility to QN. Fifty-six to 78 % of these resistant parasites were reversed by the synthetic DEAs. The reversal activity on QN resistance was more similar for all ten chemosensitizers with a range of 56 to 78% for the reversion rate. Only verapamil [26, 27] and chlorpromazine [11] showed *in vitro* reversal ability against QN resistant parasites. BG 958, BG920 and BG 918 were among the five best QN resistance reversal drugs.

The reversal activity for MQ resistance was less pronounced and was similar regardless of the molecule being tested, with rates of reversion ranging from 42% for ketotifen to 58% for reserpine, nicardipine, verapamil and cyproheptadine. The four DEAs decreased the MQ $IC_{50}$ significantly in 63 to 72% of the strains with a reduced susceptibility to MQ and achieved a 50 to 55% reversion of the parasites. Not one of these ten compounds was previously evaluated in combination with MQ.

The DEAs seem to be very efficient in reversing quinoline resistance for drugs such as CQ, MDAQ, QN, and in a less pronounced way, MQ. Resistance to amodiaquine is less documented but it could be compared to CQ rather than QN or MQ based on previous reports [45-47]. The profile of the reversal responses to CQ resistance is close to that of MDAQ and different from those of QN and MQ. Such differences may be explained by changes in mechanisms of resistance to CQ, MDAQ, QN and MQ. Moreover, verapamil reserpine, nicardipine and chlorpromazine had very different effects on the CQ $IC_{50}$ and on the MDAQ $IC_{50}$ in CQ and MDAQ susceptible strains, respectively. In addition, the ten chemosensitizers could be implicated in different manners according to the different quinolines. Nicardipine, which was ineffective in combination with CQ or MDAQ, reversed most *P. falciparum* resistant parasites in combination with QN and MQ. BG920 and BG958 were the most efficient drugs for CQ and MDAQ resistance reversal. Most previous studies on mechanisms of resistance have focused on transporters that can expel quinoline from the parasite digestive vacuole. Three relevant proteins, P-glycoprotein homologue 1 (Pgh1), encoded by the *P. falciparum* multidrug resistance gene (*pfmdr1*) [48], and *P. falciparum* CQ resistance transporter protein (PfCRT), encoded by the *pfcrt* gene [49], and the *P. falciparum* multidrug resistance protein (PfMRP), encoded by the *pfmrp* gene [50], have been identified as candidates that participate in quinoline resistance. However, it is not clear if they transport quinolines themselves or if they modify their conditions of action in the digestive vacuole. The quinoline resistant phenotype is complex and is probably affected by multiple genes, which could explain the observed differences in quinoline resistance reversal.

The use of these compounds, such as BG920 and BG958, in combination with quinoline seems to be a promising strategy to combat the development of drug resistant strains and to treat patients in drug resistant areas.

## MATERIALS AND METHODS

### *Plasmodium falciparum* Cultures

27 parasite strains or isolates from a wide panel of countries (Indochina, Thailand, Cambodia, Gambia, Djibouti, Nigeria, Senegal, Comoros, Uganda, Sierra Leone, Cameroon, Republic of Congo, Sudan, French Guyana, Brazil and Honduras) were adapted and maintained in culture in RPMI 1640 (Invitrogen, Paisley, United Kingdom), supplemented with 10 % human serum (Abcys S.A. Paris, France) and buffered with 25 mM HEPES and 25 mM $NaHCO_3$. Parasites were grown in A-positive human blood under controlled atmosphere consisting of 10 % $O_2$, 5 % $CO_2$ and 85 %

**Table 5. Distribution of *P. falciparum* Strains According to the Effects on QN $IC_{50}$ with the Combination of QN with BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM**

| Compounds | Synergy | | | | No effect | | | | Antagonism | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | Median Ratio | Q1 Ratio | Q3 Ratio | no | Median Ratio | Q1 Ratio | Q3 Ratio | no | Median Ratio | Q1 Ratio | Q3 Ratio |
| BG920 | 17 | 0.35 | 0.22 | 0.52 | 3 | 0.89 | 0.86 | 1.13 | 5 | 2.28 | 1.74 | 2.33 |
| BG958 | 17 | 0.35 | 0.23 | 0.61 | 3 | 1.10 | 0.89 | 1.17 | 5 | 1.89 | 1.56 | 2.70 |
| BG918 | 16 | 0.38 | 0.29 | 0.64 | 3 | 0.86 | 0.81 | 1.00 | 5 | 1.90 | 1.65 | 2.65 |
| BG1001 | 16 | 0.52 | 0.39 | 0.64 | 5 | 0.88 | 0.84 | 0.91 | 3 | 2.79 | 1.49 | 4.64 |
| Verapamil | 19 | 0.45 | 0.27 | 0.60 | 0 | | | | 5 | 2.03 | 1.67 | 2.47 |
| Cyproheptadine | 19 | 0.45 | 0.28 | 0.57 | 2 | 1.01 | 0.88 | 1.15 | 2 | 2.34 | 1.50 | 3.17 |
| Ketotifen | 16 | 0.29 | 0.29 | 0.57 | 3 | 0.97 | 0.94 | 0.99 | 5 | 1.46 | 1.39 | 2.08 |
| Nicardipine | 16 | 0.46 | 0.30 | 0.61 | 3 | 0.93 | 0.89 | 1.01 | 4 | 1.56 | 1.54 | 2.91 |
| Chlorpromazine | 14 | 0.44 | 0.27 | 0.68 | 3 | 0.94 | 0.86 | 0.94 | 6 | 1.89 | 1.57 | 2.34 |
| Reserpine | 14 | 0.58 | 0.23 | 0.67 | 4 | 1.05 | 0.97 | 1.10 | 5 | 1.95 | 1.76 | 1.96 |

Synergy = decreased $IC_{50}$, No effect = stable $IC_{50}$, Antagonism = increased $IC_{50}$.

Ratio = QN $IC_{50}$ with QN in combination with a chemosensitizer / QN $IC_{50}$ with QN alone.

Q1 = 25%-interquartile, Q3 = 75%-interquartile.

$N_2$ at 37 °C with a humidity of 95 %. Among the 27 *P. falciparum* parasites, 11 were CQ resistant, i.e., CQ $IC_{50}$>100 nM [50], 10 had reduced susceptibility to QN, i.e., QN $IC_{50}$>500 nM [51], 10 had reduced susceptibility to MQ, namely IC50>30 nM [53] and 13 were MDAQ resistant, i.e., MDAQ $IC_{50}$>80 nM [54].

These strains were selected to represent a wide range of origins and susceptibilities to CQ, MDAQ, QN and MQ.

## Drugs

The synthesis of BG918, BG920, BG958 and BG1001 (Fig. **1**), performed by the Medicinal Chemistry Laboratory

**Table 6. Distribution of the QN $IC_{50}$ According to the Effect (Decrease, Stable and Increase) of BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM and According to the Basic Susceptibility of the Strains to QN (Susceptible, Intermediate and Resistant Strains)**

| Compounds | Susceptible Strains $IC_{50}$ < 400 nM | | | | Intermediate Strains 400 < $IC_{50}$ < 500 nM | | | | Resistant Strains $IC_{50}$ > 500 nM | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | D | S | I | no | D | S | I | no | D | Rev | S | I |
| BG920 | 13 | 8 | 0 | 5 | 3 | 2 | 1 | 0 | 9 | 7 | 6 | 2 | 0 |
| BG958 | 12 | 6 | 2 | 4 | 3 | 3 | 0 | 0 | 9 | 7 | 7 | 2 | 0 |
| BG918 | 12 | 6 | 2 | 4 | 3 | 3 | 0 | 0 | 9 | 7 | 6 | 1 | 1 |
| BG1001 | 12 | 6 | 4 | 2 | 3 | 3 | 0 | 0 | 9 | 7 | 5 | 1 | 1 |
| Verapamil | 12 | 8 | 0 | 4 | 3 | 2 | 0 | 1 | 9 | 9 | 8 | 0 | 0 |
| Cyproheptadine | 11 | 8 | 1 | 2 | 3 | 3 | 0 | 0 | 9 | 8 | 7 | 1 | 0 |
| Ketotifen | 12 | 7 | 1 | 4 | 3 | 3 | 0 | 0 | 9 | 6 | 5 | 2 | 1 |
| Nicardipine | 11 | 8 | 1 | 2 | 3 | 2 | 1 | 0 | 9 | 8 | 8 | 1 | 0 |
| Chlorpromazine | 11 | 5 | 2 | 4 | 3 | 3 | 0 | 0 | 9 | 6 | 5 | 1 | 2 |
| Reserpine | 11 | 6 | 1 | 4 | 3 | 1 | 1 | 1 | 9 | 7 | 5 | 2 | 0 |

D = decrease, Rev = reversal, S = stable, I = increase.

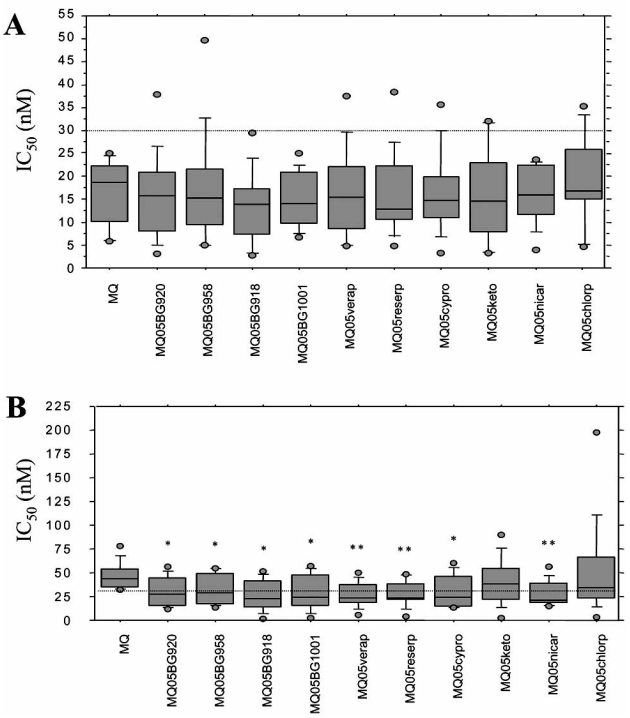


**Fig. (5).** Box-and-whisker plots showing activities of 0.5-µM modulator molecules on the MQ $IC_{50}$ in 13 MQ susceptible (A) and 12 MQ resistant strains (B). Lines in the boxes represent the 75$^{th}$ percentile, median and 25$^{th}$ percentile of the $IC_{50}$ values ( in nM); whiskers represent the lower and upper adjacent values, and dots represent outside values. Asterisks indicate a significant difference between $IC_{50}$ of MQ alone and $IC_{50}$ in presence of modulator molecules (*: $p<0.05$; **: $p<0.01$) according to Wilcoxon signed-rank test. The resistance threshold is figured by the dotted line.

of UMR-MD1, was previously described [38, 55]. Verapamil, reserpine, cyproheptadine, ketotifen, nicardipine, and chlorpromazine (Fig. **1**) were obtained from Sigma chemical (Saint Louis, MO). The 10 putative sensitizers were tested at 0.5 µM, at which no intrinsic activity on *P. falciparum* strains was observed (data not shown). Chlorpromazine, ketotifen and nicardipine were resuspended in water and diluted in RPMI to obtain a concentration of 0.5 µM. All other putative modulators were resuspended in a solution of 20 % of methanol and 80 % water before dilution in RPMI to obtain the final concentration of 0.5 µM for each molecule. CQ and QN were purchased from Sigma, MDAQ was obtained from the World Health Organization (Geneva, Switzerland) and MQ was purchased from Roche (France, Paris). CQ was resuspended in water; the concentration range being 0.3 to 200 nM for CQ susceptible strains and 5 to 3200 nM for CQR strains. QN and MDAQ were dissolved first in methanol and then diluted in water to obtain final concentration ranges from 0.125 to 400 nM for QN susceptible strains, from 5.3 to 3387 nM for QN resistant strains, from 0.06 to 407 nM for MDAQ susceptible strains and from 3.12 to 2000 nM for MDAQ resistant strains. MQ was dissolved first in methanol and then diluted in water to obtain concentration ranges from 0.125 to 400 nM. Each drug concentration was tested in triplicate in each assay.

### *In Vitro* Assay

For *in vitro* isotopic microtests, 25 µL/well of one of the 4 drugs, 25 µL/well of one of the 10 tested molecules, and 200 µL/well of the suspension of parasitized red blood cells (final parasitemia, between 0.5 and 1 % with a majority of young trophozoite stage; final hematocrit, 1.5%) were distributed in 96-well plates. Parasite growth was assessed by adding 1 µCi of [$^3$H]hypoxanthine with a specific activity of 14.1 Ci/mmol (Perkin-Elmer, Courtaboeuf, France) to each well. The plates were then incubated for 42 h in a controlled atmosphere as previously described. Immediately after incubation, plates were frozen and then thawed to lyse the erythrocytes. The content of each well was collected on standard filter microplates (Unifilter GF/B; Perkin-Elmer) and washed by using a cell harvester (Filter-Mate Cell Harvester; Perkin-Elmer). Filter microplates were dried, and 25 µL of scintillation cocktail (Microscint O; Perkin-Elmer) was placed in each well. Radioactivity incorporated by the parasites was measured with a scintillation counter (Top Count; Perkin-Elmer).

The $IC_{50}$, i.e., the drug concentration corresponding to 50 % of the uptake of [$^3$H]hypoxanthine by parasites in drug-free control wells, was determined by nonlinear regression analysis of log-dose/response curves (Riasmart$^{TM}$, Packard, Meriden, USA). Data were analyzed after logarithmic transformation and expressed as the geometric mean $IC_{50}$.

The effect of CQ, MDAQ, QN and MQ combined to 10 putative chemosensitizers were investigated respectively on 15 CQ susceptible strains and 12 CQ resistant strains, on 15 MDAQ susceptible and 12 MDAQ resistant strains, on 16 QN susceptible and 9 QN resistant strains, and 13 MQ susceptible and 12 MQ resistant strains.

### Evaluation of Drug Effect

The parasites were arranged into two groups: susceptible or resistant to the assessed conventional drugs. To evaluate the modulation of basic drug $IC_{50}$ values by associated molecules, box-and-whisker plots were constructed. The results were considered as significant or not, according to Wilcoxon signed-rank *T* test for paired data.

We analyzed the distribution of the ratio $IC_{50}$ of the quinoline in combination with a chemosensitizer / $IC_{50}$ of the same quinoline alone for each isolate. In order to classify the effect of each chemosensitizer on quinoline $IC_{50}$, we evaluated the coefficient of variation for each quinoline $IC_{50}$ (square root of variance / mean) in 8 *in vitro* CQ, MDAQ, QN or MQ $IC_{50}$ experiments against 4 strains, with half of them being susceptible. The coefficient of variation was about 10% for each quinoline. Then, strains were arranged into three groups according to the effect of the molecules on the quinoline $IC_{50}$: decreasing $IC_{50}$ (D, synergy) when $IC_{50}$ of a strain in combination with a chemosensitizer decreased by 20% with regard to the $IC_{50}$ of the quinoline alone (under 80% of the $IC_{50}$ of the quinoline alone), stable $IC_{50}$ (S, no effect) when the quinoline $IC_{50}$ of the combination is in the interval ± 20% with respect to $IC_{50}$ of the quinoline alone (80 to 120% of the $IC_{50}$ of the quinoline alone), and increasing $IC_{50}$ (I, antagonism) when $IC_{50}$ is up to 120 of the $IC_{50}$ of the quinoline alone.

**Table 7. Distribution of *P. falciparum* Strains According to the Effects on MQ $IC_{50}$ with the Combination of MQ with BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM**

| Compounds | Synergy | | | | No effect | | | | Antagonism | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | Median ratio | Q1 ratio | Q3 ratio | no | Median ratio | Q1 ratio | Q3 ratio | no | Median ratio | Q1 ratio | Q3 ratio |
| BG920 | 15 | 0.59 | 0.42 | 0.71 | 6 | 1.06 | 0.93 | 1.12 | 3 | 1.70 | 1.60 | 1.87 |
| BG958 | 12 | 0.51 | 0.39 | 0.66 | 7 | 0.88 | 0.85 | 1.03 | 6 | 1.48 | 1.28 | 2.23 |
| BG918 | 15 | 0.44 | 0.28 | 0.69 | 6 | 1.11 | 1.00 | 1.14 | 3 | 2.02 | 1.48 | 2.32 |
| BG1001 | 12 | 0.52 | 0.45 | 0.65 | 7 | 0.95 | 0.89 | 1.04 | 5 | 1.72 | 1.51 | 2.10 |
| Verapamil | 13 | 0.43 | 0.37 | 0.49 | 7 | 0.99 | 0.88 | 1.14 | 5 | 1.54 | 1.23 | 2.03 |
| Cyproheptadine | 14 | 0.55 | 0.36 | 0.74 | 6 | 1.03 | 0.83 | 1.06 | 5 | 1.60 | 1.43 | 1.90 |
| Ketotifen | 10 | 0.55 | 0.33 | 0.64 | 9 | 0.95 | 0.89 | 1.08 | 6 | 1.46 | 1.30 | 2.47 |
| Nicardipine | 12 | 0.46 | 0.41 | 0.65 | 10 | 1.01 | 0.97 | 1.09 | 3 | 1.48 | 1.26 | 3.19 |
| Chlorpromazine | 10 | 0.61 | 0.38 | 0.72 | 5 | 1.06 | 0.95 | 1.12 | 10 | 1.56 | 1.45 | 2.60 |
| Reserpine | 14 | 0.56 | 0.42 | 0.70 | 6 | 0.99 | 0.94 | 1.05 | 5 | 1.57 | 1.51 | 1.78 |

Synergy = decreased $IC_{50}$, No effect = stable $IC_{50}$, Antagonism = increased $IC_{50}$.

Ratio = MQ $IC_{50}$ with MQ in combination with a chemosensitizer / MQ $IC_{50}$ with MQ alone.

Q1 = 25%-interquartile, Q3 = 75%-interquartile.

This classification is more restrictive than other modes of potentiation interpretation normally used, such as the response modification index (RMI) defined as the ratio of $IC_{50}$ of drug A in combination with drug B to the $IC_{50}$ of the drug A alone. For example, a RMI of 0.81 ($IC_{50}$ of drug A in combination with drug B = 81 nM and the $IC_{50}$ of the drug A alone = 100) indicates sensitisation including synergy, while in our model, there is no effect.

Among drug resistant strains, an additional group represents molecules able to decrease $IC_{50}$ to below the resistance threshold (reversal activity).

**Table 8. Distribution of the MQ $IC_{50}$ According to the Effect (Decrease, Stable and Increase) of BG920, BG958, BG918, BG1001, Verapamil, Reserpine, Cyproheptadine, Ketotifen, Nicardipine and Chlorpromazine at 0.5 µM and According to the Basic Susceptibility of the Strains to MQ (Susceptible, Intermediate and Resistant Strains)**

| Compounds | Susceptible Strains $IC_{50} < 20$ nM | | | | Intermediate Strains $20 < IC_{50} < 30$ nM | | | | Resistant Strains $IC_{50} > 30$ nM | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | no | D | S | I | no | D | S | I | no | D | Rev | S | I |
| BG920 | 7 | 4 | 1 | 2 | 6 | 3 | 2 | 1 | 11 | 8 | 6 | 3 | 0 |
| BG958 | 7 | 2 | 3 | 2 | 6 | 2 | 3 | 1 | 12 | 8 | 6 | 2 | 2 |
| BG918 | 7 | 0 | 5 | 2 | 6 | 3 | 2 | 1 | 11 | 7 | 6 | 4 | 0 |
| BG1001 | 7 | 3 | 1 | 3 | 6 | 2 | 4 | 0 | 11 | 7 | 6 | 2 | 2 |
| Verapamil | 7 | 3 | 1 | 3 | 6 | 1 | 3 | 2 | 12 | 9 | 7 | 3 | 0 |
| Cyproheptadine | 7 | 3 | 2 | 2 | 6 | 3 | 1 | 2 | 12 | 8 | 7 | 3 | 1 |
| Ketotifen | 7 | 2 | 4 | 1 | 6 | 1 | 2 | 3 | 12 | 7 | 5 | 3 | 2 |
| Nicardipine | 7 | 2 | 2 | 3 | 6 | 1 | 5 | 0 | 12 | 9 | 7 | 3 | 0 |
| Chlorpromazine | 7 | 0 | 3 | 4 | 6 | 2 | 0 | 4 | 12 | 6 | 6 | 2 | 2 |
| Reserpine | 7 | 2 | 3 | 2 | 6 | 2 | 2 | 2 | 12 | 10 | 7 | 1 | 1 |

D = decrease, Rev = reversal, S = stable, I = increase.

## ACKNOWLEDGEMENTS

This work was supported by grant n°03CO006-05 from the Délégation Générale pour l'Armement (French Ministry of Defense) and was partially carried out through the COST B16 European Program.

## TRANSPARENCY DECLARATIONS

There is no conflict of interest. The authors do not own stocks or shares in a company that might financially be affected by the conclusions of this article.

## ABBREVIATIONS

CQ = Chloroquine

D = Decreased $IC_{50}$ (synergy)

DEAs = Dihydroethanoanthracenes

I = Increased $IC_{50}$ (antagonism)

$IC_{50}$ = Inhibitory concentration 50% (concentration of inhibitor that affords 50% of inhibition)

MDAQ = Monodesethylamodiaquine

MQ = Mefloquine

*pfcrt* = *P. falciparum* chloroquine resistance transporter gene

*pfmdr1* = *P. falciparum* multidrug 1 gene

*pfmrp* = *P. falciparum* multidrug resistance gene

QN = Quinine

S = Stable $IC_{50}$ (no effect)

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**Received: 02 August, 2007** **Revised: 09 January, 2008** **Accepted: 09 January, 2008**

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