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In vitro assessment of the pharmacodynamic properties of DB75, piperaquine, OZ277 and OZ401 in cultures of *Plasmodium falciparum*

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***Objectives*: Using synchronized cultures of *Plasmodium falciparum*, the time- and concentration-dependent growth changes of erythrocytic parasite stages to DB75, piperaquine, OZ277 and OZ401 were investigated *in vitro* over a concentration range of ~1–100× the IC_{50} of piperaquine, OZ277 and OZ401 and ~10–1000× the IC_{50} of DB75.**

***Methods*: The effects of timed *in vitro* exposure (1, 6, 12 or 24 h) were monitored by the incorporation of [^{3}H]hypoxanthine into the parasite nucleic acids.**

***Results*: After 1 h of exposure to the highest concentration of the compound followed by removal of the compound, the growth of all stages of *P. falciparum* was reduced to <34% for DB75 and 15% for piperaquine, OZ277 and OZ401 compared with untreated control parasites. At this time point, no stage-specific effects were observed at any of the concentrations. Strong inhibition (≤10% growth) of all parasite stages was observed when the parasites were exposed to 10× or 100× the IC_{50} of OZ277 and OZ401 for ≥6 h. At the 6 h incubation time point, DB75 was more active against mature parasite stages, with the IC_{50}s of young ring forms elevated up to 7-fold. This trend was observed up to 12 h, but was only statistically significant at the lowest concentration. Interestingly, the stage-specific effect of DB75 on ring forms was not detectable when washing procedures were omitted. This indicates a cytostatic action of DB75 on *P. falciparum* ring forms.**

***Conclusions*: The current study suggests that *P. falciparum* ring stages are less susceptible to DB75. A milder and often statistically insignificant stage-specific trend was observed for piperaquine, whereas OZ277 and OZ401 were equally active against the erythrocytic parasite stages.**

Keywords: speed of antimalarial drug action, stage specificity, [^{3}H]hypoxanthine

Introduction

The spread of resistance of the mosquito vector to currently available insecticides, the growing resistance of the parasites to treatment and the limited success of potential antimalarial vaccines have led to the urgent need to optimize existing drugs and to find new chemotherapeutic agents for the treatment of malaria, especially agents against *Plasmodium falciparum*.[1]

The Medicines for Malaria Venture (MMV), founded in 1999 as a not-for-profit Swiss foundation, aims to discover, develop and deliver, through effective public–private partnerships, new affordable antimalarial drugs that cure patients with a 3 day treatment regimen.[2] One former MMV project focused on an entirely new class of antimalarial compounds, the diamidines (www.MMV.org). Diamidines, such as DB75, are known to be active *in vitro* against chloroquine-sensitive (NF54) and chloroquine-resistant (K1) strains of *P. falciparum* as well as against the blood stage forms of *Plasmodium vivax*.[3] A second project sponsored by MMV is the 'ozonide' project, which has the aim of providing fully synthetic peroxides for patients with uncomplicated *P. falciparum* malaria. The pre-clinical candidate OZ277 (RBx11160) was described by Vennerstrom *et al.*[4] and is currently being developed by Ranbaxy Laboratories Limited in combination with partner drugs such as piperaquine (MMV Annual Report 2006; www.

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mmv.org). Other ozonides, such as OZ401 investigated here, originate out of the 'OZ next generation' project.

To our knowledge, the present study describes for the first time the pharmacodynamic effects of DB75, piperaquine and OZ401 on *P. falciparum* cultures by assessing their stage specificity and rate of action in comparison to those of OZ277, which was analysed previously.[5] A comprehensive knowledge about their pharmacodynamic properties may help to promote the development of new antiplasmodial agents and drug combinations, and improve the efficacy of available drugs.

Materials and methods

Parasite cultivation

The drug-sensitive isolate of *P. falciparum*, NF54, from the Netherlands and its clone 3D7 were used for the *in vitro* assays. All *Plasmodium* strains were provided by F. Hoffmann-La Roche Ltd (Basel, Switzerland) and were cultured as described previously.[5,6] When required, parasites were synchronized twice with 5% D-Sorbitol.[7] The second treatment was performed 6–8 h after the first synchronization. This procedure provided a parasite culture containing $\geq$80% young trophozoites (20–24 h old), $\geq$75% young schizonts (36–40 h old) and $\geq$90% young ring forms (52–56 h old, which equals 4–8 h of the next cycle).

Chemicals and materials

DB75 (MW: 377) was obtained from Immtech Pharmaceuticals, Inc. (Vernon Hills, IL USA), piperaquine tetraphosphate (MW: 928) from Ranbaxy Laboratories Limited (Gurgaon, India), OZ277 tosylate (MW: 565) and OZ401 mesylate (MW: 566) were provided by J. L. Vennerstrom (University of Nebraska Medical Center, NE, USA), pyrimethamine (MW: 249) was a gift from F. Hoffmann-La Roche Ltd (Basel, Switzerland) and [8-^{3}H]hypoxanthine was purchased from Amersham Bioscience (Buckinghamshire, UK). Antimalarial compounds were dissolved in DMSO at 10 mg/mL. The stock solutions were kept at 4°C for not more than 6 months. Dilutions were prepared from the stock solution immediately before use. The DMSO concentration in experiments had no inhibitory effect on parasite cultures.

In vitro growth inhibition assay and washing procedure

P. falciparum growth was assessed by measuring incorporation of the nucleic acid precursor [^{3}H]hypoxanthine.[8] IC_{50} values for NF54 were found to be 17 $\pm$ 0.3 ng/mL for DB75,[3] 8.5 $\pm$ 0.1 ng/mL for piperaquine,[9] 0.91 $\pm$ 0.12 ng/mL for OZ277,[4] 1.0 $\pm$ 0.0 ng/mL for OZ401 and 5.6 $\pm$ 0.5 ng/mL for pyrimethamine.[5] Synchronized cultures of young NF54 trophozoites with parasite counts of 0.15% and a haematocrit of 5% were divided into three 10 cm Petri dishes. Two dishes were further incubated for 16 or 32 h at 37°C for maturation into early schizonts or early ring stages. The third dish with the early trophozoites was used immediately for a 1, 6, 12 or 24 h exposure to the following four antimalarial compounds: DB75 (final concentrations 20 000, 10 000, 5000, 2500, 1250, 625 and 313 ng/mL), piperaquine (final concentrations 1000, 500, 250, 125, 63, 31 and 15.6 ng/mL), and OZ277 and OZ401 (final concentrations 100, 50, 25, 13, 6, 3 and 1.6 ng/mL). The 1 and 24 h time points and the 6 and 12 h time points were performed separately. As a validation of our methodology, the stage-specific effect of 24 h of pyrimethamine incubation (final concentrations 500, 250, 125, 63, 31, 15.6 and 8 ng/mL) was also investigated over a concentration range of $\sim$1–100$\times$ the IC_{50} of the compound. Similar to earlier findings,[5,10] pyrimethamine was found to be ineffective against ring and trophozoite stages (data not shown). The only sensitive parasite blood forms were the schizonts. After the respective incubation times for the parasite-compound mixture, the plates were washed four times resulting in a >1000-fold dilution of free compound. After another incubation period of 24 h at 37°C in the atmosphere described above, the plates were frozen at $-$20°C or directly processed as described.[5] With DB75, three additional assays were performed, where after 12 h the compound was not removed by washing. Two assays were performed with young 3D7 ring forms and one assay with young NF54 ring forms, giving very similar results.

Results

Assessment of stage-specific drug activity

The *in vitro* stage-specific effects of 1, 6, 12 or 24 h of compound exposure were investigated with synchronous cultures of *P. falciparum* NF54. Figure 1 shows parasite growth plotted against incubation time for three selected compound concentrations: $\sim$10$\times$ IC_{50}, $\sim$100$\times$ IC_{50} and $\sim$1000$\times$ IC_{50} (for DB75), and $\sim$1$\times$ IC_{50}, $\sim$10$\times$ IC_{50} and $\sim$100$\times$ IC_{50} (for piperaquine, OZ277 and OZ401). Table 1 demonstrates the comparison of the IC_{50} values of DB75, piperaquine, OZ277 and OZ401 evaluated for ring, trophozoite and schizont stages.

After 1 h of exposure at the highest compound concentration ($\sim$100$\times$ the IC_{50} for piperaquine, OZ277 and OZ401, and $\sim$1000$\times$ the IC_{50} for DB75) followed by the removal of the compound, the growth of all parasite stages was rapidly reduced and with no obvious stage specificity (Figure 1). For piperaquine, OZ277 and OZ401, the growth decreased to <15% compared with untreated control parasites. In the case of DB75, the growth ranged from 16% to 34%.

When the parasites were exposed to OZ277 and OZ401 for $\geq$6 h, again all stages were similarly affected. For DB75, however, the young ring forms were less susceptible than the mature stages (Figure 1). At the 6 h time point, the *P* values for the difference between the ring and trophozoite stages or ring and schizont stages were 0.0003 and 0.00001 (medium concentrations), and 0.02 and 0.02 (low concentrations). At the 12 h time point, the *P* values for DB75 were only statistical significant at the lowest concentration (0.03 and 0.02), respectively. After 24 h, we observed no more stage-specific profiles. A similar, however mostly statistically insignificant stage-specific trend was found for piperaquine (Figure 1).

Analysis of the IC_{50} values of the different parasite stages (Table 1) showed that for DB75 the IC_{50} values of young ring stages were up to 7$\times$ higher than those of trophozoites and schizonts at the 6 h time point ($P = 0.01$ for both). A mostly statistically insignificant stage-specific activity was found for piperaquine ($P = 0.04$ and 0.05, respectively). This trend could be observed up to 12 h, in particular for DB75, but without being statistically significant for either molecule.

Interestingly, the stage-specific effect of DB75 on ring forms could no longer be observed when washing procedures were omitted. Three independent 12 h experiments resulted in an average IC_{50} value of 180 ng/mL for young ring forms, which is 9-fold lower than the IC_{50} value obtained when the parasitized cells were washed (Table 1, 1638 ng/mL) and which is similar

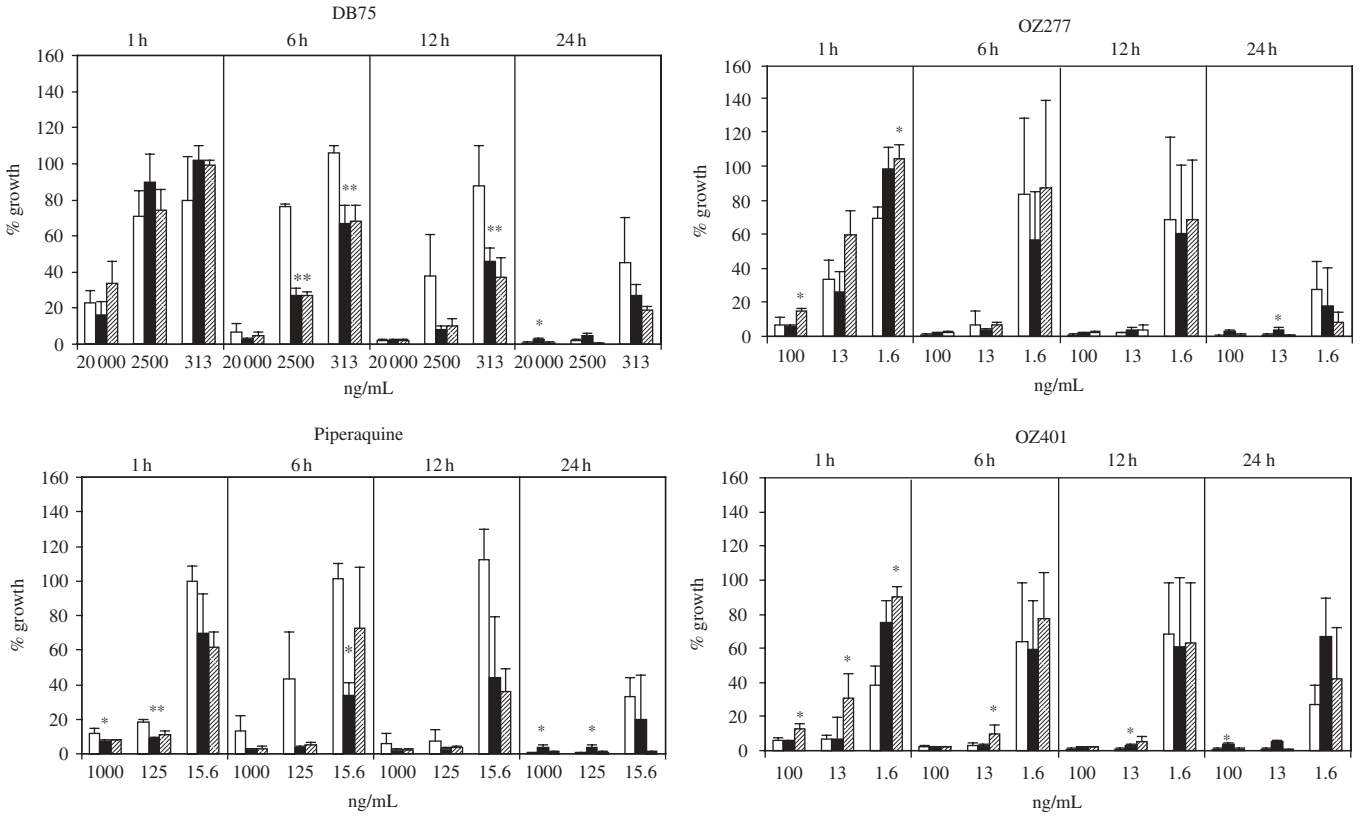

Figure 1. Stage-dependent effects of DB75 (∼10×, ∼100× and ∼1000× the IC_{50}), piperaquine, OZ277 and OZ401 (∼1×, ∼10× and ∼100× the IC_{50}) on [^{3}H]hypoxanthine incorporation in synchronous cultures of *P. falciparum* strain NF54. Compounds were added for 1, 6, 12 or 24 h. After removal of the compounds, parasites were incubated for another 24 h in the presence of [^{3}H]hypoxanthine. Compound effects are expressed as the percentage of growth of the respective development stage relative to an untreated control. The open bar is the ring stage, the filled bar is the trophozoite stage and the hatched bar is the schizont stage. Each bar represents the mean ± SD of $n = 3$ independent experiments. Significant differences between ring and trophozoite stages ($^*P < 0.05$), or ring and schizont stages ($^*P < 0.05$) were determined by Student's *t*-tests.

to the IC_{50} values of the trophozoite and schizont stages (Table 1, 304 and 270 ng/mL).

Discussion

To our knowledge, this is the first report on the pharmacodynamic effects of DB75, piperaquine and OZ401 on *P. falciparum*. Understanding the stage specificity and onset of action of new chemotherapeutic agents for the treatment of malaria is considered to be of clinical importance, particularly in view of the increasing development of resistance in parasites to known antimalarial agents.

In our pharmacodynamic studies, we found that the growth of all parasite stages was similarly affected after 1 h of exposure to DB75, piperaquine, OZ277 and OZ401 at all evaluated concentrations. However, exposure of piperaquine, and especially DB75, to malarial parasites for 6 or 12 h showed that they were more active against the mature parasite stages (up to 7-fold lower IC_{50}s) than against the young ring forms (Figure 1 and Table 1).

The University of Chapel Hill performed similar [^{3}H]hypoxanthine incorporation studies with DB75.[11] For the trophozoite parasite stages, the Meshnick IC_{50} data were very similar to our 12 h IC_{50} data [268 ng/mL (711 nM) versus 304 ng/mL], but differed ∼30× for the ring stages [54 ng/mL (143 nM) versus 1638 ng/mL]. In order to determine whether the washing steps omitted by the Chapel Hill group could be the explanation for the different IC_{50} values between the two labs, we performed three independent 12 h experiments without the removal of DB75. Indeed, removing the washing step resulted in IC_{50} values for young ring forms that were very similar between the two laboratories [54 ng/mL (143 nM) versus 180 ng/mL]. This indicates a cytostatic action of DB75 on *P. falciparum* ring forms.

For DB75, it should also be noted that comparable growth inhibitions relative to the other three compounds could only be achieved with a 10× higher compound concentration. We conclude that DB75 can act as fast as the other three compounds, but only at a 10× higher concentration.

For OZ277 and OZ401, we found that the growth of all parasite stages was affected in a similar way at all time points and concentrations. These observations are mostly in line with what was reported earlier for OZ277.[5] The only difference was found at the highest concentration at the 1 h time point, where our earlier findings at the highest OZ277 concentration indicated that young ring forms were ∼2-fold less sensitive compared with mature stages.[5] A possible explanation could be that the data in the previous study were from two independent experiments, whereas in the present study three independent experiments were performed. Another factor that possibly could have contributed to

Table 1. Mean IC_{50} values for DB75, piperaquine, OZ277 and OZ401 determined by the [^{3}H]hypoxanthine assay for synchronized cultures of *P. falciparum* strain NF54

Compound/ incubation time (h)	Mean IC_{50} [mean ± SD (ng/mL)]		
	ring	trophozoite	schizont
DB75			
1	10 587 ± 2275	7267 ± 3270	10 352 ± 5446
6	5577 ± 1799	942 ± 525*	822 ± 433*
12	1638 ± 943	304 ± 27	270 ± 26
24	346 ± 98	285 ± 9	263 ± 15
Piperaquine			
1	35 ± 8	22 ± 7	18 ± 2*
6	55 ± 10	17 ± 11*	25 ± 16
12	39 ± 24	21 ± 6	19 ± 7
24	13 ± 1.2	16 ± 8	10 ± 0.6*
OZ277			
1	6.3 ± 3.5	6.2 ± 1.4	17 ± 11
6	3.7 ± 3.0	1.9 ± 0.6	3.4 ± 1.6
12	3.0 ± 1.5	2.0 ± 1.0	3.0 ± 2.1
24	1.4 ± 0.1	1.3 ± 0.3	1.2 ± 0.2
OZ401			
1	1.4 ± 0.1	2.1 ± 0.5	6.1 ± 2.9
6	1.9 ± 0.6	1.8 ± 0.2	6.3 ± 2.6
12	2.2 ± 0.9	2.0 ± 1.0	4.3 ± 2.5
24	1.4 ± 0.1	2.0 ± 0.3	1.6 ± 0.3

Cultures were initiated at a parasitaemia of 0.15% and a hematocrit of 5%, and incubated for 1, 6, 12 and 24 h. After removal of the compounds, parasites were incubated for another 24 h in the presence of [^{3}H]hypoxanthine. Data are the means ± SD of $n = 3$ independent experiments. Significant differences between ring and trophozoite stages, or ring and schizont stages were determined by Student's *t*-tests (*$P < 0.05$).

the observed data variability is the very short compound incubation time (1 h).

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Transparency declarations

None to declare.

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