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Molecular Epidemiology of Malaria in Cameroon. XXV. *In Vitro* Activity of Fosmidomycin and its Derivatives against Fresh Clinical Isolates of *Plasmodium falciparum* and Sequence Analysis of 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase

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Abstract. The *in vitro* activities of fosmidomycin derivatives, chloroquine, and pyrimethamine were assessed by the radioisotopic assay in clinical isolates of *Plasmodium falciparum*. In a series of experiments with RPMI 1640 medium–10% fetal bovine serum, the geometric mean 50% inhibitory concentrations (IC_{50}s) (n = 34) for fosmidomycin and FR900098 were 301 nM and 118 nM, respectively. In another series of experiments, the geometric mean IC_{50}s (n = 33) for fosmidomycin and TH II46 were 413 nM and 249 nM, respectively. The IC_{50}s were 2–3 times lower with RPMI–10% fetal bovine serum than the IC_{50}s obtained with RPMI–10% human serum. FR900098 and TH II46 were 2.6 and 1.7 times more potent, respectively, than fosmidomycin. There was no correlation between chloroquine or pyrimethamine and fosmidomycin, which suggested the absence of *in vitro* cross-resistance. Sequence analysis showed five amino acid substitutions, but their possible relationship with the response to fosmidomycin is not clear. Fosmidomycin derivatives are promising candidates for further development.

INTRODUCTION

One of the strategies to combat the spread of drug-resistant malaria is to introduce novel drugs, which may be either newly synthesized drugs or old drugs that had been originally developed for diseases other than malaria. The search for the latter group of drugs may be facilitated and developed on a rational basis by taking advantage of the available genomic information. *Plasmodium falciparum* possesses three genomes: 14 chromosomes in the nuclear genome, a 6-kb mitochondrial DNA, and a 35-kb plastid-like genome in the apicoplast.[1–3] The complete sequence of these genomes is known. The apicoplast is present in most, but not all, apicomplexans. The genome of this unique non-photosynthetic organelle shares homologous sequences with the plastid genome in plants although its genome is reduced compared with that of the plastid. Most apicoplast-located proteins are nuclear-encoded and transported into the organelle, where several metabolic pathways have been localized.[4] One of these pathways is the 1-deoxy-D-xylulose 5-phosphate (DOXP) isoprenoid biosynthetic pathway (also called the 2-*C*-methyl-D-erythritol 4-phosphate [MEP] pathway), which is common to eubacteria (most Gram-negative and some Gram-positive bacteria), algae, plants, and apicomplexans (*P. falciparum*, *Babesia spp*., *Toxoplasma gondii*, *Theileria spp*.) but absent in humans.[5–8] An alternative isoprenoid pathway, known as the mevalonate pathway, is used by archaebacteria, some eubacteria, fungi, and mammals, including humans. Isoprenoids include sterols, ubiquinones, and carotenoids. Dolichols and ubiquinones were identified because they were the most important isoprenoids of *P. falciparum*.

Fosmidomycin (FR-31564) is a natural phosphonic acid antibiotic isolated from *Streptomyces lavendulae*.[9] It specifically inhibits DOXP reductoisomerase (EC 1.1.1.267), also known as 2-*C*-methyl-D-erythritol synthase and IspC, which catalyzes the conversion of DOXP to MEP in the second step of the DOXP pathway. Phase I and phase II clinical studies conducted in the 1980s in healthy volunteers and patients with urinary tract infections demonstrated the tolerance and safety of fosmidomycin in humans and its antibacterial activity, but its use as an antibiotic was not developed further.[10,11] Recent clinical studies have confirmed its safety and tolerance in *P. falciparum*-infected adult patients, but the recrudescence rate on day 28 was high (50%; in 7 of 9 Thai patients and 2 of 9 Gabonese patients), even after 7-day oral fosmidomycin monotherapy.[12,13] However, a three-day regimen of fosmidomycin–clindamycin and fosmidomycin–artesunate combination therapies was highly effective, except in very young children less than three years of age, during the 28-day follow-up in a small number of symptomatic Gabonese children.[14–16] These data indicate that DOXP reductoisomerase is a specific target for new drugs for the treatment of malaria, and possibly infections with other apicomplexan protozoa, and that fosmidomycin, or its derivatives, may be a highly promising antimalarial drug candidate for further development.

The *in vitro* antimalarial activities of fosmidomycin and its derivatives have so far been evaluated only in laboratory-adapted reference clones of *P. falciparum*. The present study was conducted with the aim to assess the *in vitro* activities of fosmidomycin, FR900098 (the natural acetyl derivative of fosmidomycin isolated from *Streptomyces rubellomurinus*),[17] and TH II46, the α-(*p*-methyl-phenyl) synthetic derivative of FR900098, against fresh clinical isolates of *P. falciparum* and evaluate the potential for *in vitro* cross-resistance with chloroquine and pyrimethamine. Furthermore, sequence data of the DOXP reductoisomerase gene of these *P. falciparum* isolates were compared with those of the reference clones with the aim to study sequence variations and detect mutation(s) that may be associated with the *in vitro* response to fosmidomycin.

PATIENTS AND METHODS

Patients. After informed consent was obtained, fresh clinical isolates of *P. falciparum* were collected by venipuncture (5–10 mL of blood) from symptomatic patients ≥ 12 years of

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age who came to the Nlongkak Catholic missionary dispensary in Yaoundé in 2001–2005 into EDTA-coated tubes. The inclusion criteria were as follows: parasitemia ≥ 0.1%, absence of other *Plasmodium* species, and denial of recent self-medication with an antimalarial drug confirmed by the Saker-Solomons urine test.[18] Young children less than 12 years of age, pregnant women, and patients with signs and symptoms of severe and complicated malaria were excluded. The enrolled patients were treated with amodiaquine monotherapy or an amodiaquine–sulfadoxine-pyrimethamine combination. The study was reviewed and approved by the Cameroonian National Ethics Committee and Cameroonian Ministry of Public Health.

Drugs. Chloroquine diphosphate and pyrimethamine base were obtained from the Sigma Chemical Co. (St. Louis, MO). Fosmidomycin monosodium salt and its derivatives FR900098 and TH II46 were kindly provided by Dr. Hassan Jomaa (Justus-Liebig-University, Giessen, Germany) and Professor Serge van Calenbergh (Ghent University, Ghent, Belgium), respectively. Stock and working solutions of chloroquine and fosmidomycin derivatives were prepared in sterile distilled water. Stock and working solutions of pyrimethamine were prepared in absolute ethanol. Ninety-six–well culture plates were pre-coated in duplicate with seven 2-fold dilutions of chloroquine (25–1,600 nM final concentrations). The plates were also pre-coated in triplicate with eleven 4-fold dilutions of pyrimethamine (0.0488–51 200 nM final concentrations). The test plates were dried in a laminar flow hood.

Preliminary studies had suggested that fosmidomycin binds to polystyrene when it is used to pre-coat test plates and the plates are dried, resulting in high 50% inhibitory concentration (IC_{50}) values. To avoid physical binding of the drug to plastic, fresh solutions of fosmidomycin derivatives in two-fold dilutions (25 μL/well; final concentrations, range = 25–25,600 nM) were distributed in duplicate just before performing the assays.

***In vitro* assay.** The *in vitro* response was determined by the isotopic microtest described by Desjardins and others.[19] Venous blood samples were transported without ice to our laboratory within 1–2 hours after collection. Infected erythrocytes were washed three times with RPMI 1640 medium buffered with 25 mM HEPES and 25 mM $NaHCO_3$ by centrifugation and suspended in the complete RPMI 1640 medium with either 10% non-immune type AB^+ human serum (pooled from donors at the Blood Transfusion Center, Strasbourg, France) or 10% mycoplasma-free fetal bovine serum (batch no. 5-41201; Integro b. v., Amsterdam, The Netherlands) at a 1.5% hematocrit. The suspension (200 μL) was distributed into each well. Parasitemia was adjusted to 0.6% by adding fresh uninfected erythrocytes if the initial parasitemia was ≥ 1%. The culture plates were incubated at 37°C in 5% CO_2 incubator. For fosmidomycin and its derivatives, parasite growth was assessed by adding ^{3}H-hypoxanthine (1 μCi/well; Amersham International, Little Chalfont, United Kingdom) to the test plate after 42 hours. Cultures were incubated for 72 hours. For chloroquine and pyrimethamine, ^{3}H-hypoxanthine was added at the beginning of the assay and test plates were incubated for 42 hours. The plates were frozen to terminate the *in vitro* assay. Incorporation of ^{3}H-hypoxanthine was measured with a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden). The IC_{50}, defined as the drug concentration at which 50% of the incorporation of ^{3}H-hypoxanthine is inhibited compared with that of drug-free control wells, was calculated by a nonlinear regression analysis using Prism™ software (GraphPad Software, Inc., San Diego, CA).

DNA extraction, polymerase chain reaction (PCR), and DNA sequencing. An aliquot of 2 mL of red blood cell pellet was used for extraction of parasite DNA. Infected erythrocytes were suspended in 15 mL of lysis buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5 [NET buffer], 0.015% saponin). The lysate was centrifuged at 2,000 × *g* for 10 minutes, and the pellet was resuspended in 500 μL of NET buffer in a 1.5-mL microfuge tube. The mixture was incubated with 1% sarkosyl and 200 μg/mL proteinase K at 50°C for 1 hr. DNA was extracted three times in equilibrated phenol (pH 8), phenol-chloroform (1:1 [v/v]), and chloroform and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried and resuspended in 50 μL of 10 mM Tris–1 mM EDTA (TE) buffer.

The entire coding region of the DOXP reductoisomerase gene was amplified by PCR. Synthetic oligonucleotides were designed from the complete DNA sequence of the gene (1,467 basepairs [bp]) of the HB3/Honduras clone (GenBank accession no. AF111813).[5] The gene encoding DOXP reductoisomerase is located on chromosome 14. The reaction mixture for the primary PCR consisted of 3 μL of DNA, 15 pmol of primer pairs (DXR-1 forward primer, 5′-ATG-AAGAAATATATTTATATATATTTTTTC-3′ and DXR-2R reverse primer, 5′-CTATGAAGAATTATGTTTGTTG-TATATATC-3′), 200 μM of deoxynucleoside triphosphate mixture (dGTP, dATP, dTTP, and dCTP), 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris buffer, pH 8.4, and 1 unit of *Taq* DNA polymerase (Roche Diagnostics, Meylan, France) in a final volume of 50 μL. The PCR was performed using the PTC-100 thermal cycler (MJ Research, Watertown, MA) under the following conditions: denaturation at 94°C for 2 minutes for the first cycle and 1 minute in subsequent cycles, annealing at 50°C for 2 minutes for the first cycle and 1 minutes in subsequent cycles, and extension at 72°C for 3 minutes for all cycles, for a total of 30 cycles.

The secondary nested PCR was performed with a similar PCR mixture as for the primary amplification using 1 μL of the primary product and 30 pmol of internal primer pairs DXR-3 (5′-TTCATCACAATAACTATTAATGATT-TAGTA-3′) and DXR-8R (5′-GTTAAATTTTGAAATG-GACCTCCAG-3′) or primer pairs DXR-5 (5′-GTTGC-GTTAGCTAATAAAGAATCCATTG-3′) and DXR-4R (5′-GGTAGCTTTATCTTTGGCCCAAGAATGTAT-3′) in a final volume of 100 μL. The following thermal cycling program was used: denaturation at 94°C for 2 minutes for the first cycle and 1 minute in subsequent cycles, annealing at 50°C for 1 minute in all cycles, and extension at 72°C for 1 minute for all cycles, for a total of 35 cycles. The amplified products were visualized by agarose gel electrophoresis and ultraviolet transillumination. The secondary amplification using DXR-3 and DXR-8R primer pairs and DXR-5 and DXR-4R primer pairs yield 803-bp and 840-bp products, respectively. These two products share an overlapping fragment spanning 236 bp. Each secondary amplification product was sequenced from both 5′- and 3′-ends using the ABI Prism automated DNA sequencer (Perkin Elmer Corp., Les Ulis, France).

Data analysis. Data were expressed as the geometric mean

IC_{50}. Based on our previous studies,[20,21] the arbitrary cut-offs for *in vitro* resistance to both chloroquine and pyrimethamine were fixed at an $IC_{50} \geq 100$ nM. Threshold values for fosmidomycin and its derivatives are not established. For the chloroquine-susceptible isolates and the chloroquine-resistant isolates, the mean logarithmic IC_{50} values of fosmidomycin were compared by the two-tailed, unpaired *t*-test. The coefficients of correlation (r) of the IC_{50} values were calculated by the Spearman rank correlation test. The significance level (P) was fixed at 0.05 for all statistical tests.

The deduced amino acid sequence of the isolates was compared with that of HB3/Honduras *P. falciparum* clone (GenBank accession no. AF111813). Alignment of the deduced amino acid sequence of *P. falciparum* DOXP reductoisomerase with the sequence from the homologous enzyme of *Escherichia coli* was based on published data.[5] If a mutation resulting in an amino acid substitution was detected among the isolates, DNA was re-extracted, and the nested PCR was performed using *Pfu* DNA polymerase (Promega France; Charbonnière-les-Bains, France), which has a proofreading activity. The PCR product was re-sequenced to confirm the presence of the mutation.

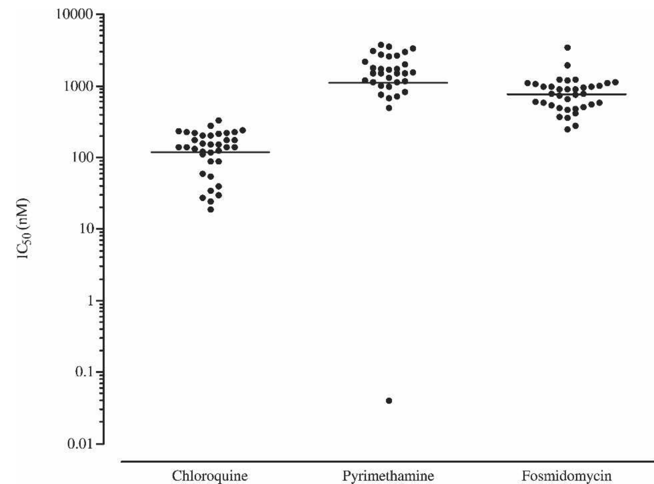

FIGURE 1. Response of 36 Cameroonian clinical isolates of *Plasmodium falciparum* to chloroquine, pyrimethamine, and fosmidomycin using RPMI 1640 medium–10% non-immune human serum. Horizontal bars indicate the geometric mean 50% inhibitory concentration (IC_{50}).

RESULTS

Preliminary studies were performed with 58 isolates collected in 2001–2002 (geometric mean parasitemia = 59,500 asexual parasites/μL of blood, range = 5,000–325,000 asexual parasites/μL before dilution, 3 isolates were excluded because of technical errors) using RPMI–10% non-immune human serum. The control thin blood films prepared after 42 hours of incubation showed that 8 isolates failed to grow during the first 48 hours because of a massive transformation into gametocytes or failure of schizont maturation, possibly due to an undetected presence of antimalarial drugs in the patients' plasma. The remaining 47 isolates developed into schizonts within the first 42 hours of incubation. Eleven of 47 isolates developed poorly during the second intraerythrocytic cycle between 42 and 72 hours, leading to a low quantity (< 1,000 counts per minute) of ^{3}H-hypoxanthine being incorporated into the parasite DNA in drug-free wells and uninterpretable results. Among isolates with satisfactory schizont maturation, the addition of ^{3}H-hypoxanthine at the beginning of the assay and an incubation period of 42 hours did not yield an interpretable result using the initial concentration range of fosmidomycin (50–3,200 nM in triplicate). The optimal assay conditions for fosmidomycin were obtained by using the concentration range 25–25,600 nM in duplicate and by adding ^{3}H-hypoxanthine 42 hours after the initial incubation, followed by an additional 30-hour incubation, for a total incubation of 72 hours. Based on the results of 36 isolates (geometric mean parasitemia = 67,500 asexual parasites/μL of blood, range = 5,000–300,000 asexual parasites/μL before dilution) with satisfactory parasite growth during both 48-hour and 72-hour incubation periods, the geometric mean IC_{50} (95% confidence interval [CI]) for fosmidomycin determined after 72-hr incubation was 756 nM (633–904 nM) (Figure 1).

In 2001–2002, there were 10 (28%) chloroquine-susceptible ($IC_{50} < 100$ nM) and 26 (72%) chloroquine-resistant ($IC_{50} \geq 100$ nM) isolates. The geometric mean IC_{50} (95% CI) of chloroquine-susceptible isolates was 41.2 nM (28.1–60.4 nM). The geometric mean IC_{50} (95% CI) of chloroquine-resistant isolates was 177 nM (158–199 nM). All isolates, except one (IC_{50} = 0.040 nM), were pyrimethamine-resistant ($IC_{50} \geq 100$ nM). The geometric mean IC_{50} of resistant isolates was 1,560 nM (range = 494–3,760 nM).

Further experiments suggested that the use of fetal bovine serum results in a higher invasion rate of merozoites and higher success rate of initiating the second intraerythrocytic cycle. Based on these findings, the *in vitro* activity of fosmidomycin was compared with that of its derivative FR900098 against 35 fresh isolates of *P. falciparum* in 2003. Of 35 isolates, 34 (97%, geometric mean parasitemia = 51,200 asexual parasites/μL of blood, range = 8,000–550,000 asexual parasites/μL before dilution) developed into schizonts using RPMI–10% fetal bovine serum within 42 hours and yielded interpretable results after 72 hours. One isolate (parasitemia = 30,000 asexual parasites/μL) failed to grow with both human serum and fetal bovine serum supplements during the first intraerythrocytic cycle because of self-medication with chloroquine reported by the patient but undetected by the urine screening test. The geometric mean IC_{50}s (95% CIs, n = 34) for fosmidomycin and FR900098 using fetal bovine serum were 301 nM (245–370 nM) and 118 nM (93.3–149 nM), respectively (Figure 2). The relative potency of FR900098 versus fosmidomycin was 2.6. Of these isolates, 12 (35%) were chloroquine-susceptible (geometric mean, 95% CI = 56.0 nM, 43.5–72.2 nM) and 22 (65%) were chloroquine-resistant (geometric mean, 95% CI = 197 nM, 158–247 nM). Assays for chloroquine response were performed with the standard RPMI–10% human serum, and chloroquine IC_{50}s were determined after a 42-hour incubation throughout the present study. The *in vitro* response to pyrimethamine was not determined for these 34 isolates because most parasites in Yaoundé have become pyrimethamine-resistant, as seen in our preliminary studies in 2001–2002 described above.

The comparison of assays using 10% human serum and 10% fetal bovine serum yielded the following geometric mean IC_{50}s (95% CIs, n = 9 isolates, results from 6 additional isolates were excluded because of poor growth in RPMI–

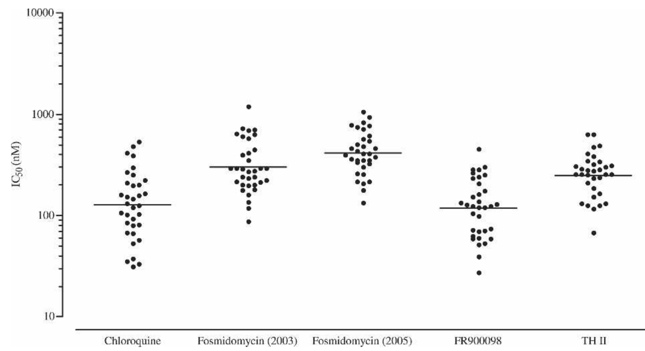

FIGURE 2. Response of Cameroonian clinical isolates of *Plasmodium falciparum* to fosmidomycin and its derivatives FR900098 and TH II46 (TH II), using RPMI 1640 medium–10% fetal bovine serum. Response to chloroquine was determined by using RPMI–10% non-immune human serum. Activities of chloroquine, fosmidomycin (2003), and FR900098 were evaluated in the isolates collected in 2003. Fosmidomycin (2005) and TH II46 were evaluated in the isolates collected in 2005. Horizontal bars indicate the geometric mean 50% inhibitory concentration (IC_{50}).

human serum during the second intraerythrocytic cycle): fosmidomycin/human serum, 973 nM (501–1,890 nM), FR900098/human serum, 583 nM (292–1,164 nM), fosmidomycin/fetal bovine serum, 451 nM (289–702 nM), and FR900098/fetal bovine serum, 187 nM (130–269 nM). The IC_{50}s for fosmidomycin and FR900098 determined after a 72-hour incubation with RPMI–fetal bovine serum were an average 2.2 and 3.1 times lower than the IC_{50}s determined with RPMI–human serum, respectively.

The fosmidomycin derivative TH II46 was synthesized in 2004. Its activity was compared with that of fosmidomycin against 37 isolates (geometric mean parasitemia = 20,500 asexual parasites/μL of blood, range = 5,000–100,000 asexual parasites/μL before dilution) in 2005. Four assays were uninterpretable because of poor growth. Based on the response of 33 isolates (geometric mean parasitemia = 24,400 asexual parasites/μL of blood, range = 5,000–100,000 asexual parasites/μL) that yielded interpretable results with RPMI–10% fetal bovine serum after a 72-hour incubation, the geometric mean IC_{50}s (95% CIs) were 413 nM (346–493 nM) and 249 nM (208–297 nM) for fosmidomycin and TH II46, respectively (Figure 2). TH II46 was an average 1.7 times more potent than the parent compound. For this series of experiments, the *in vitro* responses to chloroquine and pyrimethamine were not determined.

The comparison of logarithmic IC_{50} values for fosmidomycin between chloroquine-susceptible isolates (n = 22) and chloroquine-resistant isolates (n = 48) showed no statistical difference between the mean IC_{50}s ($P > 0.05$), which indicated that fosmidomycin activity is not influenced by the response to chloroquine (statistical test not done for pyrimethamine because of the skewed distribution of pyrimethamine-resistant isolates). The *in vitro* responses to chloroquine and fosmidomycin (r = 0.043, $P > 0.05$) and pyrimethamine and fosmidomycin (r = –0.103 $P > 0.05$) were not correlated.

The DOXP reductoisomerase gene was successful amplified by PCR from all 70 isolates with interpretable *in vitro* assays during 2001–2003. DNA from isolates tested in 2005 was not used. Isolates from each of the subgroups (2001–2002 and 2003) were listed according to the ascending order of IC_{50}s, and PCR products of every other isolate were sequenced. Sequence data were available from 33 isolates. The use of internal primers within the coding region to perform the nested PCR resulted in undetermined sequences of approximately 40 amino acid residues (total number of deduced amino acids = 489) in both the 5′ and 3′ ends of the gene in most isolates. The deduced amino acid sequences of the 33 isolates were highly conserved. Five amino acid substitutions were observed in five different isolates and confirmed by the second PCR amplification using *Pfu* DNA polymerase, followed by direct sequencing: Arg126Ile, Val148Ala, Ser173Cys, Asp326Glu, and mixed codon Gln/Arg361 (Table 1). Two of these isolates (Yaoundé 119/01 and 21/03) had relatively high IC_{50}s for fosmidomycin (1,940 nM and 1,920 nM, respectively) when the *in vitro* response was determined with RPMI–human serum. For isolate Yaoundé 21/03 (not done for Yaoundé 119/01), the corresponding IC_{50} determined with RPMI–fetal bovine serum was much lower (292 nM).

DISCUSSION

The results of the present *in vitro* study suggest that fosmidomycin is moderately active against both chloroquine-susceptible isolates and the chloroquine-resistant isolates of *P. falciparum*. Highly active antimalarial drugs, such as mefloquine, lumefantrine, atovaquone, and artemisinin derivatives, had IC_{50}s within a low nanomolar range.[22,23] Fosmidomycin is at least 50–100 times less potent than highly active drugs when tested in RPMI–human serum. Although the *in*

TABLE 1
In vitro drug response of *Plasmodium falciparum* isolates with amino acid substitutions in DOXP reductoisomerase*

Isolate	Amino acid changes†	IC_{50} (nM) and growth in RPMI1640 medium–human serum‡		IC_{50} (nM) and growth in RPMI–fetal bovine serum‡	
		Fosmidomycin	FR900098	Fosmidomycin	FR900098
Yaoundé 119/01	Arg126Ile	1,940 (4,670 cpm)	ND	ND	ND
Yaoundé 04/02	Ser173Cys	492 (2,400 cpm)	ND	ND	ND
Yaoundé 21/03	Val148Ala	1,920 (1,610 cpm)	1,280 (1,710 cpm)	292 (8,040 cpm)	133 (8,720 cpm)
Yaoundé 66/03	Mixed Gln/Arg361	ND	ND	212 (9,550 cpm)	70.5 (11,200 cpm)
Yaoundé 88/03	Asp326Glu	548 (1,620 cpm)	639 (1,420 cpm)	574 (4,140 cpm)	241 (4,360 cpm)

* DOXP = 1-deoxy-D-xylulose 5-phosphate; IC_{50} = 50% inhibitory concentration; ND = not done.
† GenBank accession nos.: Yaoundé 119/01, EF545153; Yaoundé 04/02, EF545154; Yaoundé 21/03, EF545155; Yaoundé 66/03, EF545156; Yaoundé 88/03, EF545157.
‡ The IC_{50}s were determined after a 72-hour incubation. Among the isolates tested in 2001–2002, wild-type isolates (n = 34) yielded a geometric mean fosmidomycin IC_{50} of 745 nM (range = 251–3,520 nM), while two mutant isolates had IC_{50}s of 1,940 nM and 492 nM. Among the isolates tested in 2003, wild-type isolates (n = 31) had geometric mean fosmidomycin and FR900098 IC_{50}s of 298 nM (range = 86.8–1,200 nM) and 116 nM (range = 27.5–452 nM) using fetal bovine serum, respectively. Growth denotes the quantity of ^{3}H-hypoxanthine incorporated by the parasites during part of the second intraerythrocytic cycle. The mean counts per minute (cpm) in duplicate drug-free wells for parasites cultivated in RPMI–human serum and/or RPMI–fetal bovine serum is shown in parentheses. Growth was arbitrarily considered to be satisfactory for ≥ 1,000 cpm.

vitro response cannot be extrapolated to predict *in vivo* response because the latter is partly determined by pharmacodynamic and pharmacokinetic parameters and the level of host immunity, the failure to obtain a satisfactory cure rate 14–28 days after 3–7-day fosmidomycin monotherapy is consistent with the moderate level of fosmidomycin activity.[12,13]

In a previous study using reference clones of *P. falciparum*,[5] the following fosmidomycin IC_{50}s (± SD) were reported: 350 ± 170 nM for HB3/Honduras, 370 ± 45 nM for A2/The Gambia, and 290 ± 130 nM for Dd2/Indochina. In a later study by the same investigators,[24] higher mean IC_{50}s for fosmidomycin were reported for the same clones and 3D7 reference clone (geometric mean = 732 nM, range = 488–1,171 nM), with an overall range of 224–1,268 nM for the four clones. The *in vitro* activity of fosmidomycin against the Cameroonian clinical isolates is within the range of activity against the reference clones. In our study, many factors, including incubation period, duration of ^{3}H-hypoxanthine pulsing, starting parasitemia, and use of RPMI–human serum, were similar to those of the previous studies. These similarities enabled a direct comparison of *in vitro* responses of fresh clinical isolates to those of laboratory-adapted reference clones. Our studies are also in agreement with previous findings that FR900098 and TH II46 are approximately 2–3 times more active than the parent compound fosmidomycin.[5,25]

Higher activities of fosmidomycin derivatives, as well as a higher success rate of growth during the second intraerythrocytic cycle, were obtained when RPMI medium was supplemented with fetal bovine serum instead of with human serum. This observation depends on the batch of fetal bovine serum because some batches do not support parasite growth.[26] The possible explanations for lower IC_{50}s when using fetal bovine serum were not explored. The lower values may be due to differential drug-protein affinity, facilitated drug transport, or other biochemical processes. A 2–3-fold difference in the IC_{50} obtained when using fetal bovine serum instead of human serum has also been observed with other antimalarial drugs.[27,28] The same culture conditions (use of human serum, 42-hour incubation, pulsing with ^{3}H-hypoxanthine, and other factors) were maintained for the chloroquine assay throughout the present study to avoid large differences in IC_{50}s.

A wide variation in the *in vitro* fosmidomycin response was observed in clinical isolates in the present study and also against the reference clones in previous studies.[5,24] Based on the limited number of isolates with a single amino acid change observed in the present study, it cannot be concluded whether the relatively high IC_{50} for fosmidomycin determined with RPMI–human serum in two isolates is due to a natural phenotype variation, as observed with other antimalarial drugs, reduced response to fosmidomycin, or artifacts related to a relatively poor growth rate during the second intraerythrocytic cycle with RPMI–human serum. Although a single mutation within a 1,467-bp sequence may be the result of an erroneous DNA replication and amplification by *Taq* DNA polymerase, which lacks proofreading activity, the second PCR amplification using *Pfu* and sequencing confirmed the presence of these mutations in five isolates. A definite proof that the amino acid substitutions observed in this study are associated with a reduced response to fosmidomycin would require further experiments with the mutant genes produced by mutagenesis and characterization of the expressed protein.

Crystallographic studies of *E. coli* DOXP reductoisomerase have shown that fosmidomycin can be superimposed on the natural substrate DOXP and that the following amino acid residues, which are highly conserved in different organisms, may form the putative active site (positions in the *P. falciparum* DOXP reductoisomerase with the corresponding positions in *E. coli* DOXP reductoisomerase in parentheses): Ile89 (Ile13), Asp 231 (Asp150), Glu233 (Glu152), His234 (His153), His293 (His209), Met298 (Met214), Ile302 (Ile218), Glu315 (Glu231), Glu318 (Glu234), His341 (His257), and Met360 (Met276).[29–32] In all 33 *P. falciparum* isolates sequenced in the present study, there was no amino acid substitution at these positions. Furthermore, these crystallographic data indicate that the *E. coli* DOXP reductoisomerase is V-shaped and consists of three domains: amino-terminal NADPH binding domain (residues 1–150), the central domain containing the active site (residues 151–311), and the carboxyl-terminal alpha-helical domain (residues 312–398). The corresponding positions in the *P. falciparum* enzyme are residues 1–231, residues 232–395, and residues 396–489. The amino acid substitutions observed in the present study are located within the putative NADPH-binding domain and the putative central domain. However, this correspondence remains speculative until the *P. falciparum* DOXP reductoisomerase is crystallized and characterized.

The results of the present study suggest that fosmidomycin is moderately active *in vitro* against both chloroquine-susceptible and chloroquine-resistant *P. falciparum* isolates. Two of its derivatives tested in this study were more potent than the parent compound. Fosmidomycin inhibits a novel target, DOXP reductoisomerase, that is specific for malaria and some other apicomplexans, most eubacteria, and plants but is absent in humans. The sequence of the target enzyme was highly conserved in *P. falciparum* isolates. Furthermore, recent studies have suggested that fosmidomycin is safe and well-tolerated in malaria-infected patients and may be of clinical use, particularly if it is used in combination with artesunate or clindamycin.[12–16] Other fosmidomycin derivatives have been synthesized to improve drug absorption via the oral route and to increase schizontocidal potency and are currently under evaluation in preclinical studies.[25,33–35] Available data suggest that fosmidomycin and its derivatives are promising candidates for further clinical development.

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