Novel K540N Mutation in Plasmodium falciparum Dihydropteroate Synthetase Confers a Lower Level of Sulfa Drug Resistance than Does a K540E Mutation

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Sulfadoxine (SDX) and sulfamethoxazole (SMX) each inhibit the Plasmodium falciparum dihydropteroate synthetase (DHPS), and certain point mutations in this enzyme yield the drug-resistant parasite. Using a simple Escherichia coli model system, we describe here the effect of the recently reported novel K540N mutation in DHPS on the level of SDX/SMX resistance. The survival rate of the transformed E. coli (DHPS-deficient strain) under different SDX or SMX concentrations revealed that the K540N mutation confers a lower level of drug resistance than its contemporary K540E mutation. Further, SMX was more effective than SDX in the E. coli system.

Plasmodium falciparum can cause the most severe form of malaria and death in humans. Antifolates are commonly used for P. falciparum malaria treatment. These drugs can specifically inhibit the enzymes involved in the parasite folate biosynthesis pathway. Both sulfadoxine (SDX) and sulfamethoxazole (SMX) target dihydropteroate synthetase (DHPS) by competing with its substrate para-aminobenzoic acid. Five point mutations in the P. falciparum DHPS (PfDHPS) at codons 436, 437, 540, 581, and 613 had been reported to be associated with resistance to sulfonamides (7–9). Different PfDHPS mutant alleles have been reported from different parts of the world, including India. Recently, we have reported a novel K540N mutation in PfDHPS among P. falciparum isolates from Andaman and Nicobar Islands of India (6). This K540N mutation in PfDHPS has now also been reported from other countries (10). Using the Escherichia coli system, we have evaluated here the in vitro effect of this novel PfDHPS mutation on drug resistance levels compared to that of its contemporary K540E mutation.

Various cell-based inhibition studies had earlier been used to complement the dhps gene from Mycobacterium, Pneumocystis, and Plasmodium (1, 4, 5). Therefore, we used here the model E. coli system to evaluate the effect of the different mutant Plasmodium falciparum pyrophosphokinase (PfPPPK)-DHPS alleles on PfPPPK-DHPS gene was amplified from cDNA by high-fidelity proofreading enzyme Platinum PfX DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) using the primers pGEXF (5’-CAAGAATTCTACATTTCTTGAGG-3’) and pGEXR (5’-CTCAGGTACCTTGCTATTTTG-3’). The cDNA was denatured at 94°C for 5 min, and PCR amplification was carried out for 35 cycles under the following conditions. The primers were annealed at 48°C for 1 min, with extension for 2 min at 60°C and cycling denaturation at 94°C for 30 s. The final extension was carried out at 60°C for 10 min. The PCR product was subcloned in pGEM-T (Promega Corp., Madison, WI) and then transferred to pGEX 4T-1 (glutathione S-transferase-tagged) (GE Healthcare Life Sciences, Buckinghamshire, NA, England) vector for expression. The resulting pGEX 4T-1–PfPPPK-DHPS was used as a template to modify the gene for improved stability through site-directed mutagenesis using primers InsF (5’-GAATATGAACTAAATTAATGAGAAGAAGAAACAAATTCATAC-3’) and InsR (5’-CTACTAAGATATCCCTCTCTCTTATAATTTATTATATATATTT-3’) as described in reference 9. This PCR product was digested with DpnI and then used to transform the E. coli DH5α strain. The amino acid sequence encoded by the PfPPPK-DHPS gene was S436A437K540A581A613 as confirmed by nucleotide sequencing (the mutated amino acid is in boldface). This was used as a template for one-step mutagenesis to create alleles encoding the sequences S436A437K540A581A613 and S436A437N540A581A613 and A436G437S540A581A613 by using the primers A436F (5’-ACTTCTCGCTCTTTTTGTTTACC-3’) and A437R (5’-ACTAATTTTTTTAGTACATTACC-3’) for S436A437K540A581A613, A436F (5’-ATCCCTGTTCTCCCTCTCTCTTTTACC-3’) and A437R for S436A437N540A581A613, and N540F (5’-GATAATTCAAAATTTATGTTAC-3’) and E540R (5’-TTATATCAAAATTTATGTTAC-3’) for S436A437N540A581A613 and the gene tailor mutagenesis kit (Invitrogen Life Technologies, Carlsbad, CA). The PfPPPK-DHPS amino acid sequence A436G437S540A581A613 was used as a template to create alleles encoding the A436G437E439A581A613 and A436G437N540A581A613 sequences using primers E540F (5’-A439F (5’-CTCAGGTACCTTGCTATTTTG-3’), and A439R for S436A437N540A581A613 and the gene tailor mutagenesis kit (Invitrogen Life Technologies, Carlsbad, CA). The PfPPPK-DHPS amino acid sequence A436G437S540A581A613 was used as a template to create alleles encoding the A436G437E439A581A613 and A436G437N540A581A613 sequences using primers E540F (5’-
GATGAAGCAACAAATTTATGATAAATC^3)/E540R and N540F/ES436R, respectively. The PCR conditions were the same as those described above except that only 20 instead of 35 cycles of amplification were performed here. The pGEX 4T-1–IPPPK–DHPS constructs were precultured overnight in LB medium containing 100 μg/ml ampicillin. The next day, the culture was diluted with LB medium to an optical density at 600 nm of ~0.5. Twenty microliters of this cell suspension was then seeded in 1 ml of Mueller-Hilton medium (Difco, BD Biosciences, San Jose, CA) in 24-well culture plates (Corning Incorporated, Corning, NY) supplemented with various concentrations of SDX (0, 32.26, 64.52, 121.29, 252.81, 322.58, 645.16, 1,412.90, 2,258.06, or 3,225.81 μM) or SMX (0, 39.53, 79.05, 197.63, 276.68, 395.25, 790.51, 1,976.28, 2,766.80, or 3,952.57 μM) dissolved in dimethyl sulfoxide (DMSO). The culture plates were then grown at 37°C with shaking for 6 h before the growth was measured at A600. The A600 values were plotted against the log of drug concentrations, and 50% cell growth inhibition of variance (ANOVA) was done to determine the significant differences between the IC50s of different constructs.

The IC50s of SDX or SMX for wild-type and mutant PfDHPS alleles are shown in Table 1. These IC50s of SMX were significantly lower (P < 0.05) than those of SDX for all the PfDHPS alleles except for the allele encoding the A346G S436K540A581A613 sequence. Lower IC50s for SMX than SDX have also been reported by the in vitro P. falciparum culture system (11). The IC50s of SDX or SMX for the wild-type allele encoding the S436G A540K S581A613 sequence were insignificantly lower than the IC50s for the allele encoding the S346A347N 540A581A613 sequence. But the IC50s of both drugs for the wild-type allele were significantly lower than that of the other mutant PfDHPS alleles (P < 0.05). For example, the IC50s of SDX and SMX for the single-mutant allele encoding the S346A347G K540A S581A613 sequence were 5.59- and 4.04-fold higher, respectively, than that of the wild-type PfDHPS allele. Additional mutations S456A and K540E in PfDHPS significantly increased the drug resistance further (P < 0.05). Thus, the triple-mutant PfDHPS allele encoding the A346G S457E 540A 581A 613 sequence showed the highest IC50 of SDX or SMX. These results were similar to those of earlier reports where additional mutations in this enzyme caused higher levels of SDX resistance (1, 6, 9). However, the novel mutant allele encoding the A346G S457N 540A 581A 613 sequence showed significantly lower IC50 values than the mutant allele encoding the A346G S457E 540A 581A 613 sequence (P < 0.05) (Table 1). This result indicates that the novel mutation confers less resistance to SDX or SMX than its contemporary mutation in the pfdhps gene. The mutation K540E causes a loss in the hydrophobic interactions with side chains of lysine and sulfadoxine methoxy groups and thus increases the hydrophilicity and the loop widening to expose the active site (2, 6). In the case of the K540N mutation, binding of SDX is altered because a hydrogen bond could not be formed with the drug due to the short side chain of asparagine compared to that of lysine (6).

Earlier, we described the selection of the parasite population with this novel K540N mutation in PfDHPS which was predicted to be due to an unusual drug pressure in the field (6). Although the K540N mutation containing the parasite could be less SDX/SMX resistant than the K540E mutation, its association with two other mutations (triple PfDHPS mutant alleles) among the majority of the isolates is a cause of concern.

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