

# Isoprenoid Biosynthesis of the Apicoplast as Drug Target

Jochen Wiesner and Hassan Jomaa\*

Universitätsklinikum Giessen und Marburg, Institut für Klinische Chemie und Pathobiochemie, Gaffkystrasse 11, 35392 Giessen, Germany

\*Address correspondence to this author at the Universitätsklinikum Giessen und Marburg, Institut für Klinische Chemie und Pathobiochemie, Gaffkystrasse 11, 35392 Giessen, Germany; Fax: (49)-641-9947579; E-mail: hassan.jomaa@uniklinikum-giessen.de

**Abstract:** In *Plasmodium falciparum* the biosynthesis of isoprenoids is achieved by the mevalonate-independent 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway. The enzymes of the DOXP pathway are localised inside the plastid-like organelle (apicoplast). Fosmidomycin inhibits DOXP reductoisomerase, the second enzyme of this pathway. The antimalarial activity of fosmidomycin was established *in vitro* and in a rodent malaria model. Fosmidomycin alone or in combination with clindamycin was evaluated for the treatment of acute uncomplicated *P. falciparum* malaria in early phase II studies. Fosmidomycin monotherapy led to a fast parasite and fever clearance but was inefficient in radical elimination of the parasites. With the fosmidomycin-clindamycin combinations the cure ratio on day 28 was 100 % (10/10) with treatment durations of 5 and 4 days. The cure ratio was 90 % (9/10) with treatment duration of 3 days.

**Key Words:** Malaria; apicoplast; isoprenoid biosynthesis, DOXP reductoisomerase, fosmidomycin, FR900098.

## INTRODUCTION

Fosmidomycin (**1**) was originally isolated as natural antibiotic from *Streptomyces lavendulae* [1] and developed through early clinical trials for the treatment of urinary tract infections by Fujisawa Pharmaceuticals Company (Osaka, Japan) in the early 1980s (Fig. (1)) [2]. This development was abandoned, probably due to relatively low efficacy compared to other upcoming new classes of antibiotics. The mechanism of action of fosmidomycin was largely unknown despite some observations of effects on the isoprenoid content of bacteria [3]. The molecular target of fosmidomycin was finally elucidated with the discovery of a mevalonate-independent pathway of isoprenoid biosynthesis, the so-called 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway [2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, nonmevalonate pathway, Rohmer pathway] [4]. This pathway is found in most eubacteria and the plastids of plant [5,6]. Fosmidomycin was demonstrated to represent a highly specific inhibitor of DOXP reductoisomerase, the second enzyme of the DOXP pathway [7,8]. Inspired by the finding that malaria parasites of the genus *Plasmodium* possess a non-photosynthetic plastid-like organelle (apicoplast), which according to a widely accepted hypothesis had been acquired by secondary endosymbiosis of an alga [9-13], it was investigated whether the isoprenoid biosynthesis of malaria parasite might also depend on the DOXP pathway [14,15]. At that time only the first two enzymes of the DOXP pathway (DOXP synthase and DOXP reductoisomerase) were known and the corresponding genes could be identified in the emerging *P. falciparum* genome sequence database. The deduced polypeptides were predicted to be apicoplast-localised due to characteristic N-terminal targeting sequences. When the antimalarial potency of fosmidomycin was evaluated considerable activity was found *in vitro* and in mice infected with the rodent parasite *P. vinckei*. In the meantime, several clinical phase II studies for the treatment of *P. falciparum* malaria with fosmidomycin and fosmidomycin combinations have been conducted.

Even though the DOXP pathway is likely to be present in most other *Apicomplexa* detailed biochemical data are only available for

*Plasmodium*. Also, among the several enzymes of the DOXP pathway inhibitors are only known for DOXP reductoisomerase at present, most of them fosmidomycin derivatives. For those reasons, this review focuses on the current knowledge on the isoprenoid biosynthesis of *P. falciparum* and the evaluation of DOXP reductoisomerase inhibitors as antimalarial drugs.

## ISOPRENOID BIOSYNTHESIS IN *P. FALCIPARUM*

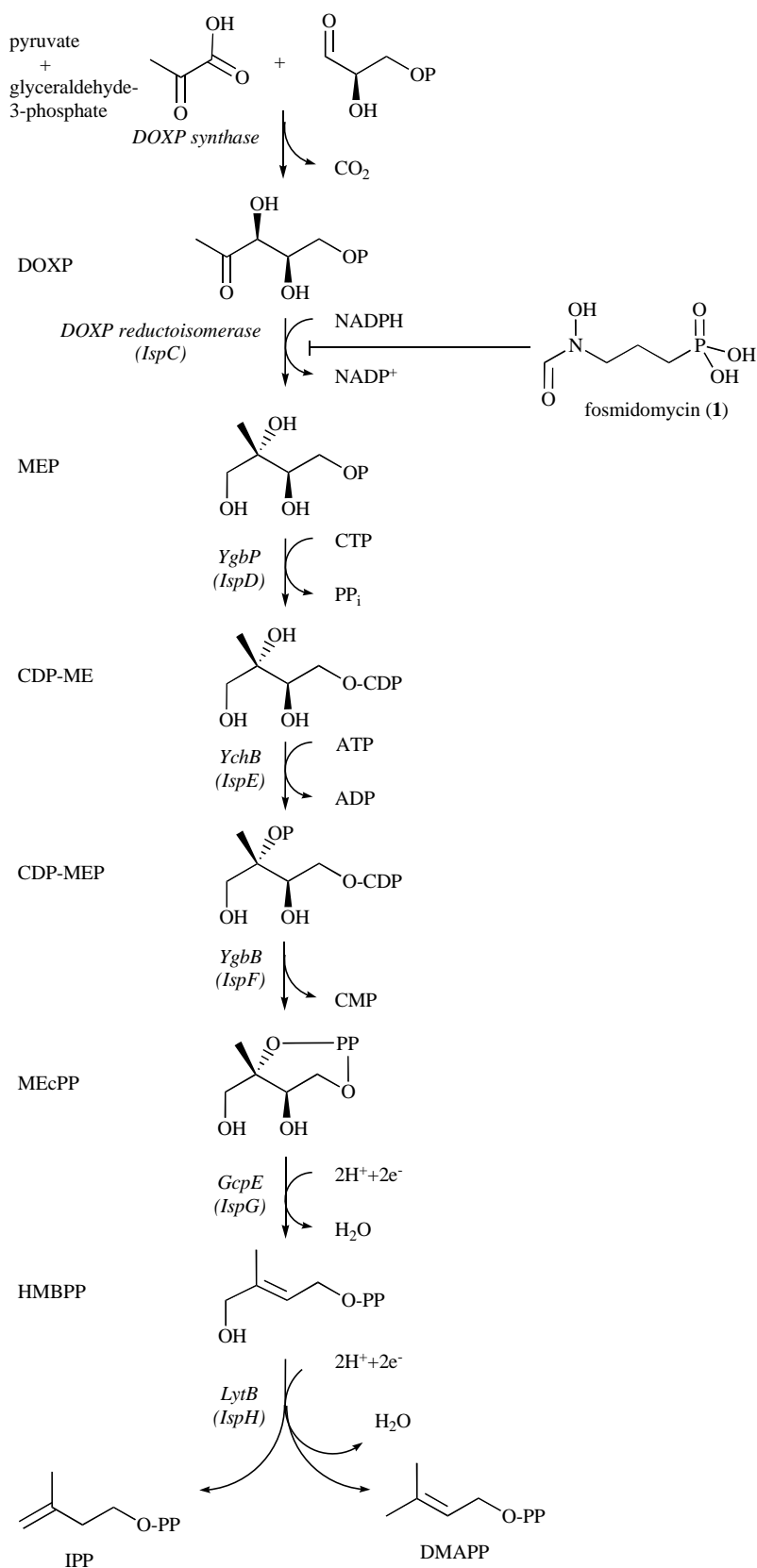
### Overview of the DOXP Pathway

All isoprenoids are assembled from five-carbon building blocks derived from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). Until quite recently it was assumed that the well-characterised mevalonate pathway is the only possible biosynthetic route leading to IPP and DMAPP. The mevalonate pathway starts with the condensation of three molecules acetyl-CoA and is used by animals, fungi, archaeobacteria, and some eubacteria [16]. In 1993 evidence was provided for the existence of an alternative completely unrelated pathway for the synthesis of IPP and DMAPP leading to the cloning of DOXP synthase as the first enzyme of the DOXP pathway in 1997 [4,17-23] while the last two reaction steps were elucidated in 2002 [24-26]. The DOXP pathway is used by most eubacteria as well as by the plastids of algae and higher plants. In plants both pathways are present with the DOXP pathway being operative in the plastids and the mevalonate pathway in the cytosol. In green algae the cytosolic mevalonate pathway is missing and isoprenoid supply solely depends on the plastidial DOXP pathway while in *Euglena* only the mevalonate pathway seems to be present [27]. In some eubacteria the presence of both, a functional DOXP and a mevalonate pathway has been demonstrated [28,29].

The DOXP pathway is initiated by the reaction of glyceraldehyde 3-phosphate and pyruvate to 1-deoxy-D-xylulose 5-phosphate (DOXP) catalysed by DOXP synthase (Fig. (1)).

In addition to isoprenoids DOXP can also serve as precursor for the synthesis of the cofactors thiamine pyrophosphate and pyridoxal phosphate. As the first step unique to isoprenoid synthesis DOXP is converted to 2-C-methyl-D-erythritol 4-phosphate (MEP) by DOXP reductoisomerase. Three additional well-characterised enzymatic steps lead to the formation of 2-C-methyl-D-erythritol-2,4-cyclo-diphosphate (MEcPP) [30]. The last two steps leading to IPP and DMAPP with (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate

\*Address correspondence to this author at the Universitätsklinikum Giessen und Marburg, Institut für Klinische Chemie und Pathobiochemie, Gaffkystrasse 11, 35392 Giessen, Germany; Fax: (49)-641-9947579; E-mail: hassan.jomaa@uniklinikum-giessen.de



**Fig. (1).** The reaction steps of the DOXP pathway. Fosmidomycin (1) inhibits the second step catalysed by DOXP reductoisomerase. CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate; DMAPP, dimethylallyl diphosphate; DOXP, 1-deoxy-D-xylulose-5-phosphate; HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate; IPP, isopentenyl diphosphate; MEcPP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate.

(HMBPP) as intermediate are less well understood. The involved enzymes GcpE and LytB were characterised as oxygen sensitive iron-sulphur proteins most likely containing [4Fe-4S] clusters [24-26,31,32]. Both reactions depend on the transfer of two single electrons. In *E. coli* it was demonstrated that flavodoxin together with flavodoxin reductase and NADPH can serve as electron donor for both GcpE and LytB [25,31,32] while in the cyanobacterium *Thermosynechococcus elongatus* ferredoxin was identified as electron donor for GcpE [33]. The nature of the electron shuttle systems in the plant plastids and the plastid-like organelle of apicomplexan parasites are unknown. Although not yet proven it has been hypothesised that the ferredoxin/ferredoxin reductase system recently identified in *P. falciparum*, *Toxoplasma gondii* and other *Apicomplexa* may play a role in this process [34]. LytB leads to the simultaneous formation of IPP and DMAPP in a ratio of approximately 5:1 from HMBPP. This ratio resembles the requirement of an excess of IPP for the downstream synthesis of isoprenoids. The LytB catalysed reaction is in sharp contrast to the last step of the mevalonate pathway which exclusively leads to IPP which is then converted to DMAPP by an IPP isomerase.

Remarkably, HMBPP was demonstrated to activate human  $\gamma/\delta$  T cells in the sub-nanomolar range and, therefore, is likely to be responsible for the elevated level of  $\gamma/\delta$  T cells in the blood of patients suffering from malaria and other infections with microbial pathogens known to possess the DOXP pathway [35,36]. The role of this activation of  $\gamma/\delta$  T cells for the disease and its possible implications for the development of antimicrobial therapeutics targeting the DOXP pathway are unknown. Both a protective function of the  $\gamma/\delta$  T cells and a role in the induction of immunological tolerance have been discussed [37].

#### The DOXP Pathway in *P. falciparum* in Detail

Already before the discovery of the DOXP pathway different studies were performed on the isoprenoid biosynthesis in malaria parasites. These studies were designed under the assumption that *de novo* synthesis of isoprenoids can only occur via the mevalonate pathway. Thus, cell-free extracts of *P. falciparum* and *P. knowlesi* were analysed for HMG-CoA reductase (a key enzyme of the mevalonate pathway) activity, leading to a negative result [38]. Nevertheless, HMG-CoA reductase inhibitors commonly used for the treatment of hypercholesterolemia were found to inhibit the *in vitro* growth of *P. falciparum* [39]. However, high inhibitor concentrations which are not of pharmaceutical relevance were required. Radioactively labelled acetate but not mevalonate was reported to be incorporated into isoprenoids by *P. falciparum* infected erythrocytes while another study claimed also the incorporation of mevalonate into farnesyl pyrophosphate using a cell-free parasite extract [40,41].

With the completion of the sequencing of the genome of *P. falciparum* the genes of all enzymes of the DOXP pathway could be identified while there are no homologies to any of the genes encoding enzymes of the mevalonate pathway. The *P. falciparum* derived genes encoding DOXP reductoisomerase and YgbB could be expressed in *E. coli* resulting in functional enzymes [14,42]. In addition,

using a sensitive biochemical assay DOXP reductoisomerase activity could be detected in crude and partially purified protein extracts from *P. falciparum* [43]. Most intermediates of the DOXP pathway could be detected by electrospray mass spectrometry in extracts from cultured *P. falciparum* parasites which were fractionated by HPLC [44]. Radiolabelled DOXP and acetate were found to be efficiently incorporated into these intermediates. In addition, incorporation of radiolabelled DOXP into pyridoxine phosphate was demonstrated. All enzymes of the DOXP pathway are encoded by nuclear genes of *P. falciparum*. The predicted amino acid sequences of these enzymes display characteristic N-terminal extensions which are recognised as apicoplast targeting signals [9]. Experimental evidence for the targeting of the DOXP reductoisomerase of *P. falciparum* to the apicoplast was provided using a plasmid construct encoding the leader sequenced fused to green fluorescent protein (GFP). Transient transfection of *T. gondii* (an apicomplexan parasite which can be easier manipulated than *P. falciparum*) with this construct resulted in an accumulation of GFP in the apicoplast [14].

Little is known about the isoprenoid metabolism in *P. falciparum* downstream of IPP and DMAPP. Genes encoding several prenyl diphosphate synthases responsible for the assembly of higher isoprenoids from IPP and DMAPP have been found in the *P. falciparum* genome [12]. Since these enzymes are apparently cytosolically located it must be anticipated that IPP and DMAPP are transported out of the apicoplast by a poorly understood mechanism. Ubiquinones and dolichols probably represent the most dominant extraplastidic end products of the isoprenoid biosynthesis pathway [40,45,46]. Ubiquinones are involved in the mitochondrial electron transport while dolichols are essential for protein glycosylation. The dolichol dependent transfer of glycosylphosphatidyl inositol (GPI) anchors onto membrane-bound proteins is essential for most, if not all, *P. falciparum* surface proteins and apparently represents the only glycoconjugate formation present in this organism [46,47]. Prenylation of proteins, i.e. the attachment of a farnesyl or geranylgeranyl residue, was identified as another essential role of isoprene chains in the cytosol of *P. falciparum* [48-50]. In addition to the synthesis of cytosolic isoprenoids the DOXP pathway has been predicted to play a role in the syntheses of isopentenylated tRNAs inside the apicoplast [12]. Four tRNAs encoded by the apicoplast genome are likely to require an isopentenyladenosine residue in the anticodon loop for correct translation of the apicoplast DNA [51]. An apicoplast targeted homologue of tRNA isopentenyltransferase, which probably uses DMAPP as substrate, has been identified in the *P. falciparum* genome. Early reports on an increased cholesterol content of erythrocytes infected with malaria parasites could not be confirmed, and *de novo* synthesis of cholesterol and other steroids by malaria parasites is obviously missing [38,52].

Genes encoding enzymes of the DOXP pathway can also be found in the genomes of other *Apicomplexa* such as *Babesia*, *Theileria*, *Toxoplasma* and *Neospora*. DOXP reductoisomerase activity was also demonstrated by a functional assay in cell extracts of *T. gondii* (J. Wiesner and D. Soldati, unpublished observations). This activity was inhibited by fosmidomycin. The apparent failure of

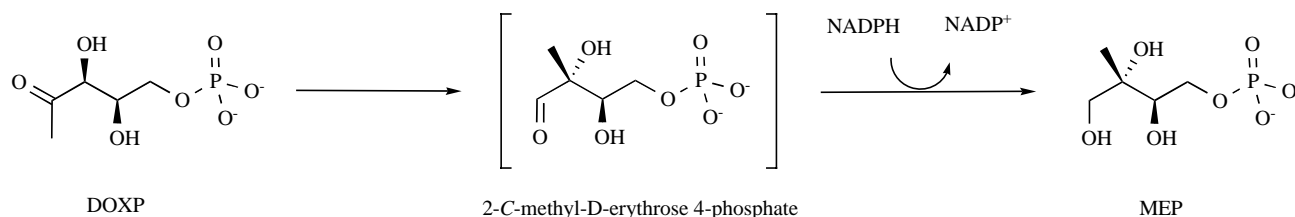


Fig. (2). Conversion of DOXP into MEP by DOXP reductoisomerase via the putative intermediate 2-C-methyl-D-erythrose 4-phosphate.

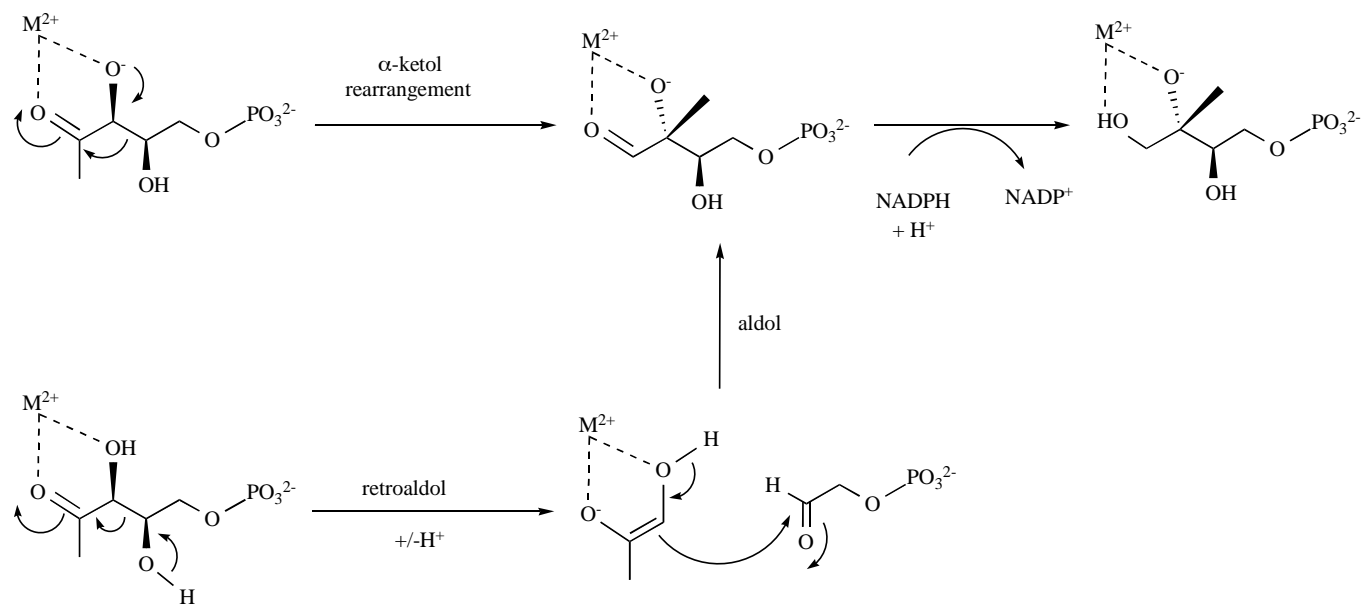


Fig. (3). Hypothetical mechanisms for the intramolecular rearrangement catalysed by DOXP reductoisomerase.

fosmidomycin to inhibit the growth of *T. gondii* may be due to insufficient access of the drug to the parasite (H. Jomaa and D. Soldati, unpublished observations). In *Cryptosporidium*, another apicomplexan parasite, the DOXP pathway is apparently missing. There is compelling evidence from the recently finished genomic sequences of both *C. parvum* and *C. hominis* that these organisms have lost the apicoplast and the plastid-associated metabolic pathways in course of the evolution [53,54]. The presence of genes encoding some enzymes of the downstream isoprenoid metabolism such as farnesyl and geranyl-geranyl transferase may suggest that isoprenoid precursors are scavenged from the medium.

## DOXP REDUCTOISOMERASE AS DRUG TARGET

### Catalytic Mechanism of DOXP Reductoisomerase

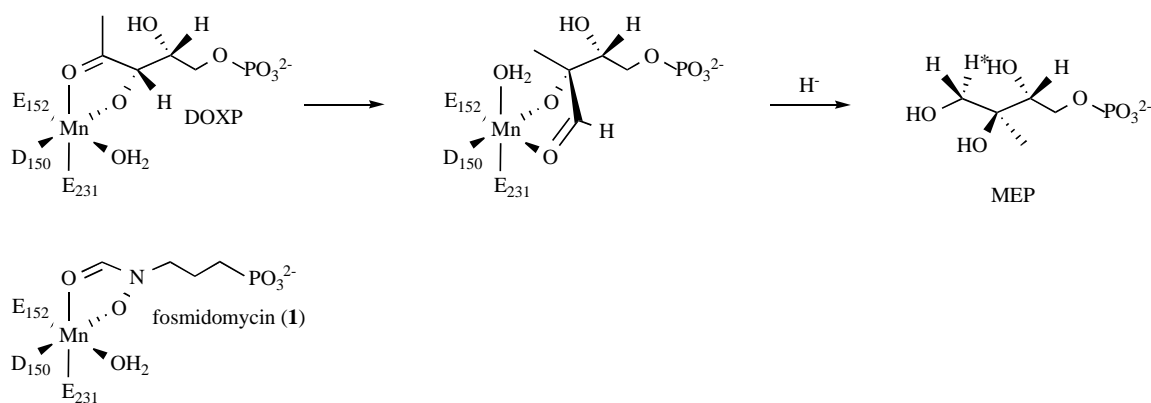
Most studies on DOXP reductoisomerase were performed with the recombinant enzyme of *E. coli* [55]. In addition, recombinant DOXP reductoisomerase has been obtained from a variety of bacteria (*Pseudomonas aeruginosa* [56], *Zymomonas mobilis* [57], *Synechococcus leopoliensis* [58], *Streptomyces coelicolor* [59], *Synechocystis sp.* PCC6803 [60], *Mycobacterium tuberculosis* [61]) and plants (*Arabidopsis thaliana* [62], *Mentha piperita* [63], *Zea mays* [64]). Also, DOXP reductoisomerase of *P. falciparum* was produced as recombinant protein using a fully synthetic gene adapted to the preferred codon usage of *E. coli* [14]. Nevertheless, the accessibility of DOXP reductoisomerase of *P. falciparum* is limited since only small amounts of soluble protein were obtained, and the enzyme turned out to be very unstable. With respect to the highly conserved primary structure of DOXP reductoisomerase and the available experimental data it can be assumed that the homologous enzymes from different organisms rely on quite similar catalytic mechanisms.

The DOXP reductoisomerase catalysed conversion of DOXP into MEP depends on divalent metal cations, under physiological conditions most likely  $Mg^{2+}$  or  $Mn^{2+}$ . The reaction proceeds via an intramolecular rearrangement of the carbon skeleton of DOXP leading to the putative intermediate 2-C-methyl-D-erythrose 4-phosphate followed by an NADPH dependent reduction step (Fig. (2)). Even though the existence of 2-C-methyl-D-erythrose 4-phosphate as transient intermediate could not be proven directly, synthetic 2-C-methyl-D-erythrose 4-phosphate was accepted as substrate and converted into MEP in the presence of NADPH and  $Mg^{2+}$  or  $Mn^{2+}$  [65]. In contrast, it was not possible to separate the isomerisation of

DOXP to 2-C-methyl-D-erythrose 4-phosphate from the reduction reaction. Conversion of DOXP was only observed in the presence of NADPH. Even with dihydro-NADPH, an NADPH analogue which is not a reducing cofactor, there was no reaction. Dihydro-NADPH was shown to inhibit DOXP reductoisomerase as a competitive inhibitor for NADPH, thus expected to bind to the enzyme very similar to the natural coenzyme [66]. It was therefore concluded that the reduction step seems to represent the driving force to perform the rearrangement.

The mechanism of the rearrangement of the carbon backbone is not yet fully understood. Two different ways for the formation of 2-C-methyl-D-erythrose 4-phosphate from DOXP seem to be possible [61,65,67] (Fig. (3)): (1) A hypothetical  $\alpha$ -ketol rearrangement mechanism would involve the deprotonation of the hydroxyl group at C-3 of DOXP followed by the migration of the phosphate-bearing C2 subunit. (2) Alternatively, a retroaldol/aldol type mechanism has been suggested involving the deprotonation of the C-4 hydroxyl group of DOXP, followed by the cleavage of the carbon-carbon bond between the carbon atoms C-3 and C-4 to give the enolate of hydroxyacetone and glycolaldehyde phosphate. Rearrangement via an aldol addition by formation of a new carbon-carbon bond between the carbon atoms derived from C-2 and C-4 of DOXP would afford 2-C-methyl-D-erythrose 4-phosphate. At present, there are no unambiguous experimental results in favour of one of the two mechanisms.

Crystallographic analysis of DOXP reductoisomerase as complex with  $Mn^{2+}$  and fosmidomycin revealed that the hydroxamic acid moiety of fosmidomycin replaces two water molecules in the octahedral coordination sphere of the  $Mn^{2+}$  while the phosphono group is anchored by several hydrogen bonds [68]. Fosmidomycin is supposed to bind in a similar way as the substrate DOXP (Fig. (4)). With respect to the rearrangement reaction it has been deduced from stereochemical considerations based on isotope labelling experiments that the migrating group and the reducing cofactor are located on opposite faces of the plane defined by the metal coordinated  $\alpha$ -hydroxycarbonyl substructure [69]. Therefore, it was postulated that the putative 2-C-methyl-D-erythrose 4-phosphate intermediate does not relax to an extended substrate-like conformation and the phosphate group remains essentially anchored in its binding site. Notably, 1-deoxy-D-xylulose does not act as a substrate for DOXP reductoisomerase [65].



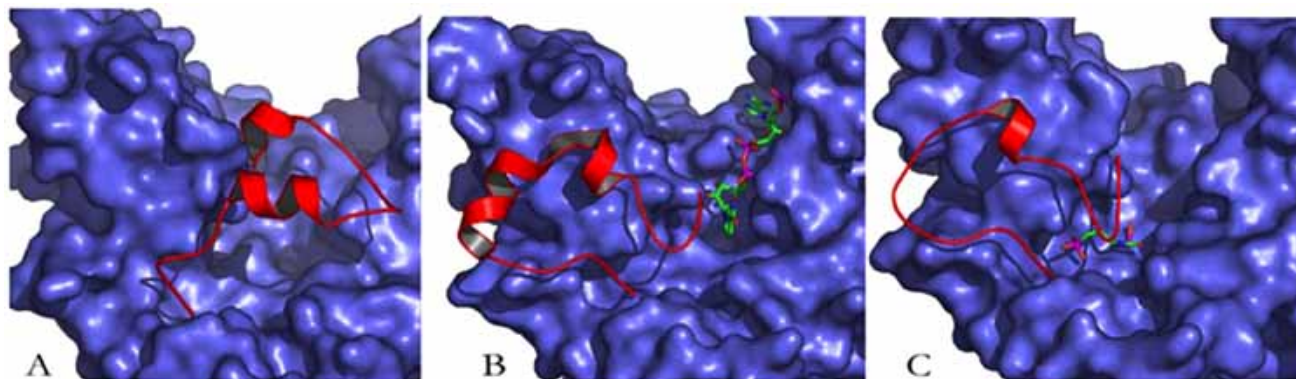
**Fig. (4).** Stereochemical course of the reaction catalysed by DOXP reductoisomerase. The asterisk indicates the hydride transferred from NADPH. Fosmidomycin (**1**) binds to the manganese ion similar to DOXP.

Kinetic analysis of the *E. coli* DOXP reductoisomerase suggested a mandatory ordered sequence with NADPH binding first and DOXP second [66]. In contrast, studies on product inhibition and isotope effects performed with DOXP reductoisomerase from *M. tuberculosis* suggested that the enzyme utilises a steady-state random mechanism [61]. Additional evidence that supports a random mechanism is provided by crystallographic studies with *E. coli* DOXP reductoisomerase. The structures of a metal-free enzyme-NADPH complex [70] and an enzyme-Mn<sup>2+</sup>-fosmidomycin complex [68,71] have been solved. These data suggest that the enzyme can bind NADPH regardless of whether the metal ion and fosmidomycin (which mimics DOXP) are bound, and vice versa.

### Three-Dimensional Structure and Conformational Dynamics of DOXP Reductoisomerase

Different crystal structures of *E. coli* DOXP reductoisomerase are available, representing the apo-enzyme [72], a complex with NADPH and a sulphate ion replacing the phosphate group of DOXP [70], a complex with fosmidomycin and Mn<sup>2+</sup> [68], two complexes with bisphosphonate inhibitors and manganese [73] and finally a complex with fosmidomycin and NADPH [74]. In addition, the crystal structure of the DOXP reductoisomerase of *Zymomonas mobilis* is available, both in the apo-form and as binary complex with NADPH [71]. The enzyme forms an elongated homodimer with a pronounced cleft-like structure in each monomer that is covered by a flexible active site loop. Each monomer can be subdivided into basically three domains. The N-terminal domain is a member of the classical dinucleotide binding fold and serves as an

anchor for NADPH. The central catalytic domain harbours the binding site for the divalent metal cation, the phosphate binding site and the catalytic loop. The C-terminal four-helix bundle domain is connected to the catalytic domain by a linker region that spans the entire monomer. The C-terminal domain appears to have a structural role in supporting the catalytic domain. In the structure of the apo-enzyme, the catalytic and the four helix bundle domains showed significant mobility upon superposition of the dinucleotide binding domains of the three conformational states present in the asymmetric unit of the crystal (Fig. (5A)). A still more pronounced flexibility was observed for the catalytic loop, which adopted two completely different conformations within the three protein conformers. The flexible nature of the structure provided strong evidence that an induced fit could occur after substrate binding with the catalytic loop folding over the active site in order to shield the reactants completely from the solvent environment. In the crystal structure of the NADPH-sulphate complex the domain conformations were fixed. The loop was placed over the putative catalytic pocket containing the sulphate ion (Fig. (5B)). The loop region showed higher B-factor values than other parts of the protein indicative for a higher degree of flexibility. Also for the complex with Mn<sup>2+</sup> and fosmidomycin the loop could be fitted to a closed conformation covering the active site pocket (Fig. (5C)). Nevertheless, parts of the loop remained disordered and it seems likely that it adopts more than one conformation. It was concluded that complete ordering of the catalytic loop very likely requires the presence of both NADPH and DOXP. Analysis of the *Z. mobilis* DOXP reductoisomerase crystal structure basically reflected the findings ob-



**Fig. (5).** Three different crystal structures of *E. coli* DOXP reductoisomerase. A Apo-enzyme (pdb entry code: 1k5h), B Complex with NADPH and a sulphate ion (pdb entry code: 1jrs), C Complex with fosmidomycin and Mn<sup>2+</sup> (pdb entry code: 1ONP). The catalytic loop is indicated in red. Figure courtesy of C. Richter, Wolfgang Goethe-University Frankfurt.

tained with the *E. coli* enzyme. Further evidence for conformational flexibility of DOXP reductoisomerase comes from mutation analysis [75]. Replacement of His209 that is part of the flexible loop for Gln resulted in an enzyme with markedly reduced affinity for DOXP indicating that this residue must participate in the constitution of the active site pocket, even though this residue is found in considerable distance from the active site cleft in the apo-enzyme.

### Inhibitors of DOXP Reductoisomerase

Fosmidomycin (Fig. (6), (1)) [3-(N-formyl-N-hydroxyamino)-propylphosphonic acid mono-sodium salt, FR-31564], together with some structurally highly similar derivatives, still represents the most potent inhibitor of DOXP reductoisomerase known at present. Kinetic analysis of *E. coli* DOXP reductoisomerase revealed that fosmidomycin is an uncompetitive inhibitor against NADPH and a slow, tight-binding competitive inhibitor against DOXP. The  $K_i$  values of fosmidomycin against DOXP were 215 nM and 21 nM when determined for initial ( $v_o$ ) and final ( $v_s$ ) velocity, respectively. The existence of two distinguishable  $K_i$  values is believed to reflect an initial binding step, followed by isomerisation of the enzyme to a state which binds the inhibitor more tightly [66].

Fosmidomycin (1) was identified in the culture broth of *S. lavendulae*, and three derivatives were also isolated from natural sources: FR-900098 (2) (*S. rubellomurinus*) FR-32863 (3) (*S. lavendulae*) and FR-33289 (4) (*S. rubellomurinus*) (Fig. (6)) [1,76-78]. The acetyl (5) and formyl (6) derivatives of FR-32863 (3) and FR-33289 (4), respectively, were prepared by chemical synthesis [79]. All these compounds were found to possess antibacterial activity with fosmidomycin being most active against most isolates. In a recent study a set of 42 fosmidomycin derivatives has been analysed for structure-activity relationships based on the inhibition values against DOXP reductoisomerase of *E. coli* [80]. All published compounds were significantly less active than fosmidomycin with exception of three compounds which displayed activity in the same order of magnitude. These most active compounds comprised FR-900098 (2) and 5 which had already been analysed for antibacterial activity previously [79]. In addition, 7 was similarly potent as fosmidomycin. In a more detailed study the compounds 8 and 9, which formally represent derivatives of fosmidomycin (1) and

FR900098 (2), respectively, with the hydroxamate group in an inverted orientation, were analysed for inhibition of DOXP reductoisomerase of *E. coli* [81]. Compound 9 was only 1.5-fold less active than fosmidomycin (1) while the activity of 8 was 5.3-fold decreased. Remarkably, 9 retained considerable activity in a growth inhibition assay using a fosmidomycin resistant *E. coli* strain.

The bisphosphonates 10 and 11 were demonstrated to inhibit *E. coli* DOXP reductoisomerase with  $IC_{50}$  values of 4 and 7  $\mu$ M, respectively (Fig. (7)). The crystallographic structures of two complexes of these compounds bound to DOXP reductoisomerase were solved [73]. Several adenosine derivatives which possibly could bind to the NADPH binding site of DOXP reductoisomerase were reported to inhibit the enzyme with  $IC_{50}$  values around 30  $\mu$ M [82]. It seems unlikely that the reported moderate antimalarial activity of these compounds is related to the inhibition of DOXP reductoisomerase. A library of 32,000 compounds was screened for DOXP reductoisomerase inhibitors using an assay based on the replacement of a surrogate peptide ligand, resulting in five hits with  $IC_{50}$  values of 0.4 – 19  $\mu$ M [83]. The chemical structures of these compounds were not disclosed. A series of new fosmidomycin derivatives was tested for effects on monoterpenoid indole alkaloid production in *Catharanthus roseus* cells [84]. However, from the results obtained it cannot be concluded whether or not these derivatives inhibit DOXP reductoisomerase.

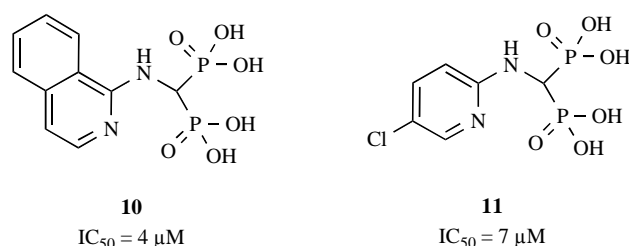


Fig. (7). Bisphosphonates.  $IC_{50}$  values obtained with recombinant DOXP reductoisomerase of *E. coli* are indicated.

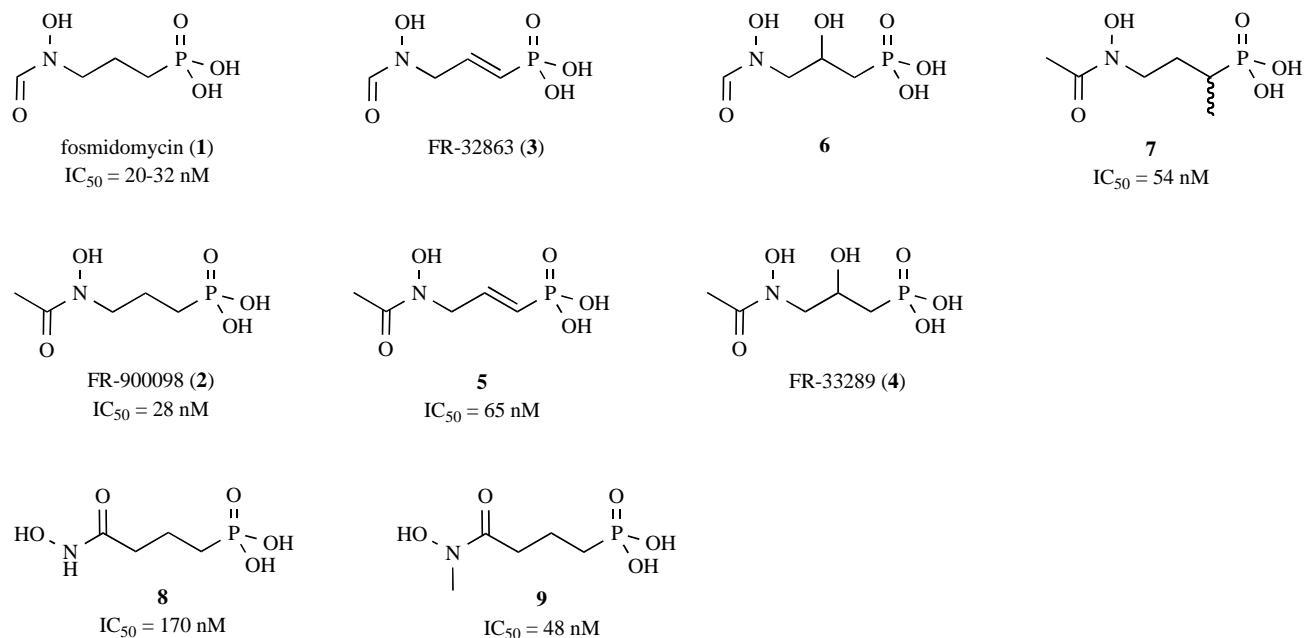


Fig. (6). Fosmidomycin (1) derivatives. If available,  $IC_{50}$  values obtained with recombinant DOXP reductoisomerase of *E. coli* are indicated. Data on the stereochemistry of 4 and 6 are not reported.

In the context of studies on the catalytic mechanism of DOXP reductoisomerase several compounds closely related to the substrate DOXP were synthesised and evaluated for enzyme inhibition. The isosteric phosphono analogue **12** (Fig. (8)) was shown to be converted by DOXP reductoisomerase, albeit with 40-fold decreased efficacy [85,86]. Also, the fluorine analogue **13** was converted with 7-fold decreased efficacy [67]. The DOXP analogues **14-20** were shown to be weak inhibitors which are not converted by the enzyme [67,86] (Fig. (8)). D-erythrose 4-phosphate was found to be neither a substrate nor an inhibitor of DOXP reductoisomerase in one study [65] while another study reports a slow turnover [66].

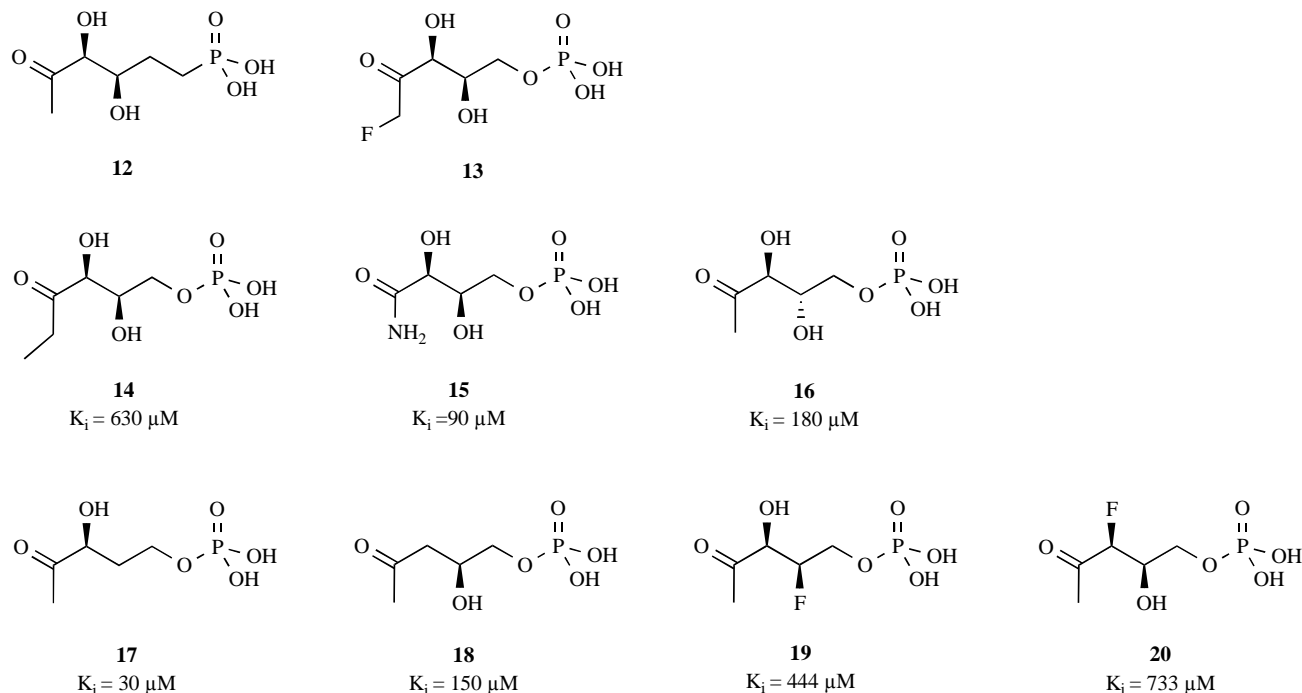
## FOSMIDOMYCIN AS AN ANTIMALARIAL DRUG

### Antimalarial Activity *in Vitro* and in Animals

Antimalarial drugs targeting the apicoplast are not new and in part well established. It has been recognised that some classical antibiotics commonly used in antibacterial therapy possess activity against apicomplexan parasites including *P. falciparum* (reviewed in [87]). The antiparasitic activity of these antibiotics is now commonly accepted to be related to the bacteria-like nature of the apicoplast's DNA replication, transcription and translation machinery. Thus, quinolones and fluoroquinolones are believed to inhibit the apicoplast-localised gyrase resulting in an arrest of the organellar DNA replication. The antiparasitic activity of rifampicin is most likely linked to inhibition of the apicoplast's RNA polymerase, and a variety of tetracyclines, lincosamides, macrolides and chloramphenicol in addition to some peptide antibiotics, all of which are translation inhibitors of prokaryotic systems, are considered to inhibit protein synthesis inside the apicoplast. A common characteristic of most, if not all, of these antibiotics is a so-called delayed kill effect. This describes the observation that treatment with such antibiotics has virtually no effect on parasite growth at pharmacologically relevant concentrations during the first intracellular cycle, whereas the parasites die during the second replicative cycle after reinvasion of new host cells. The exact reason for the delayed kill effect is unknown. This phenomenon seems to be related to the fact that the likely targets of the mentioned antibiotics are primarily

essential for replication of the apicoplast giving rise to apicoplast deficient daughter cells during intracellular division, which are viable but fail to invade new host cells. In clinical practice, amelioration of symptoms is observed around day four after treatment with an antibiotic compared to day two with a conventional antimalarial drug, resulting in a potentially life-threatening delay. Therefore, those antibiotics cannot be used for the treatment of acute malaria. Nevertheless, doxycycline, tetracycline and to some extent clindamycin are routinely used for malaria prophylaxis or in combination with a fast-acting other antimalarial drug, in particular in areas with prevalence of multi-resistant *P. falciparum* strains.

In sharp contrast to the above mentioned antibiotics fosmidomycin was found to be fast-acting comparable to most established antimalarial drugs. The reason may be that exposure to fosmidomycin immediately shuts down the supply of isoprenoids involved in a variety of cellular functions instead of being merely required for maintenance of the apicoplast. The *in vitro* growth of different *P. falciparum* strains was inhibited by fosmidomycin with IC<sub>50</sub> values in the range of 300-1200 nM [14,88]. Microscopic inspection revealed that the parasite growth is arrested in the schizont stage (J. Wiesner, unpublished data). There is no evidence for cross-resistance with any other antimalarial drug. In order to examine its *in vivo* antimalarial activity fosmidomycin was administered to mice infected with the rodent parasite *P. vinckei* [14]. With respect to the short plasma half-life of fosmidomycin, the drug was administered three times per day for four days resulting in ED<sub>90</sub> values of approximately 5 mg/kg and 20 mg/kg after intraperitoneal (i.p.) and oral (p.o.) administration, respectively. The treatment was also effective when started at a high parasitaemia of approximately 40%. This potent *in vivo* activity seems remarkable with respect to the only moderate *in vitro* activity with IC<sub>50</sub> values being at least one order of magnitude higher than those determined with conventional antimalarial drugs under comparable conditions. This may possibly be due to relatively high plasma levels of fosmidomycin being readily reached in the *in vivo* system, probably due to the high water solubility of the molecule [89]. How fosmidomycin, a highly soluble negatively charged molecule, is able to enter the parasite finally



**Fig. (8).** DOXP analogues. Compounds **12** and **13** were converted by DOXP reductoisomerase with decreased efficacy.  $K_i$  values were obtained with DOXP reductoisomerase of *Synechocystis* sp. PCC6803 (**14-18**) and *E. coli* (**19, 20**).

reaching the apicoplast, thereby passing seven biological membranes, is unknown. It was suggested that in *E. coli* fosmidomycin is imported *via* the glycerol-3-phosphate transporter [90]. It is not known whether this is also the case in *P. falciparum*.

The interactions of fosmidomycin with a number of antimalarial drugs in current use were investigated in a series of *in vitro* experiments [88]. The combination of fosmidomycin with most drugs (chloroquine, mefloquine, halofantrine, lumefantrine, artemisinin, atovaquone, proguanil, rifampicin) resulted in an indifferent effect most likely reflecting the absence of a specific interaction. Quinine, doxycycline, ciprofloxacin and azithromycin were additive. Synergy was only observed with the lincosamide antibiotic clindamycin and its natural precursor, lincomycin. The efficacy of fosmidomycin in combination with clindamycin was further confirmed in the *P. vinckei* mouse model. One possible explanation for this synergistic effect would be that clindamycin inhibits the replication of the apicoplast, the organelle harbouring the DOXP reductoisomerase. However, this does not explain why synergy was only seen with the lincosamides but not with several other antibiotics also inhibiting apicoplast function.

FR-900098 (**2**) was shown to be two to threefold more active against *P. falciparum* *in vitro* and in *P. vinckei* infected mice [14]. Remarkably, all bacteria species tested so far were less sensitive to FR-900098 (**2**) than fosmidomycin [79] (S.F. Biketov, H. Jomaa, unpublished data). This finding was confirmed at the enzyme level where the recombinant DOXP reductoisomerase of *P. falciparum* was inhibited more efficiently by FR-900098 (**2**) ( $IC_{50} = 17$  nM) than by fosmidomycin (**1**) ( $IC_{50} = 35$  nM). In contrast, there was no measurable difference in the sensitivity of DOXP reductoisomerase of *E. coli* against FR-900098 (**2**) and fosmidomycin (**1**) [14] (H. Jomaa, S. Sanderbrand, unpublished data).

In order to increase the oral bioavailability of FR-900098 (**2**) prodrugs were synthesised and tested in the *P. vinckei* mouse model. The charged phosphonate group of FR-900098 (**2**) was either masked by biolabile aryl esters (**21**) or with double esters (**22**) (Fig. (9)). With both types of prodrugs twofold increased efficacy after oral administration was achieved compared to the parent FR-900098 (**2**) [91,92].

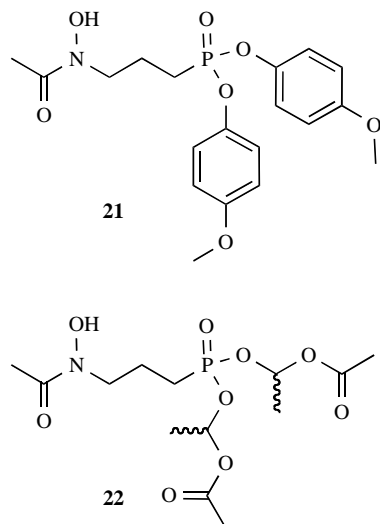


Fig. (9). Prodrugs of FR-900098 (**2**).

### Pharmacokinetics and Toxicology

Previous pharmacokinetic studies of fosmidomycin in animals were based on the use of  $^{14}C$ -labelled drug [89]. For human pharmacokinetic studies a bioassay based on the growth inhibition of

the bacterium *Enterobacter cloacae* as test organism was applied [2]. A method for the determination of fosmidomycin in blood and urine based on high performance capillary electrophoresis (HPCE) has recently been published but not yet applied for pharmacokinetic studies [93].

Pharmacokinetic studies in rats revealed that after i.v. administration approx. 90 % of the dose were excreted in the urine within 72 h, and less than 1 % were excreted in the expired air ( $^{14}CO_2$ ) and bile, which suggests the absence of enterohepatic circulation [89]. After oral administration to rats, 34 % and 61 % of the dose were excreted in the urine and faeces, respectively, suggesting about 30% gastro-intestinal absorption. The serum level data after i.v. administration closely fitted a 3-compartment open model with first order kinetics. The half-lives of the serum level curves for the early, midway, and terminal phases were: 0.13, 0.51 and 17.3 h, respectively in rats; and 0.44, 0.75 and 2.0 h, respectively, in dogs. Fosmidomycin was rapidly distributed in the tissues of rats and was maintained in high concentrations in the liver, kidney and bones. The serum protein binding in mice, rats and dogs was maximally 4%. No metabolites were detected in the urine.

Human pharmacokinetic studies were performed with healthy male volunteers through single and repeated dose regimes [2,94]. The gastrointestinal absorption rate after an oral dosing of 7.5 mg/kg was between 20 % and 40 %. The absorption was found to be slow and moderate. In single-dose studies fosmidomycin was administered by the p.o. (7.5 mg/kg), i.m. (7.5 mg/kg) and i.v. (30 mg/kg) route. The mean peak serum concentrations were 2.45  $\mu g/ml$ , 12.3  $\mu g/ml$  and 157  $\mu g/ml$  after p.o., i.m. and i.v. doses, respectively. The serum half-lives were 1.87 h (p.o.), 1.58 h (i.m.) and 1.65 h (i.v.). The recovery rate in urine was 26% (p.o.), 66.4% (i.m.), 85.5% (i.v.). In repeated dose studies no accumulation could be observed. The serum protein binding was less than 1%. Unmetabolised fosmidomycin was the only bioactive substance found in the urine.

In mice and rats the acute  $LD_{50}$  of fosmidomycin was higher than 11,000 mg/kg and approx. 8,000 mg/kg after p.o. and s.c. administration, respectively. As a mean toxic effect diarrhoea was observed at 2,500 and 12,500 mg/kg after p.o. administration. Pilo-errection was only observed at the 12,500 mg/kg dose level in both mice and rats.

Phase I studies with fosmidomycin were conducted in 127 healthy male volunteers [95]. When administered in repeated doses of 8 g/day i.v. for 7 days (2 g every 6 h), 4 g/day i.m. for 5 days (1 g every 6 h) and 4 g/day p.o. for 7 days (1 g every 6 h), no adverse events were reported, except for mild to moderate irritation at the site of injection. No side-effects were reported in the group receiving the drug orally. No changes of the haematological and biochemical parameters were observed. A total of 70 patients with acute urinary tract infection were treated with fosmidomycin in a pilot phase II trial [2]. Tolerance and efficacy were reported to be good although in some cases nausea, vomiting and loose stools occurred. In a recent phase II study 20 subjects with *P. falciparum* malaria received 1.2 g fosmidomycin orally three times daily for seven days [96]. Generally the drug was well tolerated although mild gastrointestinal side-effects with symptoms of loose stools, flatulence and diarrhoea were recorded in five subjects. Alteration of the intestinal flora due to poor absorption and the prolonged treatment regimen may have been a causative factor. Haematological and clinical chemistry analysis showed no clinically significant changes throughout the study.

### Clinical Efficacy of Fosmidomycin Monotherapy

The efficacy and safety of fosmidomycin for the treatment of *P. falciparum* malaria was evaluated in adult patients from Gabon and Thailand [96]. 10 subjects at each study site received 1.2 g fosmidomycin orally three times daily for seven days. All subjects were clinically and parasitologically cured on day 7. The mean parasite

clearance time (PCT) was 46 h in Gabon and 48 h in Thailand. The fever clearance time (FCT) was 24 h in Gabon and 47 h in Thailand. On day 28, 2 subjects in Gabon and 8 subjects in Thailand had experienced a recrudescence. The variation in cure rates between Gabon and Thailand may reflect the differences in immunity between a populations living in a hyperendemic area in Central Africa and a hypoendemic area in South-East Asia. In an additional study, adult Gabonese patients were treated with the same dosing regime, and the treatment time was sequentially shortened [97]. The cure rates by day 14 were 8/9, 7/8 and 6/10 for treatment durations of 5, 4 and 3 days, respectively. This study demonstrated that in Gabon the minimum effective treatment duration defined by a cure rate > 80 % on day 14 is 4 days. However, with respect to the complicated dosing regimen and the comparably high recrudescence rate fosmidomycin monotherapy seems not to be a realistic treatment option for malaria.

### Clinical Efficacy of Fosmidomycin-Clindamycin Combination Therapy

A combination of fosmidomycin and clindamycin was tested in a first clinical trial for clearance of asymptomatic *P. falciparum* infections in Gabonese school children [98]. Fosmidomycin (30 mg/kg) plus clindamycin (5 mg/kg) were administered every 12 hours for 5 days compared to the two drugs alone. Groups of each 12 children aged 7-14 years were treated. As expected, the parasite clearance time (PCT) in the fosmidomycin-clindamycin (PCE = 18 h) and the fosmidomycin (PCT = 25 h) group was significantly shorter than in the clindamycin group (PCT = 71 h). Treatment with fosmidomycin-clindamycin and clindamycin alone led to a 100% cure rate on day 28 while parasites reappeared in 5 out of the 12 children treated with fosmidomycin alone. All regimens were well tolerated, and no serious adverse events occurred. No difference was seen in the incidence of gastrointestinal adverse reactions between the fosmidomycin-clindamycin group and the clindamycin-alone group, whereas treatment with fosmidomycin-clindamycin compared to fosmidomycin alone led to a non-significant increase in the incidence rate of gastrointestinal adverse effects. This study has demonstrated that the combination of fosmidomycin and clindamycin is well tolerated and superior to either agent on its own with respect to the rapid and radical parasite clearance.

In another study conducted in Gabon children aged 7-14 years with acute uncomplicated *P. falciparum* malaria were treated with a combination of fosmidomycin (30 mg/kg) and clindamycin (10 mg/kg) twice daily [99]. Starting with 5 days of treatment, the duration of treatment was incrementally shortened in intervals of 1 day if > 85 % of the patients in a cohort were cured by day 14. The cure ratio on day 14 was 100 % with treatment durations of 5, 4, 3 and 2 days. The cure ratios on day 28 were 10/10, 10/10, 9/10 and 7/10 with treatment durations of 5, 4, 3 and 2 days, respectively. One day of treatment led to a cure ratio of 5/10 on day 14 and 1/10 on day 28. The mean parasite clearance time was 41 h, 38 h, 39 h, 35 h and 63 h, respectively, in the five cohorts. The efficacy of the 3 day regimen, which is generally assumed to be in accordance with patients' compliance, indicates that fosmidomycin-clindamycin potentially could serve as a new treatment for malaria in Africa.

### FUTURE PERSPECTIVES

Although additional studies are still required, the current data suggest that fosmidomycin in combination with clindamycin principally meets the requirements for a new first-line treatment against malaria. In addition to its potential use as an effective treatment for uncomplicated malaria caused by parasites resistant against conventional drugs fosmidomycin-clindamycin may be of particular value for the treatment of severe malaria when patients are not able to tolerate oral medication. In phase I volunteer studies intravenous fosmidomycin resulted in high plasma levels without significant side-effects [95]. Also, clindamycin, in form of its phosphoric acid

ester, is available as an intravenous formulation. Thus, the development of an intravenous formulation of a combination of fosmidomycin with clindamycin for the treatment of severe malaria should be technically feasible. Depending on the outcome of more detailed developmental toxicity studies on fosmidomycin the combination with clindamycin, which is generally considered to be safe in pregnancy, may be used for the treatment of malaria in pregnant women, thus providing a true alternative to the artemisinins which are contraindicated during the first trimester [100]. It remains to be seen whether the more potent FR-900098 or a prodrug thereof finally will enter clinical development. The more ambiguous goal would be the development of a drug combination simultaneously inhibiting at least two enzymes involved in the DOXP pathway. Such a combination is expected to exert a highly synergistic effect similar as it is the case with the commonly used antifolate combinations. Even though all the other enzymes involved in the DOXP pathway, in addition to DOXP reductoisomerase, represent potential new targets specific inhibitors are not yet available for any of them. Fluoropyruvate was used as an experimental inhibitor of DOXP synthase in order to validate a photometric enzyme inhibition assay [56]. However, this compound is not expected to represent a useful lead compound due to its rather unspecific mechanism of action also inhibiting the mammalian pyruvate dehydrogenase. In addition to DOXP reductoisomerase crystal structures have been solved for the enzymes YgbP, YchB and YgbB of *E. coli* [101-105]. Since no inhibitors are known at present it is difficult to predict whether or not a design process solely based on that structural information will lead to new drugs. The search for inhibitors of the last two enzymes of the DOXP pathway, GcpE and LytB, may be facilitated by the availability of relatively convenient photometric assays based on the use of methylviologen as artificial electron donors [24,26]. However, these assays require the strict exclusion of oxygen and, thus, it remains to be established whether or not they can be used for a larger screening approach.

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### ABBREVIATIONS

DMAPP	=	dimethylallyl diphosphate
DOXP	=	1-deoxy-D-xylulose-5-phosphate
MEP	=	2-C-methyl-D-erythritol 4-phosphate
HMBPP	=	(E)-4-hydroxy-3-methyl-but-2-enyl diphosphate
IPP	=	isopentenyl diphosphate
MEcPP	=	2-C-methyl-D-erythritol-2,4-cyclodiphosphate

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