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Inhibition of the Non-Mevalonate Isoprenoid Pathway by Reverse Hydroxamate Analogues of Fosmidomycin

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Abstract

The non-mevalonate (methylerythritol phosphate, MEP) pathway for isoprenoid biosynthesis is essential in *Plasmodium* spp., but is absent in the human host. The pathway is a clinically validated antimalarial target on basis of studies with the antibiotic fosmidomycin, an inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr, IspC), which catalyses the first committed step of the MEP-pathway. In this review, we report on reverse, hydroxamate-based fosmidomycin analogues, which are studied by enzyme kinetics, parasite growth inhibition and crystallography in order to identify compounds with enhanced antiplasmodial activity.

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Keywords: α-arylated reverse fosmidomycin derivatives; 1-deoxy-D-xylulose 5-phosphatereductoisomerase; fosmidomycin; IspC protein; nonmevalonate pathway of isoprenoid biosynthesis

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1. Introduction

Malaria is estimated to claim at least 600.000 lifes per year[1]. Classical drugs and insecticides used to control the disease and its vectors are subject to rapidly progressing attrition by the emergence and spread of parasite and vector resistance. There is thus an urgent need for novel antimalarial drugs, but none have been brought to the market in recent decades.

The causative *Plasmodium* species have more than 5 000 genes, but the number of druggable targets is unknown. In order to counter the deleterious consequences of resistance spreading, it appears important to identify and study antimalarial drug targets that are not subject to cross-resistance with currently used antimalarial drugs. The non-mevalonate isoprenoid biosynthesis pathway that was discovered in in the final years of the 20th century was immediately recognised as a potential therapeutic opportunity[2,3]. The pathway is essential in the malaria parasites for the biosynthesis of vitally important terpenoids but is absent in mammalian hosts who generates isoprenoids exclusively via the mevalonate pathway[4,5]; drugs addressing enzymes of the MEP pathway should therefore be exempt from target-related toxicity. Also of note, the enzymes of the non-mevalonate pathway are located in the apicoplast of malaria parasites, an ancient organelle that is believed to be evolutionarily related to plant chloroplasts; whereas apicoplasts host only small numbers of proteins, they are believed to comprise attractive drug targets[6–8].

The concept of targeting the non-mevalonate pathway of *Plasmodium* received a tremendous boost when Jomaa et al. showed in 1999 that the antibiotic fosmidomycin (cf. Fig. 2) inhibits IspC (Dxr)[2], which catalyses the first committed step of the non-mevalonate pathway and is believed to be rate-limiting. More specifically, IspC catalyses a skeletal rearrangement of 1-deoxy-D-xylulose 5-phosphate (DOXP) and a subsequent reduction of the branched aldose intermediate, thus affording 2*C*-methyl-D-erithritol 4-phosphate (MEP). IspC requires a divalent cation (Mg^{2+} or Mn^{2+}) and NADPH for catalytic activity (Fig. 1).

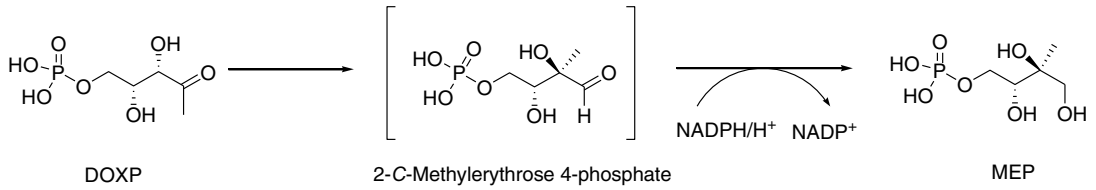


Fig. 1. IspC-catalysed conversion of DOXP to MEP.

The antibiotic, fosmidomycin, which was initially isolated from *Streptomyces lavendulae*[9,10], is a close structural analogue of the IspC substrate DOXP but carries a phosphonate residue instead of the phosphate residue of the enzyme substrate and is not subject to hydrolysis by cellular phosphatases[11]. Fosmidomycin had been under clinical development in the 1980s as an antibacterial agent but was subsequently abandoned due to its less than ideal pharmacokinetics[9,10,12]. However, due to the earlier pharmacological exploration, the compound could be rapidly admitted to several clinical studies conducted in Africa and Asia, which confirmed beyond doubt its potential to cure human malaria. Unfortunately, recrudescence of parasites was a frequent problem and the compound was shifted, in subsequent work, to combination treatment with clindamycin, an established antimalarial drug[13–16]. Even in the form of the combination therapy, the market introduction of fosmidomycin has not been accomplished. While mechanism of action and toxicity profile are favourable, the pharmacokinetic properties of the drug, with a plasma half-life of only about 2 h and a gastrointestinal absorption rate after oral administration of 20 % to 40 %, cause significant drawbacks[17,18].

The shortcomings of fosmidomycin that have so far hampered its market introduction, despite its apparent merits, have prompted attempts by several research groups to improve the structure activity relation by chemical modification[19–29]. These studies are based on a fairly thorough understanding of fosmidomycin's mode of action including more than 50 X-ray structures of IspC orthologs from a variety of pathogenic and non-pathogenic species, in the absence of or in complex with substrates, coenzymes and/or inhibitors. Thus, it is clear that the phosphonate type terminus of fosmidomycin closely mimics the phosphate ester motif of the substrate, and the hydroxamate

moiety coordinates the essential divalent cation[30–32]. Notably, however, a flexible loop forms part of the active site cavity[33], and the way from protein structure to the design of improved ligands is less than straightforward.

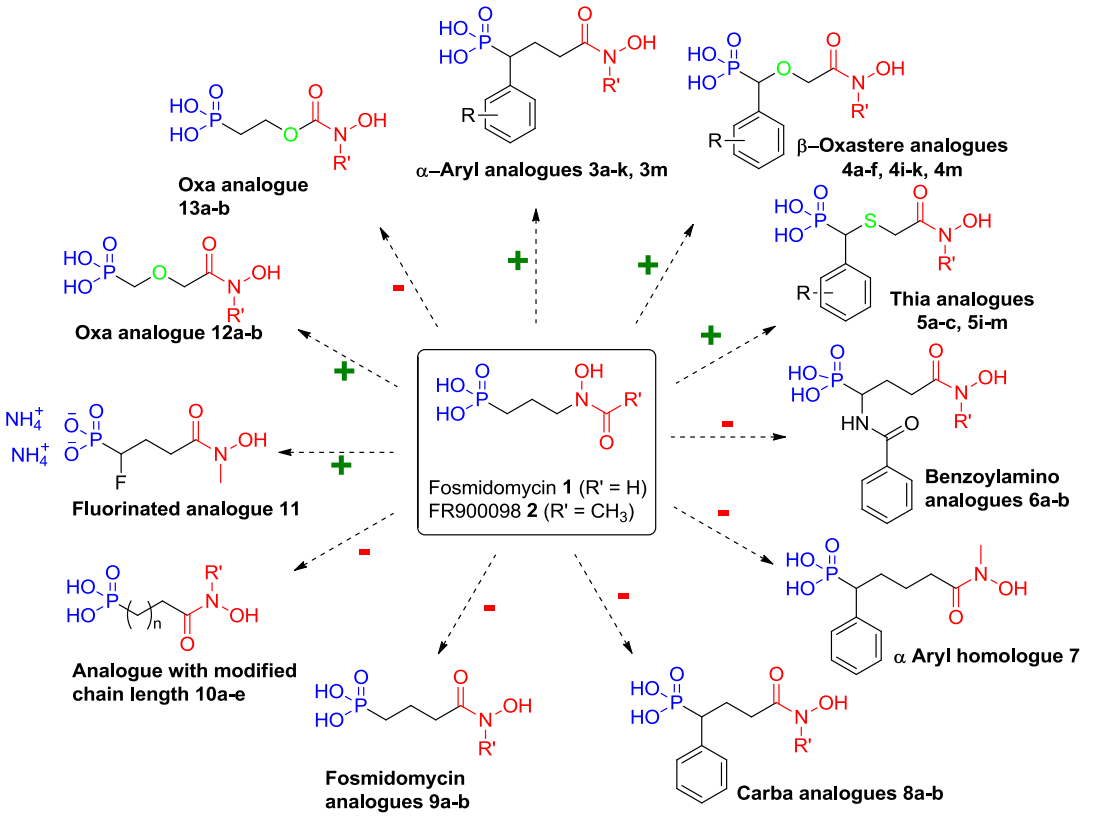


Fig. 2. Fosmidomycin as lead structure and reverse analogues [26-29, 34, 40-42]. +: antiplasmodial activity, -: no or weak antiplasmodial activity **3a**, **4a**, **5a**: R = H, R' = CH_3; **3b**, **4b**, **5b**: R = H, R' = H; **3c**, **4c**, **5c**: R = 4-CH_3, R' = CH_3; **3d**, **4d**: R = 4-CH_3, R' = H; **3e**, **4e**: R = 4-OCH_3, R' = CH_3; **3f**, **4f**: R = 4-OCH_3, R' = H; **3g**: R = 3,4-OCH_3, R' = CH_3; **3h**: R = 3,4-OCH_3, R' = H; **3i**, **4i**, **5i**: R = 3,4-F, R' = CH_3; **3j**, **4j**, **5j**: R = 3,4-F, R' = H; **3k**, **4k**, **5k**: R = 3,4-Cl, R' = CH_3; **5l**: R = 3,4-Cl, R' = H; **3m**, **4m**, **5m**: R = naphthalene-1-yl, R' = CH_3; **6a**: R' = CH_3; **6b**: R' = H; **8a**: R' = CH_2CH_3; **8b**: R' = $CH(CH_3)_2$; **9a**: R' = CH_3; **9b**: R' = H; **10a-b**: n = 1, R' = CH_3 (**10a**), H (**10b**); **10c**: n = 2, R' = CH_2CH_3; **10d-e**: n = 3, R' = CH_3 (**10d**), H (**10e**); **12a**: R' = CH_3; **12b**: R' = H; **13a**: R' = CH_3; **13b**: R' = H.

Structural modification of fosmidomycin was focused on four main areas: (i) replacement of the phosphonate motif by (bio)isosteres, synthesis of phosphonate prodrugs; (ii) replacement or modification of the hydroxamate moiety that chelates the catalytically essential divalent cation; (iii) modification of the aliphatic chain by introduction of (typically aromatic) substituents and (iv) modulation of the aliphatic linker between the anionic anchor group and the chelating head group (shortening, lengthening, isosteric replacement). The impact of the structural modification of the inhibitor can be monitored by enzyme kinetics using recombinant IspC from a variety of pathogens including *Plasmodium falciparum*, *Mycobacterium tuberculosis* and *Escherichia coli*, by growth inhibition assays of *P. falciparum*, and by X-ray crystallography. Some results are summarized below.

- The replacement of the phosphonate by (bio)isosteric moieties did not result in improved activity and was generally not a very encouraging approach. Several prodrugs displayed improved in vitro activity and in case of FR900098-prodrugs enhanced in vivo activity[34–36].
- The replacement of the hydroxamate motif by other chelating groups has not resulted in significantly improved activity[37–39]. Importantly, however, Rohmer et al.[3] were the first to synthesize retroisosteric analogues of

fosmidomycin and FR900098 comprising a reoriented hydroxamate moiety. One compound was at least as active towards IspC from *E. coli* as the parent compound, FR900098[22,34]. Retroisosteric hydroxamates (short designation, ("reverse fosmidomycin analogues") will be addressed in more detail below.

- The introduction of aromatic side chains was first explored by Kurz et al.[20] and later by Schlüter et al.[24] and afforded compounds with improved antiplasmodial activity.
- Changing the distance between the phosphonate anchor and the chelator group typically abolished the inhibitory activity[34,40].

Subsequently, the two most promising approaches that had emerged from earlier investigations, namely the retroisosteric repositioning of the hydroxamate motif and the introduction of (substituted) aryl groups in the α position (α with respect to the phosphonate group) were combined. Moreover, the effect of *N*-substitution in the retroisosteric compound series was investigated[28–30,40]. Importantly, the retroisosteric series also enabled the detailed exploration of the isosteric replacement of a methylene group in the aliphatic linker by oxygen resp. sulphur (Fig. 2)[41,42]. By work with a thia isosteric compound, the enantioselectivity of IspC for a chiral, α phenylated derivative[42] could be established.

2. Results and discussion

2.1. Reverse fosmidomycin derivatives with α aryl substituents

The synthesis of reverse hydroxamate derivatives of fosmidomycin carrying aryl substituents in the α position started from benzyl phosphonate esters resp. from benzaldehyde derivatives (Fig. 3, cf. Ref. 28 for details). Enzyme inhibition was studied using recombinant IspC from *P. falciparum* (*Pf*IspC), *M. tuberculosis* (*Mt*IspC) and *E. coli* (*Ec*IspC). Specifically, the dehydrogenation of the coenzyme, NADPH, was monitored photometrically, and initial rates were extracted from individual progression curves. IC_{50} values were obtained using Dynafit software. Typical dose response curves are shown in Fig. 4[28,40–42].

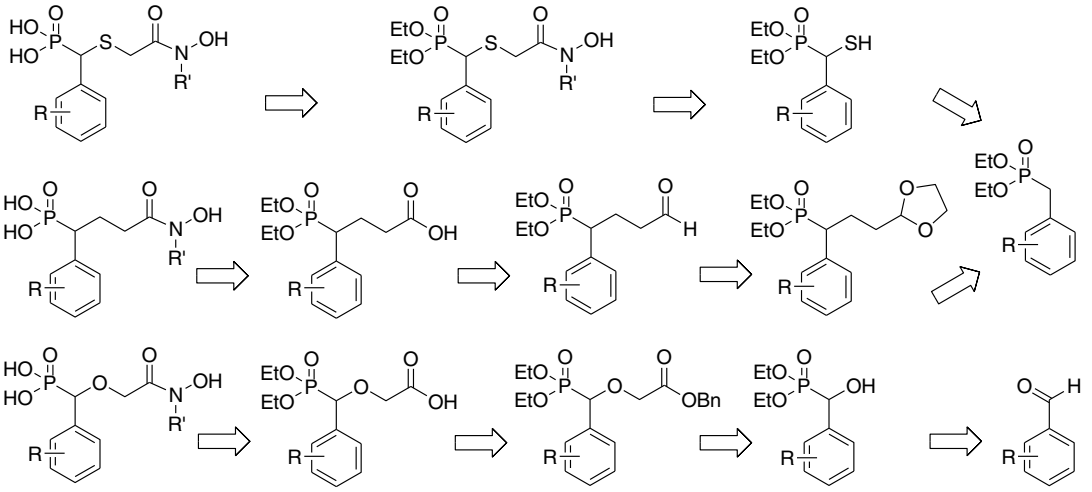


Fig. 3. Stategies for preparation of reverse fosmidomycin analogues.

Generally, the activity of reverse fosmidomycin derivatives against the *Plasmodium* enzyme exceeds that against the *M. tuberculosis* enzyme by about two orders of magnitude and the activity against the *E. coli* enzyme by about one order of magnitude. The inhibition of *P. falciparum* proliferation in erythrocytes was monitored by ELISA of histidine rich protein 2 (HRP2) using different parasite strains that were sensitive or resistant to chloroquine. A typical dose response curve is shown in Fig. 4.

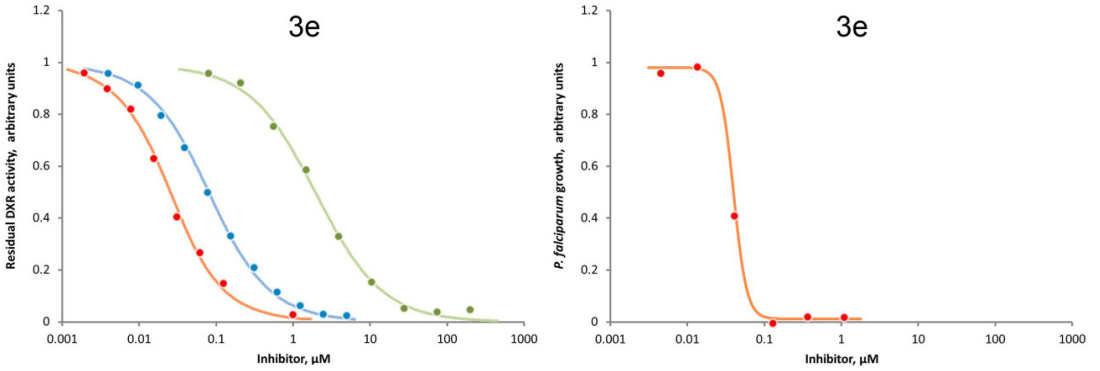
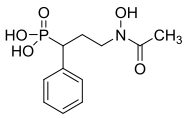
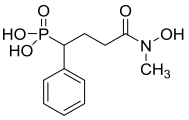
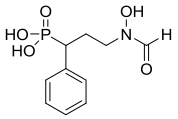
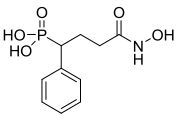
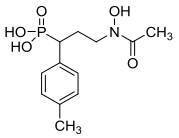
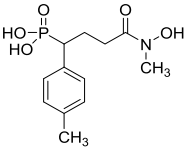
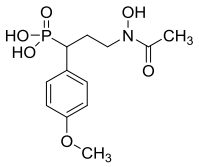
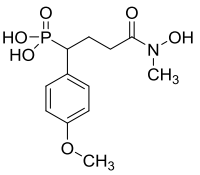
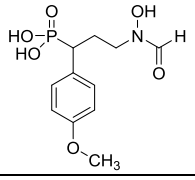
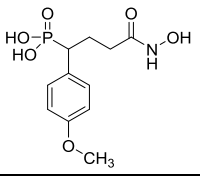


Fig. 4. Left, inhibition of IspC orthologs (*Pf* IspC, red; *Ec*IspC, blue; *Mt*IspC, green); Right, inhibiton of *P. falciparum* blood stages. Modified and reprinted with permission from reference[40]. Copyright 2014 American Chemical Society

The in vitro antiplasmodial activity of the study compounds against different parasite strains was similar. Table 1 compares the inhibition efficacy of isomeric pairs of fosmidomycin type resp. reverse fosmidomycin analogues. The activities of individual isomer pairs, as assessed against different parasite strains, differ by up to one order of magnitude but show no unequivocal trend. Moreover, pilot in vivo experiments with reverse hydroxamates using the *P. berghei* mouse model have shown some potential in the reverse carba series[29].

Table 1. Antiplasmodial activity (IC_{50}) of isomeric hydroxamate and reverse hydroxamate pairs[20, 24, 28, 40].

	Hydroxamate	*Pf*3D7 (nM)	*Pf*Dd2 (nM)		Reverse hydroxamate	*Pf*3D7 (nM)	*Pf*Dd2 (nM)
14a		550	350	**3a**		90	74
14b			400	**3b**		400	570
14c		950	220	**3c**		210	250
14d		850	270	**3e**		100	300
14e		360	200	**3f**		1 700	3 600

The IC_{50} values observed in the parasite growth assay showed good correlation with the enzyme inhibition effects, but were typically about one order of magnitude larger as compared to the IC_{50} values obtained in enzyme assays. Notably, however, the dose response curves observed in the parasite assays typically show a much steeper descent than the enzyme inhibition curves. Thus, at about 90 % inhibition level, the impact of the compounds is similar in the enzyme assays and growth assays (in terms of therapeutic potential, a steep curve shape for the inhibition of the living parasite is a definitive advantage since therapy is not targeted at 50 % reduction but at efficacious suppression of parasite growth)[28,40–42].

Fig. 5 shows the topology of fosmidomycin bound at the active site of *Pf*IspC in comparison with the bound reverse fosmidomycin analogue **3e** (Fig. 2; notably, the reverse analogue shown is considerably more bulky, due to its phenyl substituent). In both structures, the phosphonate moiety is coordinated by hydrogen bonds involving the backbone nitrogen as well as the β hydroxy group of Ser270. Moreover, in both structures, the phosphonate oxygens coordinate two water molecules. However, a significant difference in the embedding of the phosphonate motif arises by the implication of the respective side chains of His290 in case of fosmidomycin but of Ser306 in case of the reverse fosmidomycin analogue **3e**[40]. In both structures under comparison, the magnesium ion coordinates both oxygen atoms of the respective hydroxamate motifs, even though their connectivities are changed. Moreover, in both structures, the coordination of the magnesium ion by the terminal carboxylate groups Asp231, Asp233 and Glu315 appears essentially invariant. The terminal amide group of Asn311 interacts with the phosphonate motif and also with the respective oxygen of the hydroxamate moiety. Also of note, Ser232 can form two hydrogen bonds with one of the hydroxamate oxygens in each respective structure under comparison. In summary, the comparison of these structures documents the considerable flexibility of the IspC active site that had been noted early on[40].

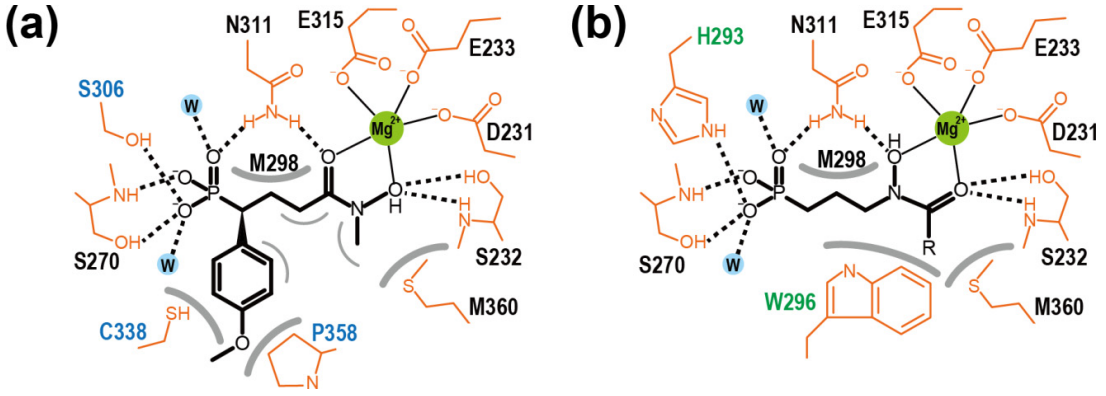


Fig. 5. Interactions of inhibitors in the active site of *Pf*IspC. Metal coordination (2.0-2.1 Å) and possible hydrogen bonds (2.7-3.1 Å) are shown as solid and dashed lines, respectively. Intra- and intermolecular van der Waals contacts are shown as thin and thick gray arcs, respectively. (a) **3e** complex. Residues uniquely involved in direct interactions with the bound inhibitor in the **3e** complex are shown in blue. (b) Fosmidomycin complex (R = H). Residues uniquely involved in direct interactions with the bound inhibitor in the fosmidomycin complex are shown in green. Reprinted with permission from reference[40]. Copyright 2014 American Chemical Society.

2.2. Oxa and thia isosters

All attempts to replace the hydroxamate motif by other chelating motifs have hitherto resulted in loss of inhibitory activity. It has also been shown that decreasing or increasing the distance between the phosphanate anchor and the hydroxamate chelator is invariably detrimental. However, Verbrugghen et al.[26] have shown that a methylene group connecting the phosphonate and hydroxamate moiety in FR900098 can be replaced by oxygen with impunity. In order to analyse in more detail the isosteric replacement of a methylene group by either oxygen or sulfur, it was preferable, for obvious technical reasons, to work with the reverse aryl derivatives.

Specifically, in order to unequivocally assess the impact of the replacement of the β methylene group in α arylated reverse fosmidomycin derivatives, sets of compounds were synthesised, which differed exclusively by

carrying either methylene, oxygen or sulphur in the β position. All compounds were analysed using IspC from *P. falciparum*, *M. tuberculosis* and *E. coli*. In case of the *P. falciparum* enzyme, the carba compounds were stronger inhibitors than both the oxa and thia isosters. Conversely, with the enzymes from bacterial origin, the thia analogues were stronger inhibitors than the carba compounds whereas the oxa isosters also had a lower activity[28,29,40–42].

2.3. Enantioselectivity

The thia analogue **5a** (Fig. 2) in complex with *Pf*IspC afforded an X-ray structure at a resolution of 2.0 Å where the electron-rich sulphur atom provides exceptionally good contrast for the molecular environment of the chiral center[42]. For the first time, the structure established unequivocally that the enzyme selectively binds the *S*-enantiomer from the racemic mixture.

The enantiomers of **5a** were subsequently separated by chiral chromatography and were shown to differ by more than three orders of magnitude with respect to their IC_{50} values toward *Pf*IspC (IC_{50}, 9.4 nM resp. 12.000 nM). The combination of chromatographic resolution and crystal structure analysis assigned the more active enantiomer as the *S*-enantiomer. Notably, the enantiomer separation affords a formal increase of inhibitor activity by a factor of two[42].

3. Conclusion

The seminal discovery of the natural products, fosmidomycin and FR900098, were made empirically in the 1970s when even the existence of the non-mevalonate pathway was still unknown[9,10,12]. The natural product was assigned its target, IspC, in 1999, and the same paper also demonstrated that fosmidomycin could cure *Plasmodium vinckei* infected mice[2]. Work that had been done already in the 1980s, in context of the failed development of fosmidomycin as an antibacterial drug, facilitated the transition to clinical phase III studies documenting that fosmidomycin can cure human malaria.

The partially successful repositioning of the abandoned antibacterial drug, fosmidomycin, as an antimalarial drug has triggered substantial efforts by academic and corporate groups to improve the activity profile of fosmidomycin by rational drug design. Most notably, the introduction of α aryl substituents improved the inhibitory potential both at the level of the isolated *P. falciparum* enzyme and of the parasite growth assay by at least one order of magnitude. In parallel, attempts to target other pathogens, notably *M. tuberculosis*, have resulted in several dozen research papers as well as at least 50 X-ray structures including several different IspC orthologs; near-atomic resolution of 1.65 Å has been achieved. The resolutions of currently available X-ray structures of the *P. falciparum* enzyme extend to 1.86 Å. However, the data presented in this paper have also shown that work with the living parasite is an important complement to work at the molecular dimension.

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