The Non-Mevalonate Pathway to Isoprenoid Biosynthesis: A Potential Source of New Drug Targets

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Dedicated to Professor Andreas Pfaltz on the occasion of his 60th birthday

Abstract: Isoprenoids are an essential class of natural products with a myriad of biological functions. All isoprenoids are assembled using two common five-carbon precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) that are biosynthesized via two completely independent routes: the mevalonate and the non-mevalonate pathway. While the former is used by humans, the latter is employed exclusively by a number of important pathogens such as the malarial Plasmodium falciparum parasite or the tuberculosis-causing Mycobacterium tuberculosis bacterium. Hence, the constituent enzymes are potentially very interesting drug targets in the ongoing fight against diseases, whose treatment has been complicated by the emergence of multi-drug resistance. After a short description of the biosynthetic pathway, an overview will be given on the known inhibitors of the individual enzymes.

Keywords: Anti-infective agents · Drug design · Inhibitors · Isoprenoid biosynthesis · Non-mevalonate pathway

1. Introduction

Isoprenoids form an extremely large and diverse class of natural products. The more than 35,000 members of the isoprenoid group fulfill a number of important biological functions, including protein degradation, meiosis, apoptosis, electron transport and regulation of transcription and post-translational processes. In spite of their striking structural diversity, all isoprenoids are biosynthesized from the same, rather simple two five-carbon building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). Until recently, only one route to these precursors was known, the so-called mevalonate pathway.[1] In the early 1990s, an alternative to this well-established biosynthetic route was discovered. It is now known as the non-mevalonate pathway.[2] 

Interestingly, the two pathways are very clearly distributed amongst organisms. On the one hand, the mevalonate pathway is used by most eukaryotes (importantly all mammals), a few eubacteria, fungi, the cytosol and mitochondria of plants, as well as some parasites such as Trypanosoma and Leishmania.[3] The non-mevalonate pathway, on the other hand, provides the precursors in algae, cyanobacteria, most eubacteria, chloroplasts of plants and apicomplexan parasites.[4]

The fact that the non-mevalonate pathway is exclusively used by important pathogens and not by humans means that the constituent enzymes may be new attractive drug targets. Accordingly, the past two decades witnessed intense research activity aimed at the elucidation of the structures and mechanisms of the involved enzymes. Today, most of the structures have been solved and detailed mechanistic data exist.[5] In particular, the component enzymes have been genetically validated as drug targets.[6] The emergence of multi-drug resistance both of the malarial parasites and the tuberculosis-causing bacteria has driven research to identify anti-infectives with a novel mode of action.[7,8] To achieve this, a number of the enzymes of the non-mevalonate pathway have been chosen as targets. This mini-review will give an update on each of the enzymes, for which inhibitors or clinical candidates are known.

2. Biosynthetic Route

In contrast to the mevalonate pathway that exclusively uses two-carbon building blocks, the non-mevalonate pathway utilizes two-carbon and three-carbon building blocks for the biosynthesis of the isoprenoid precursors IPP and DMAPP. These are assembled by the action of a total of seven enzymes (Scheme). As shown by deuterium-
labelling studies, IPP and DMAPP are both generated in the last enzymatic reaction. In spite of this, one of the two types of IPP isomerase (EC 5.3.3.2) can be present as an eighth enzyme in species utilizing the non-mevalonate pathway but it was shown not to be essential in *Escherichia coli*.

![Fig. 1. Inhibitors of the enzyme DXS](image)

**3. Known Inhibitors**

### 3.1. 1-Deoxy-α-xylulose-5-phosphate Synthase (DXS)

The non-mevalonate pathway starts with the head-to-tail condensation of glyceraldehyde 3-phosphate and pyruvate via a thiamine-pyrophosphate-dependent decarboxylation catalyzed by 1-deoxy-α-xylulose-5-phosphate synthase (EC 2.2.1.7). Two inhibitors are known of the first enzyme. 5-Ketoclonazone (1), a metabolite of the known herbicide clomazone (2), has an IC\textsubscript{50} value of 0.1 mm (*Chlamydomonas* DXS) (Fig. 1). Interestingly, clomazone itself does not inhibit DXS, implying that the breakdown product 1 might be the active agent showing the herbicidal activity.

Fluoropyruvate (3) has IC\textsubscript{50} values of 400 µM and 80 µM for DXS from *Pseudomonas aeruginosa* and *E. coli*, respectively (Fig. 1). It is supposed that fluoropyruvate binds covalently to the active site of DXS.

### 3.2. 1-Deoxy-α-xylulose-5-phosphate Reductoisomerase (IspC)

The second enzyme of the pathway, 1-deoxy-α-xylulose-5-phosphate reductoisomerase (EC 1.1.1.267), catalyzes both the rearrangement and reduction of 1-deoxy-α-xylulose-5-phosphate, yielding 2C-methyl-α-erythritol 4-phosphate (14). Fosmidomycin (4) and the acetyl derivative FR-900098 (5) were the first reported inhibitors of the non-mevalonate pathway and were identified to inhibit IspC (Fig. 2). They were discovered through a database search aimed at the identification of compounds active against bacteria utilizing the non-mevalonate pathway and inactive against species, which rely on the mevalonate pathway. Compounds 4 and 5 were characterized as highly potent, slow, tight-binding inhibitors with a good selectivity for IspC from bacteria, plants and *P. falciparum*. In fact, fosmidomycin was shown to cure malaria in rodents. As combinations of 4 with the existing antimalarial clindamycin or arsunate are even more efficient, they are currently being tested in clinical trials.

The fact that fosmidomycin is a very cheap and safe drug that is well tolerated and has high efficacy makes it a very promising candidate for standard treatment in regions where resistance against the recommended sulfadoxine–pyrimethamine combination is growing or where the price of the superior or artemisinin combinations is the limiting factor. X-ray crystal structure analysis verified the binding mode of fosmidomycin. A co-crystal structure of 4 and *E. coli* IspC could be solved in complex with a Mn\textsuperscript{2+} cation (PDB code: 1OPN). Fosmidomycin was shown to bind in an analogous manner to the substrate (Fig. 3).

The phosphate group is bound by the polar or charged amino acids Ser186, Ser222, Asn227 and Lys228. The (N-formyl-N-hydroxy)amino moiety is held in place through a chelating interaction with the co-crystallized Mn\textsuperscript{2+} cation using the O atom of both the formyl and the N-hydroxy group. A crystal-bound water molecule and the side chains

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**Fig. 2. Known inhibitors of IspC,\textsuperscript{15–20}** *K*\textsubscript{i} = inhibition constant. Footnotes to Table: \textsuperscript{a} The IC\textsubscript{50} value represents the conversion to the tightly bound complex, taking into account that fosmidomycin is a slow, tight-binding inhibitor. \textsuperscript{b} The IC\textsubscript{50} value is given as no *K*\textsubscript{i} value has been published.
LAUREATES: AWARDS AND HONORS SCS FALL MEETING 2007

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of Asp150, Glu152 and Glu231 provide the remaining four ligands for the octahedrally coordinated Mn$_{2}^{2+}$ cation.

Several derivatives of 4 and 5 were conceived to improve the binding affinity, achieve a better understanding of the binding mode and optimize the drug-like properties (Fig. 2)."\textsuperscript{[20]}" Woo et al. analyzed a series of systematically modified derivatives. While replacement of the phosphonate moiety by a phosphate group led to even more potent inhibitors (compounds 6a,b), however at the cost of pharmacokinetic stability,\"\textsuperscript{[20c]}" introduction of a carbobate or sulfamate group (compounds 6c,d) produced a decrease in affinity. The reduced affinity of the N-methyl analogue 6e suggests that the Mn$_{2}^{2+}$-coordinating N-hydroxy moiety (expectedly) makes a substantial contribution to the binding of fosmidomycin. Van Calenbergh and coworkers found that rigidification of the rather flexible propyl linker using a cyclopropyl ring (in (1R,2S)-7) did not enhance but rather reduced the binding affinity as compared to 4.\"\textsuperscript{[20d]}" Introduction of aromatic rings α to the phosphate moiety led to one interesting derivative (±)-8 with a comparable $K_i$ value to fosmidomycin against \textit{E. coli} IspC but a significantly enhanced activity between the rings of Pro182 and Tyr25. According to the modeling, both enantiomers seem to fit equally well into the active site of Asp150, Glu152 and Glu231 provide the remaining four ligands for the octahedrally coordinated Mn$_{2}^{2+}$ cation.

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Fig. 3. Co-crystal structure of fosmidomycin and \textit{E. coli} IspC (PDB code: IONP).\textsuperscript{[19]}" Color code: protein skeleton: C; gray; inhibitor skeleton: C, green; S: red; D: blue; P: orange; Mn$_{2}^{2+}$: violet sphere, H$_2$O: red sphere. Hydrogen-bond distances are given in Å.

3.3. 4-Diphosphocytidyl-2C-methyl-d-erythritol Kinase (IspE)

The central enzyme of the non-mevalonate pathway, 4-diphosphocytidyl-2C-methyl-d-erythritol kinase (EC 2.7.1.148), phosphorylates 4-diphosphocytidyl-2C-methyl-d-erythritol to afford 4-diphosphocytidyl-2C-methyl-d-erythritol 2-phosphate.\"\textsuperscript{[21]}" The first inhibitors of this kinase were recently reported by Hirsch et al. Using structure-based design, they were developed to target the cytidine- (of CDP-ME, see Scheme) rather than the adenosine- (of ATP) binding pocket, in order to achieve selectivity against other kinases.

The first-generation inhibitors (±)-10a-e and (±)-11a-f (Fig. 4a) of \textit{E. coli} IspE achieved $K_i$ values in the nanomolar range without the need for a phosphate or ribose moiety. The introduction of phosphate or phosphonate groups was avoided in order to enhance the drug-like character of the ligands. They feature a central cytosine scaffold decorated with different ribose substituents (in (±)-10a-e) and a rather rigid propargylic sulfonamide linker, directing its alkyl substituent into a small, rather hydrophobic pocket lined by Phe185, Leu15 and Leu28. This pocket is not occupied by either substrate or cofactor. The most potent inhibitor to date (compound (±)-11a, $K_i = 290$ nM) features a tetrahydrothiophenyl ring as ribose analogue and a cyclopropyl substituent to fill the small, hydrophobic pocket.\"\textsuperscript{[22]}" The proposed, modeling-based binding mode of (±)-11a is illustrated in Fig. 4b. The cytosine scaffold of (±)-11a is postulated to be sandwiched between Tyr25 and Phe185 and to form three hydrogen bonds to both the backbone and the side chain of His26. The tetrahydrothiophenyl ring is stabilized by being sandwiched between the rings of Pro182 and Tyr25. According to the modeling, both enantiomers seem to fit equally well into the active site and the $S$ atom of the tetrahydrothiophenyl ring seems to engage in a beneficial $S$-aromatic interaction with the phenolic ring of Tyr25. The sulfonamide moiety is proposed to form hydrogen bonds to the catalytically...
important residues Lys10 and Asp144; investigations on the energetics for these polar contacts are ongoing. Both cyclopropyl (in (±)-11a) and trifluoroethyl (in (±)-11b) residues seem optimal to fill the newly discovered hydrophobic pocket. Smaller (as in (±)-11c,d) or larger (as in (±)-11e,f) residues seem to fit less well, leading to a strong reduction in binding affinity.

Cytosine derivatives 12 and 13 with a benzimidazole tail were originally developed as inhibitors of IspD. However, they were also shown to have moderate activity against E. coli IspD, with $K_i$ values of 71 $\mu$M and 86 $\mu$M for 12 and 13, respectively (Fig. 4a). Their substantial water-solubility allowed co-crystallization with IspE from the marine thermophile Aquifex aeolicus providing the first co-crystal structure analyses of an IspE enzyme with synthetic ligands. This structural information should provide valuable guidance in the future development of new anti-infecteds: In A. aeolicus IspE, similar to the kinases from P. falciparum and M. tuberculosis, the Phe185 residue, lining the small, apolar pocket in the E. coli enzyme, is substituted by a tyrosine residue and it seems important to address its OH group.

### 3.4. 2C-Methyl-D-erythritol-2,4-cyclodiphosphate Synthase (IspF)

Conversion of 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate into 2C-methyl-D-erythritol 2,4-cyclodiphosphate is catalyzed by 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (EC 4.6.1.12). The first generation of substrate-based inhibitors was developed by Crane et al. with the aim of establishing a fluorescence-based binding assay (Fig. 5a, compounds 14a,b and 15). It was expected that the cytidine moiety in the ligands should occupy pocket III, in a comparable manner to the natural substrate. Through a suitable linker, two different fluorescent aromatic substituents, anthranilate (2-amino-4-nitrobenzoate) and dansyl (5,5-dimethylaminonapthalenesulfonylamid), were introduced to be accommodated by the hydrophobic cleft in pocket II and to endow the molecules with the required fluorescence properties. A co-crystal structure of 14a and IspF validated the proposed binding mode (PDB code: 2AMT, Fig. 5b). While the cytidine moiety is anchored by four hydrogen bonds to the backbone of the Ala100–Leu106 fragment, the ribose is held in place by two hydrogen bonds with Asp56. The β-phosphate provides the fourth ligand for the tetrahedrally coordinated Zn$^{2+}$ ion.

To improve the drug-like character and the affinity of the second-generation inhibitors, cytosine derivatives (16a-c) and analogues 17a,b were designed, featuring an appropriate vector to direct the terminal aromatic rings into pocket II (Fig. 5a).[26]

The best inhibitors have IC$_{50}$ values in the upper micromolar range, representing an improvement by an order of magnitude over the first-generation inhibitors. Hence, it is possible to inhibit IspF without addressing the highly polar diphosphate or ribose binding sites. As pocket II is rather flexible, most of the binding free enthalpy seems to be derived from the occupation of the cytosine-binding site. A new hydrophobic pocket, proposed to host the central naphthyl or methylphenyl moiety of the ligands, was identified and its optimal filling is subject of ongoing investigations with the aim to further enhance the binding affinity. Inhibitors of IspG and IspH have not been described so far.

### 3.5. IPP Isomerase

Several potent inhibitors of the IPP isomerase (type I) have been known for some time, ranging from fluorinated substrate analogues, over mechanism-based epoxides, to an ammonium derivative, which could act as a transition state analogue.[27] Some of these inhibitors were crucial in the determination of the mechanism of IPP isomerase.[28] Depending on which type of isomerase is present in a given species, this enzyme may or may not be a suitable target for drug design due to the potential risk of selectivity issues.

### 4. Conclusions

Significant advances in terms of the elucidation of the structure and mechanism of the various enzymes of the non- mevalonate pathway over the past two decades have set the stage for the exploitation of the constituent enzymes as targets in the development of drugs with a novel mode of action. This is of particular importance in light of the emergence of multi-drug resistance in a number of important pathogens such as the malarial parasite P. falciparum or the causative agent of tuberculosis, M. tuberculosis. While inhibitors of IspC such as fosmidomycin have validated the pathway as an antimalarial target and are tested in clinical trials, progress has also been made in the identification of drug-like, non-phospho-lead for the central enzymes of the pathway, IspE and IspF. There is no doubt that the coming years will see further en-
hanced efforts to block the enzymes of the non-mevalonate pathway and to shut down isoprenoid biosynthesis in the malarial and tuberculosis parasites.

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