Hijacking the biosynthesis of coenzyme A for antimicrobial drug development

Vitamin imposters

Humankind’s struggle to find cures for infectious diseases is as old as humanity itself. During the last century, we have probably made the greatest advance in our battle against these diseases: the discovery of antibiotics. Importantly, the first antimicrobial agents introduced for clinical use in 1937, the sulfonamide drugs, act by hijacking the disease-causing organism’s biosynthetic pathway for making folic acid, a vitamin that is required in the synthesis of DNA and RNA. Since then, many other antibiotics with diverse mechanisms of action have been discovered, and, by the early 1960s, it seemed as if any infection could be treated successfully with a course of antibiotics. However, since the first introduction of these drugs, we have also started to suffer our greatest defeat: bacterial strains that show resistance against nearly every antibiotic were often isolated within a few years of their first clinical use. We have been forced back to the drawing board to come up with new antimicrobials, and this has led us to revisit the antimetabolite inhibition strategy used by the sulfonamide drugs. This article discusses the recent advances that have made in discovering compounds that interfere with the biosynthesis of the essential metabolic cofactor coenzyme A (CoA) from pantothenate (vitamin B₅).

CoA biosynthesis as a drug target

Pantothenate is an essential nutrient required for the survival of all organisms¹,². Most bacteria, fungi and plants have the capability to synthesize the vitamin themselves; however, animals, eukaryotic pathogens (such as the Plasmodium parasites that cause malaria) and some bacteria must obtain it from exogenous sources. The essential requirement for pantothenate is based on it acting as the biosynthetic precursor for CoA, a cofactor that is required by metabolic reactions that involves the transfer, condensation or breakdown of acyl groups (such as the acetyl group, derived from acetate). In fact, it is estimated that up to 9% of all known enzyme activities involve CoA in some or other form¹.

CoA is formed in a pathway that consists of five enzymatic steps in which pantothenate, cysteine and three equivalents of ATP is used to produce the final cofactor. The five steps of the CoA pathway can be summarized as follows (Figure 1): first, pantothenate is phosphorylated by pantothenate kinase (PanK) to form 4΄-phosphopantothenate (PPan) with one equivalent of ATP serving as phosphate donor. This is followed by the Mg⁺⁺-dependent formation of 4΄-phosphopantothenoylcysteine (PPC) from PPan and L-cysteine by phosphopantothenoylcysteine synthetase (PPCS) using either CTP (in bacteria) or ATP (in eukaryotes) for activation. After decarboxylation of PPC by phosphopantothenoylcysteine decarboxylase (PPCDC) to give 4΄-phosphopantetheine (PPantSH), the fourth enzyme in the pathway, phosphopantetheine adenyltransferase (PPAT) catalyses the reversible Mg⁺⁺-dependent adenylylation of 4΄-phosphopantetheine to form dephospho-CoA (dPCoA) and pyrophosphate as products. Finally, dephospho-coenzyme A kinase (DPCK) catalyses the selective MgATP-dependent phosphorylation of dephospho-CoA’s 3΄-hydroxy group on its ribose moiety to form CoA.

The essential requirement for CoA means that the inhibition of its biosynthesis from pantothenate in a specific organism would result in its death; as such, the CoA biosynthetic enzymes are all obvious targets for antimicrobial drug development¹,². However, not all the enzymes are equally ‘druggable’; PPCDC, for example, is a complex multimeric enzyme with a small enclosed active site that does not suggest an obvious approach whereby inhibition can be achieved. Additionally, the pursuit of lead compounds that inhibit the CoA biosynthetic enzymes would only make sense as a strategy if the equivalent human enzymes remain unaffected (or much less affected), i.e. if selective inhibition is possible. Fortunately the

Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; DPCK, dephospho-coenzyme A kinase; DHPS, dihydropteroate synthase; dPCoA, dephospho-CoA; MIC, minimum inhibitory concentration; N5-Pan, N⁵-pentylpantothenamide; N7-Pan, N⁷-heptylpantothenamide; PABA, p-aminobenzoic acid; PanAm, pantothenamide; PanK, pantothenate kinase; PPan, 4΄-phosphopantothenate; PPAT, phosphopantetheine adenyltransferase; PPC, 4΄-phosphopantothenoylcysteine; PPCDC, phosphopantothenoylcysteine decarboxylase; PPCS, phosphopantothenoylcysteine synthetase.

Key words: antimetabolite, antimicrobial agent, biosynthesis, coenzyme A, enzyme inhibitor, pantothenamide
Coenzymes

CoA biosynthetic pathway shows remarkable diversity between different organisms on the enzyme level, and consequently three enzymes, i.e. PanK, PPCS and PPAT, have been highlighted as the most promising targets to be pursued for antimicrobial drug design. These particular enzymes each show significant differences in regard to their structures and/or mechanism when the human and bacterial counterparts are compared; for example, PanK occurs as three distinct types, two of which predominate in bacteria whereas the other is mainly found in eukaryotes (including humans). In some cases, the selective inhibition of these enzymes has already been demonstrated experimentally, validating them as targets for the development of selective antimicrobial targets.

Apart from the direct inhibition of the CoA biosynthetic enzymes themselves, the pathway also offers another method whereby inhibition can be achieved: it is possible for compounds that resemble pantothenate to hijack the CoA biosynthetic pathway and to be transformed into CoA analogues. These so-called antimetabolites act as structural mimics of CoA, but lack its important catalytic features. As such, these compounds can interfere with any number of essential CoA-dependent reactions and ultimately cause cell death. An antimetabolite-based inhibition strategy poses several advantages over the target-based approach described above: first, the structural similarity of the antimetabolite precursor to the vitamin in question means that often its uptake takes place through the same routes used by the vitamin, so that permeability is less of a consideration. Secondly, differences between the CoA pathway enzymes of the human host and the targeted pathogen is a non-issue as these enzymes do not serve as the targets of inhibition, but act as metabolic activators of the actual inhibitor. Lastly, the pool of targets becomes much larger, as any CoA-dependent process can potentially act as the point of inhibition. However, this does pose a potential problem to establish selectivity a priori; instead, in vitro and in vivo toxicity tests must be performed to show that such antimetabolites are selective in their inhibition.

To demonstrate the advances that have been made in CoA-directed antimicrobial drug discovery, we discuss two examples: that of the natural product CJ-15,801 and of the pantothenamide-derived antimetabolites.

**CJ-15,801: an antimetabolite in the mould of the sulfonamides**

The discovery of the mode of action of the sulfonamides, the first clinically used antimicrobial agents as alluded to above, has served as cornerstone for much antimetabolite-focused drug discovery. The sulfonamide sulfanilamide acts by mimicking the structure of \( p \)-aminobenzoic acid (PABA) and competitively inhibiting the enzyme dihydropteroate synthase (DHPS), which converts PABA into 7,8-dihydropteroate in the folate biosynthesis pathway (Figure 2a). Consequently, the sulfonamides interfere with the targeted organisms’ ability to produce folic acid from PABA, and since they cannot source exogenous folic acid from elsewhere, this leads to cell death.

Following from this work, sulfonamide analogues of several other vitamins, including pantothenate, have been prepared and tested as antimicrobials. However, although some of these compounds did show some promise in in vitro and even in vivo studies, it was unclear whether they acted by inhibiting the formation of CoA in a manner similar to the inhibition of folic acid biosynthesis by the sulfonamides. In fact, until recently, no pantothenate...
analogue has been shown to directly inhibit any of the CoA biosynthetic enzymes. This changed with the elucidation of the mode of action of the natural product CJ-15,801, a fungal metabolite that was discovered by Pfizer in 2001. The compound was shown to inhibit drug-resistant strains of *Staphylococcus aureus* (a notorious source of hospital-associated infections) with micromolar minimum inhibitory concentration (MIC) values, but not any other bacterial species. Interestingly, the compound is nearly a structural copy of pantothenate, with the notable exception of an added trans-substituted double bond in the β-alanine moiety. This close structural similarity raised the question as to the basis for its unique selectivity, and the mechanistic basis of its inhibition.

Through detailed biochemical analyses of CJ-15,801’s interaction with the CoA biosynthetic enzymes, it was shown in 2012 that the compound in fact acted in a manner very similar to the sulfonamides (Figure 2b). First, it is accepted as alternative substrate by the PanK enzyme of *S. aureus*, which phosphorylates it and turns it into an alternative substrate for the next pathway enzyme, PPCS. Upon cytidylylation (activation of its carboxyl group by cytidylate transfer) by this enzyme, a tight-binding structural mimic of the native PPCS reaction intermediate was found to be formed. This mimic showed nanomolar $K_i$ values for the *S. aureus* enzyme and prevented it from performing its usual role in CoA biosynthesis. Importantly, the inhibitor’s unique selectivity for *S. aureus* was found to reside in the substrate of its PanK enzyme, as no other bacterial PanK that was tested phosphorylated CJ-15,801 to activate it as inhibitor of PPCS. From a mode of action perspective, CJ-15,801 therefore acts as a pantothenate antimetabolite that requires activation by the very pathway that it inhibits.

CJ-15,801 has also been tested on the human malaria parasite *Plasmodium falciparum*, showing promising inhibition (IC$_{50}$ of 39 µM). However, its mode of action in this organism has not yet been determined, although it is likely to also target its PPCS enzyme in a similar fashion to what was found in *S. aureus*.

Unfortunately, the inhibitory potency of CJ-15,801 is not sufficiently high to warrant its development as an antibacterial or antimalarial agent. Nonetheless, it has highlighted the potential of the PPCS enzyme as a drug target; this is being actively pursued as part of continuing inhibitor development studies in our group.

**Promise and potential of the pantothenamides**

Among the many pantothenate analogues that have been tested for antimicrobial activity, the $N$-substituted pantothenamides (PanAms) have shown the most
potential. These compounds are formed when a primary amine is coupled to the carboxy group of pantothenate, and were initially described as growth inhibitors of selected lactic acid bacteria and *Escherichia coli* in 1970. However, their potential as antimicrobials have been investigated with renewed interest since 2002 when it was shown that *N*-pentylpantothenamide (N5-Pan, the prototypical example of this class of compounds), is transformed into a CoA antimetabolite (an anti-CoA). However, the exact mode of action of the PanAms still remains a point of debate, and may in fact be different in the various organisms that it inhibits. Two main inhibitory pathways have been suggested (Figure 3): in the first, the production of the anti-CoAs is proposed to lower CoA levels since it was found that their biosynthesis occurs faster than that of CoA. The second pathway proposes that the anti-CoAs serve to modify and inactivate the holo-acyl carrier proteins (holo-ACPs) that are an essential part of type II fatty acid synthase systems in bacteria. This happens when the ACP synthase (AcpS) enzyme that normally uses CoA to activate apo-ACPs by transfer of its 4’-phosphopantetheine group in a post-translational modification, uses an anti-CoA instead. This leads to the formation of so-called crypto-ACPs that lack the thiol required for activation of the fatty acids are formed instead; and (3) PanK, the first enzyme in the CoA pathway, can be inhibited directly to reduce CoA levels, as has been shown to occur in *S. aureus*.

In bacteria, N5-Pan and its heptyl counterpart, N7-Pan, remains the most potent growth inhibitors discovered to date, with N7-Pan especially showing promise against *S. aureus*, with an MIC of 78 nM. The PanAms have also been investigated as antiplasmodial agents, especially since the pathway has been highlighted as an attractive drug target in the malaria parasite *P. falciparum* based on its essential requirement for pantothenate in the blood stage of its life cycle. *N*-phenethyl PanAm, the most potent antiplasmodial PanAm identified to date, was found to have a potency rivalling that of the reference
antimalarial chloroquine (IC$_{50}$ of 20 nM)$^{10}$. However, studies of the antiplasmodial activity of the PanAms revealed a significant hurdle to their use in an in vivo context: pantetheinases, enzymes from the Vanin family of proteins that normally degrade pantetheine (itself a CoA degradation product) to form pantothenate and cysteamine, also degrade the PanAms. Since these enzymes are ubiquitously present in serum, they substantially reduce the potency of these compounds; for example, in normal serum (i.e. with pantetheinase activity present) the IC$_{50}$ of N-phenethyl PanAm increases to more than 60 μM, a 3000-fold difference. Consequently, the major challenge now is either to develop PanAm variants that are stable in the presence of pantetheinase, or to develop combination strategies in which the PanAms are used in combination with pantetheinase inhibitors to prevent them from being broken down. Both of these strategies have already been pursued with varying levels of success.$^{10,21}$

Taken together, the PanAms clearly show significant promise and realizable potential as antimicrobial agents. However, they still require improvement from a medicinal chemistry perspective. Also, a clearer understanding of their mode of action in the various target organisms would help in improving their potency and selectivity even further.

**Outlook**

The recent discoveries (and re-discoveries) of CoA biosynthesis inhibitors have reignited interest in this process. The recent discoveries (and re-discoveries) of CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have reignited interest in CoA biosynthesis inhibitors have rein...