Experiences with the shikimate-pathway enzymes as targets for rational drug design

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Abstract

The background and current context of work on the shikimate-pathway enzymes as potential targets for anti-bacterial, anti-fungal and anti-parasitic drugs is reviewed. Recent work on the third enzyme of the pathway, dehydroquinase, which occurs in two structurally and mechanistically distinct forms, is used to illustrate the present state of studies into rational drug design.

Background and current context

In plants and micro-organisms, all the key aromatic compounds involved in primary metabolism, including the three aromatic amino acids found in proteins, are produced via the shikimate pathway [1,2] (Scheme 1). Animals, in contrast, have to derive their aromatic compounds from their diet. For this reason, there has been interest, extending back more than 25 years, in the shikimate-pathway enzymes as potential targets for non-toxic herbicides and anti-microbial compounds.

In 1972, the first report that inhibitors of the aromatic amino acid biosynthetic pathway had herbicidal activity appeared [3,4]. One of these papers from Jaworski's group [3] at Monsanto heralded a new era in the herbicide field. Their lead compound, glyphosate (*N*-phosphonomethylglycine), proved to be a billion dollar herbicide, and in the 1980s, more commercially important herbicides that targeted other amino acid biosynthetic pathways, for example the sulphonylureas and the imidazolinones, were developed [5–8].

The precise enzyme target for glyphosate, 5-enoylpyruvyl shikimate phosphate synthase (EPSP synthase), was identified by Steinrucken and Amrhein [9], and insight into the reason for its potency through formation of a dead-end ternary complex of enzyme, shikimate phosphate and glyphosate was provided by Boocock and Coggins [10]. Subsequent mechanistic, kinetic and structural studies on EPSP synthase have extended our understanding [11–13], but interestingly no more commercially important inhibitors of this enzyme have emerged.

The shikimate pathway was elucidated first in bacteria [14,15] largely by studying mutants lacking the individual enzyme activities. The pathway is essential since mutations completely block growth in culture unless aromatic supplements are provided. Mutation or deletion of one of the shikimate-pathway genes, such as *aroA*, which encodes EPSP synthase, in pathogen species such as *Salmonella*, *Aeromonas* and *Shigella* results in highly attenuated strains that are unable to survive *in vivo* and cause infection; this has been exploited for the development of vaccines [16,17]. Bacteria require the shikimate pathway for the synthesis of many crucial intermediates, including the aromatic amino acids, vitamin K, ubiquinone and enterochelin (Scheme 1). Mammals, lacking the shikimate pathway, depend on their diet for these componds. For pathogenic bacteria to grow *in vivo* and maintain infection they must use their own biosynthetic capacity to provide these aromatic compounds. The most critical nutrient appears to be *p*-aminobenzoic acid [18], the precursor of folate, which is essential for nucleotide biosynthesis.

It has long been known that glyphosate inhibits bacterial growth *in vitro* [3,19]. The inhibition of the growth of apicomplexan parasites *in vitro* by glyphosate provided the first evidence of the occurrence of the shikimate pathway in this important group of mammalian pathogens [20,20a]. The presence of the shikimate pathway in fungi has been well established since the pioneering work of Giles (reviewed in [15]). Recent genome studies confirm that the pathway is indeed present in many pathogens [18] and there is certainly scope for developing broad-spectrum compounds with efficacy against a variety of pathogens. It is possible that a single compound that inhibits the shikimate pathway or combinations of synergistic compounds that inhibit various enzyme targets in this and connected pathways could be used to treat patients with multiple infections. This would be of great value in the treatment of opportunistic pathogens such as *Pneumocystis carinii*, *Mycobacterium tuberculosis*, *Cryptosporidium parvum* and *Toxoplasma gondii*, which may simultaneously infect AIDS and other immunocompromised patients [21].

First steps in inhibitor development

As a tool to investigate the mechanisms of the shikimate-pathway enzymes and to develop the first generation of potentially useful inhibitors, a wide variety of fluorine- and

Key words: dehydroquinone, dehydroquinone inhibitor, 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase), 6-fluoroshikimate, rational drug design, shikimate pathway.

Abbreviations used: EPSP synthase, 5-enolpyruvylshikimate-3-phosphate synthase; DHQase, dehydroquinase.

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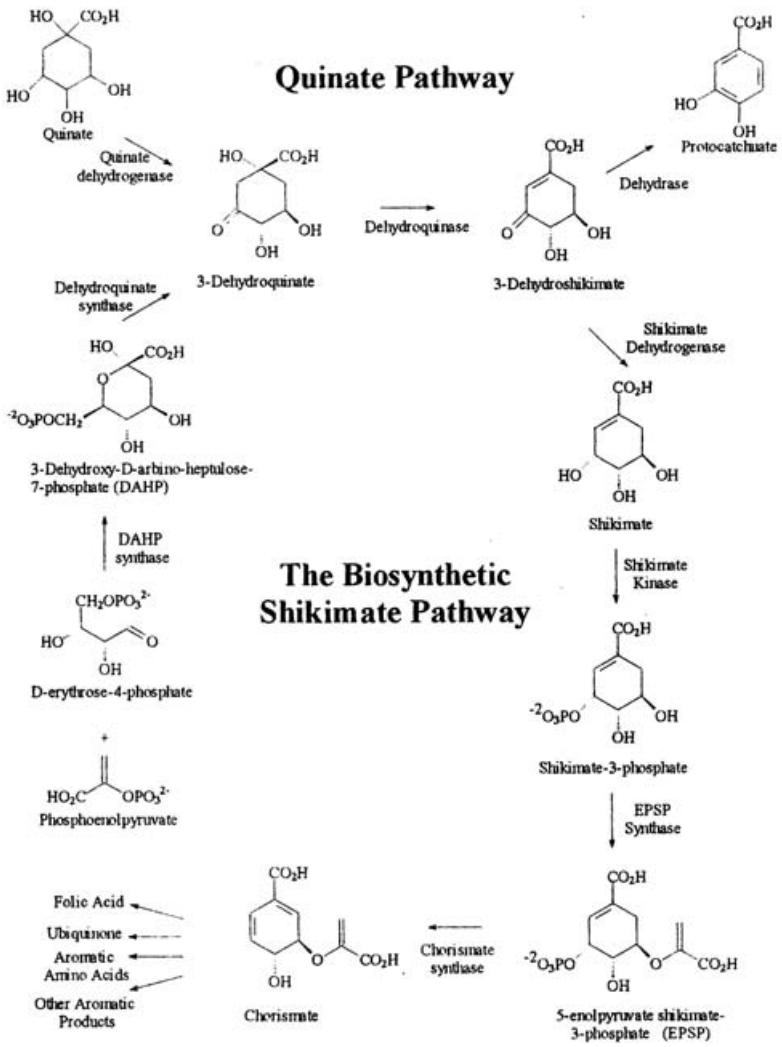
Scheme 1 | The biosynthetic shikimate and the catabolic quinate pathways, showing the inter-relationship between the pathways and how folate, ubiquinone and the aromatic amino acids arise

The quinate pathway provides a means for soil organisms, including bacteria and fungi, to use the abundant plant metabolite quinic acid. More details of the pathways and particularly the biosynthetic products derived from the shikimate pathway are given in [1,2].



other halogen-substituted shikimate-pathway intermediates were prepared either by chemical synthesis [22] or by a combination of chemical synthesis and biotransformation [23,24]. Among the first compounds made were the two stereoisomers of 6-fluoroshikimate, which were transformed into the corresponding 6-fluoroEPSPs and shown to be inhibitors of chorismate synthase [25]. The first evidence that shikimate-pathway enzyme inhibitors had anti-bacterial activity in minimal medium and efficacy in animal-infection models came from studies with (6*S*)-6-fluoroshikimate [26]. It appears that this compound is converted by the subsequent enzymes in the pathway to 6-fluorochorismate, which is believed to inhibit the biosynthesis of *p*-aminobenzoic acid [27]. Unfortunately, spontaneous resistance to (6*S*)-6-fluoroshikimate occurred at high frequency because of mutations in the transport system required for the uptake of the drug and it has therefore not been developed further [28]. This resistance mechanism is specfic to (6*S*)-6-fluoroshikimate and related compounds and should not be a general problem for inhibitors of shikimate-pathway enzymes [18].

Studies on dehydroquinase (DHQase)

DHQase (3-dehydroquinate dehydratase) catalyses the reversible dehydration of 3-dehydroquinic acid to 3-dehydroshikimic acid (Scheme 1). This reaction is common to two metabolic pathways, the biosynthetic shikimate pathway and the catabolic quinate pathway (Scheme 1). Two distinct classes of DHQases (types I and II) are responsible for catalysing this transformation by different

Scheme 2 | The proposed mechanism of the type II DHQases [36]
DHQ, 3-dehydroquinate; DHS, dehydroshikimate.

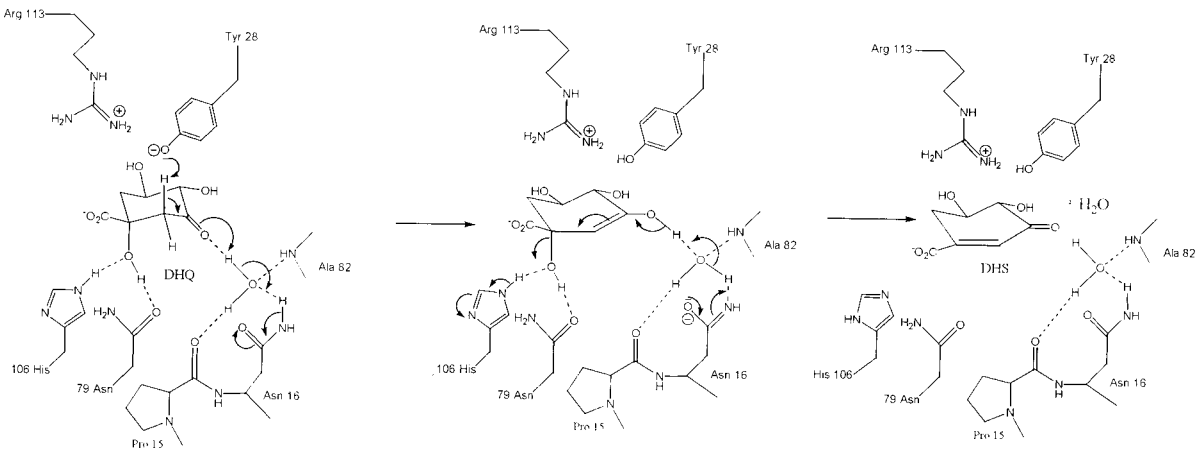
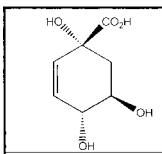
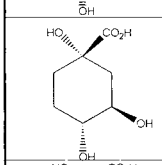
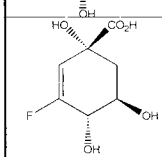
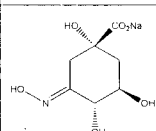


Table 1 | Inhibition constants (μM) for inhibitors against type I and type II DHQases
The K_m values were measured for the respective enzymes with dehydroquinic acid [37,38].

	Type I *S. typhi*	Type II *A. nidulans*	Type II *M. tuberculosis*	Type II *S. coelicolor*
	3000 ± 1000μM	60 ± 10μM	200 ± 20μM	30 ± 10μM
	4500 ± 500μM	1500 ± 200μM	1200 ± 200μM	600 ± 200μM
	1500 ± 500μM	50 ± 5μM	10 ± 2μM	15 ± 2μM
	> 25000μM	15 ± 1μM	20 ± 2μM	500 ± 0.2μM

mechanisms [29]. Type I DHQases operate in the shikimate pathway, whereas type II DHQases have both biosynthetic and catabolic roles [30]. The two types of DHQase have completely different subunit architectures. The overall topology of the type I DHQase is an eight-stranded α/β-barrel while the type II DHQase subunit consists of a five-stranded β-sheet core flanked by four α-helices [31]. The type I enzymes catalyse a *cis*(*syn*)-dehydration involving loss of the 2-pro-*R* hydrogen via a covalent imine intermediate [32,33], while the type II enzymes catalyse a *trans*(*anti*)-dehydration which results in the loss of the 2-pro-*S* hydrogen, probably via an enolate intermediate [34].

The occurrence of two mechanistically and structurally distinct forms of DHQase offers the possibility of developing selective inhibitors for the two types of enzyme. This could be very valuable in the selective therapy of slowly growing organisms such as *M. tuberculosis* and *Helicobacter pylori*, which only have type II enzymes, in contrast with gut organisms such as *Escherichia coli*, which only have type I enzymes [31]. The first type-specific inhibitors had halogen substituents replacing the 2-pro-*R* hydrogen in dehydroquinic acid and were therefore expected to inhibit the type I enzymes [35]. These compounds, exemplified by (2*R*)-2-bromo-3-dehydroquinic acid, were substrates for the

type II DHQases since they retained the 2-pro-*S* hydrogen, but were indeed irreversible inhibitors of the type I enzymes [35].

The availability of the structure of the *M. tuberculosis* DHQase [31] and more recently the structure of the type II enzyme from *Streptomyces coelicolor* [36] has allowed us to focus on the design of specific inhibitors of the type II DHQases. The elimination proceeds by an E_1CB mechanism [34,36], via a postulated enolate intermediate, and involves the loss of the more acidic 2-pro-*S* hydrogen of the substrate, in an *anti*-elimination of water (Scheme 2). The intermediate is characterized by a flattening of the carbocyclic ring and is likely to involve stronger hydrogen bonding to the enolate oxygen in the transition state. The first target compound was 2,3-anhydroquinic acid [37] (Table 1, row 1). This is structurally similar to the substrate but lacks the carbonyl group necessary to form an imine with the type I enzymes. In addition, the C-2–C-3 double bond mimics the flattening of the ring in the enolate intermediate. To assess the contribution that this subtle conformational restriction makes to binding, the reduced compound 3-deoxyquinic acid was also tested [37] (Table 1, row 2). The second design strategy was to look for extra binding affinity in the carbonyl-binding pocket of the type II DHQases, where it is believed that stabilization is provided by the formation of the enolate intermediate. The target was the simple oxime [37] (Table 1, row 4). As a third strategy, to find an even better mimic of the geometric and electronic features of the enolate intermediate, the enolate oxyanion was replaced by its closest chemical equivalent, the isosteric and isoelectronic fluorine atom [38] (Table 1, row 3). All of the specifically designed inhibitors were very much better competitive inhibitors of the type II DHQases than of the type I enzymes (Table 1). The structures of enzyme–inhibitor complexes with the type II DHQase from *S. coelicolor* have now been determined [36] and this, with further structural studies on other enzyme–ligand complexes [39], will facilitate improvements in inhibitor design and hopefully lead to compounds with therapeutic utility.

Future work

There is no doubt that the shikimate-pathway enzymes offer many important targets for the development of drugs against bacterial, fungal and apicomplexan parasitic diseases. All of the enzymic steps are of potential interest and the availablity of structures for almost all of the pathway enzymes will allow rational inhibitor design to progress more rapidly [40–44].

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