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# Enoyl Reductases as Targets for the Development of Anti-Tubercular and Anti-Malarial Agents

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**Abstract:** Tuberculosis (TB) and Malaria are neglected diseases, which continue to be major causes of morbidity and mortality worldwide, killing together around 5 million people each year. Mycolic acids, the hallmark of mycobacteria, are high-molecular-weight α-alkyl, β-hydroxy fatty acids. Biochemical and genetic experimental data have shown that the product of the *M. tuberculosis inhA* structural gene (InhA) is the primary target of isoniazid mode of action, the most prescribed anti-tubercular agent. InhA was identified as an NADH-dependent enoyl-ACP(CoA) reductase specific for long-chain enoyl thioesters and is a member of the Type II fatty acid biosynthesis system, which elongates acyl fatty acid precursors of mycolic acids. *M. tuberculosis* and *P. falciparum* enoyl reductases are targets for the development of anti-tubercular and antimalarial agents. Here we present a brief description of the mechanism of action of, and resistance to, isoniazid. In addition, data on inhibition of mycobacterial and plasmodial enoyl reductases by triclosan are presented. We also describe recent efforts to develop inhibitors of *M. tuberculosis* and *P. falciparum* enoyl reductase enzyme activity.

**Key Words:** Tuberculosis, mycobacteria, mycolic acids, isoniazid, InhA, enoyl reductase, drugs.

## TUBERCULOSIS: EPIDEMIOLOGY AND CHEMOTHERAPY

Tuberculosis (TB) is a global health emergency that remains the leading cause of mortality due to a bacterial pathogen, *Mycobacterium tuberculosis*. The re-emergence of TB occurred, in most cases, in the late 1980s and involved the USA and some European countries due to increased poverty in urban settings and the immigration from TB high-burden countries [1]. No sustainable control of TB epidemics can thus be reached in any country without properly addressing the global epidemic. It is estimated that 8.2 million new TB cases occurred worldwide in the year 2000, with approximately 1.8 million deaths in the same year, which translates into more than 4,900 deaths per day, and more than 95 % of these were in developing countries [2]. In addition, approximately 12 % (226,000) of total deaths from TB was attributed to co-infection with *M. tuberculosis* and human immunodeficiency virus (TB-HIV). It has been estimated that approximately 39.4 million people are infected with HIV worldwide, and more than half of them live in sub-Saharan Africa and nearly about a fifth in South and South-East Asia [3]. Immune deficient patients with HIV are at increased risk of latent *M. tuberculosis* infections progressing to active disease, and being transmitted to others [4]. TB and HIV are intricately linked to malnutrition, unemployment, alcoholism, drug abuse, poverty and homelessness. The direct and indirect costs of illness due to TB and HIV has been estimated to be more than 30 % of the annual household income in developing countries and have thus a catastrophic impact on the economy in the developing world [5].

It has been estimated that 3.2 % of the world's new cases of TB, in 2000, were multidrug-resistant tuberculosis (MDR-TB), defined as resistant to at least isoniazid and rifampicin [6]. According to the 2004 Global TB Control Report of the World Health Organization (WHO), there are 300,000 new cases per year of MDR-TB worldwide, and 79 % of MDR-TB cases are "super strains", resistant to at least three of the four main drugs used to treat TB [7]. More recently, a survey of the frequency and distribution of extensively drug-resistant (XDR) TB cases, which are defined as cases in persons with TB whose isolates were resistant to isoniazid and rifampicin and at least three of the six main classes of second-line drugs, showed that during 2000-2004, of 17,690 TB isolates, 20% were MDR and 2% were XDR, and that XDR-TB has a wide geographic distribution [8]. Localized high incidence rates of MDR-TB have been found in particular regions, which have been defined as hot zones based on either areas where the prevalence of MDR-TB cases is >5 % (that is, where >5 % of current cases are MDR-TB) or areas where the incidence of MDR-TB cases is >5 % (that is, where >5 % of new cases are MDR-TB) [6]. MDR-TB is an airborne bacterium that is spread just as easily as drug-sensitive TB. An individual who is sick with any strain of TB will infect between 10 and 20 people each year with that same strain [9], thereby making the hot zones of particular concern to public health officials. A mathematical model showed that, paradoxically, areas with programs that successfully reduced wild-type pansensitive strains (as a result of high case detection and treatment rates) often evolved into hot zones [10]. Accordingly, it has been suggested that second-line drugs be quickly introduced to disrupt the amplification of resistance. However, the bacteriostatic second-line drugs are more toxic and less effective and are given for at least three times as long and at 100 times the cost of basic short-course chemotherapy regimens [11, 12]. A mathematical model of the impact of fitness of multidrug-resistant strains of *M. tuberculosis* on the emergence MDR-TB has shown that even when the average relative fitness of MDR strains is low and a well-functioning control program is in place, a small subpopulation of a relatively fit strain may eventually outcompete both the drug-sensitive strains and the less fit MDR strains [13]. These results indicate that current epidemiological measures and short-term trends in the burden of MDR-TB do not provide evidence that MDR-TB strains can be contained in the absence of specific efforts to limit transmission and combat MDR disease. The factors that most influence the emergence of drug-resistant strains include inappropriate treatment regimens, and patient noncompliance in completing the prescribed courses of therapy due to the lengthy standard "short-course" treatment or when the side effects become unbearable [14].

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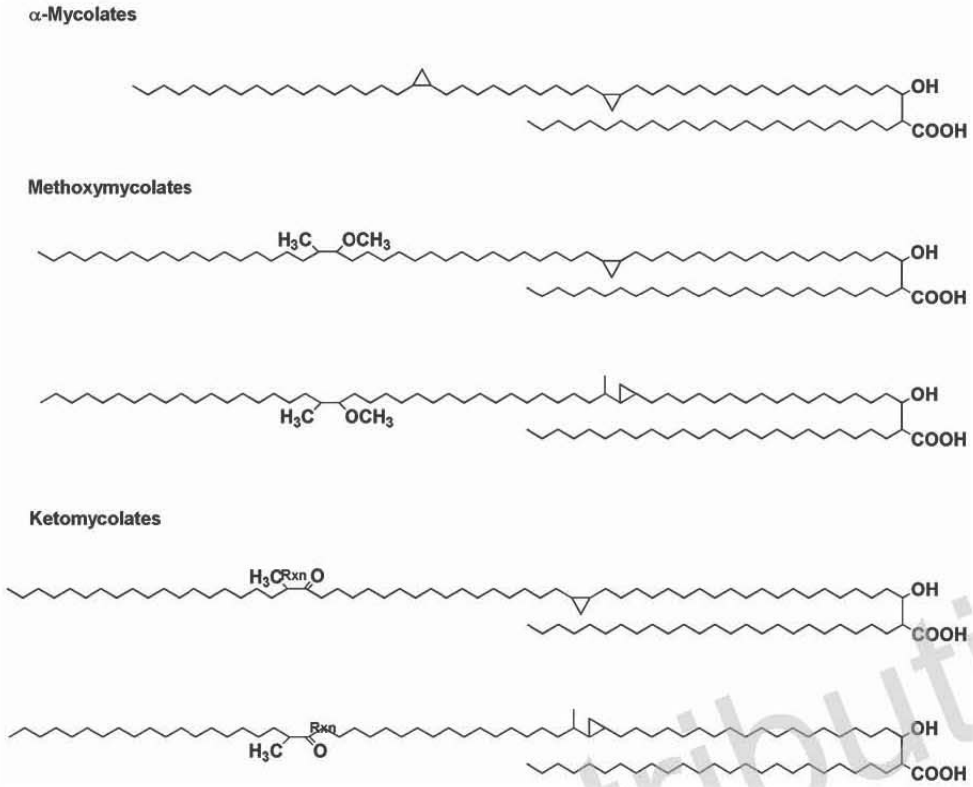



**Fig. (1).** The structures of mycolic acids identified in *M. tuberculosis*. α-Mycolates: its meromycolate chain contains two *cis*-cyclopropanes. Methoxymycolates: its meromycolate chain contains an α-methyl methyl-ether moiety in the distal position and a *cis*-cyclopropane or an α-methyl *trans*-cyclopropane in the proximal position. Ketomycolates: its meromycolate chain contains an α-methyl ketone moiety in the distal position and proximal functionalities as in the methoxy series. It should be pointed out that, more recently, unsaturations have been detected in the meromycolate chain of *M. tuberculosis* [25], which are not shown here.

Although only 5-10 % of infected individuals develop the disease because the host immune response against *M. tuberculosis* is highly effective in controlling bacterial replication, *M. tuberculosis* is almost never eradicated due to its ability to establish and maintain latency, a period during which the infected person does not have clinically apparent TB. Post-primary TB, which is predominantly a pulmonary disease, develops later in life, and can be caused either by reactivation of bacteria remaining from the initial infection or by failure to control a subsequent infection. Post-primary TB involves extensive damage to the lungs and efficient aerosol transmission of bacilli. It has been pointed out that the success of *M. tuberculosis* as a pathogen is largely attributable to its ability to persist in host tissues, where drugs that are rapidly bactericidal *in vitro* require prolonged administration to achieve comparable effects [15]. Thus effective tuberculosis chemotherapy must include early bactericidal action against rapidly growing organisms and subsequent sterilization of the semidormant and dormant populations of bacilli. The first-line drugs isoniazid, rifampicin, streptomycin and ethambutol exhibit early bactericidal activity against actively metabolizing bacilli [16]. Pyrazinamide is active against the semidormant bacilli in acidic intracellular environments. The modern, standard "short-course" therapy for TB is based on a four-drug regimen of isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin for two months, followed by treatment with a combination of isoniazid and rifampicin for an additional four months. This combination therapy must be strictly followed to prevent drug resistance and relapse and direct observation of patient compliance is the most reliable way to ensure effective treatment and prevent the acquisition of resistance. The bacteriostatic second-line drugs (amikacin, kanamycin, capreomycin, cycloserine, *para*-aminosalicylic acid, ethionamide, and fluoroquinolones) are reserved to strengthen the treatment of drug-resistant disease or when bactericidal drugs are prohibited because of toxicity [17].

It has recently been pointed out that there is a rapidly growing capability to undertake health innovation in many developing countries that have high indigenous science and technology capacity but relatively low economic strength, including India, China, Brazil, South Africa, Thailand, Argentina, Malaysia, Mexico and Indonesia [18]. Collectively, these countries invest at least U$ 2.5 billion per year in health research and it has been proposed that they should assume a leadership position in creating health innovations that target diseases of the poor because they are closer to those that are most in need for more effective health products [19]. In addition, to achieve the Millennium Development Goals (MDG), including investments in strategies to halt and reverse the spread of TB, developing country governments should adopt bold development strategies to meet the MDG targets for 2015 [20]. In particular, more effective and less toxic anti-tubercular agents are urgently needed to shorten the duration of current treatment, improve the treatment of MDR-TB and to provide effective treatment of latent tuberculosis infection.

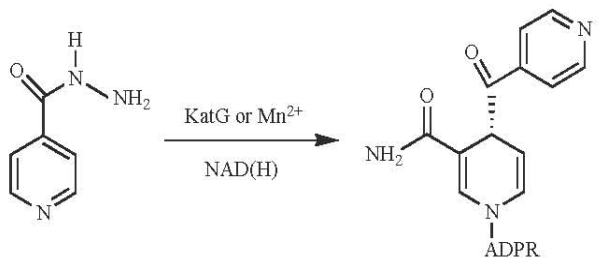
## *MYCOBACTERIUM TUBERCULOSIS* FATTY ACID BIOSYNTHESIS AND ENOYL REDUCTASE

The mycobacterial cell wall is comprised of three covalently linked macromolecules: peptidoglycan, arabinogalactan and mycolic acid, which is often described as mycolyl-arabinogalactan-peptidoglycan complex (mAGP) [21, 22, 23, 24]. Mycolic acids are high-molecular-weight α-alkyl, β-hydroxy fatty acids (Fig. **1**) that appear mostly as bound esters in tetramycolylpentaarabinosyl cluster in the mycobacterial cell wall. In the pyrolytic cleavage of my-

colic acids, the intact fatty acid released is referred to as the α branch and the aldehyde released is referred to as the meroaldehyde and the corresponding segment of the intact mycolate is thus referred to as the meromycolate branch. The meromycolate branch is functionalized at regular intervals by cyclopropyl, α-methyl ketone, or α-methyl methylethers groups, and as shown more recently, by unsaturations [25].

Fatty acid elongation occurs through repetitive cycles of condensation, β-keto reduction, dehydration and enoyl reduction which are catalyzed by, β-ketoacyl synthase (KAS, condensing enzyme), β-ketoacyl reductase (KAR; MabA in *M. tuberculosis*), β-hydroxyacyl dehydrase (DE), and enoyl reductase (ENR; InhA in *M. tuberculosis*) respectively. These chemical reactions are catalyzed by two types of fatty acid synthase systems (FAS). The FAS-I system is a multidomain polyprotein that encodes all the enzymes necessary for fatty acid synthesis in one large polypeptide and is generally present in most eukaryotes, except in plants [26]. FAS-II systems, which are present in bacteria and plants, catalyze the individual reactions by separate proteins readily purified independently of the other enzymes of the pathway and are encoded by unique genes. Mycobacteria, unlike most organisms, have both FAS-I and FAS-II systems [27]. The mycobacterial FAS-I system catalyzes the synthesis of $C_{16}$ and $C_{18}$ fatty acids, the normal products of *de novo* synthesis and elongation to produce $C_{24}$ and $C_{26}$ fatty acids [28]. The mycobacterial FAS-II is analogous to other bacterial FAS-II systems, with the notable exception of primer specificity. The mycobacterial FAS-II is not capable of *de novo* synthesis from acetate but instead elongates the "short-chain" acyl-ACP intermediates to fatty acids ranging from 24 to 56 carbons in length [29, 30]. In summary, the mycobacterial FAS-I produces a bimodal ($C_{14:0}$-$C_{16:0}$ to $C_{24:0}$-$C_{26:0}$) distribution of acyl-CoA fatty acids. The mycobacterial FAS-I system would provide the "shorter" acyl-CoA fatty acid precursors ($C_{14:0}$-$C_{16:0}$) for condensation with malonyl-ACP by mtFabH enzyme activity whose products, in turn, would be elongated by the FAS-II system, yielding the long carbon chain of the meromycolate branch (50-60 carbons) of mycolic acids. The longer chain acyl-CoA products ($C_{24:0}$-$C_{26:0}$) of FAS-I would be excluded from chain elongation and remain available to be utilized, presumably in the CoA form, as substrates for formation of the α-alkyl branch (20-26 carbons) of mycolic acids. The readers are referred to recent reviews describing mycolic acid biosynthesis and their processing into the final products for a more comprehensive description of these processes [23, 24, 31, 32].

Isoniazid (INH, isonicotinic acid hydrazide; Fig. **2**) is one of the oldest synthetic antitubercular and the most prescribed drug for active infection and prophylaxis. INH is highly active against *M. tuberculosis* with an MIC value in the range of 0.02 to 0.2 μg $mL^{-1}$, and is active against growing tubercle bacilli but not resting bacilli. Isoniazid uptake in *M. tuberculosis* has been proposed to occur by passive diffusion [33]. However, the involvement of efflux pumps in isoniazid transport has recently been reported [34]. The mechanism of action of isoniazid is complex, as mutations in a number of




Isoniazid (INH) INH-NAD Adduct

**Fig. (2).** Chemical structure of isoniazid and INH-NAD adduct that inhibits InhA enzyme activity.

different genes have been found to correlate with isoniazid resistance in *M. tuberculosis* [23, 24, 35, 36].

The product of the *M. tuberculosis inhA* structural gene (InhA or ENR) has been shown to be the primary target for isoniazid [37]. InhA was identified as an NADH-dependent 2-*trans* enoyl-ACP (acyl carrier protein) reductase enzyme that exhibits specificity for long-chain enoyl thioester substrates [38]. InhA is a member of the mycobacterial FAS-II, which elongates acyl fatty acid precursors yielding the long carbon chain of the meromycolate branch of mycolic acids, the hallmark of mycobacteria [23, 24]. Consistent with InhA as the primary target of INH mode of action, inactivation of *M. smegmatis inhA*-encoded enoyl reductase and INH treatment resulted in similar mycolic acid biosynthesis inhibition and morphological changes to the mycobacterial cell wall leading to cell lysis [39]. Transformation of *M. smegmatis*, *M. bovis* BCG and three different strains of *M. tuberculosis* with multicopy plasmids expressing *inhA* genes conferred a 20-fold resistance to INH and a 10-fold resistance to ethionamide (ETH) [40]. Further biochemical and genetic evidence has been provided showing that InhA is the primary target of INH [41]. It has recently been suggested that *M. tuberculosis* dihydrofolate reductase, an enzyme essential for nucleic acid synthesis, is a target for INH [42]. However, transfer of a single point mutation allele (S94A) within the putative target gene *inhA* in *M. tuberculosis* by specialized linkage transduction has been shown to be sufficient to confer clinically relevant levels of resistance to INH and inhibition of mycolic acid biosynthesis [43]. In agreement with these results, mutations in the *inhA* structural gene and in the *inhA* locus promoter region have been associated with isoniazid resistance [44]. Moreover, INH-resistant clinical isolates of *M. tuberculosis* harboring *inhA*-structural gene missense mutations, but lacking mutations in the *inhA* promoter region, *katG* gene and *oxyR-ahpC* region, were shown to have higher dissociation constant values for NADH than WT InhA, whereas there were only modest differences in the steady-state parameters [44]. Consistent with these results, a comparison of the crystal structure of binary complex of WT and INH-resistant mutant InhA (S94A) in complex with NADH [45] showed that disruption of a hydrogen bond network in the mutant protein could account for higher dissociation constant value for the coenzyme. In WT InhA-NADH complex structure (2.2 Å), a well-ordered water molecule mediates two hydrogen bonds between the O2 of the $P_N$ phosphate of NADH and protein that are lost in the S94A mutant protein [45]. This water molecule (WAT1) hydrogen bonds to the hydroxyl group of Ser94, the main-chain oxygen of Gly14 and the main-chain nitrogen atoms of Ala22 e Ile21. In the S94A-NADH complex structure (2.7 Å), the carbonyl group of residue Gly14 rotates away from the water molecule, breaking the hydrogen bond observed in wild-type structure (Fig. **3A**). A computational comparison of 102 high-resolution structures (≤ 1.90 Å) of enzyme-dinucleotide complexes revealed that the WAT1 water is structurally conserved in proteins that exhibit the classic Rossmann dinucleotide-binding fold motif [46]. The conserved water molecule links, through a conserved hydrogen-bonding pattern, the glycine-rich phosphate-binding loop with the dinucleotide pyrophosphate moiety [47]. In enoyl reductase enzyme the phosphate-binding loop consensus sequence has been proposed to be GXXXXXXXG(A) (residues Gly14-Ala 22 in InhA) [43]. Typically, the WAT1 water makes four hydrogen bonds and two of them are invariant. The invariable hydrogen bonds involve the dinucleotide pyrophosphate moiety and main-chain amine of the last conserved Gly of phosphate binding loop (Ala22 in InhA). Moreover, the interaction of pyrophosphate has been shown to be stereospecific. Almost without exception, the pyrophosphate atom that interacts with the structurally conserved water is O2N in the case of NAD- or NADP-binding proteins. In structures with the sequence pattern GXXXXXXXG(A), the partners for the other two hydrogen bonds involve the carbonyl group of the first conserved Gly (Gly14 in InhA) and a C-terminal residue of β4 strand (Ser94 in InhA) [46]. In our efforts to understand the structural basis of

isoniazid resistance mechanism of InhA, we have solved the crystal structures WT InhA-NADH and S94A InhA-NADH binary complexes [48] to a better resolution than previously reported [45], which were refined to, respectively, 1.9 Å and 2.3 Å. The NADH binding pocket analysis showed that in the S94A InhA-NADH structure, there was no rotation of the carbonyl group of Gly14 and only the conserved hydrogen bond of WAT1 water molecule with hydroxyl group of Ser94 was lost owing to mutation to Ala94 (Fig **3C** and **D**). Thus, this hydrogen bond disruption probably is the main cause of a sixty-fold reduction in affinity for NADH observed for the S94A InhA protein, which corresponds to approximately 2.5 kcal $mol^{-1}$ [44]. This is in agreement with estimates for energy hydrogen bonds, which are in the range of 3 to 9 kcal $mol^{-1}$ [49]. In addition, this WAT1 water has been proposed to be an integral characteristic of dinucleotide-binding Rossmann fold domains that significantly contributes to dinucleotide recognition and presumably provide a favorable enthalpic contribution to the free energy of binding [46]. We have also recently reported the crystal structures of binary complexes of WT and INH-resistant I21V and I47T mutant InhA enzymes refined to, respectively, 2.3, 1.9 and 2.0 Å resolution [48]. The I21V harbors a mutation in the glycine-rich diphosphate binding loop (Gly14 – Ala22 in InhA). In WT InhA, the CD1 of Ile21 apolar side-chain makes van der Waals contacts with the nicotinamide ring, nicotinamide ribose and phosphate oxygens [48]. In the INH-resistant I21V InhA mutant, these van der Waals contacts are lost because the Val side-chain does not have the CD1 atom. In the INH-resistant I47T mutant InhA, the slightly polar side-chain of Thr47 allows the entrance of an additional water molecule (Water4 – that is absent in the structures of WT-NADH, I21V-NADH, S94A-NADH), which promotes a direct H-bond between the OG atom of Thr47 and the carbonyl group of Ile15 from the glycine-rich diphosphate binding loop (Gly14-Ala22). This results in significantly larger H-bond distances between the carbonyl group of Ile15 and Water1, and Water1 with 2'-OH and 3'-OH atoms of adenine ribose, thereby weakening the interaction between Water1 hydroxyl groups of adenine ribose [48]. Pre-steady state kinetics results have shown that the limiting rate constant values for NADH dissociation from the InhA-NADH binary complexes were eleven, five, and tenfold higher for, I21V, I47T, and S94A INH-resistant mutants respectively,of InhA as compared to INH-sensitive WT InhA [48]. Accordingly, these results were proposed to be able to account for the reduction in affinity for NADH for the INH-resistant InhA enzymes thereby providing a mechanism for the mechanism of INH drug resistance in *M. tuberculosis*. In agreement with this proposal, molecular dynamics simulations of fully solvated WT and INH-resistant clinical isolates of *M. tuberculosis* in complex with NADH showed that mutations of the glycine-rich loop residues I21V and I16T resulted in a change in the pattern of direct hydrogen bond contacts with the pyrophosphate moiety of NADH [50]. The NADH pyrophosphate moiety undergoes considerable conformational changes, reducing its interactions with InhA binding site and probably indicating the initial phase of ligand expulsion in which NADH moves apart from its binding site [50].

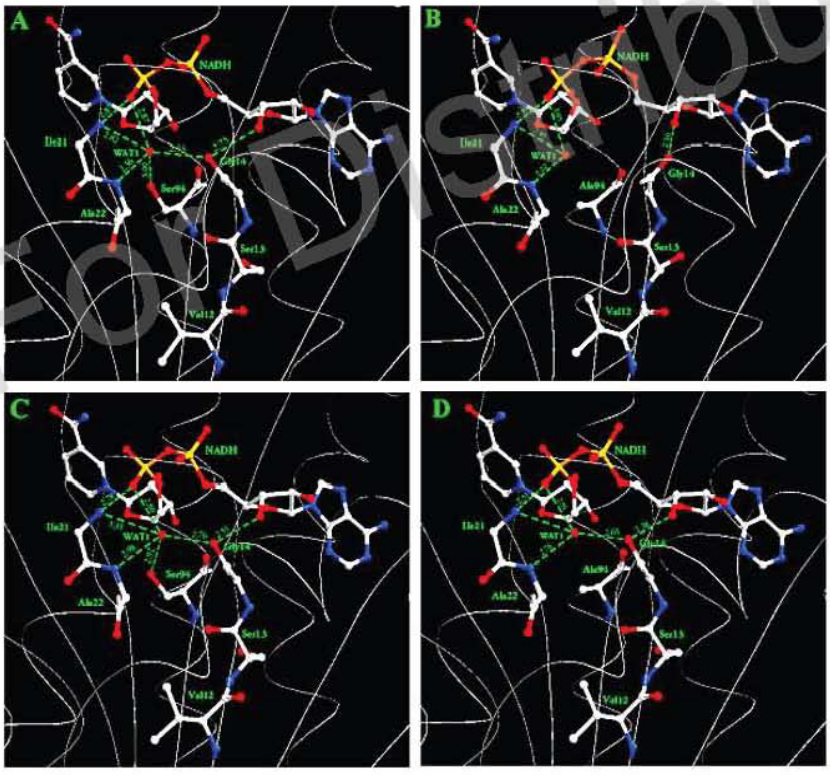



**Fig. (3).** Differences in hydrogen bond network between the crystal structures of WT and INH-resistant mutant InhA (S94A) complexed with NADH.
**Top**: Crystal structures of WT InhA-NADH (**A**) and S94A InhA-NADH (**B**) binary binary complexes refined to, respectively, 2.2 Å and 2.7 Å [45].
**Bottom**: Crystal structures of WT InhA-NADH (**C**) and S94A InhA-NADH (**D**) binary complexes solved to a better resolution than previously reported [45], which were refined to, respectively, 1.9 Å and 2.3 Å [48]. Only the conserved hydrogen bond between the WAT1 water and hydroxyl group of residue S94 is lost owing to mutation to Ala94. Both structures were refined with X-PLOR-NIH program [51, 52]. The completeness values for all data were 97.7% and 95.5% for, respectively, WT InhA and S94A InhA proteins. The final $R_{Factor}$ and $R_{Free}$ values calculated for structure of WT InhA-NADH complex were, respectively, 21.5% and 25.1%. For the structure of S94A InhA-NADH complex, the final $R_{Factor}$ and $R_{Free}$ values were, respectively, 20.8% and 27.4%. Crystals for both wild type and mutant were hexagonal, space group $P6_222$, with on molecule per assymetric unit. The figures were prepared using the program Swiss-PDBViewer v3.7 (www.expasy.org/spdbv) [53]. For clarity, only residues Val12, Ser13, Gly14, Ile21, Ala22, and WAT1 water and NADH molecules are shown. Direct and water-mediated hydrogen bonds between the protein and NADH pyrophosphate moiety are represented as broken green lines. The interactions distances are in Å. The atoms are colored white for carbon, red for oxygen, blue for nitrogen, and yellow for phosphorus. The InhA strucures are represented as thin-line ribbons colored gray.

The increase in the NADH dissociation constant observed for the I21V and I16T isoniazid-resistant mutants [44] may thus be attributed to a decrease in the number of H-bond interactions between NADH and amino acids in the binding pocket and between the cofactor molecule and water molecules that mediate interactions with the enzyme [48]. In agreement, estimates of NADH free energy of binding by molecular docking experiments showed that the WT enzyme has higher affinity for NADH than the mutant enzymes [50]. The correlation between the NADH binding properties in solution, crystal structures, molecular dynamics simulation and docking studies and resistance to INH inactivation of *M. tuberculosis* strains harboring *inhA*-structural gene mutations provides a mechanism of resistance at molecular level for this clinically important drug. Accordingly, current experimental data point to InhA as the primary target for INH mode of action in *M. tuberculosis*.

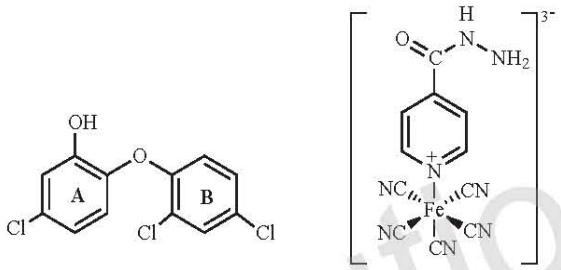
Approximately 50 % of INH-resistant clinical isolates of *M. tuberculosis* harbor deletions of, or missense mutations in the *katG* gene that codes for a catalase-peroxidase enzyme [36, 54]. The S315T KatG mutant enzyme, which is one of the most commonly encountered substitutions in clinical INH-resistant strains, has been shown by isothermal titration calorimetry to have reduced affinity for INH as compared to WT KatG enzyme [55]. These authors have shown that although S315T KatG maintains reasonably good steady-state catalytic rates, poor binding of INH to the mutant enzyme would reduce INH activation and bring about drug resistance [55]. INH is a pro-drug that is activated by the mycobacterial *katG*-encoded catalase-peroxidase enzyme in the presence of manganese ions, NAD(H) and oxygen [56, 57, 58, 59]. Data from X-ray crystallography and mass spectrometry showed that the KatG-produced acylpyridine fragment of isoniazid binds covalently to the C4 of the nicotinamide ring of NADH (Fig. **2**), forming a binary complex with InhA [60]. This isonicotinyl-$NAD^+$ adduct has been shown to bind to InhA with a dissociation constant value lower than 0.4 nM [61]. This adduct has recently been shown to be a slow, tight-binding competitive inhibitor of WT InhA [62]. The initial rapidly reversible weak binding of isonicotinyl-$NAD^+$ adduct to WT InhA ($K_i$ = 16 nM) is followed by a slow isomerization of the enzyme-inhibitor binary complex with an overall dissociation constant ($K_i$*) value of 0.75 nM. Interestingly, the kinetic and thermodynamic parameters for the interaction of isonicotinyl-$NAD^+$ adduct with INH-resistant I21V, I47T and S94A InhA mutant enzymes were found to be similar to those of the wild-type enzyme [62]. These results prompted the authors to suggest an alternative hypothesis to explain for INH resistance mechanism in strains harboring *inhA*-structural gene mutations. A high molecular weight FAS-II system (fatty acid synthase system II) that catalyzes the ACP-dependent synthesis of long chain fatty acids and that contains both enoyl reductase (ENR) and β-ketoacyl reductase (KAR) activities has been purified from *M. smegmatis* [63]. The *mabA* (*fabG1*)-encoded β-ketoacyl-ACP (CoA) reductase (MabA or KAR) has been shown to be a member of FAS-II in mycobacteria [64]. Based on these findings, it has been proposed that mycobacterial ENR (InhA in *M. tuberculosis*) may interact directly with other components of the FAS-II system [62]. Accordingly, the resistance-associated mutations in the *inhA* structural gene would affect the susceptibility of InhA to INH inhibition only in the context of the multienzyme complex and not when mycobacterial ENR (InhA) is tested in isolation as in *in vitro* assays. More recently, yeast two-hybrid and co-immunoprecipitation approaches have been employed to study protein-protein interactions between known components of FAS-II system [65]. These authors reported several types of FAS-II complexes and proposed that either these complexes might coexist or the quaternary structure of a "unique" FAS-II might change from one composition to another during the time and according to the degree of elongation of the substrate. In particular, *M. tuberculosis* InhA was shown to interact with KasA (β-ketoacyl synthase A) and this protein-protein interaction has been suggested as a probable explanation to occurrence of INH-resistant mutant in KasA, even if InhA is indeed the only primary target of INH [65]. However, it remains to be shown whether *inhA* structural gene mutations identified in INH-clinical isolates of *M. tuberculosis* will affect the inhibition of InhA by INH in the context of, for instance, InhA-KasA multienzyme complex. In agreement with InhA as the primary target for INH mode of action, recessive mutations in *M. smegmatis* and *M. bovis* BCG *ndh* gene, which code for a type II NADH dehydrogenase (NdhII), have been found to increase intracellular NADH/NAD ratios [66]. Increasing NADH levels protected InhA against inhibition by the INH-NAD adduct formed upon KatG activation of INH. Hence, mutations in mycobacterial *ndh* gene resulted in increased intracellular NADH concentrations, which competitively inhibited the binding of INH-NAD adduct to InhA [66], in agreement with the higher dissociation constant values for NADH found for INH-resistant clinical isolates harboring *inhA*-structural gene mutations as compared to WT InhA [44].

The INH mechanism of action requires KatG-catalyzed conversion of INH into a number of electrophilic intermediates [56]. Formation of the isonicotinyl-$NAD^+$ adduct has been proposed to be through addition of either an isonicotinic acyl anion to $NAD^+$ or an isonicotinic acyl radical to an $NAD^{\cdot}$ radical [60]. Using a combination of spectroscopic, biochemical and computational techniques, an enzyme-catalyzed mechanism for INH activation has recently been proposed, leading to isonicotinoyl radical formation (thought to be the activated form of INH) and the production of the amide end product via a diazene intermediate [67]. In the proposed mechanism, the oxyferryl group of compound I of KatG, generated after reaction with peroxide, is reduced by INH in a single electron transfer to the heme. In concert, a proton is lost from the hydrazide moiety and could be accepted by His108. In the following step, the C-N bond of hydrazide is broken, yielding a diazene and the acyl radical. The diazene intermediate (that may be stabilized by Trp107, Asp137 or the oxyferryl group) is reduced to hydrazine and ammonia, which may involve deprotonation of His108, Asp137 or Arg104 of KatG, although Arg104 may also play a role. The peroxidase activity of KatG catalyzes the conversion of $Mn^{2+}$ to $Mn^{3+}$ [68]. Rapid freeze-quench electron paramagnetic resonance spectroscopy experiments have shown that hydrogen atoms abstractions may be initiated by a KatG tyrosil radical [69]. It has been shown that the yield of isonicotinoyl-NAD adduct is about the same for oxidation of isoniazid by KatG as it is by $Mn^{3+}$ [70]. Accordingly, oxidation by $Mn^{3+}$-pyrophosphate has been proposed as an alternative method for nonenzymatic INH activation for simple chemical synthesis of various INH derivatives that mimic the isonicotinyl-NAD adduct [71]. X-ray data show a single isonicotinoyl-NAD(H) adduct in the open form with a 4*S* configuration (Fig. **2**) bound to InhA [60]. However, oxidation of isoniazid by the nonenzymatic $Mn^{3+}$-pyrophosphate method has been shown to generate a family of isomeric INH-NAD(H) adducts [72]. Proton and carbon NMR characterization showed that there are four main species of adducts: two are open diastereoisomers consisting of the covalent attachment of the isonicotinoyl radical at C4 of the nicotinamide moiety and the other two result from cyclization of the amide group of the nicotinamide moiety of NAD(H) and the carbonyl group of the isonicotinoyl radical yielding diastereisomeric hemiamidals. These results prompted the authors to raise the question whether there is only one active form of INH-NAD(H) adduct or there are several forms able to inhibit InhA activity. However, production of INH-NAD adduct in the active site of InhA and analysis of purified adduct by reverse-phase HPLC showed two peaks, whose production has been ascribed to the experimental conditions used for HPLC that may promote interconversions of the INH-NAD adduct into a number of different forms [62]. Moreover, molecular modeling analysis showed that the C4 (*R*)enantiomeric INH-NAD adduct is stereochemically forbidden due to severe van der Waals repulsion around Gly192, Pro193 and Ile194 residues of InhA [73]. In any case, the nonenzymatic synthesis of compounds that mimics the isonicotinyl-NAD adduct [71] should allow the synthesis of new

InhA inhibitors with potential antitubercular activity. Interestingly, formation of isonicotinyl adducts by the $Mn^{3+}$-pyrophosphate method and InhA inhibition studies suggest that the adenylic moiety and the amide function at position 3 of the pyridinium ring may play a role in inhibitor binding [74].

The current understanding of INH mode of action has provided clues into critical and unique biosynthetic pathways in mycobacteria. Owing to the INH chemical simplicity, oral availability and favorable toxicology profile, INH analogs appear worthy of examination as anti-tubercular agents. The mechanism of activation for isoniazid has been proposed to occur *via* an electron transfer reaction [75]. More recently, a redox reversible probe metal center has been used to show the involvement of an inner sphere pathway for activation of thionamide-containing pro-drugs [76]. An approach has thus been put forward for the design of self-activating drugs for the treatment of strains of *M. tuberculosis* resistant to drugs that require activation. These drugs would be activated by electron transfer reactions before interacting with its cellular target. Most of the INH resistance is associated with *katG* structural gene alterations resulting in catalase-peroxidase mutant enzymes with impaired ability to form activated-INH intermediates that will form the INH-NAD adduct and ensue inhibition of InhA enzyme activity. In this context, the use of a redox reversible metal complex coordinated to the pro-drug appears as a very first system. Accordingly, we have recently shown that a pentacyano(isoniazid)ferrateII complex (Fig. **4**) inhibits enzyme activity of both wild-type InhA and I21V mutant InhA identified in isoniazid-resistant clinical isolates of *M. tuberculosis* [77]. The *in vitro* kinetics of inactivation indicate that this process requires no activation by KatG, no need for the presence of NADH, and is also effective against INH-resistant mutant InhA. An MIC value of 0.2 μg $mL^{-1}$ for this inorganic complex was determined by the radiometric BACTEC AFB system for *M. tuberculosis* H37Rv strain, and toxicity assays in HL60 leukemia and MCS-7 breast cancer cells yielded an $IC_{50}$ value > 25 μg $mL^{-1}$; thereby indicating a good selectivity index (SI = $IC_{50}$/MIC > 125; as suggested by the Tuberculosis Antimicrobial Acquisition & Coordinating Facility of USA, for a compound to move forward through screening programs SI should be larger than 10). More recently, we have shown that the pentacyano(isoniazid)ferrateII complex is a slow-onset inhibitor of *M. tuberculosis* InhA enzyme activity, with a true overall dissociation constant value of 70 nM [78]. In this mechanism of action an initial enzyme-inhibitor complex is rapidly formed, which then undergoes a slow isomerization reaction of an enzyme-inhibitor binary complex in which the inhibitor is more tightly bound to enzyme. The weakness in the use of classical enzyme inhibitors as drugs for clinical conditions is that inhibition results in the upstream accumulation of the substrate for the enzyme, which may overcome the inhibition. By contrast, the build up of substrate cannot have any effect on the isomerization of enzyme-inhibitor complex typical of the slow-onset mechanism and hence reversal of the inhibition [79]. In addition, a half-time value of 630 min (10.5 hours) for the limiting step for inhibitor dissociation from the binary complex is a desirable feature, since it may be expected to enhance inhibitor's effectiveness [80]. Drug optimization based on drug-target binary complex residence time has recently been proposed to confer advantages in terms of duration of pharmacological effect and target selectivity [81]. For *in vivo* situations, the duration of efficacy of a ligand most critically depends on the rate dissociation rate constant of the receptor-ligand complex, which can be assessed *in vitro* by experimental methods for measuring dissociative half-life such as recovery of biological activity. A long dissociative half-life from an intracellular receptor would be expected to translate into sustained efficacy in a cell after removal of the ligand supply from the medium, that is, the longer the *in vitro* dissociation rate constant value for a ligand the better its *in vivo* duration of action (efficacy). By maximizing the dissociative half-life one can approach "ultimate physiological inhibition", in which recovery from inhibition only occurs as the result of new target synthesis by the organism. Accordingly, this inorganic complex may represent a new class of lead compounds to the development of anti-tubercular agents having long residence time, aiming at the inhibition of a validated target and that is effective against INH-resistant strains.

Triclosan (TCL; 2,4,4'-trichloro-2'-hydroxyphenyl ether; Fig. **4**) is a chlorinated bisphenol that possesses broad spectrum antimicrobial action and has a favorable satefy profile [82]. Accordingly, TCL has been used in many contemporary consumer and professional health care products, including hand soaps, shower gels, deodorant soaps, health care personnel hand washes, hand lotions and creams, toothpastes, mouth washes, underarm deodorants, surgical scrubs and drapes and hospital over-the-bed table tops.




**Triclosan(TCL)** **Pentacyano(isoniazid)ferrateII**

**Fig. (4).** Chemical structures of enoyl reductase inhibitors:Triclosan(TCL) and Pentaciano(isoniazid)ferrateII.

Strains of *Escherichia coli* that were selected for resistance to TCL harbored mutation in the enoyl-ACP reductase-encoding *fabI* gene provided evidence that TCL inhibits lipid biosynthesis [83], and the G93S enoyl reductase mutant was shown to have higher $IC_{50}$ values than the wild-type enzyme for TLC [84]. Triclosan increases the affinity of *E. coli* FabI for $NAD^+$, leading to the formation of a stable FabI-$NAD^+$-TCL ternary complex through non-covalent interactions with amino acid residues in the enzyme active site [85, 86]. TCL has been shown to act as a slow-onset inhibitor of *E. coli* FabI and that TCL binds to FabI-$NAD^+$ complex with a dissociation constant value of 20 - 40 pM [87]. The inhibition of *E. coli* enoyl reductase prompted studies on its mycobacterial counterpart. Genetic studies have thus shown that the enoyl-ACP reductase-encoding *inhA* gene is the target for triclosan in *M. smegmatis* [88] and *M. tuberculosis* [41]. TCL has been shown to be a submicromolar uncompetitive inhibitor of *M. tuberculosis* InhA and that the M161V and A124V mutants have significantly higher inhibition constants for triclosan [89]. *In vitro* whole-cell assays demonstrated TCL activity against INH-resistant and -sensitive *M. tuberculosis* strains with MIC values ranging from 20 to 60 μM and a value of 8.5 μM for the inhibition constant of *M. tuberculosis* InhA [90]. The 2.6 Å-resolution three-dimensional structure of *M. tuberculosis* InhA, $NAD^+$ and triclosan complex shows that the hydroxyl-substituted ring of triclosan (Fig. **4**; the "A" ring) stacked with the nicotinamide ring of $NAD^+$ and formed a conserved hydrogen-bonding pattern with the 2'-OH group of $NAD^+$ and with Tyr158 in the catalytic active site [90]. The dichlorophenyl ring (Fig. **4**; the "B" ring) was oriented orthogonally to the "A" ring and the chlorine atoms were projected toward the solvent. Interestingly, two molecules of triclosan were found by X-ray crystallography to bind to *M. tuberculosis* enoyl reductase [90]; such mode of binding has not been observed for any TCL-enoyl reductase complex solved to date. The first TCL molecule occupies the same site as the singly-bound enzyme, whereas the second molecule is in an inverted orientation relative to the first TCL molecule, residing in an almost

entirely hydrophobic site within the binding cavity and making van der Waals interactions between themselves and the protein. The presence of two molecules of triclosan in the active site of *M. tuberculosis* enoyl reductase has been attributed to the specificity for long chain fatty acyl substrates. To accommodate long fatty acids, *M. tuberculosis* enoyl reductase possesses a hydrophobic substrate-binding loop (residues 197-226) that is approximately 10 residues longer (residues 203-212) than the corresponding loop in *E. coli* and *B. napus* FabI. These data suggest that selective targeting of *M. tuberculosis* enoyl reductase may be achieved by a dual complex between triclosan molecules. Synthesis of triclosan analogs and high-throughput screening efforts to target enoyl-ACP reductase activity may lead to the development of new anti-tubercular agents.

It has recently been reported that the *M. tuberculosis iniA* (for isoniazid-inducible gene A) gene participates in the development of tolerance to isoniazid and ethambutol [91]. Since an *M. tuberculosis* strain containing an *iniA* deletion showed increased susceptibility to INH, it has been suggested that drugs designed to inhibit the *iniA* gene product may shorten the time required to treat TB and may help prevent the clinical emergence of drug resistance [91]. However, it remains to be shown whether this proposal will have an impact on TB treatment.

## MALARIA: EPIDEMIOLOGY AND CHEMOTHERAPY

Malaria continues to be a major cause of morbidity and mortality throughout the world with up to three million deaths and approximately five billion episodes of clinical illness possibly meriting anti-malarial therapy [92]. The vast majority of deaths occur in sub-Saharan Africa where the malaria caused by *Plasmodium falciparum* affects mainly young children, with pregnant women also forming a particularly vulnerable risk group. More recently, an empirical approach to estimating the number of clinical events caused by *Plasmodium falciparum* worldwide, by using a combination of epidemiological, geographical and demographic data, has estimated that there were 515 (range 300-600) million episodes of clinical *P. falciparum* malaria in 2002 [93]. These global estimates were up to 50 % higher than those reported by the World Health Organization. Furthermore, travelers to malaria-endemic areas have a high risk to acquire the disease [94]. The crude risk for travelers vary from 1 per 100,000 travellers to Central America and Caribean to 357 per 100,000 in central Africa.

Malaria is caused by protozoan parasites of genus *Plasmodium* and transmitted by the *Anopheles* mosquito. Four species account for almost all human infections (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) [95]. Amongst them, *P. falciparum and P. vivax* vie for the greatest prevalence in the world today. *Plasmodium falciparum* is justifiably regarded as the greater menace because of its responsibility for the most severe forms of malaria that frequently can be a deadly disease if not conveniently treated, its widespread resistance to anti-malarial drugs, and its dominance in the world's most malarious continent, Africa [95]. Malaria starts by the inoculation of the *Plasmodium* parasite sporozoite stage that invades hepatocytes. Sporozoites transform in liver stages and subsequent liver-stage development ultimately resulting in release of pathogenic merozoites that invade erythrocytes in the blood. In erythrocytes, the parasites present cycles of asexual reproduction liberating new merozoites that invade and destroy new erythrocytes. These repeated cycles of asexual reproduction of the parasite are responsible for the disease while the sequestration of mature forms of the *P. falciparum* parasites inside the erythrocytes at the endothelial cells of capillary vessels is responsible for severe malaria forms like cerebral and pulmonary malaria [96].

Treatment of *P. falciparum* malaria has depended for decades on the use of quinine. Synthetic anti-malaria drugs, inspired by the knowledge of quinine structure (Fig. **5**) were developed in the 30's and 40's of the 20th century and permitted the development of the WHO world "erradication" program starting in the 50's with large success in Europe and North America and many areas of Latin America, including malaria eradication in Coast areas of Brazil. Two important groups of anti-malaria drugs were particularly effective: the 4-aminoquinoline derivatives like chloroquine and amodiaquine and the synergistic association of anti-folate (pyrimethamine) and sulfadoxine (Fig. **5**) [97]. Sulfadoxine is a sulfonamide that interferes with the action of dihydroopteroate synthase (an enzyme in the folate biosynthesis pathway) and 2,4-diaminopyrimidine pyrimethamine is an inhibitor of dihydrofolate reductase enzyme activity [98]. Malaria had been sheltered for many years from the dangers of resistance because of the outstanding properties of chloroquine and the slow speed at which resistance developed to this drug [99]. However, resistance of *Plasmodium falciparum* to chloroquine, starting from the 60's spread on a global scale has exposed the ease with which resistance may develop to other drugs such as the anti-folates [100]. Control of malaria is becoming increasingly difficult because of rapid spread of resistance of both the parasite and the mosquito vector to few currently available anti-malarial drugs and insecticides [101]. In sub-Sahara Africa, the risk of parasitological treatment failure with the use of chloroquine is almost uniformly greater than 40%. Elsewhere, the rates of parasitological failure in Asia vary from 46% (southern Asia) to 85 % (Southeast Asia) and in South America the rate is 66% [102]. Furthermore, resistance to chloroquine in *P. vivax* has emerged, apparently having originated in New Guinea, where the failure rates now approach 100% [103]. Currently, chloroquine-resistant *P. vivax* has now spread through Southeast Asia and South America [99].Thus, the well-documented impact of drug resistance on *P. falciparum* chemotherapy [104] that has limied the effectiveness of standard drugs such as chloroquine and sulfadoxine-pyrimethamine is beginning to be observed also for *P. vivax*. Accordingly, the urgent need to undertake appropriate biological research, medicinal chemistry, and clinical research to discover and develop new drugs for *P. falciparum* is now also becoming a priority for *P.vivax* [105]. However, the technical difficulties in studying *P. vivax* seriously hamper definitive studies [99].

The risk of resistance to sulfadoxine-pyrimethamine is relatively high in Southeast Asia and eastern Africa. However, good efficacy persists in some regions as in southwestern Asia and on the Horn of Africa, where no parasitological failures were reported among 362 evaluations. In South America the treatment failure rates vary between 6% and 14% [102].

It has been pointed out that continued and sustainable improvements in anti-malarial medicines through focused research and development are essential for the future ability to treat and control malaria worldwide [104]. However, experience has shown that resistance eventually curtails the life-span of anti-malarial drugs. Therefore, controlling resistance is crucial to ensuring that the investiment put into anti-malarial drugs is not wasted. Considering increasing resistance to available agents, there is a broad consensus that there is a need to develop new anti-malarials drugs. Antimalarial drug development can follow several strategies, ranging from minor modifications of existing agents to the design of novel agents that act against new targets [106]. Increasingly, drug combinations that have independent modes of action are seen as a way of enhancing efficacy while ensuring mutual protection against resistance. Most research work has focused on the use of artesunate combined with currently used standard drugs, namely, mefloquine, amodiaquine, sulfadoxine/pyrimethamine and chloroquine [107]. Artemisinin-based combination therapy for malaria is rapidly gaining acceptance as an effective approach for countering the spread and intensity of *P. falciparum* resistance [107]. However, artemisinin derivatives are significantly more expensive than traditional anti-malarials such as chloroquine and sulfadoxine/pyrimethamine. Consequently, the major impediment to using artemisinin-based combination therapy is its cost, currently $ 1.20 - 2.50 per adult

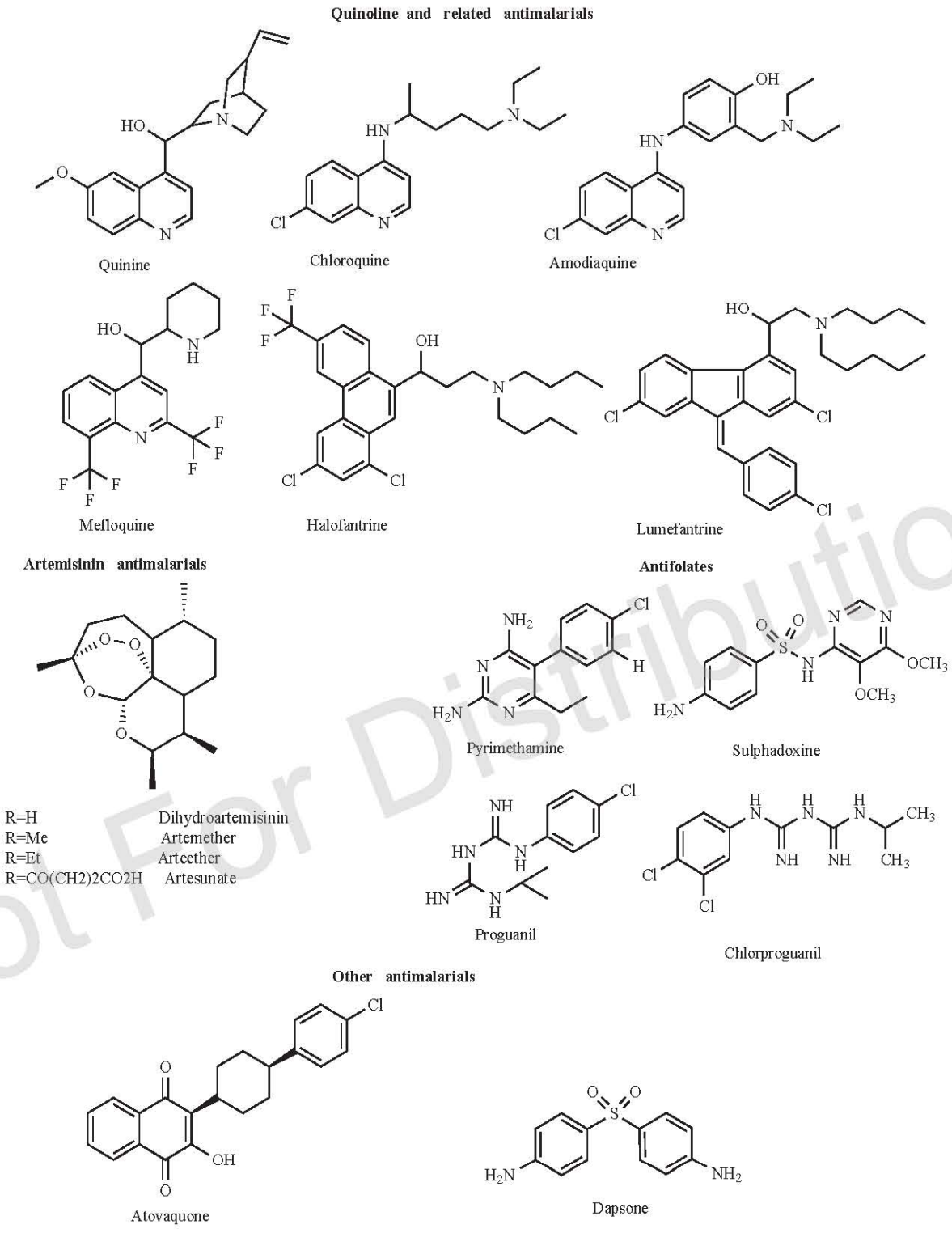



**Fig. (5).** Structures of currently available antimalarial drugs. Chloroquine, quinine, mefloquine are 4-substituted quinolines that interfere with heme polymerization; sulfadoxine, pyrimethamine, proguanil and chlorproguanil are substrate analogs that interfere with folate metabolism; artemisinin antimalarials undergo oxidoreductive cleavage of their peroxide bond in the parasite food vacuole, most probably through interaction with Fe(II) haem, generating fatal-free-radical-induced damage to the parasite; and atovaquone is a hydroxynaphthoquinone that interferes with mitochondrial electron transport [98, 99, 117].

treatment compared with $ 0.10 - 0.20 per adult treatment for chloroquine and sulfadoxine/pyrimethamine [92]. To be widely useful, anti-malarial drugs must be very inexpensive so that they are routinely available to populations in need in developing countries. Because chloroquine and sulfadoxine/pyrimethamine are inexpensive, they have been used and recommended even in the face of poor efficacy [108].

Currently, the options for malaria treatment are confined to the following drugs (Fig. **5**): chloroquine, amodiaquine, sulfadoxine/pyrimethamine, mefloquine, artesunate, dihydroartemisinin; the

artemisinin combinations, atovaquone/proguanil, dihydroartemisinin-piperaquine, and artemether/lumefantrine; and the recently registered combination chlorproguanuil/dapsone (LapDap) [104, 108]. Unfortunately, malaria is a disease of poverty and despite a wealth of scientific knowledge, there is insufficient market incentive to generate the competitive, business-driven industrial anti-malarial drug research and development that is normally needed to deliver new products. Only four of the nearly 1400 drugs registered worldwide during 1975-1999 were anti-malarials [109]. Mechanisms of partnering with industry should be established to overcome this obstacle and to open up and build on scientific opportunities for improved chemotherapy in the future. In the meantime, the best prospect for drug development and use in the short term is the government commitment to mechanisms and projects to ensure that improved drugs are sustainably discovered and developed and new targets for anti-malarial agents are unveiled.

Anti-malarial drug development is constrained by the same factors as any drug development program in that new agents must demonstrate efficacy, safety, easy manufacturing (low cost is crucial for anti-malarials), stablility, bioavailablility (that is, extensively adsorbed from the gut and avoiding first pass metabolism in the liver to achieve effective concentrations), in the systemic circulation), have an appropriate half-life can be given orally and be effective with single-daily dosing, and that curative regimens should be short, ideally 1-3 days to an adequate patient adherence [102, 104, 106].

Apicomplexan parasites are a large phylum of unicellular and obligate intracellular organisms of great medical importance. They include the human pathogens *Plasmodium* spp., the causative agent of malaria), and *Toxoplasma gondii*, an opportunistic parasite of immunosuppressed individuals and a common cause of congenital disease. Completion of genome sequence of both *P. falciparum* [110] and *P. vivax* [111], availability of a *plasmodium* genome database (http://www.PlasmoDB.org) [112] and growing sequence databases for other *Plasmodium* species, *Toxoplasma gondii*, *Eimeria tenella*, *Theileria parva* and *Theileria annulata* should provide valuable tools for whole-genome analysis of Aplicomplexa to allow identification of new targets for drug development [113]. The search for new and effective drugs against these pathogens has been boosted during the last years by an unexpected finding. Through molecular and cell biological analysis, it was realized that probably most members of this phylum harbor a plastid-like organelle, called the apicoplast, which probably is derived from the engulfment of a red algae in ancient times. Although the apicoplast itself contains a small circular genome, most of the proteome of this organelle is encoded in the nuclear genome and the proteins are subsequently transported to the apicoplast [114, 115]. It is assumed to contain a number of unique metabolic pathways not found in the vertebrate host, making it an ideal source of drug targets (reviewed by Seeber) [116]. Amongst them are three enzymes of the plant-like fatty acid synthesis machinery (acetyl-CoA carboxylase, β-ketoacyl-ACP synthase and enoyl-ACP reductase), suggesting that fatty acid and lipid biosynthesis is a major function of the apicoplast.

## *PLASMODIUM FALCIPARUM* FATTY ACID BIOSYNTHESIS AND ENOYL REDUCTASE

Fatty acid biosynthesis of apicomplexan parasites is an attractive target for drug development, since its inhibition kills the parasite rapidly unlike the "delayed death" phenotype exhibited by the inhibition of the other apicoplast processes [118]. It should be pointed out that no homologous sequence to FAS-I has been identified in the genome of *P. falciparum* [119], thereby indicating that enzymes of the FAS-II system should be attractive targets for the development of antimalarial agents with low toxicity. Enoyl-ACP reductase, a component of FAS-II system, catalyzes the rate-determining step in fatty acid biosynthesis and thus represents an attractive molecular target for anti-malarial agent development. Triclosan (TCL; Fig. **4**) has been reported to inhibit the *in vitro* growth of *Plasmodium falciparum* with an $IC_{50}$ value of 150-2000 ng $mL^{-1}$ [120]. Triclosan has also been shown to offer protection against blood stages of malaria and that the drug target is an enoyl-ACP reductase from *P. falciparum* [121]. Homology modeling showed that *P. falciparum* enoyl reductase uses NADH rather than NADPH as co-substrate [122]. Steady-state kinetics studies determined Michaelis-Menten constant ($K_m$) values of 36 μM for NADH and 165 μM for crotonyl-CoA and a value of 1.62 $s^{-1}$ for the catalytic constant [123]. *P. falciparum* enoyl-ACP reductase inhibition studies showed that triclosan is competitive with respect to NADH and uncompetitive with respect to $NAD^+$ and binding of $NAD^+$ to the enzyme promotes the binding of triclosan [123]. More recently, TCL has been shown to act as a slow-tight binding inhibitor of the *P. falciparum* enoyl-ACP reductase enzyme activity with an overall inhibition constant value of 96 pM [124]. Surface plasmon resonance analysis has been reported for *P. falciparum* enoyl-ACP reductase interaction with substrates and triclosan [125]. The 300-fold increase in the binding constant of TCL in the presence of NAD has been attributed to a 17-fold increase in the association rate constant and a 16-fold decrease in the dissociation rate constant, translating into an overall dissociation constant of approximately 33 nM for TCL in the presence of $NAD^+$ as compared to approximately 9 μM in the absence of $NAD^+$ [125]. Determination of the three-dimensional structure of malarial enoyl reductase-triclosan-$NAD^+$ ternary complex has provided a structural framework that sheds light on the mode of binding of triclosan [126]. However, analysis of the structural features of *P. falciparum* enoyl reductase responsible for the high affinity of the enzyme for triclosan in the presence of $NAD^+$ has only recently been reported [124]. It has shown that the enhancement of TCL affinity for *P. falciparum* enoyl reductase in the presence of $NAD^+$ can be explained by increased van der Waals contacts in the ternary complex, which is facilitated by the movement of residues 318-324 of the substrate-binding loop and the nicotinamide ring of $NAD^+$.

Synthesis of triclosan analogs and high-throughput screening efforts have recently been made to target enoyl-ACP reductase activity aiming at anti-tubercular and anti-malarial agents development. Replacement of the oxygen bridge with a sulfur bridge (thioether) in triclosan or with a carbon bridge in hydroxydiphenylether (yielding a hydroxydiphenylmethane) has been shown to dramatically reduce inhibitory activity against *E. coli* [84, 127]. The hydroxyl group of triclosan appears to make an important contribution to affinity, since the *des*-hydroxyl analogue shows a 10,000-fold decrease in affinity for the *E. coli* enoyl reductase [128]. The chloro substituent at C-9 appears to make an important contribution since replacing it with an OH group, yielding 2,2'-dihydroxydiphenyl ether, makes binding to enoyl reductase weaker [122]. Synthesis of twenty analogs of triclosan, which were originally designed to target the closely related *M. tuberculosis* enoyl reductase and subsequent screening against *P. falciparum* enoyl reductase revealed that enzyme inhibition was sensitive to the hydroxyl group [126]. The hydroxyl group could not be replaced with methoxy groups or sulfur derivatives, as observed for the bacterial enzymes. A triclosan analogue carrying a nitrogen as a bridging atom, *N*-(2,2-dichlorophenyl)-2'-hydroxyaniline, had an inhibition constant value of 14.3 μM against *P. falciparum* enoyl reductase. A naphthalene derivative of triclosan, 4-chloro-2-hydroxyphenyl 6'-hydroxynaphtyl ether, had an inhibition constant value of 0.15 μM [126]. The inhibition constant value for triclosan is 0.05 μM against *P. falciparum* enoyl reductase and, thus, these analogs are poor substitutes for triclosan. Nevertheless, the structural information for these analogs provides important data on structure-activity relationships that should help the design of triclosan analogs with improved activity against *P. falciparum* and/or *M. tuberculosis* enoyl reductase(s).

Flavonoids comprise a large group of polyphenolic secondary metabolites that are widespread throughout the plant kingdom and are all based on the flavan skeleton. A library of flavonoids has recently been tested as inhibitors of *P. falciparum* enoyl reductase (FabI) enzyme activity [129]. The strongest inhibitors of *P. falciparum* enoyl reductase enzyme activity were C-3 gallic acid esters of catechins, with sub micromolar $IC_{50}$ values. Interestingly, these polyphenols showed *in vitro* activity against chloroquine-sensitive (NF-54) and –resistant (K1) *P. falciparum* strains in the low to submicromolar range [129]. Since flavonoids display very low toxicity to humans, these compounds may represent leads to the development of drugs with increased efficacy to treat malaria.

## CONCLUDING REMARKS

*M. tuberculosis* and *P. falciparum* enoyl-ACP reductases are validated targets for anti-malarial agents development to be used in drug screening efforts. In addition, owing the TCL and INH chemical simplicity and favorable toxicology profile, analogs of TCL and INH appear worthy of examination as anti-malarial and/or anti-tubercular agents. An analog of INH, 1-isonicotinyl-2-nonaoyl hydrazine, has recently been shown to exhibit a two-fold increase in the degree of susceptibility against *M. tuberculosis* H37Rv [130]. Enzymatic acetylation of INH by *N*-acetyltransferase reduces the therapeutic activity of this drug, resulting in underdosing, decreased bioavailability and the consequent possibility of acquired INH resistance. The lipophilic Schiff base $N^2$-cyclohexylidenyl isonicotinic acid hydrazide, in which the hydrazine moiety is blocked toward acetylation, has shown activity against both intracellular and extracellular *M. tuberculosis* (Erdman strain) *in vitro* and excellent bioavailability [131]. However, a 25-fold increase in the MIC value of this compound for INH-susceptible *M. tuberculosis* strain (0.03 $\mu g\ mL^{-1}$) as compared to an INH-resistant strain (> 0.75 $\mu g\ mL^{-1}$) casts doubt on its usefulness for the treatment of patients infected with resistant strains. A completely new approach, which was mentioned above, to INH analog design based on inorganic atoms attached to the nitrogen atom of the heterocyclic ring of isoniazid has provided promising results [77, 78]. Two novel inhibitors, named Genz-10850 and Genz-8575, have been identified in a high-throughput screen against *M. tuberculosis* enoyl reductase with $IC_{50}$ values in the submicromolar range [90]. The compound Genz-8575 showed good activity in whole-cell assays against several drug-susceptible and drug-resistant strains of *M. tuberculosis*, with MIC values ranging from 1.25 to 30 μM (MIC = 1.5 μm for isoniazid against *M. tuberculosis* H37Rv strain). The X-ray crystal structure of the *M. tuberculosis* enoyl reductase and Genz-10850 binary complex showed that Genz-10850 has hydrogen-bonding interactions with $NAD^+$ and the catalytic residue Tyr158 similar to the binary complex formed between the enzyme and triclosan. The crystal structure should help optimization of these compounds. However, whether or not Genz-10850 and Genz-8575 target(s) the *M. tuberculosis* enoyl reductase *in vivo* as well as any effect they may have on mycolic acid biosynthesis, will have to await for further studies.

Aminopyridine-based inhibitors of *Staphylococcus aureus* and *Hemophilus influenzae* enoyl reductase (FabI) enzymes have been developed through a combination of iterative medicinal chemistry and X-ray crystal structure based design [132]. One of these compounds, 3-(6-aminopyridin-3-yl)-*N*-methyl-*N*-[(1-methyl-1*H*-indol-2-yl)methyl]acrylamide, was shown to be a low micromolar inhibitor of FabI from *S. aureus* ($IC_{50}$ = 2.4 μM) and *H. influenzae* ($IC_{50}$ = 4.2 μM). In addition, this compound has shown good *in vitro* anti-bacterial activity against *S. aureus* (MIC = 0.5 $\mu g\ mL^{-1}$). Even though the inhibitory activity of this compound, if any, against *M. tuberculosis* enoyl reductase still needs to be shown, it may represent a new lead for anti-tubercular agent development. A series of 1,4-disubstituted imidazoles have shown *in vitro* inhibitory activity against *S. aureus* and *E. coli* enoyl reductase enzyme, and whole-cell antimicrobial activity against *S. aureus* determined by a broth microdilution assay [133]. Compounds based on 2,9-disubstituted 1,2,3,4-tetrahydropyrido[3,4-*b*]indole have been synthesized and their inhibitory activity against *S. aureus* and *E. coli* enoyl reductase assessed *in vitro* [134]. The compounds showing submicromolar $IC_{50}$ against enoyl reductase activity were tested for *in vitro* anti-bacterial activity with MIC values ranging from 0.5 to 4.0 $\mu g\ mL^{-1}$ against *S. aureus*. A naphthyridinone-based series of compounds was found to have inhibitory activity against *S. aureus* enoyl reductase with $IC_{50}$ and MIC values lower than those for triclosan [134]. The activity of these compounds could be determined against *M. tuberculosis* and *P. falciparum* to evaluate both their potential as mycobacterial or malarial enoyl reductase inhibitors and as lead compounds to the development of anti-tubercular and anti-malarial agents.

*M. tuberculosis* and *P. falciparum* enoyl reductases may represent molecular targets for immobilization on solid support in natural-product screening efforts. Plant-derived natural compounds represent an alternative approach that can benefit from knowledge of medicinal plants among natives from malarious regions. Therefore, as a great improvement over random screening, a plant product with specific clinical activity can be the starting point for a medicinal chemistry effort [135, 136]. Extensive evaluations of plant natural-products as potential new therapies for malaria are underway [137]. Moreover, natural-products of plants are the sources for the two most important drugs currently available to treat severe falciparum malariae, quinine and derivatives of artemisinin [106]. Very recently, the anti-plasmodial activity-guided fractionation of the endemic Turkish plant *Phlomis brunneogaleata* (Lamiaceae) led to the isolation of new secondary metabolites that showed anti-plasmodial activity and inhibitory activity against *P. falciparum* enoyl reductase [138]. The compounds luteolin 7- O-beta- D-glucopyranoside and chrysoeriol 7-O-beta- D-glucopyranoside were determined to be the major anti-malarial principles of the crude extract, which showed $IC_{50}$ values of 2.4 and 5.9 $\mu g\ mL^{-1}$, respectively. The former *Phlomis brunneogaleata* compound showed promising inhibiting effect ($IC_{50}$ = 10 $\mu g\ .\ mL^{-1}$) against *P. falciparum* enoyl reductase and has been reported as the first anti-malarial natural product targeting enoyl reductase of *P. falciparum* [138]. More recently, gallic acid esters of catechins have also shown to inhibit *P. falciparum* enoyl reductase activity and to be effective against chloroquine-sensitive and –resistant strains [129].

The determination of the complete genome sequence of *M. tuberculosis* H37Rv strain has had a striking impact on researchers in the TB field [139]. In addition, completion of the *Plasmodium falciparum* genome sequence [110] and availability of a *plasmodium* genome database (http://www.PlasmoDB.org) [112] should provide valuable tools for discovery of new drug targets.

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## ABBREVIATIONS

| | | |
|---|---|---|
| ACP | = | Acyl carrier protein |
| ADR | = | Adenine ribose |
| BSA | = | Bovine serum albumin |
| CoA | = | Coenzyme A |
| CQ | = | Chloroquine |
| ENR | = | Enoyl reductase |

| | | |
|---|---|---|
| ETH | = | Ethionamide |
| FabI | = | Enoyl reductase |
| FAS-II | = | Type II dissociated fatty acid biosynthesis system |
| HPLC | = | High Performance Liquid Chromatography |
| INH | = | Isoniazid, isonicotinic acid hydrazide |
| InhA | = | 2-*trans* enoyl-ACP (CoA) reductase |
| KAR | = | β-ketoacyl reductase |
| KatG | = | Catalase-peroxidase |
| KAS | = | β-ketoacyl synthase |
| MabA | = | β-ketoacyl reductase |
| mAGP | = | Mycolyl-arabinogalactan-peptidoglycan complex |
| MDG | = | Millennium Development Goals |
| MDR-TB | = | Multidrug-resistant tuberculosis, defined as resistant to at least isoniazid and rifampicin |
| MIC | = | Minimum inhibitory concentration |
| SI | = | Selectivity index (SI = $IC_{50}$/MIC) |
| TB | = | Tuberculosis |
| TCL | = | Triclosan (2,4,4'-trichloro-2'-hydroxyphenyl ether) |
| XDR-TB | = | Extensively drug-resistant tuberculosis, defined as resistant to isoniazid and rifampicin and at least three of the six main classes of second-line drugs |
| WT | = | Wild type |

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