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An inorganic iron complex that inhibits wild-type and an isoniazid-resistant mutant 2-*trans*-enoyl-ACP (CoA) reductase from *Mycobacterium tuberculosis*

Jaim S. Oliveira,[a] Eduardo H. S. Sousa,[b] Luiz A. Basso,*[a] Moisés Palaci,[c] Reynaldo Dietze,[c] Diógenes S. Santos[d] and Ícaro S. Moreira*[b]

[a] *Universidade Federal do Rio Grande do Sul, Departamento de Biologia Molecular e Biotecnologia, Av. Bento Gonçalves 9500, Porto Alegre, RS 91501-970, Brazil. E-mail: labasso@dna.cbiot.ufrgs.br*

[b] *Universidade Federal do Ceará, Departamento de Química Orgânica e Inorgânica, Campus Pici, Fortaleza, CE 60455-760, Brazil. E-mail: icarosm@dqoi.ufc.br*

[c] *Universidade Federal do Espírito Santo, Núcleo de Doenças Infecciosas, Av. Marechal Campos 1468, Vitória, ES 29040-091, Brazil*

[d] *Faculdade de Farmácia, Instituto de Pesquisas Biomédicas, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, CEP 90619-900, Brazil. E-mail: diogenes@dna.cbiot.ufrgs.br*

Received (in Cambridge, UK) 28th October 2003, Accepted 24th November 2003
First published as an Advance Article on the web 7th January 2004

The *in vitro* kinetics of inactivation of both wild-type and I21V InhA enzymes by $[Fe^{II}(CN)_5(INH)]^{3-}$ indicate that this process requires no activation by KatG, and no need for the presence of NADH. This inorganic complex may represent a new class of lead compounds to the development of anti-tubercular agents aiming at inhibition of a validated target.

In 1952 isoniazid (INH, isonicotinic acid hydrazide) was first reported to be effective in the treatment of tuberculosis.[1] However, strains of *Mycobacterium tuberculosis*, the causative agent of the disease, resistant to INH were reported shortly after its introduction.[2] The primary mechanism of multiple drug resistance in *M. tuberculosis* is the accumulation of mutations in individual drug target genes.[3] The mechanism of action of INH is complex, as mutations in at least five different genes (*katG*, *inhA*, *ahpC*, *kasA*, and *ndh*) have been found to correlate with isoniazid resistance.[4] The primary target of INH has been shown to be the product of the *inhA* structural gene.[5] The *inhA* gene codes for an NADH-dependent 2-*trans*-enoyl-ACP (CoA) reductase that exhibits specificity for long chain ($C_{18} > C_{16}$) enoyl thioester substrates,[6] consistent with its role in mycobacterial cell wall biosynthesis.[7] Missense mutations in the *inhA* structural gene, but lacking mutations in the *inhA* promoter region, *katG* gene and *oxyR*-*ahpC* region, were identified in INH-resistant clinical isolates of *M. tuberculosis*[8] and shown to correlate with changes in the NADH binding properties of enoyl reductase.[9] Moreover, deletions of, or missense mutations in, the *katG* gene have been associated with decreased susceptibility to INH in approximately 50% of clinical isolates of *M. tuberculosis*.[10] The isoniazid mechanism of action involves the conversion of INH by the mycobacterial *katG*-encoded catalase-peroxidase into a number of electrophilic intermediates.[11] Although isoniazid does not bind to the *inhA*-encoded enoyl reductase,[12] the KatG-activated drug intermediate binds to, and inhibits the enoyl reductase activity in the presence of NAD^+ or NADH.[13] The three-dimensional structure determination of WT InhA, NADH, and activated isoniazid intermediate ternary complex has shown that the acylpyridine fragment of isoniazid is covalently attached to the C4 position of NADH.[14] This isonicotinyl-NAD^+ adduct binds to WT InhA with a dissociation constant value lower than 0.4 nM.[15] Isoniazid is, therefore, a KatG-activated pro-drug, that upon formation of an isonicotinyl-NAD^+ adduct inhibits the *M. tuberculosis* enoyl reductase, resulting in reduction of mycolic acid synthesis. In trying to find better alternatives to INH, we have investigated an INH analog that contains a cyanoferrate moiety (**1**) and tested its ability to inhibit both WT and isoniazid-resistant I21V mutant enoyl reductases from *M. tuberculosis*. Incubation of WT InhA with $Na_3[Fe^{II}(CN)_5(INH)]\cdot 4H_2O$[16] in the absence of NADH resulted in the time-dependent inactivation of the enzyme with an apparent first-order rate constant value of 327 (± 34) × 10^{-3} min^{-1} (Fig. 1A, λ inset; $t_{1/2}$ = 2.1 ± 0.2 min).

The rate constant values were 65 (± 4) × 10^{-3} min^{-1} (Fig. 1A, ■ inset; $t_{1/2}$ = 10.7 ± 0.7 min) in the presence of 10 μM NADH and 15.7 (± 0.7) × 10^{-3} min^{-1} (Fig. 1A, ▲ inset; $t_{1/2}$ = 44 ± 2 min) in the presence of 100 μM NADH. Inactivation of WT InhA by oxidized INH derivatives produced by KatG in the absence of NADH has been reported not to occur at detectable levels in the time range tested here (25–30 min).[9,21] Moreover, a value of 8.9 × 10^{-3} min^{-1} has been reported for the rate constant for WT InhA enzyme inactivation by KatG-activated isoniazid in the presence of 100 μM NADH.[9]

The results presented here clearly demonstrate that WT InhA inactivation by $[Fe^{II}(CN)_5(INH)]^{3-}$ requires no activation by KatG,

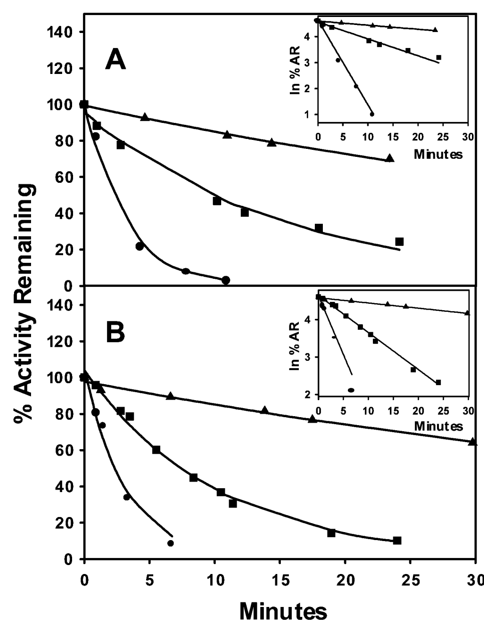

Fig. 1 (A) Inactivation of WT InhA (3 μM) by $[Fe^{II}(CN)_5(INH)]^{3-}$ (100 μM):[18] ●, no NADH; ■, 10 μM NADH; ▲, 100 μM NADH. The inset shows a plot of the natural log of the percentage of WT InhA activity remaining (%AR) *versus* time in the absence (●), presence of 10 μM NADH (■), and presence of 100 μM NADH (▲). (B) Inactivation of I21V InhA under the same experimental conditions described in part A (except that WT InhA was replaced for I21V InhA): no NADH (●), 10 μM NADH (■); and 100 μM NADH (▲). The inset shows a plot of the natural log of the percentage of I21V InhA activity remaining (%AR) *versus* time in the absence (●), presence of 10 μM NADH (■), and presence of 100 μM NADH (▲).

DOI: 10.1039/b313592f

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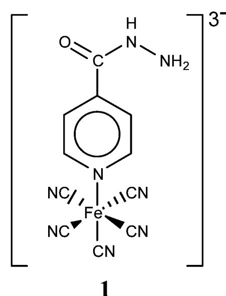

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no need for the presence of NADH, and its mechanism of action probably involves interaction with the NADH binding site of the enzyme. The *in vitro* kinetics of inactivation of the I21V mutant enzyme, under identical conditions, proceeded with apparent first-order rate constant values of 315 (± 38) × 10^{-3} min^{-1} (Fig. 1B, ● inset; $t_{1/2}$ = 2.2 ± 0.3 min) in the absence of NADH, 99 (± 4) × 10^{-3} min^{-1} (Fig. 1B, ■ inset; $t_{1/2}$ = 7.0 ± 0.3 min) in the presence of 10 μM NADH, and 14 (± 1) × 10^{-3} min^{-1} (Fig. 1B, ▲ inset; $t_{1/2}$ = 50 ± 3 min) in the presence of 100 μM NADH. As for the WT InhA enzyme, these results demonstrate that inactivation of I21V mutant enzyme from a INH-resistant clinical isolate of *M. tuberculosis* by $[Fe^{II}(CN)_5(INH)]^{3-}$ requires no activation by KatG, no need for NADH, and probably the same site of interaction. Interestingly, since the *in vivo* NADH concentration in *M. tuberculosis* H37Rv has been estimated to be < 10 μM,[22] the inorganic complex would probably display a better efficacy against isoniazid-resistant *M. tuberculosis* strains harboring *inhA* structural gene mutations than WT InhA strains. In order to verify if slow formation of a covalent binary compound between $[Fe^{II}(CN)_5(INH)]^{3-}$ and NADH could result in inactivation of WT and I21V enzymes, pre-incubation experiments[23] were performed and the kinetics of inactivation followed for WT and I21V enzymes. Apparent first-order rate constant of inactivation values of 15 (± 1) × 10^{-3} min^{-1} and 13 (± 2) × 10^{-3} min^{-1} were obtained for, respectively, WT and I21V InhA enzymes (data not shown). These values are within standard error of the ones determined with no pre-incubation suggesting that there is no slow formation of an intermediate compound capable of inactivating WT and I21V enzymatic activities.

An MIC value of 0.2 μg mL^{-1} for the $[Fe^{II}(CN)_5(INH)]^{3-}$ compound was determined by the radiometric BACTEC AFB system.[24] *M. tuberculosis* is susceptible to isoniazid in the range of 0.02–0.2 μg mL^{-1}.[26] Accordingly, the $[Fe^{II}(CN)_5(INH)]^{3-}$ compound appears to be a promising candidate for further anti-tubercular drug development and may represent a new class of lead compounds. Efforts to obtain crystals of the binary complex formed between this inorganic compound and either WT or I21V InhA are currently underway and should assist in the design of a new class of antimycobacterial agents.

Financial support for this work was provided by QuatroG Pesquisa e Desenvolvimento to D.S.S., L.A.B., and I.S.M. Part of this work was also supported by Millennium Initiative Program MCT-CNPq, Ministry of Health, Secretary of Health Policy (Brazil) grant awarded to D.S.S., R.D., L.A.B. and M.P.

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