Bioorganic & Medicinal Chemistry Letters 23 (2013) 3096–3100

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Isoniazid: Radical-induced oxidation and reduction chemistry

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ARTICLE INFO

Article history:
Received 8 March 2012
Revised 27 February 2013
Accepted 4 March 2013
Available online 13 March 2013

Keywords:
Isoniazid
Oxidation
Reduction
Kinetics
Isonicotinoyl radical

ABSTRACT

Isoniazid is a potent and selective therapeutic prodrug agent used to treat infections by *Mycobacterium tuberculosis*. Although it has been used clinically for over five decades its full mechanism of action is still being elucidated. Essential to its mechanism of action is the activation of isoniazid to a reactive intermediate, the isonicotinyl acyl radical, by the catalase-peroxidase KatG. The isonicotinyl acyl radical then reacts with NAD producing an inhibitor of the NADH-dependent enoyl ACP reductase responsible for mycolic acid synthesis as its primary target. However, the initial oxidation of isoniazid by KatG has also revealed alternative reaction pathways leading to an array of carbon-, oxygen-, and nitrogen-centered radical intermediates. It has also been reported that isoniazid produces nitric oxide in the presence of KatG and hydrogen peroxide. In this study, the temperature-dependent rate constants for the hydroxyl radical oxidation and solvated electron reduction of isoniazid and two model compounds have been studied. Based on these data the initial oxidation of isoniazid by the hydroxyl radical has been shown to predominantly occur at the primary nitrogen of the hydrazyl moiety, consistent with the postulated mechanism for the formation of the isonicotinyl radical. The hydrated electron reduction occurred mostly at the pyridine ring. Concomitant EPR spin-trap measurements under a variety of oxidizing and reducing conditions did not show any evidence of nitric oxide production as had been previously reported. Finally, examination of the transient absorption spectra obtained for hydrated electron reaction with isoniazid demonstrated for the first time an initial reduced transient identified as the isonicotinyl acyl radical produced from isoniazid.

Tuberculosis is the second major cause of death from an infectious disease globally and the World Health Organization estimates that one-third of the world population is infected with *Mycobacterium tuberculosis*.[1] Presently the front-line treatment for infections by *M. tuberculosis* is isoniazid (Fig. 1), a potent and selective agent that has been the centerpiece of tuberculosis therapy for over half-a-century. Unfortunately, despite its simple chemical structure and therapeutic use for over half-a-century the complex and possibly synergistic mechanisms of action of isoniazid remains unclear.[2,3]

Despite the lack of understanding over the complete mechanism of action of isoniazid (INH) there is general agreement concerning certain aspects of its action. Isoniazid is a prodrug that is activated by the *M. tuberculosis* catalase-peroxidase KatG to generate an isonicotinoyl acyl radical.[4–6] This isonicotinoyl radical then adds to NAD^+ to generate an INH–NADH adduct that produces antituberculosis activity.[7] The INH–NADH adduct is a potent inhibitor of InhA, an enoyl acyl-carrier-protein reductase, that is responsible for cell wall biosynthesis in *M. tuberculosis*.[8] However, it has also been suggested that the antituberculosis activity may, in part, arise from the initial oxidation of isoniazid by KatG to produce a series of

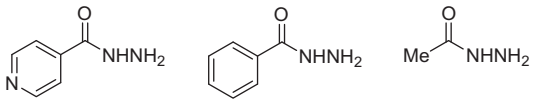

Figure 1. Structures of hydrazides investigated in this study.

reactive intermediates. Several studies have detected carbon-, oxygen-, and nitrogen-centered radical isoniazid intermediates[9] and secondary free radical decomposition products including superoxide,[10] hydroxyl radical,[11–13] and nitric oxide have been observed by electron paramagnetic resonance (EPR) spectroscopy.[14,15]

Despite these available EPR data on the free radical intermediates, a variety of inconsistencies and questions still remain on the roles these species play in the antituberculosis activity of isoniazid. First, these experiments, whether biological or chemical, were all conducted under heterogeneous conditions containing species that could themselves react with isoniazid to produce free radical products (peroxide sources for enzymatic experiments and metal ions or hypochlorous acid[16] for chemical experiments). Therefore, it would be useful in the elucidation of the isoniazid mechanism of action to precisely determine the structure of the reactive inter-

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http://dx.doi.org/10.1016/j.bmcl.2013.03.006

mediates produced by oxidation. Second, the hydrazyl radical formed from the initial oxidation of isoniazid could reside on either the primary (1°) or secondary (2°) nitrogen atom in isoniazid with even the same authors[9,14] reporting either depending on the experimental data presented. Although mechanisms can describe the decomposition of both radicals to the isonicotinoyl acyl radical the nature of the resulting hydrazine moiety would be different for each initially formed radical species. The primary hydrazyl radical would produce a diazene species while the secondary radical produces a hydrazide radical species. And last, no evidence for the production of the isonicotinoyl acyl radical from isoniazid has been directly observed.[17]

In this study, we quantitatively establish temperature-dependent rate constants for the hydroxyl radical induced oxidation and hydrated electron reduction of isoniazid and two hydrazide model compounds (for structures see Table 1). Our concomitant transient absorption spectra for the reactions of these two radicals demonstrated the formation of the isonicotinoyl radical only under reducing conditions. Additionally, electron paramagnetic resonance (EPR) measurements did not show any free nitric oxide production, contradicting previous reports for this oxidation occurring in the presence of oxygen.[14,15]

The reaction rates of the hydroxyl radical with the three hydrazides of this study were determined using SCN^- competition kinetics, utilizing the decrease in absorption intensity of the $(SCN)_2^{\cdot-}$ transient at 475 nm with increasing hydrazide concentration (Fig. 2a). Based on the competing hydroxyl radical reactions:

$$^{\cdot}OH + RC(O)NHNH_2 \rightarrow H_2O + {}^{\cdot}RC(O)NHNH_2/RC(O)NHN^{\cdot}H \quad k_6 \qquad (6)$$

$$^{\cdot}OH + SCN^- (+SCN^-) \rightarrow OH^- + (SCN)_2^{\cdot-} \quad k_7 = 1.05 \times 10^{10}\ M^{-1}\ s^{-1} \qquad (7)$$

the analytical expression for the change in yield of the product $(SCN)_2^{\cdot-}$ can be derived:[18]

$$\frac{Abs^{\circ}(SCN)_2^{\cdot-}}{Abs(SCN)_2^{\cdot-}} = 1 + \frac{k_6[\text{hydrazide}]}{k_7[SCN^-]} \qquad (8)$$

In this equation $Abs^{\circ}(SCN)^{\cdot-}$ is the peak absorption measured for the $(SCN)_2^{\cdot-}$ transient and $Abs(SCN)^{\cdot-}$ is the lower absorption due to the presence of the added hydrazide. A plot of this absorption ratio against the concentration ratio $[RC(O)NHNH_2]/[(SCN)_2^{\cdot-}]$ gives a straight line of slope k_6/k_7. A typical transformed plot for isoniazid is shown in Figure 2b. From the established rate constant for hydroxyl radical reaction with thiocyanate ($k_7 = 1.05 \times 10^{10}\ M^{-1}\ s^{-1}$ at our temperature)[18] the value of $k_6 = (8.63 \pm 0.16) \times 10^9\ M^{-1}\ s^{-1}$ is readily determined.

No rate constant data for the aqueous oxidation of isonazid by the hydroxyl radical was found in the literature. Additional measurements for two model compounds (Fig. 1), benzoic hydrazide

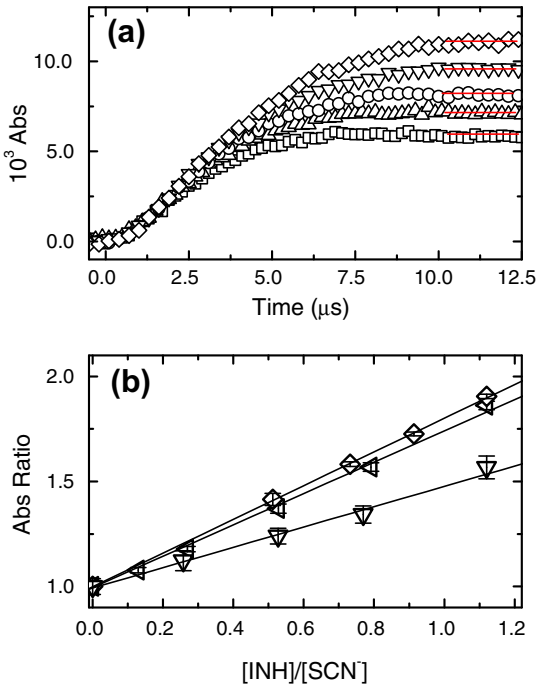

Figure 2. (a) Intensity decrease for $(SCN)_2^{\cdot-}$ radical transient absorbance at 475 nm for 38.07 μM KSCN in N_2O-saturated solution at natural pH containing 42.66 (□), 30.12 (△), 20.10 (○), 10.27 (▽), and 4.94 (◇) μM isoniazid, respectively. (b) Competition kinetics transformed peak intensities of (a) at 8.8 (◇), 23.3 (○), and 38.6 (▽) °C. Solid lines are weighted linear fits, corresponding to the second order reaction rate constants of $(6.87 \pm 0.08) \times 10^9$, $(8.6 \pm 0.16) \times 10^9$, and $(1.11 \pm 0.09) \times 10^{10}\ M^{-1}\ s^{-1}$, respectively.

($(1.61 \pm 0.11) \times 10^{10}\ M^{-1}\ s^{-1}$) and acetic hydrazide ($(9.75 \pm 0.70) \times 10^9\ M^{-1}\ s^{-1}$) were faster than for isoniazid, implying that not all the oxidation occurred at the hydrazine moiety in INH (see Table 1). This hypothesis is supported by the literature rate constant for 1,1-dimethylhydrazine ($1.6 \times 10^{10}\ M^{-1}\ s^{-1}$),[19] and the considerably slower value for pyridine in water ($3.0 \times 10^9\ M^{-1}\ s^{-1}$).[20] Further insight into the mechanism of reaction was gained from comparison to the slower hydroxyl radical reaction rate constant for isonicotinamide ($1.6 \times 10^9\ M^{-1}\ s^{-1}$),[21] which suggests that oxidation of isoniazid occurs predominantly at the primary nitrogen of the hydrazine group. The minor hydroxyl radical reaction with the pyridine ring might occur by either addition or hydrogen atom abstraction, whereas the reaction at the nitrogen would result in hydrogen atom abstraction to form a hydrazyl radical ($RC(O)NHN^{\cdot}H$).

This finding contradicts a previous study[14] of the mechanism of action of isoniazid that had postulated the following reaction sequence:

Table 1
Summary of radical reaction rate constants for isoniazid and model compounds

Compound	$k_{\cdot OH}$ (°C) $M^{-1}\ s^{-1}$	k_e^- (°C) $M^{-1}\ s^{-1}$
Isoniazid	$(6.87 \pm 0.08) \times 10^9$ (8.8)	$(1.36 \pm 0.02) \times 10^{10}$ (3.5)
	$(7.53 \pm 0.21) \times 10^9$ (13.8)	$(1.68 \pm 0.02) \times 10^{10}$ (8.5)
	$(8.63 \pm 0.16) \times 10^9$ (23.3)	$(2.35 \pm 0.10) \times 10^{10}$ (20.7)
	$(1.02 \pm 0.05) \times 10^{10}$ (31.4)	$(2.97 \pm 0.05) \times 10^{10}$ (27.8)
	$(1.11 \pm 0.09) \times 10^{10}$ (38.6)	$(3.39 \pm 0.05) \times 10^{10}$ (35.0)
Benzoic hydrazide	$(1.61 \pm 0.11) \times 10^{10}$ (22.8)	$(1.55 \pm 0.01) \times 10^{10}$ (20.6)
Acetic hydrazide	$(9.75 \pm 0.70) \times 10^9$ (24.2)	$< 6 \times 10^7$ (22.5)
Pyridine	3.0×10^9	1.4×10^{10} [28]
1,1-Dimethylhydrazine	1.6×10^{10} [24]	2.4×10^7 [24]
Isonicotinamide	1.6×10^9 [29]	3.2×10^{10}