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METABOLISM OF ISONIAZID BY NEUTROPHIL MYELOPEROXIDASE LEADS TO INH-NAD$^+$ ADDUCT FORMATION: A COMPARISON OF THE REACTIVITY OF ISONIAZID WITH ITS KNOWN HUMAN METABOLITES

Authors: Saifur R. Khan[1], Andrew G.M. Morgan[1], Karim Michail[1], Randy M. Whital[2], Naif Aljuhani[1,3], and Arno G. Siraki[1*]

Affiliations:

[1] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada.

[2]Department of Chemistry, Faculty of Sciences, University of Alberta, Edmonton, Canada.

[3]Pharmacology and Toxicology Department, Faculty of Pharmacy, Taibah University, Madinah, Saudi Arabia

[*]Corresponding Author: Arno G. Siraki, PhD; Faculty of Pharmacy and Pharmaceutical Sciences, Katz Group Centre for Pharmacy and Health Research, Room 2-020H, University of Alberta, Edmonton, AB T6G 2E1, Canada. Telephone: +1-780-248-1591, Fax: +1-780-492-3575, E-mail address: siraki@ualberta.ca.

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ABSTRACT

The formation of isonicotinyl-nicotinamide adenine dinucleotide (INH-NAD^+) via the mycobacterial catalase-peroxidase enzyme, katG, has been described as the major component of the mode of action of isoniazid (INH). However, there are numerous of human peroxidases that may catalyze this reaction. The role of neutrophil myeloperoxidase (MPO) in INH metabolism and INH-NAD^+ adduct formation has never been explored; this is important, as neutrophils are recruited at the site of tuberculosis infection. In our studies, we showed that neutrophil MPO is capable of INH metabolism using electron paramagnetic resonance (EPR) spin-trapping and UV-Vis spectroscopy. MPO or activated human neutrophils (by phorbol myristate acetate) catalyzed the oxidation of INH and formed several free radical intermediates; the inclusion of superoxide dismutase revealed a carbon-centered radical which is considered to be the reactive metabolite that binds with NAD^+. Other human metabolites, including N-acetyl-INH, N-acetylhydrazine, and hydrazine did not show formation of carbon-centered radicals, and either produced no detectable free radicals, N-centered free radicals, or superoxide, respectively. A comparison of these free radical products indicated that only the carbon-centered radical from INH is reducing in nature, based on UV-Vis measurement of nitroblue tetrazolium reduction. Furthermore, only INH oxidation by MPO led to a new product (λ_{max} = 326 nm) in the presence of NAD^+. This adduct was confirmed to be isonicotinyl-NAD^+ using LC-MS analysis where the intact adduct was detected (m/z = 769). The findings of this study suggest that neutrophil MPO may also play a role in INH pharmacological activity.

1. INTRODUCTION

Although isoniazid (INH) was introduced into tuberculosis (TB) therapy in 1952, its mode of action still remains elusive. It is generally accepted that INH is a prodrug and is oxidized to the isonicotinyl radical ($INH^{\bullet}$) through peroxidation by the bacterial catalase-peroxidase, KatG; $INH^{\bullet}$ then reacts with the oxidized form of nicotinamide adenine dinucleotide (NAD^{+}) producing the INH-NAD^{+} adduct. This adduct inhibits mycolic acid biosynthesis of *Mycobacterium tuberculosis* (*Mtb*) cell wall by blocking an essential enzyme named enoyl acyl-carrier-protein reductase [1, 2]. A number of limitations such as effectiveness of INH in latency (where *Mtb* does not possess typical cell wall), low penetration of INH into granuloma (where *Mtb* resides) and exceptionally long treatment duration as an antibiotic (as opposed to *in vitro* experiments) have been identified [3-5], which suggests that there are other additional mechanism(s) of action of INH.

Two studies reported that INH at suprapharmacological concentrations (35 mM) produces nitric oxide (NO) through the peroxidation cycle of KatG, which in turn is proposed to kill *Mtb* [6, 7]. The oxidation of the hydrazide nitrogen atom proximal to the carbonyl of INH was proposed as the primary site of oxidation in this study, which is decomposed to NO and $INH^{\bullet}$. Recently a study on the temperature-dependent rate constants for the hydroxyl radical oxidation and solvated electron reduction of INH revealed that the initial oxidation of INH by hydroxyl radical is the distal nitrogen of hydrazyl moiety. Therefore, the decomposition product is not expected to be NO, but rather would be diazene (HN=NH) and $INH^{\bullet}$ [8]. This study contradicted the report of NO generation, which has not been reported elsewhere. However, $INH^{\bullet}$ was produced in both cases of nitrogen atom oxidation either that is proximal or distal to the carbonyl of INH. In addition, the role of oxidation in the formation of $INH^{\bullet}$ has not been

challenged, where the oxidation can be enzymatic or non-enzymatic auto-oxidation. Auto-oxidation for $INH^{\bullet}$ formation requires a longer time period has been reported in several studies [9, 10].

There are several enzymes which usually catalyze xenobiotic oxidation, including the cytochrome P-450 enzymes, and peroxidase enzymes such as neutrophil myeloperoxidase (MPO). Recently, an INH-$NADP^+$ adduct (m/z 851.0) was identified in human liver microsomes through LC-MS experiments in an *in vitro* study that concluded cytochrome P450 was involved [9]. MPO has been shown to oxidize INH, but there has been no report of a subsequent interaction with NAD^+ or $NADP^+$ [11, 12]. MPO is a human catalase-peroxidase enzyme which has some resemblance to bacterial KatG in terms of activity. However, a comparison between KatG and the plant peroxidase horseradish peroxidase (HRP) showed that KatG had very poor peroxidase activity (5.1± 0.5 units/mg) compared to HRP (6405 ± 170 units/mg) [13]. In another study, the capacity of tyrosine nitration from nitrite was compared between MPO and HRP and it was found that the peroxidation capacity of MPO was at least ten time more than that of HRP [14]. Therefore, MPO possesses approximately 10^4 times stronger peroxidase activity than KatG. Due to the high peroxidase activity of MPO, it is likely that MPO could rapidly oxidize INH to $INH^{\bullet}$, which has been previously been reported by others [11, 15]. However, the role of MPO or neutrophils in generating INH-NAD^+ adducts has not been explored.

Recently, a metabolomics study found the INH-nicotinamide adduct in urine from both TB patients and healthy mice treated with INH; it was argued that INH-nicotinamide could be a break-down product of the INH-NAD^+ adduct, which indirectly suggests the existence of an INH-NAD^+ adduct *in vivo* [16]. Furthermore, this study suggested that INH can be activated by a host peroxidase, for example, lactoperoxidase. The cause of poor effectiveness of INH in the

case of KatG mutants of TB (even though host peroxidases can activate INH) has been explained due to the distal proximity of host INH activation, which leads to other reaction pathways. It causes the degradation and modification of INH-NAD^+ adduct *in vivo* [16]. However, it is unknown if recruited neutrophils at site of infection can activate INH. In this study, we hypothesized that neutrophil MPO is another site of INH metabolism, and can metabolize INH into $INH^{\bullet}$ and lead to INH-NAD^+ adduct formation. To test this hypothesis, INH and its main human metabolites, including N-acetylisoniazid (NAcINH), N-acetylhydrazine (NAcHZ) and hydrazine (HZ, Scheme 1), were investigated for reactive species generation through MPO oxidation using both isolated MPO and activated neutrophils.

2. MATERIALS & METHODS

2.1 Chemicals and Kits

NAcINH was purchased from Toronto Research Chemicals, Inc. (Toronto, ON). Nicotinamide adenine dinucleotide free acid form (NAD^+) was procured from Santa Cruz biotechnology, Inc. (Dallas, TX). Human neutrophil MPO was purchased from Athens Research & Technology (Athens, GA). 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), manufactured by Dojindo Molecular Technologies, Inc. was purchased from Cedarlane Laboratories Ltd (Burlington, ON). Superoxide dismutase (SOD) and glucose oxidase (GOx) were purchased from Sigma-Aldrich Canada Co. (Oakville, ON). Nicotinamide, nicotinamide adenine dinucleotide reduced form (NADH), nitrotetrazolium blue chloride (NBT), hydrogen peroxide (H_2O_2), INH, NAcHZ, HZ and all other chemicals (unless otherwise noted) were purchased from Sigma-Aldrich Canada Co.

2.2 Electron paramagnetic resonance (EPR) spin trapping and characterization

The free radical species were detected by spin trapping, where the free radical species covalently bind to the nitrone spin trap (DMPO) to produce a relatively stable paramagnetic adduct. Reactions were prepared by adding a final concentration of 2 mM of each chemical (INH, NAcINH, NAcHZ, HZ) and 100 mM DMPO in a 200 µL volume of Chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA to a micro test tube containing either 0.1 µM MPO or 6 x 10^4 neutrophils (freshly isolated from human blood – see below). To initiate the reactions, 100 µM of H_2O_2 was used in reactions with MPO, whereas 0.8 µM of phorbol 12-myristate 13-acetate (PMA) was used for neutrophil activation. SOD (2.5 µM) was used to rapidly dismutate superoxide radical. 2 mM of NAD^+ was used in reactions to study trapping of free radicals from INH (and its metabolites). Reactions were briefly vortexed prior to transferring to a Suprasil quartz ESR flat cell (Bruker Canada, Milton, ON) for spectrum recording. EPR spectra were obtained with a Bruker Elexsys E-500 spectrometer (Billerica, MA) equipped with an ER 4122 SHQ cavity operating at 9.78 GHz and 100 kHz modulation field at room temperature with the following parameters: power = 20 mW, scan rate = 0.47 G/s, modulation amplitude = 0.4 G, and receiver gain = 6.32× 10^5. Spectra were recorded as single scan.

2.3 Relative oxidation and reduction of INH and its metabolites

To identify the redox properties of INH and its metabolites, we used several approaches. The reductive capacity of the metabolite(s) of each compound (INH, NAcINH, NAcHZ, and HZ) was studied through NBT reduction, where 1 mM of each chemical was incubated with 200 µM of NBT, 50 nM of MPO, and 50 µM of H_2O_2. The absorption of the reduced NBT (formazan) was measured at 520 nm. The oxidative capacity of the compounds (INH and its metabolites) were

measured through NADH oxidation where 1 mM of each chemical was incubated with 200 µM of NADH, 50 nM of MPO, and 50 µM of H_2O_2. The absorbance was measured at 339 nm and all data were acquired by using a SpectraMax M5 microplate reader with cuvette port (Molecular Devices, Sunnyvale, CA). In all experiments 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA were used.

2.4 UV-Vis analysis for covalent adduct formation and other interactions

To identify the possible interactions between INH and endogenous molecules, 500 µM of four different nucleotides (thymine, guanine, cytosine, and adenine) nucleotide were exposed to 500 µM of INH, 1 µM of MPO, 10 milliunits (mU) glucose oxidase (GOx) and 5 mM glucose for 30 mins and the UV-spectrum was analyzed. In addition, 500 µM of each chemical (INH, NAcINH, NAcHZ, and HZ) was exposed to 500 µM of nicotinamide, adenine, or NAD^+. The reaction was initiated by the addition of 1 µM of MPO, 10 mU GOx and 5 mM glucose and run for 30 minutes to 1 h using UV-Vis kinetic spectroscopy to compare the changes in spectrum. The absorbance at specific adduct peaks were also monitored. In all experiments 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA were used.

2.5 Neutrophil (PMN) isolation from human blood

Human neutrophils were collected from healthy donors by consent granted from the Human Ethics Research Office of the University of Alberta by a methodology described elsewhere [18]. In brief, 6 mL of whole blood was layered on top of 6 mL Histopaque™1119 in 15 mL polystyrene centrifuge tubes and spin for 20 min at 800 × g with the centrifuge brake set to off (i.e., zero deceleration). It produced four distinct layers of the blood. The third pink-reddish layer was collected into 15 ml polystyrene centrifuge tubes. Cells were washed twice with 1× PBS

through centrifugation at 300 × g for 10 min. 100% Percoll™ solution was prepared by mixing 18 mL Percoll™ with 2 mL 10× PBS. From 100% Percoll™ solution, 5 mL of 85%, 80%, 75%, 70% and 65% Percoll™ gradients were prepared by using 1× PBS. 2 mL of the re-suspended cells were layered onto the each Percoll™ gradients followed by spin tubes at 800 × g for 20 min with the centrifuge brake set to off. After centrifugation the interphases were visibly distinguishable due to the highest cell density. By removing top layers, white interphase that contains neutrophils was collected into a clean 15 mL polystyrene centrifuge tube. The separated neutrophils were further washed by filling up the tube with 1x PBS followed by spin at 300 × g for 10 min. The supernatant was discarded and the cell sediment was re-suspended in 2 ml of PBS. Cells were plated at the required density in PBS.

2.6 INH-NAD^+ extraction and LC-MS analysis

For INH-NAD^+ adduct identification, 500 µM of INH was exposed to 500 µM of NAD^+, 1 µM MPO, 5 mM glucose and 10 mU GOx in 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA for 1 h while the INH-NAD^+ adduct formation was monitored at 326 nm in UV-Vis kinetic spectroscopy. After 1 h, the reaction content was passed through an Oasis® HLB 1 cc extraction cartridge followed by washing with deionized water. The final eluent was extracted using methanol. LC-MS was performed on an Agilent 1200 UHPLC with an Agilent 6130 single quadrupole mass spectrometer equipped with an electrospray source. Samples were injected onto a 2.1 × 50mm Agilent Zorbax SB-C18 column with 1.8 µm silica particles and separated using a water /acetonitrile gradient with 0.1% formic acid added as a solvent modifier at a flow rate of 0.5 mL/min. After holding at 1% acetonitrile for 0.5 min the gradient is ramped linearly to 60% acetonitrile in 5 min. Column eluent is first monitored by an Agilent G4212B diode array

detector, monitoring the UV signal at 326 nm followed by the mass spectrometer giving a small delay between the UV and MS signals of 0.02 min. The mass spectrometer is run in both positive and negative ion modes, switching modes continuously between scans. Exact mass LC-MS was performed on an Agilent 6220 time-of-flight mass spectrometer equipped with an ESI source to provide compound formulae in positive ion mode.

3. RESULTS

3.1 EPR studies for INH in MPO system and neutrophils

Studies were first carried out using commercially purchased MPO. The latter in the presence of INH showed a relatively weak but discernable DMPO/$^{\bullet}$OH spectrum using EPR (Fig. 1A). In presence of H_2O_2, which activates MPO, the DMPO/$^{\bullet}$OH signals were found to be intensified along with a mixture of DMPO/$^{\bullet}$OOH and possibly carbon centered free radicals (Fig. 1B). With the addition of SOD, DMPO/$^{\bullet}$OOH signals were eliminated and DMPO/$^{\bullet}$OH signals were further intensified (Fig. 1C). Moreover, carbon centered radical signals appeared more clearly (Fig. 1C). By the addition of NAD^+, the EPR spectrum showed a significant decline of DMPO/$^{\bullet}$OH and carbon centered radicals (DMPO/$^{\bullet}$C), and reappearance of DMPO/$^{\bullet}$OOH (Fig. 1D).

In the case of activated (PMA treated) neutrophils, EPR spectra showed a similar composite spectrum as seen with commercial MPO (Fig. 2 A-1). SOD was added to eliminate spectra arising from the trapping of superoxide ($O_2^{\bullet-}$) which is expected in activated neutrophils. The EPR spectrum of activated neutrophils incubated with SOD demonstrated no significant spin adduct (only a trace of DMPO/$^{\bullet}$OH signals were observed) (Fig 2 A-2), indicating that the spectra was attributed to oxygen activation, which is well known and shown previously by others [19]. The addition of INH to activated neutrophils (Fig. 2 E-1) was unclear due to the signals

arising from of activated neutrophils themselves. Upon addition of SOD (Fig. 2 E-2), two signals were remained: DMPO/$^{\bullet}$OH and DMPO/$^{\bullet}$C; these were similar to what was observed using MPO (Fig. 1C), albeit less intense. When NAD^+ was added to the reaction (fig 2 E-3), the previously found DMPO/$^{\bullet}$OH and carbon centered radicals both were attenuated, which was greater than that observed with MPO (Fig. 1D). From these observations, it appears that isolated MPO produces the same free radical products as activated neutrophils in the presence of SOD; also, activated neutrophils demonstrate INH metabolism.

3.2 EPR studies for the metabolites of INH in MPO and neutrophils

In the MPO system, NAcINH did not show any EPR signals in any of its reactions (Fig. 1E-H). However, NAcHZ (Fig 1I-L) and HZ (Fig 1M-P) showed detectable EPR spectra. NAcHZ showed N-centered free radical (DMPO/$^{\bullet}$N) signals which was increased in presence of H_2O_2 (Fig. 1J) and not significantly changed after addition of SOD (Fig. 1K) or NAD^+ (Fig. 1L). With HZ, we found DMPO/$^{\bullet}$OH signals which were enhanced by the addition of H_2O_2 (Fig. 1M, N). The addition of SOD caused a marked attenuation of DMPO/$^{\bullet}$OH (Fig. 1O), suggesting that the DMPO/$^{\bullet}$OH signals were generated from $O_2^{\bullet-}$. Hence, DMPO/$^{\bullet}$OOH signals did not appear in any of HZ reactions. In NAcHz and HZ reactions, we did not find EPR-based evidence of interactions between NAD^+ and any free radicals generated with these compounds (Fig. 1L,P).

Using activated neutrophils, we found DMPO/$^{\bullet}$N associated with NAcHZ and DMPO/$^{\bullet}$OH associated with HZ (Fig. 2C-1,C-2,D-1,D-2); which was similar to findings with MPO. Additionally, NAcINH did not show any EPR spectra as was found with MPO.

3.3 Chemical nature of INH and its metabolites

In the NBT reduction assay, we compared the MPO metabolites of INH with its human metabolites; only INH showed capacity to reduce NBT to form formazan which was measured at 520 nm in UV-Vis spectroscopy (Fig. 3). A previous study showed that only INH has capacity to reduce NBT in comparison other hydrazine drugs (hydralazine and iproniazid) [11]. Therefore, INH product(s) of MPO is/are reducing agent(s) which can donate electrons to NBT to form NBT-diformazan. On the other hand, all other metabolites of INH, when oxidized by MPO, did not react with NBT. Therefore, biochemically these radical metabolites were significantly different from the parent compound's initial free radical metabolite(s).

3.4 UV-Vis and LC/MS studies for INH-NAD^+ adduct formation

We carried out a series of UV-Vis studies where either INH or its metabolites (NAcINH, NAcHZ and HZ) were incubated with NAD^+/MPO/H_2O_2. After 30 minutes of each reaction, we compared the spectrum with the initial time-point spectrum to determine if new products were found. No compound, except for INH, showed a change in its spectrum (Fig 4). Based on these findings, we investigated the UV-Vis absorbance kinetics at 326 nm (fig 4A inset). These findings demonstrated that only INH oxidation led to a possible product in the presence of NAD^+. There was no change in the spectrum when NAD^+ was omitted from the reaction containing INH, MPO, and H_2O_2 (data not shown).

We further performed another series of UV-Vis studies where either NAD^+, the components of NAD^+ (nicotinamide and adenine) or NADH were added with INH and MPO/H_2O_2 (Fig. 5). After 30 minutes of each reaction, we compared the spectrum with the initial time-point spectrum to find out the interactions. No new products were detected with any of the potential reactants used, except for NAD^+ (Fig. 4A).

In another set of UV-Vis studies, four different nucleotides (guanine, cytosine, adenine and thymine) were added individually with INH in MPO/H_2O_2 system to find out the interactions. As INH is electron rich (due to the hydrazide moiety) it behaves like a reducing agent; as such, it is possible to interact with electron acceptors, for example NAD^+. However, our studies revealed that INH did not interact with any of four nucleotides (data not shown). It suggests that the reaction between INH and NAD^+ is rather unique.

3.5 Identification of INH-NAD^+ adduct in LC-MS

LC-MS was carried out after a reaction containing 500 µM INH, 500 µM NAD^+, 1 µM MPO, 5 mM glucose, and 10 mU GOx for 1 h in 0.1M phosphate buffer pH 7.4 containing 100 µM DTPA (sample preparation was described Materials & Methods). The LC chromatogram revealed three main peaks (λ = 326 nm) with retention times at 0.42, 3.70, and 5.77 min. (Fig. 6A). ESI-MS shows the INH-NADH adduct in both positive and negative ion modes at an RT of 0.44min. An extracted ion chromatogram of the positive mode ESI shows that the m/z 769 peak corresponding to INH-NAD^+ is observed only at 0.44 min (Figure 6B). Interestingly, in positive mode both the INH-NAD^+ and INH-NADH forms are observed at m/z 769 and m/z 771, respectively (Fig 6C). In negative mode, the INH-NADH form is observed (Fig. 6D). Reduction of NAD^+ to NADH can occur during the ESI process as NAD^+ has a more positive reduction potential (-0.18 V) than the iron metal (-0.41 V) in the ESI spray tip [20]; therefore, it is not surprising that both NAD^+ and NADH forms are observed, despite having added only NAD^+ to the reaction mixture. Fragmentation of INH-NAD^+ also occurs and results in the formation of fragment peaks at m/z 228, m/z 542 and m/z 664. The proposed structures of the fragments are shown in Fig. 6E and the proposed formulae were confirmed by high-resolution ESI LC-MS in a separate experiment (data not shown).

4. **DISCUSSION**

These studies demonstrated that there are fundamental differences in the behavior of INH compared to its human metabolites (NAcINH, NAcHZ and HZ). In addition, MPO is significant in the metabolism of INH and its human metabolites. By using EPR spectroscopy, a DMPO/$^{\bullet}$C (a carbon center radical) signal was identified in both MPO and isolated human neutrophils. It is assumed that this DMPO/$^{\bullet}$C signal was generated due to INH$^{\bullet}$ formation from INH via the peroxidation cycle of MPO; the similar DMPO/$^{\bullet}$C signal was also identified for KatG to propose INH$^{\bullet}$ generation [21, 22]. In addition, this study identified INH-NAD^+ adducts formation catalyzed by MPO by the addition of NAD^+. Therefore, neutrophil MPO can oxidize INH into INH$^{\bullet}$. The INH$^{\bullet}$ metabolite of MPO appeared to be chemically reducing, which made it different from all of its major metabolites; this characteristic is likely what leads it to interact with the oxidized form of nicotinamide adenine dinucleotide (NAD^+). This interaction leads to formation of INH-NAD^+ adduct which was postulated as major anti-TB component of INH [1, 2]. The INH-NAD^+ adduct was confirmed by using LC-MS in INH/MPO/H_2O_2. As INH-NAD^+ adduct was found in the MPO biochemical system and MPO was found here the main metabolic enzyme of human neutrophils, it is expected to be formed in activated neutrophils. Furthermore, our studies showed that the unique chemical properties of NAD^+ are necessary for the interaction of the INH$^{\bullet}$ since the individual components of NAD^+ (nicotinamide and adenine) did not interact with INH$^{\bullet}$ metabolite. To our knowledge, this is the first study to show that neutrophil MPO can form INH-NAD^+ adduct.

In EPR studies, the reaction between INH and MPO resulted in DMPO/$^{\bullet}$OH signals. A previous study had shown that INH in 0.1 M phosphate buffer ($pH \geq 7$) produced apparent hydroxyl radicals in presence of a potent metal chelator, phytic acid, with or without catalase;

however, in acidic pH (≤ 6) this apparent hydroxyl radical was non-detectable. Here phytic acid was used to inhibit metal-induced oxidation [23]. It suggested that the comparatively basic pH of 0.1 M phosphate buffer may play a role in the formation of hydroxyl radical of INH by facilitating slow auto-oxidation; and it may also be influenced by the reduction potential of the compounds; the higher the reduction potential higher the chance to be auto-oxidized in such reaction condition. In our studies we used 0.1 M phosphate buffer with a pH of 7.4 which caused the generation of apparent hydroxyl radical (DMPO/$^{\bullet}$OH) through slow auto-oxidation in INH and HZ reactions (Fig 1A and 1M), but not in NAcINH and NAcHZ reactions. From redox potential studies, it is known that INH has the highest redox potential (-1.5 V) [24], then HZ (-0.201 V) [25]. NAcHZ has very low redox potential (-0.077 V) [25] which makes it not suitable candidate for auto-oxidation; and NAcINH is relatively inert regarding redox potential since its acetylation blocked its H^+ transfer capacity.

In presence of H_2O_2, compound-I of MPO was formed and oxidized INH. EPR signals demonstrated at least three radical species (DMPO/$^{\bullet}$OH, DMPO/$^{\bullet}$OOH and DMPO/$^{\bullet}$C) were formed. Goodwin *et al.* were the first to show similar findings in their studies for this reaction. However, the result of SOD addition was somewhat different in terms of DMPO/$^{\bullet}$OH signal intensity [11]. In our studies, we found that DMPO/$^{\bullet}$OH signals were intensified and DMPO/$^{\bullet}$OOH signals were eliminated by the addition of SOD. However, Goodwin *et al.* showed both signals were decreased [11]. Both experiments suggested that $O_2^{\bullet-}$ was the source for DMPO/$^{\bullet}$OOH signals. However, the contradiction between DMPO/$^{\bullet}$OH signals may vary due to the variation of pH variation of the buffer systems (discussed above). We speculate that at physiological pH, INH$^{\bullet}$ may reduce H_2O_2 to hydroxyl radical as the INH-induced DMPO/$^{\bullet}$OH spectrum was insensitive (and enhanced) by SOD, though further evidence is needed. Again, as

HZ has very low redox potential in comparison to that of INH, its reduction capacity of H_2O_2 may be very weak. Therefore, SOD attenuated DMPO/$^{\bullet}$OH, arising from $O_2^{\bullet-}$, from HZ reactions.

In our studies, we introduced NAD^+ which has not been explored yet in MPO-mediated INH metabolism. The addition of NAD^+ into INH/SOD/MPO/H_2O_2 system caused a slight decrease of the entire spectrum of INH (DMPO/$^{\bullet}$C and DMPO/$^{\bullet}$OH) along with reappearance of DMPO/$^{\bullet}$OOH. This suggested that carbon centered radical of INH (INH$^{\bullet}$) reacted with NAD^+ to form INH-NAD^+ adduct which was confirmed in UV-Vis absorbance at 326 nm followed by identified in LC-MS. However, it is unknown how the formation of INH-NAD^+ adduct caused the significant decrease of DMPO/$^{\bullet}$OH signal and reappearance of DMPO/$^{\bullet}$OOH signal in EPR spectrum. The reappearance of DMPO/$^{\bullet}$OOH signal suggests that INH-NAD^+ adducts may have an inhibitory effect on SOD activity, but this needs further investigation. In the case of other reactions (NAcINH, NAcHZ and HZ), no spectral changes were observed after adding NAD^+. This again highlights the specific reaction between INH radical metabolites and NAD^+.

Similar to isolated MPO, EPR studies in activated human neutrophils (PMN) were carried out. Firstly, SOD was needed in order to visualize the INH or INH metabolite associated free radicals due to oxygen activation by the respiratory burst. Activated PMNs showed a characteristic EPR spectrum, which is composed of a mixture of DMPO/$^{\bullet}$OOH and DMPO/$^{\bullet}$OH as reported previously [26]; the addition of SOD essentially abrogated the oxygen-derived free radical spectra. In presence of INH, SOD resulted in a residual DMPO/$^{\bullet}$OH spectrum and a weak DMPO/$^{\bullet}$C. The addition of NAD^+ in the reaction caused even further attenuation. These observations were consistent with spectra from reactions using isolated MPO, except that the intensity was significantly less when using PMNs. Therefore, it is apparent that MPO of

neutrophils is playing major role in INH oxidation and free radical generation. In addition, it was shown for the first-time that neutrophils can produce carbon centered radical, which we proposed to be derived from $INH^{\bullet}$.The metabolites of INH appeared to behave quite similar as they did when using MPO as the catalyst such that NAcINH did not produce detectable free radical products, NAcHZ formed nitrogen-centered radicals, and HZ formed superoxide. A summary of these findings regarding INH oxidation via neutrophil MPO is presented in Scheme 2.

Studies evaluating the reducing or oxidizing effects of the free radical metabolites of INH and its human metabolites demonstrated that MPO metabolism of INH only led to a free radical metabolite which is reducing and can reduce NBT. This type of assay has been used in other studies that used KatG [27, 28], or even when assaying superoxide radical [29, 30]. As per its redox potential, INH is a very strong reducing agent whereas HZ and NAcHZ are very weak [25, 31]. Therefore, we speculate that the intermediate species of INH during MPO metabolism may have higher reduction potential than the intermediates of other compounds tested. This is the likely explanation of why the INH intermediate radical solely reacted with NAD^{+} (oxidized form), and others did not show any interaction. As NADH is the reduced form of NAD^{+}, it was not favourable to interact with $INH^{\bullet}$ which was also reducing in nature. These findings were characterized by LC-MS analyses. Interestingly, a recent study in TB patients and healthy mice identified similar species in urine [16].

In comparison with other immune cells, neutrophils are less well studied with respect to TB infection. Although the role of neutrophils in host defense against TB is contradictory, the early recruitment of neutrophils at the site of *mtb* infection is well documented [32]. After recruitment, they recognize *mtb* directly or via opsonisation and internalize them followed by deploying various killing mechanisms such as reactive oxygen species generation, NETs

formation and release of other antimicrobial peptides [32]. The successful killing causes the apoptosis of neutrophils which can recruit non-inflammatory macrophages and lead to disease recovery [33-35]. Many studies showed that most neutrophils killing, however, is not effective against *mtb*[32], and causes the necrosis of neutrophils which recruits more neutrophils and inflammatory macrophage on the site of infection [35]; this causes more damage rather than recovery [32, 35, 36]. Our studies showed that neutrophil MPO can produce the postulated anti-bacterial component of INH, INH-NAD^+ adduct upon INH treatment, and suggests an alternative pathway to to kill *mtb* . Future studies should be directed at exploring the relationship between the neutrophil-induced production of INH-NAD^+ and TB outcome.

5. ACKNOWLEDGEMENTS

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Figures

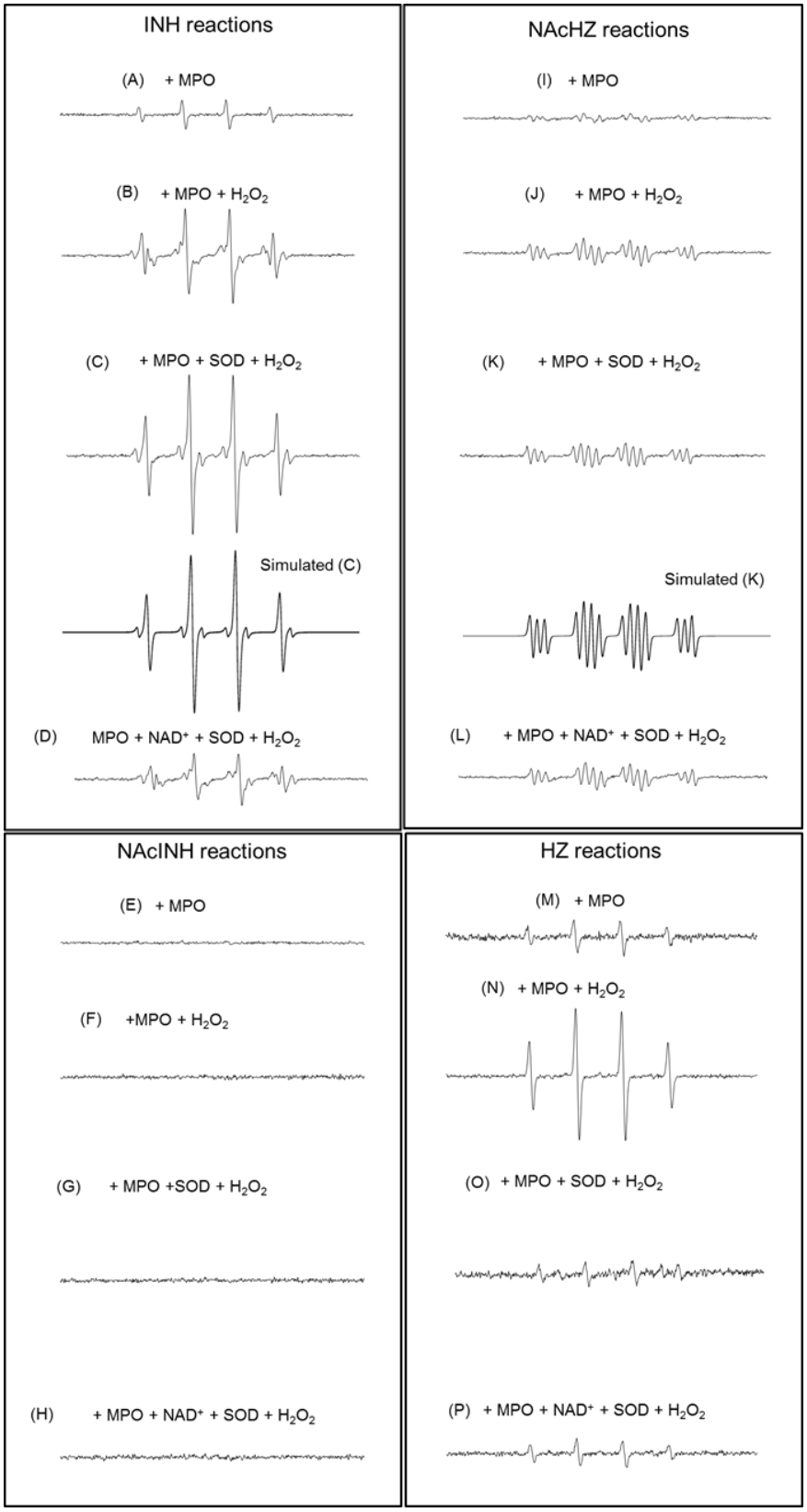


Figure 1. EPR studies of INH and its metabolites in MPO system. Each reaction was carried out in 200 µl of chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA, and has 0.1 µM of MPO with or without 100 µM of H_2O_2. 2 mM of either INH or any of its metabolites (NAcINH, NAcHZ and HZ) was used for treatment. 2.5 µM of SOD was added to eliminate spectra arising from the trapping of superoxide ($O_2^{\bullet-}$). In addition, 2 mM of NAD^+ was further used to find the role of NAD^+ in each reaction. The figure shows here INH reactions (A –D), NAcINH reactions (E-H), NAcHZ reactions (I-L) and HZ reactions (M-P). There are two simulated spectra to identify the radical species: Simulated (C) is the simulation of INH reaction C, and simulated (K) is the simulation of NAcHZ reaction K. The parameters for simulated (C) of INH reaction "C": $a^N_{nitroxide}$ = 14.69 G, a^H_{carbon} = 21.75 G, r = 0.99; the parameters for simulated (K) of AcHZ reaction "K": $a^N_{nitroxide}$ = 15.01 G, a^N = 2.32 G, a^H = 17.84 G, r = 0.97. EPR settings are described in Materials & Methods.

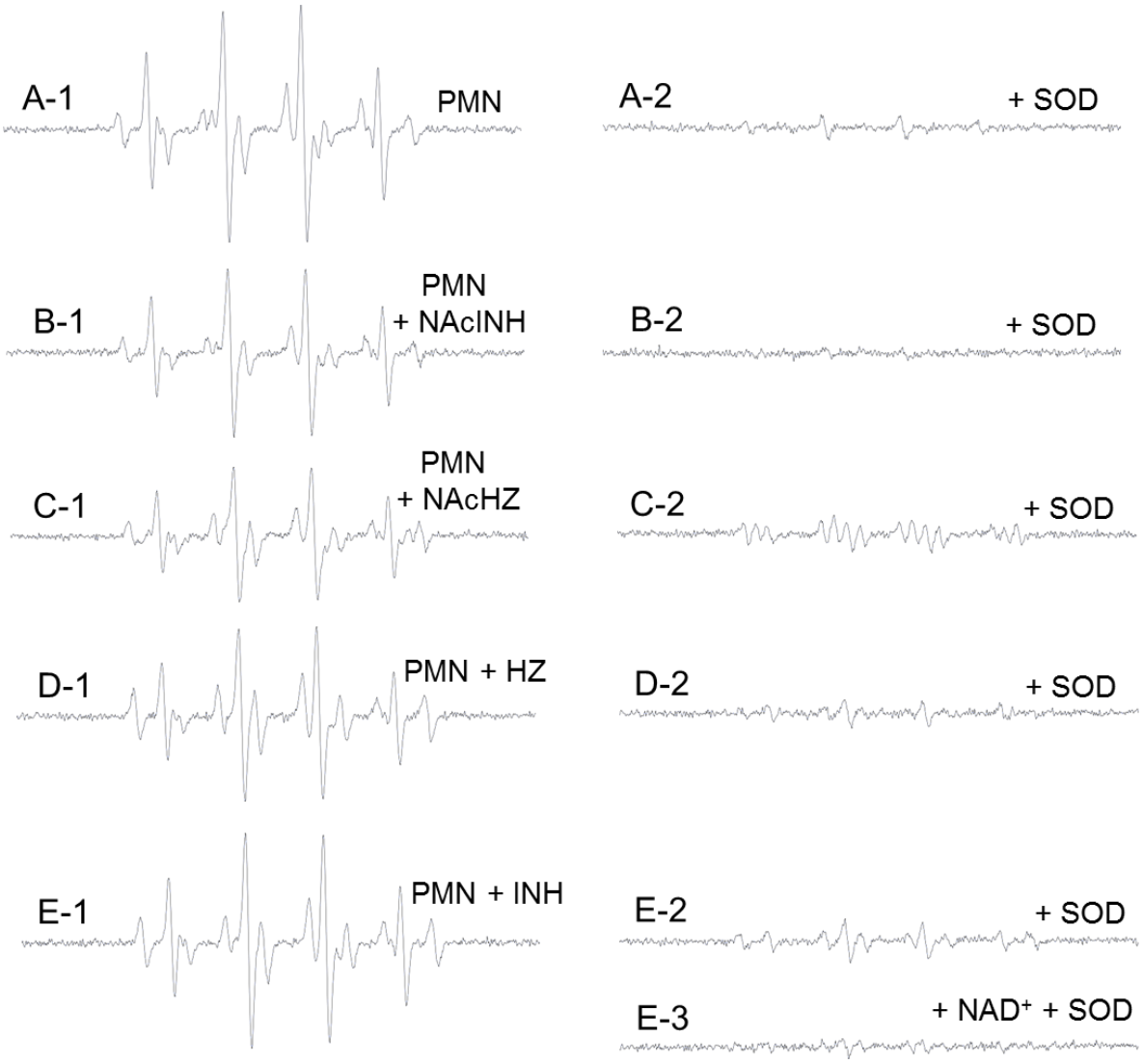


Figure 2. EPR study of INH and its metabolites in human neutrophil activated by PMA. Each 200 µl of reaction contains $6x10^4$ neutrophils activated by 0.8 µM of PMA, and 100 mM DMPO. 2 mM of either INH or any of its metabolites (NAcINH, NAcHZ and HZ) was used for treatment. 2.5 µM of SOD was added to eliminate spectra arising from the trapping of superoxide ($O_2^{\bullet-}$) due to activation of neutrophils. In addition, 2 mM of NAD^+ was further used to find the role of NAD^+ in each reaction. All reactions were carried out in chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA. A-1 is the typical spectrum of activated neutrophils, and A-2 is the same reaction after adding SOD. B-1 is the spectrum of activated neutrophils treated with NAcINH, and B-2 is the same reaction after addition of SOD. C-1 is the spectrum of activated neutrophils treated with NAcHZ, and C-2 is the same reaction after addition of SOD. D-1 is the spectrum of activated neutrophils treated with HZ, and D-2 is the same reaction after addition of SOD. E-1 is the spectrum of activated neutrophils treated with INH, and E-2 is the same reaction after addition of SOD. The further addition of NAD^+ into the reaction E-2 has been shown in E-3.

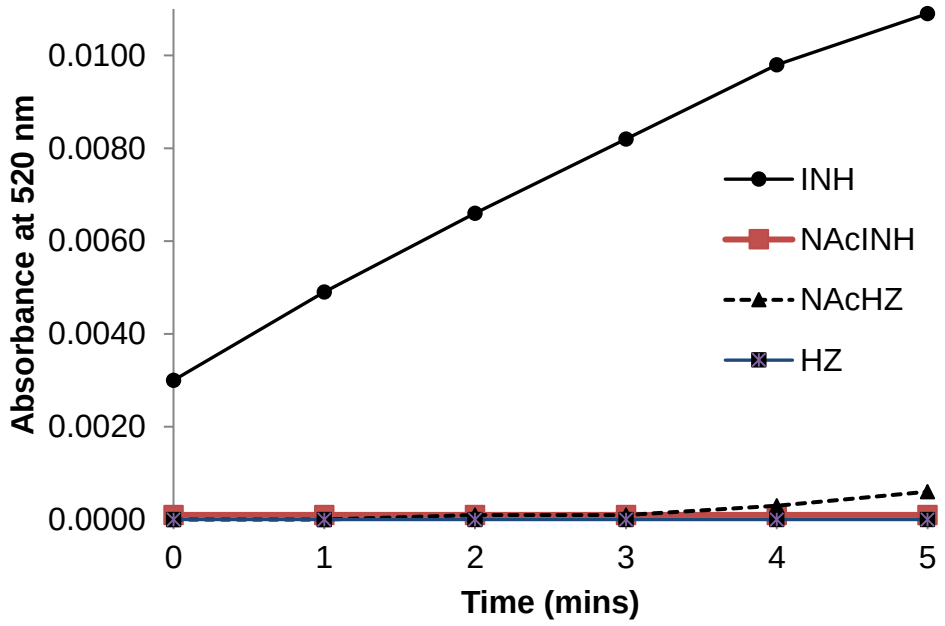


Figure 3. NBT reduction assay of INH and its human metabolites. Reactions contained 1 mM of either INH, NAcHZ, AcINH, or HZ in the mixture of 200 µM NBT, 50 nM MPO, and 50 µM H_2O_2. The absorption of the reduced NBT (formazan) was measured at 520 nm. All reactions were carried out in chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA.

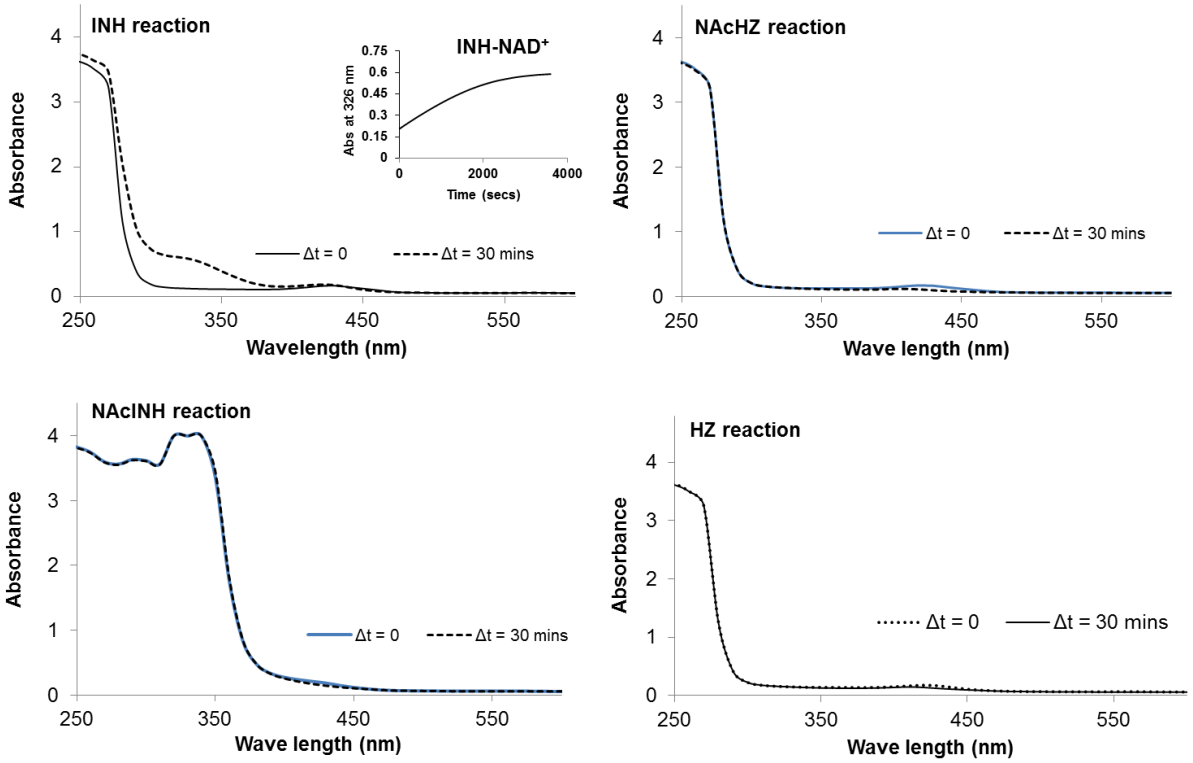


Figure 4. UV-Vis study for drug-NAD$^+$ adduct formation in MPO system. 500 µM of each chemical (INH, NAcINH, NAcHZ, and HZ) was exposed to 500 µM of NAD$^+$, 1 µM of MPO, 10 mU GOx and 5 mM glucose. Each reaction was carried for 30 minutes followed by compared the spectrum (Δt = 30 mins) with the initial time point (Δt = 0) spectrum. INH showed significant changes of spectra over time. A UV-Vis kinetic spectroscopy was carried out for INH at 326 nm (inset of INH reaction). It showed the formation of INH-NAD$^+$ adduct over time. All reactions were carried out in chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA.

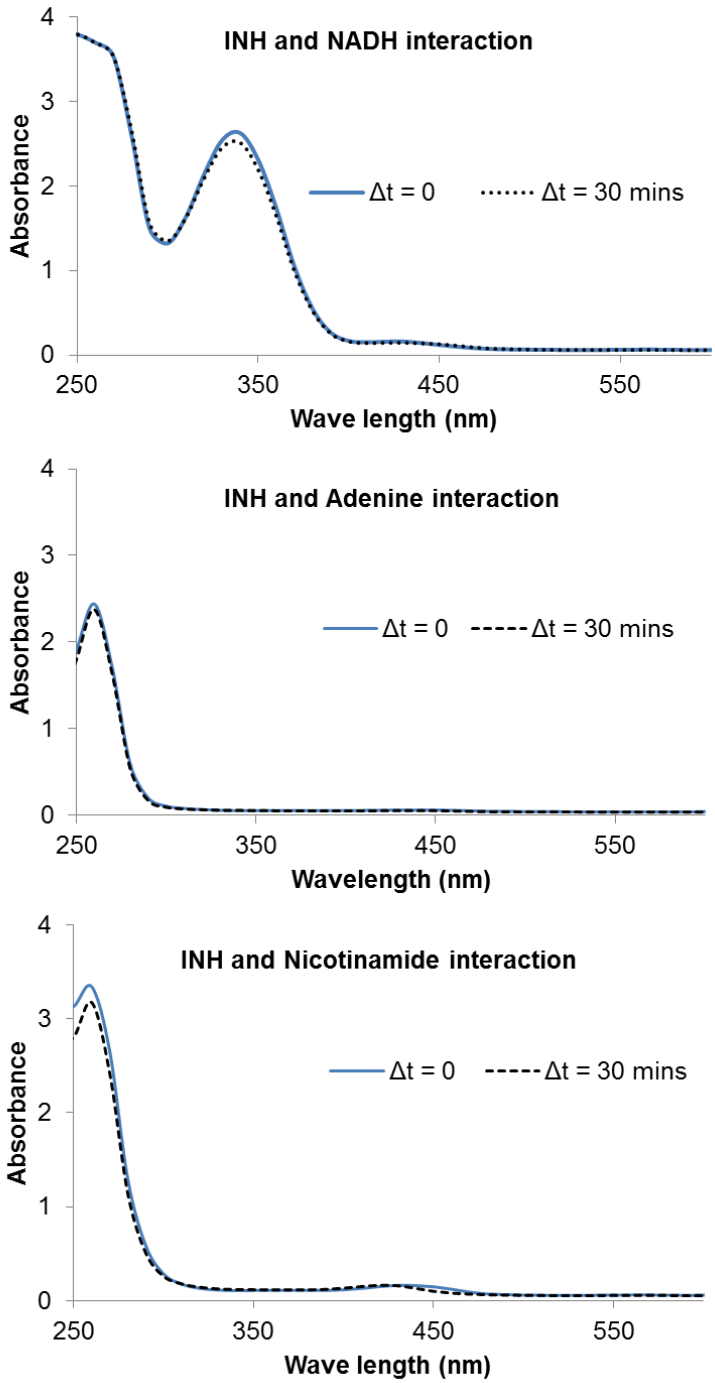


Figure 5. UV-Vis studies for INH interactions with any of these compounds (NADH, adenine and nicotinamide) in MPO system. 500 µM of each compound (NADH, adenine and nicotinamide) was exposed to 500 µM of NAD^+, 1 µM of MPO, 10 mU GOx and 5 mM glucose. Each reaction was carried for 30 minutes followed by compared the spectrum (Δt = 30 mins) with the initial time point (Δt = 0) spectrum. The results showed that none of them are reactive with INH. All reactions were carried out in chelex-100-treated 0.1 M sodium phosphate buffer (pH 7.4) containing 100 µM DTPA

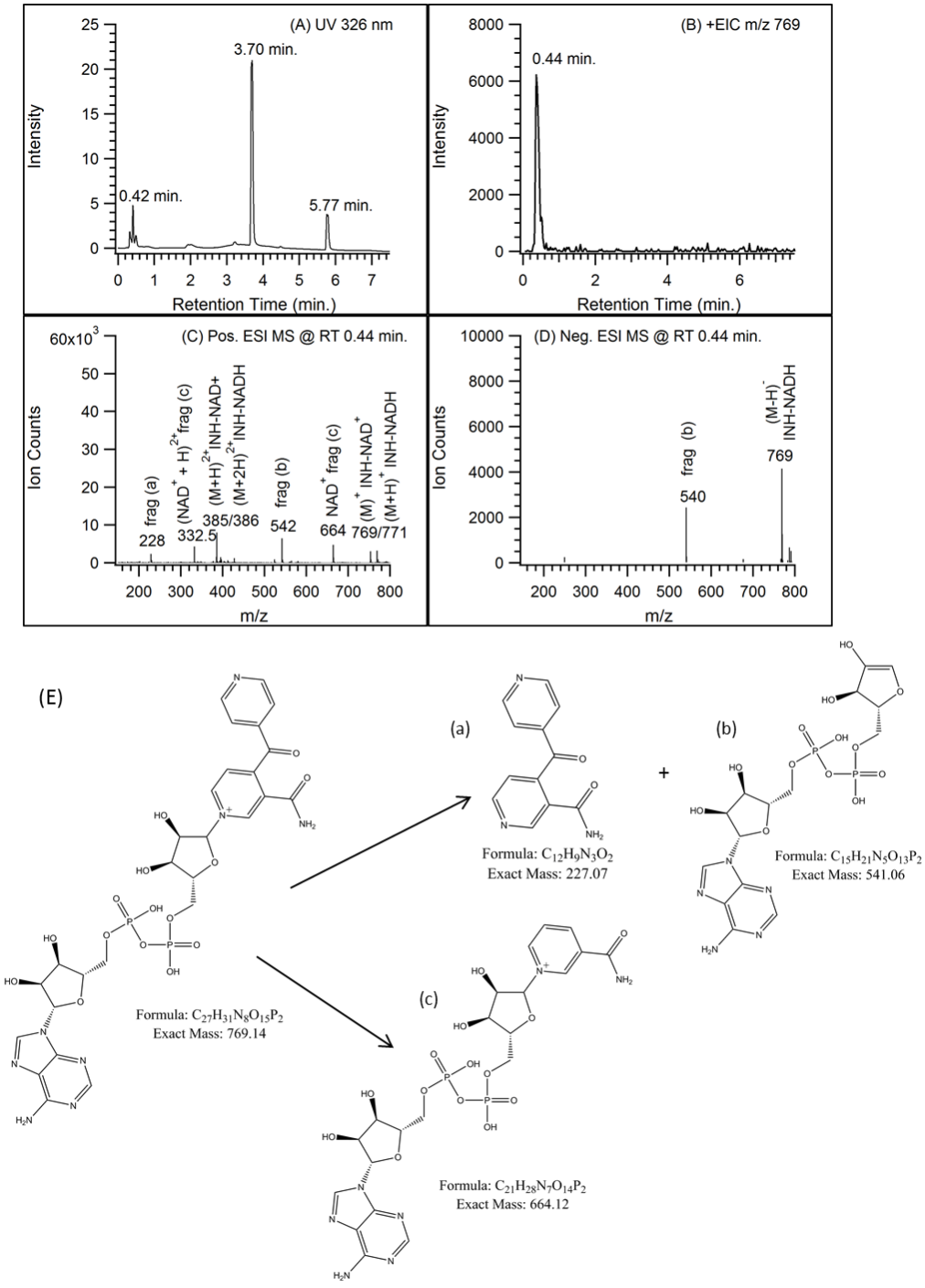
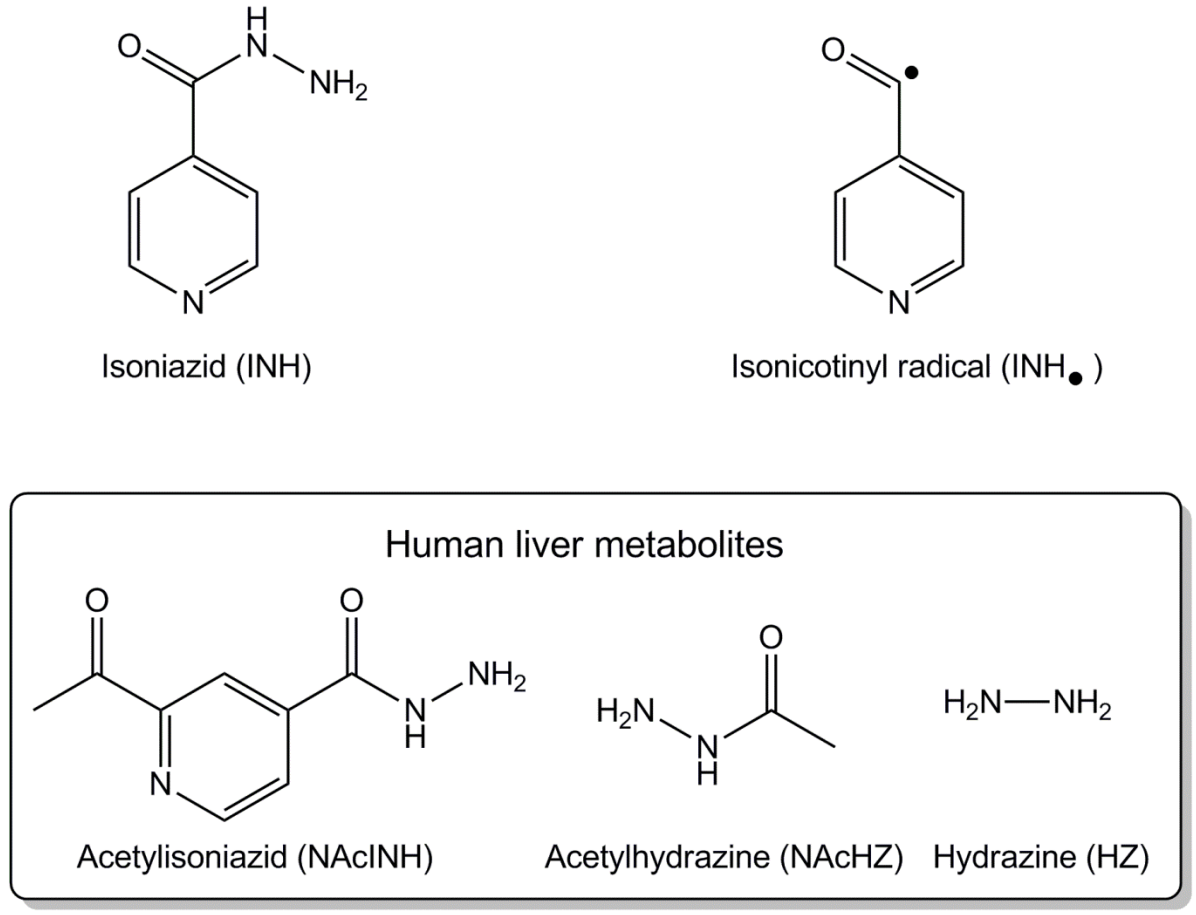
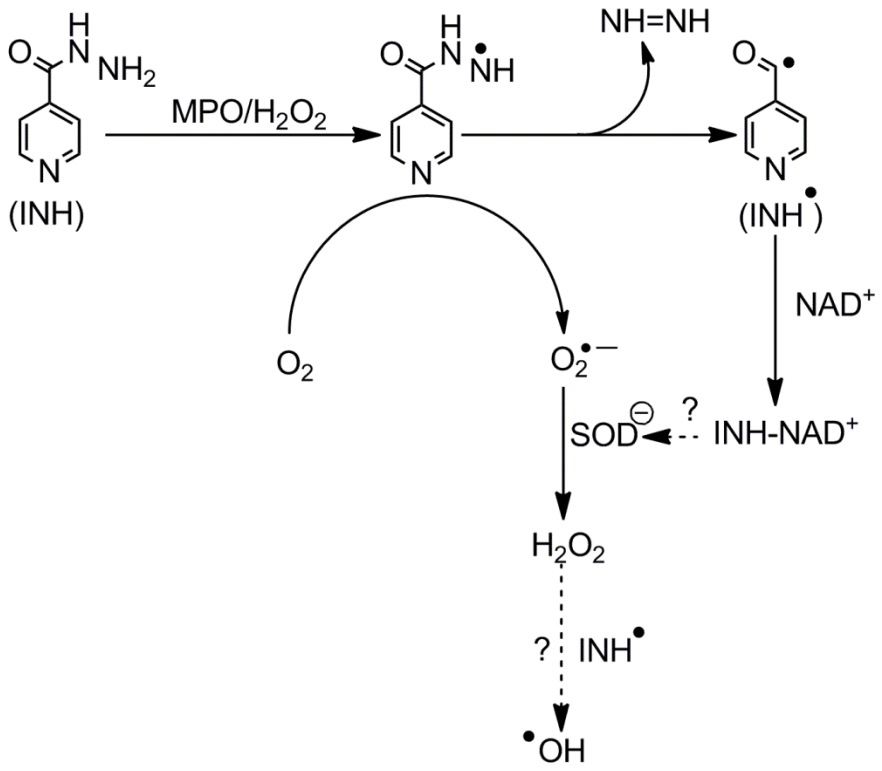


Figure 6. LC-MS study of INH-NAD$^+$ adduct formation in MPO system. (A) Three major peaks at λ = 326 nm in LC chromatogram at retention times 0.42, 3.70, and 5.77 min. (B) The LC-MS positive ion extracted-ion chromatogram for INH-NAD$^+$ at m/z 769. (C) The positive ion ESI mass spectrum at RT 0.44 min showing INH-NAD$^+$ (M^+) at m/z 769 and its doubly charged peak at m/z 385 as well as expected fragments at m/z 228, 542 and 664. INH-NADH $(M+H)^+$ at m/z 771 was also observed (see text for explanation). (D) The negative ion mass spectrum at RT 0.44 min showing INH-NADH $(M-H)^-$ at m/z 769 and the neutral-loss fragment at m/z 540 (see test for explanation). (E) The proposed structures of the INH-NAD$^+$ fragments observed in the mass spectrum.



Scheme 1. Chemical structure of compounds used in this study.



Scheme 2: The summary of INH interactions with MPO/H_2O_2 system in presence of SOD and SOD/NAD^+. INH was oxidized into isonicotinyl radical (INH•) by activated MPO. It enhanced superoxide ($O_2^{\bullet-}$) formation via oxygen reduction. In presence of SOD, $O_2^{\bullet-}$ is converted into H_2O_2 which we speculate is further reduced to •OH due to the presence of INH• (which is a strong reducing intermediate species). In presence of NAD^+, INH• forms INH-NAD^+ which may have an inhibitory effect on SOD (based on EPR spectra)**.** The dashed arrows indicate speculated pathways which require further study.