The Postulated Hepatotoxic Metabolite of Methimazole Causes Mitochondrial Dysfunction and Energy Metabolism Disturbances in Liver

Hossein Niknahad^1,2, Akram Jamshidzadeh^1,2, Reza Heidari^1*, Narges Abdoli^3, Mohammad Mehdi Ommati^4, Faezeh Jafari^2, Mehdi Zarei^2, Behnam Asadi^2

^1Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
^2Department of Pharmacology and Toxicology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.
^3Iran Food and Drug Administration (IFDA), Ministry of Health, Tehran, Iran.
^4Department of Animal Science, School of Agriculture, Shiraz University, Shiraz, Iran.

Introduction
Drug-induced liver injury (DILI) is a major clinical complication.1,2 Hepatotoxicity is a serious adverse effect associated with antithyroid drugs.3,4 Methimazole (Methyl mercapto imidazole, MMI, Figure 1) is the most prescribed drug against hyperthyroidism in human.5 On the other side, a number of adverse effects such as dermal and gastrointestinal disorders, loss of taste, and lupus-like syndrome are attributed to methimazole.6,7 Methimazole also causes dangerous and potentially fatal adverse effects such agranulocytosis and hepatotoxicity.8-10 Several cases of methimazole-induced liver injury have been reported.4,11-13 Although the precise mechanism(s) of methimazole-induced liver injury is not known, but oxidative stress and its associated events might play a role in methimazole hepatotoxicity.14-16

Figure 1. Chemical structure of methimazole (left) and N-methyl thiourea (right) as its proposed hepatotoxic metabolite.

The importance of drug bioactivation and its relevance to DILI has been widely investigated.17

*Corresponding Author: Reza Heidari, E-mail: rezaheidari@hotmail.com
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(CYP450) and flavin monooxygenases (FMO) enzyme to metabolites which are suspected to be cytotoxic.\textsuperscript{16,18} N-methyl thiourea (NMT, Figure 1) is the postulated toxic metabolite of methimazole in the liver as well as in other tissues.\textsuperscript{16,19} NMT secondary metabolites might also covalently bind to proteins, a mechanism which could be involved in olfactory system and liver injury after methimazole therapy.\textsuperscript{14,16,18}

Mitochondrial toxicity sometimes plays a pivotal role in the etiology of DILI.\textsuperscript{20,21} Drug-induced mitochondrial dysfunction might lead to cell apoptosis and finally organ injury.\textsuperscript{20} Some investigations indicate the importance of mitochondrial injury in methimazole-induced toxicity.\textsuperscript{19,22} Previously we also found that methimazole caused mitochondrial membrane potential collapse in freshly isolated rat hepatocytes.\textsuperscript{19,23}

The current study was designed to evaluate the effect of NMT, as the hepatotoxic metabolite of methimazole, on liver mitochondria in two experimental models. The data obtain from this investigation will help to clear the mechanism of methimazole-induced liver injury, and perhaps the development of preventive/therapeutic strategies against this complication.

**Materials and Methods**

**Chemicals**

Sodium succinate, 4,2-Hydroxyethyl,l-\textit{piperazine}ethanesulfonic acid (HEPES), 3-\textit{(N-morpholino)propano} acid (MOPS), N\textit{methyl thiourea, Dimethyl sulfoxide (DMSO), D-mannitol, Fatty acid-free bovine serum albumin (BSA)} fraction V, 2-Thiobarbituric acid (TBA), 3-\textit{[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dithiobis-2-nitrobenzoic acid (DTNB), Glutathione (GSH), 2',7'-Dichlorofluorescein diacetate (DCFH-DA), Malondialdehyde (MDA), Sucrose, Rhodamine123 (Rh 123), Coomassie brilliant blue, Ethylene glycol-bis (2-aminoethyl)ether)-N,N,N',N'-tetraacetic acid (EGTA), and Ethylene diamine tetra acetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acid (TCA) and hydroxyethylaminomethane hydrochloride (Tris-HCl) were obtained from Merck (Dardamstd, Germany). Kits for evaluating plasma biochemistry were obtained from Pars Azmun (Tehran, Iran). All salts for preparing buffer solutions were of analytical grade and prepared from Merck (Dardamstd, Germany).

**Animals**

Male BALB/c mice (20-25 g, n=96) were obtained from Animal Breeding Center of Shiraz University of medical sciences, Shiraz Iran. Mice were housed in cages on wood bedding at a temperature of 23±1°C and relative humidity of 40%. Animals had free access to tap water and a standard chow diet. Mice were handled according to the animal handling protocol approved by a local ethics committee at Shiraz University of Medical Sciences, Shiraz, Iran (95-01-36-11415).

**In vivo experimental design**

Mice (n=40) were treated with increasing doses of NMT (10, 20, 40 and 80 mg/kg, i.p). As there is no previous \textit{in vivo} investigation on NMT toxicity, we used lower and higher doses of this chemical to evaluate its hepatotoxic profile. Moreover, a preliminary investigation revealed that NMT caused animals death at a dose of 100 mg/kg, i.p. Five hours after NMT administration, animals were sacrificed by cervical dislocation and their liver mitochondria was isolated based on a previously reported procedure.\textsuperscript{24}

**Plasma biochemistry**

A Mindray\textsuperscript{24} BS-200 auto-analyzer and standard kits (Pars Azmun, Tehran, Iran) were used to evaluate plasma LDH, ALT, and AST in NMT-treated mice.

**In vitro experiments**

Isolated liver mitochondria from healthy mice (n=56) were incubated with different concentrations of NMT (10 µM-160 mM) and the mitochondrial indices were evaluated.\textsuperscript{24}

**Liver Mitochondria isolation**

Mice liver mitochondria were isolated as previously described.\textsuperscript{24} Briefly, animals were sacrificed by cervical dislocation and their liver was rapidly excised and washed with ice-cold saline (sodium chloride 0.9%, 4°C).\textsuperscript{24,25} The liver was homogenized in a buffer containing 75 mM mannitol, 225 mM sucrose, 0.5 mM EGTA, 2 mM MOPS, 0.1% BSA (pH = 7.4) at a 10:1 buffer to liver (v/w) ratio.\textsuperscript{24} The liver homogenate was centrifuged at 1000 g for 10 minutes at 4°C to remove intact cells, cell debris, and nuclei. The supernatants were further centrifuged (10000 g, 4°C, 10 minutes) to precipitate the heavy membrane fractions (mitochondria).\textsuperscript{24} This step was repeated three times using fresh buffer medium. As mentioned, all manipulations for liver mitochondria isolation were performed at 4°C or on ice to minimize mitochondrial injury.\textsuperscript{24}

**Mitochondrial dehydrogenase activity (MTT assay)**

The 3-\textit{(4, 5-dimethylthiazol-2-yl)-2, the 5-diphenyltetrazolium bromide (MTT) assay was applied as a colorimetric method for determination of mitochondrial dehydrogenase activity in isolated liver mitochondria.\textsuperscript{21,26} For this purpose,
Mitochondrial suspension in a buffer containing 320 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH=7.4), was incubated with 40 µl of the MTT solution (5 mg/ml) at 37°C for 30 minutes. The product of purple formazan crystals was dissolved in 1 ml dimethyl sulfoxide (DMSO). Then, 100 µl of dissolved formazan was added to 96 well plate and the optical density at 570 nm was measured with an EPOCH plate reader (BioTek Instruments, Highland Park, USA). Samples protein concentrations were determined by the Bradford method.

**Reactive oxygen species (ROS) in isolated liver mitochondria**

The mitochondrial ROS measurement was performed using the fluorescent probe DCFH-DA. Briefly, isolated liver mitochondria were incubated in a buffer containing 125 mM sucrose, 65 mM KCl, 5 mM sodium succinate, and 10 mM HEPES (pH=7.2). Following this step, DCFH-DA was added (final concentration of 10 µM) to mitochondria and incubated for 30 min at 37°C in dark. Then, the fluorescence intensity of DCF was measured using an FLUOstar Omega® multifunctional fluorescent microplate reader (λ excitation=485 nm and λ emission=525 nm).

**Mitochondrial membrane potential**

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123, was used for the estimation of mitochondrial membrane potential. The mitochondrial fractions (0.5 mg protein/ml) were incubated with 10 µM of rhodamine 123 in a buffer containing 125 mM sucrose, 65 mM KCl, 5 mM sodium succinate, and 10 mM HEPES (pH=7.2) (30 min, 37°C). After centrifugation (15000 g, 5 min, 4°C), the fluorescence intensity of samples was monitored using a FLUOstar Omega® multifunctional fluorescent microplate reader at the excitation and emission wavelength of 485 nm and 525 nm, respectively.

**Mitochondrial glutathione (GSH) content**

Mitochondrial GSH level was determined by a spectrophotometric method using 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB). Briefly, the mitochondrial suspension was treated with ice-cooled trichloroacetic acid (10% w/v) to extract mitochondrial glutathione. The mixture was centrifuged (15000 g for 10 min, 4°C) to remove denatured proteins. Afterward, 100 µl of DTNB (0.04% in phosphate buffer) was added and the intensity of the produced yellow color was recorded at 412 nm with an ultraviolet spectrophotometer (Pharmacia biotech, Uppsala, Sweden).

**Mitochondrial Swelling assay**

Mitochondrial swelling was measured as previously described. The isolated mitochondria (0.5 mg protein/ml) were suspended in a buffer containing 125 mM sucrose, 65 mM KCl, 5 mM sodium succinate, and 10 mM HEPES (pH=7.2). Light absorbance at 540 nm was measured at the two-time points (10 and 70 minutes) at 25°C. It is accepted that a decreased light absorbance is consistent with an increase in mitochondrial volume. Hence, as mitochondria are more swelled, the differences between light absorbance of two-time points are higher. The differences between the absorbance of samples were assessed (ΔOD250 nm) and compared in different experimental groups.

**Lipid peroxidation in liver mitochondria**

Thiobarbituric acid reactive substances (TBARS) were measured as an index of lipid peroxidation in isolated liver mitochondria by a previously reported procedure. Isolated mitochondria were washed to remove sucrose in an ice-cold buffer of MOPS-KCl (50 mM MOPS, 100 mM KCl, pH=7.4), and re-suspended in fresh MOPS-KCl buffer. Afterward, the mitochondrial suspension was mixed with twice its volume of a solution containing trichloroacetic acid (15% w/v), thiobarbituric acid (0.375% w/v), and 0.24 N HCl plus 0.5 mM Trolox. The mixture was heated for 15 min at 100°C. After centrifugation (15000 g, 10 min), the absorbance of the supernatant was assessed at 532 nm with an Epoch plate reader (BioTek Instruments, Highland Park, USA).

**Mitochondrial ATP level**

A luciferase–luciferin-based kit (ENLITEN® from Promega) was used to assess mitochondrial ATP content. Samples and buffer solutions were based on the kit instructions and the luminescence intensity of samples was measured at 560 nm using a FLUOstar Omega® multifunctional microplate reader.

**Statistical analysis**

Data are given as the Mean±SD. Data comparison was performed by the one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons as the post hoc test. Differences were considered statistically significant when P <0.05.

**Results**

Adverse effects of NMT on mice liver was biochemically evident as elevated plasma biomarkers of liver injury (Figure 2). It was found that plasma ALT, LDH, and AST levels were significantly increased in NMT-treated mice (Figure 2). Evaluation of liver mitochondria isolated from NMT-treated animals revealed a decrease in SDA,
and mitochondrial membrane potential collapse (Figure 3). Moreover, mitochondrial swelling and reactive oxygen species were increased in liver mitochondria of NMT-treated animals (Figure 3).

Figure 2. Plasma biomarkers of liver injury in N-methyl thiourea-treated mice. NMT: N-methyl thiourea. Data are given as Mean±SD (n=8). Asterisks indicate significantly different as compared with control (*P<0.05, **P<0.01, ***P<0.001).

Figure 3. Effect of N-methyl thiourea (NMT) on liver mitochondria isolated from drug-treated mice. NMT decreased SDA activity (A), collapsed mitochondrial membrane potential (B), increased mitochondrial swelling (C), and enhanced ROS formation (D), in mice liver mitochondria. Data are given as Mean±SD (n=8). Asterisks indicate significantly different as compared with control (*P<0.05, **P<0.01, ***P<0.001).
Further evaluation of liver mitochondria isolated from NMT-treated mice revealed decreased mitochondrial glutathione reservoirs and ATP content (Figure 4). Mitochondrial lipid peroxidation was also increased in NMT-treated (40 and 80 mg/kg) animals (Figure 4). A marked decrease in mitochondrial succinate dehydrogenase activity (MTT assay) was detected when isolated liver mitochondria were incubated with different NMT concentrations (Figure 5).

Concentrations of NMT greater than 10 µM, significantly decreased mitochondrial succinate dehydrogenase activity (Figure 5). Further assessment of liver mitochondria treated with NMT, revealed a marked increase in mitochondrial ROS formation (Figure 5). Moreover, NMT caused a collapse in mitochondrial membrane potential (Figure 5), and an increase in mitochondrial swelling (Figure 5).

**Figure 4.** Liver mitochondrial ATP level, glutathione content, and mitochondrial lipid peroxidation in NMT-treated mice. NMT: N-methylthiourea.
Data are given as Mean±SD (n=8). Asterisks indicate significantly different as compared with control (*P<0.05, **P<0.01, ***P<0.001). ns: not significant as compared to control (P > 0.05).

**Figure 5.** Effect of N-methyl thiourea (NMT) on isolated mice liver mitochondria. NMT caused a decrease in SDA activity (A), ROS formation (B), mitochondrial swelling (C), and collapse in mitochondrial membrane potential (D), in isolated mice liver mitochondria. Data are given as Mean±SD (n=8). Data in colored boxes are significantly different as compared with 0 mM NMT (P < 0.001). ns: not significant as compared to control (0 mM NMT) (P > 0.05).
It was also detected that mitochondrial glutathione stores and ATP level were decreased in NMT-treated liver mitochondria (Figure 6). NMT administration (0.5-160 mM) also increased mitochondrial lipid peroxidation (Figure 6).

Discussion

Antithyroids hepatotoxicity is a clinical complication. Methimazole is the most convenient antithyroid drug in human. However, there are several cases of liver injury induced by this drug. There is no clear idea on the mechanism of methimazole-induced liver injury. However, methimazole reactive metabolites seem to be involved in the liver injury induced by this drug. The ring cleavage metabolite of methimazole, NMT is a postulated hepatotoxic intermediate of this drug. The exact mechanism of cell injury induced by NMT has not been revealed so far. Previously, we found that liver enzyme induction drastically deteriorated methimazole-induced hepatotoxicity. All these data indicate the importance of methimazole reactive metabolites in the hepatotoxic effects of this drug.

Mitochondrial dysfunction is contributed in the mechanism of cellular injury induced by several xenobiotics. Mitochondria are also major potential targets for many drugs to induce liver injury. Mitochondrial injury plays a crucial role in cell death and apoptosis process. Hence, drugs which affect this vital organelle might finally cause organ failure.

Previous investigations indicated that anti-thyroid drugs such as methimazole might affect hepatocytes mitochondria as revealed by a collapse in mitochondrial membrane potential (ΔΨm). The effects of methimazole on cellular mitochondria might be attributed to its reactive metabolites. Previous structure-toxicity relations suggest NMT as a hepatotoxic metabolite for methimazole.

In the current investigation, we found that NMT caused significant damage toward liver mitochondria. Hence, the mitochondrial injury might serve as a mechanism for methimazole reactive metabolites to induce liver injury (Figure 7).

It has been found that NMT is further metabolized by FMO or CYP enzymes to reactive sulfonic and sulfuric acid species (Figure 7). Sulfonic and sulfuric acids are reactive electrophilic species which are capable of interacting with several intracellular targets such as different proteins. In the current study, we found that NMT administration significantly damaged liver mitochondria both in vitro and in vivo. NMT reactive metabolites might also be involved in the mitochondrial injury induced by this chemical in vivo (Figure 7).

Figure 6. Effect of N-methyl thiourea (NMT) on ATP level (A), glutathione content (B), and mitochondrial lipid peroxidation (C). Data are given as Means±SD (n=8). Data in the colored box are significantly different as compared with control (0 mM NMT) (P < 0.001). ns: not significant as compared to control (0 mM NMT) (P > 0.05).
Glyoxal is another metabolite of methimazole. Glyoxal is a well-known mitochondrial toxin. It has been reported that a part of methimazole hepatotoxicity might be mediated by glyoxal. Hence, methimazole metabolites might synergistically disturb hepatocytes mitochondrial function (Figure 7).

Propylthiouracil (PTU) is also a thionamide antithyroid drug with several cases of severe hepatotoxicity. Mitochondria seems to be a target for PTU to induce cellular damage and toxicity. It has been found that shape and size of mitochondria were changed to giant mitochondria (megamitochondria) in PTU-induced hepatic injury. Furthermore, it has been observed that the inner and outer membrane of mitochondria were fragmented and their matrices were lytic in PTU-induced hepatotoxicity. The direct effect of PTU on isolated liver mitochondria is under investigation in our department (Unpublished data). There is no investigation on the role of drug metabolism in the PTU-induced liver injury. The involvement of the thionamide metabolite of PTU and its effects on vital organelles such as mitochondria might clear the mechanisms of hepatotoxicity induced by this drug.

The toxic properties of several other thionamide-based structures are well known. Thiourea is the parent compound for many drugs and industrial agents. Some antituberculosis agents, centrally acting histamine H3 antagonists, and anti-HIV reverse transcriptase (RT) inhibitors, are among thiourea-containing drugs. Interestingly, the hepatic injury is a serious adverse effect associated with the mentioned drugs. Hence, the thiourea chemical structure and its potential adverse effects toward liver mitochondria might play a role in the mechanism of hepatotoxicity induced by these drugs.

**Conclusion**

Collectively, our data indicate that hepatocytes mitochondria might be a potential target for NMT-induced toxicity (Figure 7). These data could help to clear the mechanism of hepatotoxicity induced by methimazole and other thionamide-based drugs. Furthermore, it might help to develop therapeutic or preventive strategies in drug development and pharmacotherapy.

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**Conflict of interests**

The authors claim that there is no conflict of interest.

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