



Propylthiouracil-Induced Mitochondrial Dysfunction in Liver and its Relevance to Drug-Induced Hepatotoxicity

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ABSTRACT

Background: Propylthiouracil (PTU) administration is associated with several cases of hepatotoxicity, especially in children. The mechanism(s) of PTU-induced hepatotoxicity is obscure. In the current study, we aimed to assess the effect of PTU on hepatocytes mitochondria in different experimental models.

Methods: Mice were treated with PTU (10, 20, 40, 80, and 100 mg/kg, i.p) then, the liver mitochondria were isolated and evaluated. Moreover, liver mitochondria were isolated from normal mice and incubated with increasing concentrations of PTU (10 μ M-1 mM). Mitochondrial dehydrogenases activity, mitochondrial membrane potential, mitochondrial swelling, and mitochondrial adenosine triphosphate (ATP) content were monitored.

Results: PTU hepatotoxicity was biochemically evident in mice by increased serum biomarkers of liver injury. PTU also caused a decrease in mitochondrial dehydrogenases activity, increased mitochondrial swelling, depleted mitochondrial ATP, and caused mitochondrial depolarization both *in vitro* and *in vivo*.

Conclusion: Our data suggest mitochondrial dysfunction as a mechanism for PTU-induced hepatotoxicity.

Introduction

Drug-induced hepatotoxicity is a big challenge in pharmacotherapy and drug development.^{1,2} Antithyroid drugs-induced hepatotoxicity is a clinical complication.^{3,4} Propylthiouracil (PTU) is prescribed against hyperthyroidism especially in situations such as pregnancy.⁵ On the other hand, PTU administration is associated with several adverse drug reactions including taste loss, gastrointestinal disturbances, and lupus-like syndrome.^{6,7} PTU also causes serious adverse effects such as agranulocytosis and liver failure.⁸⁻¹⁰ Several cases of PTU-induced hepatotoxicity have been reported.^{4,11-16} Pediatrics seems to be more vulnerable to PTU-induced hepatotoxicity.¹⁷ There is no precise mechanism for PTU-induced liver injury.^{12,18}

The etiology of drug-induced liver injury sometimes underlies mitochondrial toxicity.¹⁹ Mitochondrial dysfunction could play a pivotal role in cell death and organ failure.¹⁹ Previous studies mention the role of mitochondrial dysfunction in thioamide-based drugs toxicity.^{20,21} PTU is a

thionamide antithyroid drug. Previously, we also found that methimazole as another thionamide antithyroid drug caused mitochondrial injury in isolated rat hepatocytes.^{20,22}

In the current study, we aimed to investigate the effect of PTU on hepatic mitochondria in two different experimental models. The data obtain from this study might help to provide therapeutic/preventive options against PTU-induced hepatotoxicity.

Materials and Methods

Chemicals

3-(N-morpholino)propane sulfonic acid (MOPS), Ethylene diamine tetra acetic acid (EDTA), Dimethyl sulfoxide (DMSO), Sodium succinate, 6-Propyl-2-Thio uracil (PTU), 4,2-Hydroxyethyl,1-piperazineethanesulfonic acid (HEPES), D-mannitol, Rhodamine123 (Rh 123), 2,3-[4,5dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Fatty acid-free bovine serum albumin (BSA) fraction V, Sucrose, Coomassie brilliant blue, and Ethylene glycol-bis (2-

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aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydroxymethyl aminomethane hydrochloride (Tris-HCl) and Trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). The luciferase–luciferin-based kit for ATP measurement was from Promega (ENLITEN®). The kits for serum biochemistry were obtained from Pars Azmun® (Tehran, Iran). All other chemicals used for buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

Animals

Male BALB/c mice (20-25 g, n=84) were obtained from Comparative and Experimental Medicine Research Center, Animal Breeding Department, Shiraz University of medical Sciences, Shiraz Iran. Mice were housed in cages on chip-wood bedding at a constant temperature of 23±1°C and relative humidity of 40%. Animals had free access to tap water and a typical chow diet. Mice received human care and handled according to an animal handling protocol approved by a local ethics committee at Shiraz University of Medical Sciences, Shiraz, Iran (#11453).

In vivo experiments

Mice (n=42) were treated with PTU (10, 20, 40, 80, and 160 mg/kg, i.p). The control animals received PTU vehicle (Normal saline, 5 ml/kg, i.p). Five hours later, mice were sacrificed and their liver mitochondria was isolated based on a previously reported protocol.^{23,24}

Serum biochemistry and liver histopathology

Blood samples were collected from vena cava of the anesthetized animals (Thiopental, 50 mg/kg, i.p). Samples was transferred to standard tubes (Improvacuter®, gel and clot activator-coated tubes; Guangzhou, China) and blood serum was prepared by centrifugation (3000 g, 4°C, 15 min.). The liver tissue was removed and washed in ice-cold sodium chloride solution (0.9% w:v). A Mindray® BS-200 auto-analyzer (Mindray chemistry analyzers for low-volume laboratories, Guangzhou, China) and commercial kits (Pars Azmun®, Tehran, Iran), were used to evaluate serum lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate amino transferase (AST) in PTU-treated mice.^{25,26} Liver samples were fixed in buffered formalin solution (0.4% sodium phosphate monobasic, NaH₂PO₄, 0.64% sodium phosphate dibasic, Na₂HPO₄, and 10% formaldehyde in distilled water) and used for tissue histopathological assessment.^{27,28} Paraffin-embedded blocks of liver tissue were prepared and stained with haematoxylin and eosin (H&E) before viewing by a light microscope

(Olympus CX21®, Japan).

In vitro experimental design

Increasing concentrations of PTU (10 µM-1 mM) were added to isolated liver mitochondria and several mitochondrial indices were assessed. Control mitochondria received no specific treatment and incubated at the same conditions.

Liver mitochondria isolation

Mice liver mitochondria were isolated by differential centrifugation method based on a previously reported protocol.²³ Briefly, animals were sacrificed by cervical dislocation and their liver was quickly excised and washed with cold (4°C) sodium chloride solution (0.9% w: v).^{23,29} The liver tissue (at a 10:1 buffer to liver ratio) was homogenized in a solution containing 225 mM sucrose, 75 mM mannitol, 2 mM MOPS, 0.1% BSA, and 0.5 mM EGTA (pH = 7.4).²³ Tissue homogenate was centrifuged (1000 g, 4°C, 10 minutes) to remove cell debris, intact cells, and cell nuclei. To precipitate mitochondrial fraction, the supernatants were further centrifuged at 10000 g, 4°C, for 10 minutes.²³ Using fresh buffer medium, the last step was repeated three times. As mentioned, all manipulations during mitochondrial isolation process were performed on ice (4°C) to minimize mitochondrial injury.²³

Mitochondrial dehydrogenase activity (MTT assay)

A colorimetric method based on the 3-(4, 5-dimethylthiazol-2-yl)-2, the 5-diphenyltetrazolium bromide reduction (MTT assay) was used for assessment of mitochondrial dehydrogenases activity.^{30,31} Liver 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 (1 mg/ml) at 37°C for 30 minutes in dark. 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).^{31,32} Samples protein concentrations were determined by the Bradford method.³³

Mitochondrial membrane potential

Rhodamine 123 was used as the cationic fluorescent probe for assessment of mitochondrial membrane potential.^{23,32} Briefly, fractions (0.5 mg protein/ml) of prepared liver mitochondria were incubated with rhodamine 123 (Final concentration of 10 µM) in a buffer containing 65 mM KCl, 125 mM sucrose, 10 mM HEPES and 5 mM sodium succinate (pH=7.2, 30 min, 37°C). Samples were centrifuged (15,000 g, 10 min, 4 °C), and the

fluorescence intensity of the supernatant was measured using a FLUOstar Omega® multifunctional fluorescent microplate reader ($\lambda_{excitation} = 485 \text{ nm}$ and $\lambda_{emission} = 525 \text{ nm}$).^{23,31}

Mitochondrial Swelling assay

Monitoring the decrease in light absorbance at 540 nm was used as method assess mitochondrial swelling.²³ Briefly, mitochondrial samples (0.5 mg protein/ml) were suspended in swelling buffer (125 mM sucrose, 5 mM sodium succinate, 65 mM KCl, and 10 mM HEPES, pH=7.2). To start mitochondrial swelling, calcium (200 μM) was added after 5 minutes of samples incubation. Light absorbance ($OD_{540 \text{ nm}}$) was assessed using a FLUOstar Omega® multifunctional fluorescent microplate reader with constant shaking (100 rpm orbital shaking before each read) and temperature (30°C).^{23,34} A decrease in light absorbance is directly associated with an increase in mitochondrial volume.²³

Mitochondrial ATP level

A commercial kit based on luciferase–luciferin reaction (Promega, ENLITEN® ATP Assay System, Promega Corporation, Madison, USA) was used to monitor mitochondrial ATP level.^{32,35} Samples and buffer solutions were prepared based on the kit manual and the luminescence intensity was measured using a FLUOstar Omega® multifunctional microplate reader ($\lambda=560 \text{ nm}$).

Statistical analysis

Data are presented as the Mean±SD. The one-way analysis of variance (ANOVA) with Tukey's multiple comparison test as a *post hoc* was used for data comparison. $P < 0.05$ was considered as statistically significant difference between groups.

Results

PTU-induced hepatotoxicity in mice was revealed by significant elevation in serum biomarkers of liver injury (Figure 1). It was found that serum LDH, ALT, and AST levels were significantly elevated in PTU-treated animals (Figure 1). Moreover, liver histopathological changes were evident as inflammation, tissue necrosis, and sinusoidal dilation in PTU-treated animals (Figure 1).

Evaluation of liver mitochondria isolated from PTU-treated animals revealed a decrease in mitochondrial dehydrogenases enzyme activity (MTT assay), and the collapse of mitochondrial membrane potential ($\Delta\Psi$) (Figure 2). In addition, mitochondrial ATP level was decreased in mitochondria isolated from PTU-treated animals (Figure 3). Moreover, mitochondrial swelling was increased in the PTU-treated mice (Figure 4).

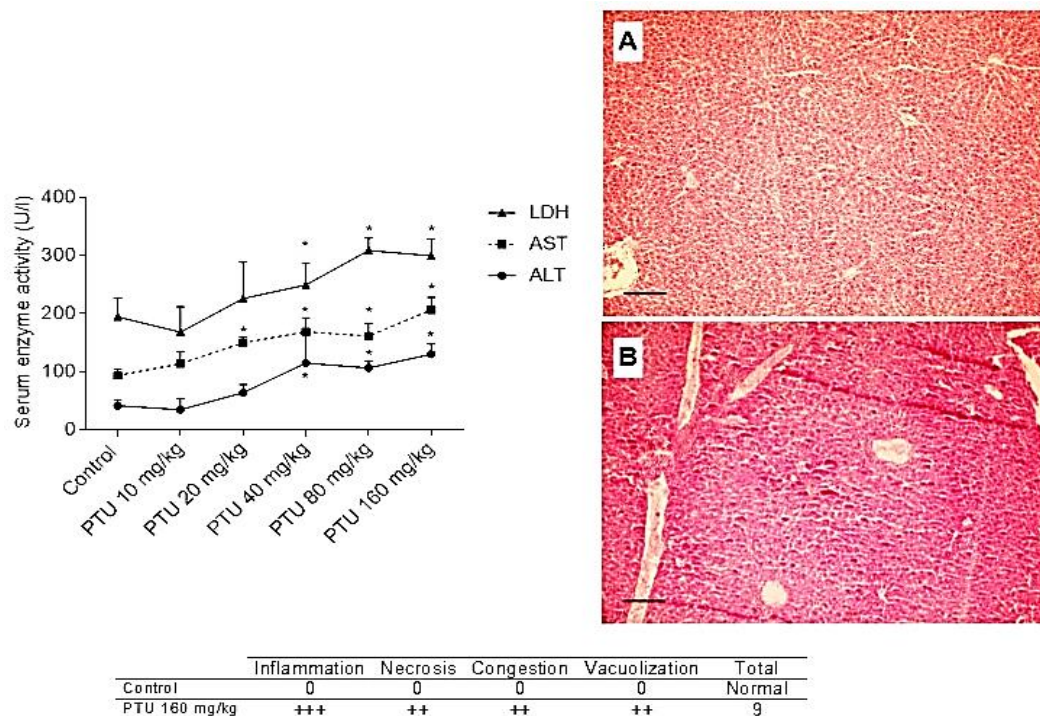


Figure 1. Serum biochemistry of PTU-treated mice. PTU: Propylthiouracil. Data are presented as Mean±SD (n=7) as assessed 5 hours after PTU administration. *Indicates significantly different as compared with control ($P < 0.01$). Histopathological evaluation (H&E staining) of mice liver revealed tissue necrosis, sinusoidal dilation and inflammatory cells aggregation in PTU-treated animals (B) in comparison with vehicle-treated group (A). Magnification 40x; Scale bar 1000 μm .

A significant decrease in mitochondrial succinate dehydrogenase activity (SDA) was also evident when isolated mitochondria were incubated with PTU (*In vitro*) (Figure 2). Further assessment of PTU-treated liver mitochondria, revealed a marked collapse of mitochondrial membrane potential

(Figure 2). It was also found that mitochondrial ATP content was depleted in PTU-treated groups (Figure 3). Moreover, when isolated liver mitochondria were incubated with PTU, an increase in mitochondrial swelling was detected (Figure 4).

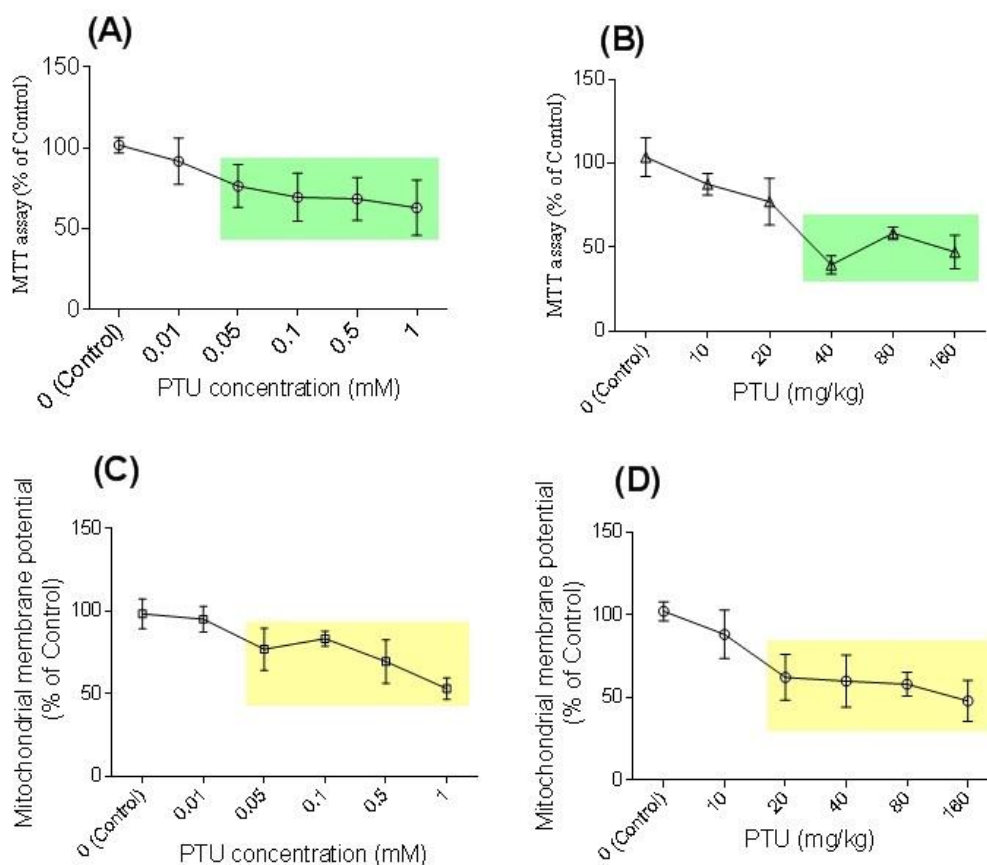


Figure 2. Effect of PTU on liver mitochondria. PTU: Propylthiouracil. PTU caused a decrease in succinate dehydrogenase (SDA) activity (MTT test) (A and B) and collapse in mitochondrial membrane potential (C and D).

Data are given as Mean±SD (n=7).

Data in colored boxes are significantly different as compared with control (P<0.001).

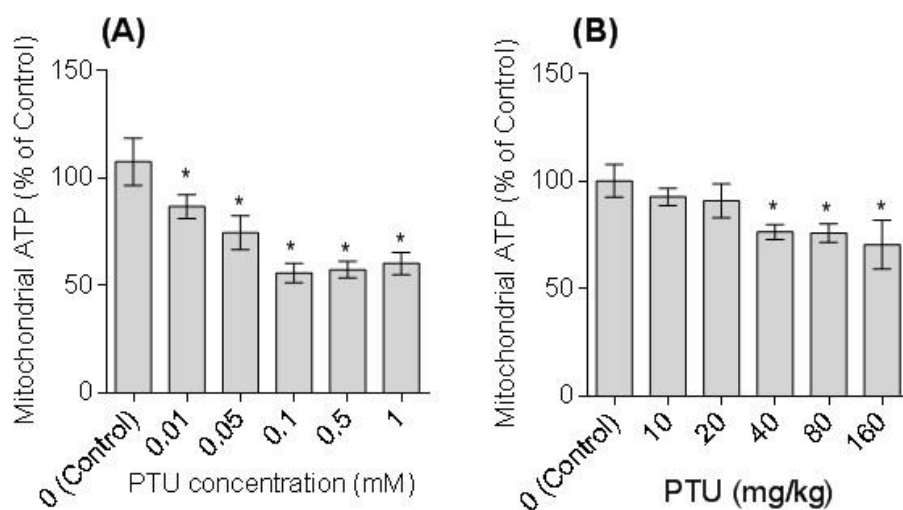


Figure 3. Effect of PTU on mitochondrial ATP level *in vitro* (A) and *in vivo* (B). PTU: Propylthiouracil. Data are given as Mean±SD (n=7).

*Indicates significantly different as compared with control (P<0.01).

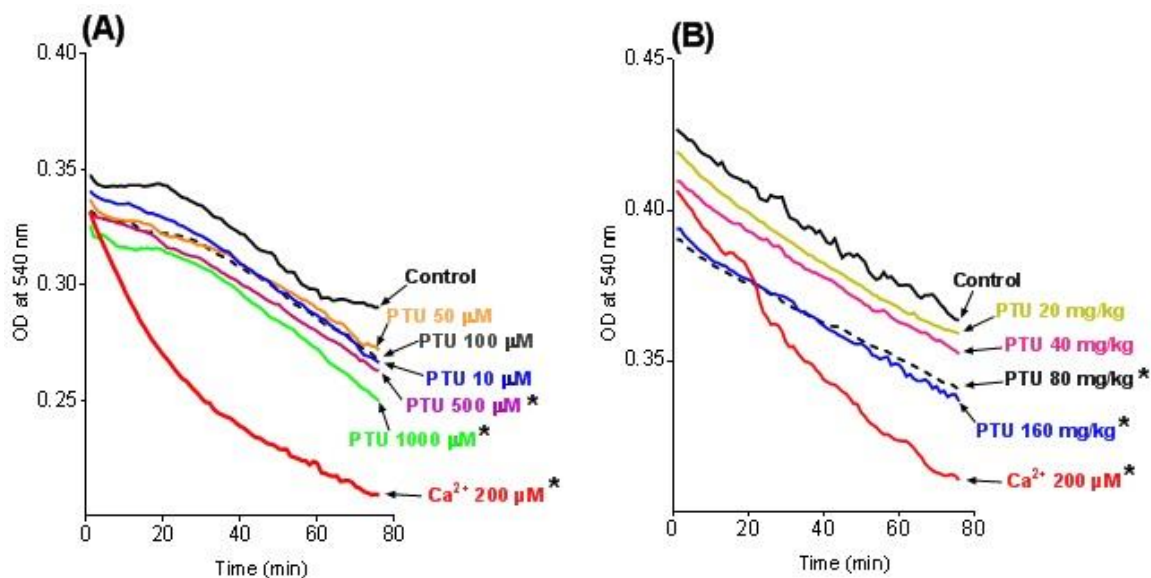


Figure 4. PTU-induced mitochondrial swelling *in vitro* (A) and *in vivo* (B). PTU: Propylthiouracil. Data are given as Mean \pm SD (n=7). *Indicates significantly different as compared with control (P<0.05).

Discussion

Hepatotoxicity is a major clinical complication associated with antithyroid drugs.^{36,37} There is several human cases of PTU-induced hepatotoxicity.^{15,38-39} The mechanism(s) of PTU hepatotoxicity is obscure. In this study, we found that PTU caused mitochondrial dysfunction both *in vitro* and *in vivo*. PTU administration caused mitochondrial depolarization, decreased succinate dehydrogenases activity and ATP level. Moreover, mitochondrial swelling was increased in PTU-treated groups.

Effects of PTU on liver could manifest as a transient elevation of serum transaminases to acute liver failure and death.^{11,13} On the other hand, clinicians advised frequent monitoring of liver function in PTU-treated patients.^{11,13,17,40-44} Hence, finding the mechanism(s) of PTU-induced hepatotoxicity might help to prevent or cure liver injury induced by this drug.

Previously, we found that PTU-induced liver injury was associated with oxidative stress and its associated complications in the liver.²⁴ Oxidative stress might play a role as a cause and/or the consequence of mitochondrial injury.⁴⁵ The mitochondrial injury is involved in the mechanism of hepatotoxicity induced by many xenobiotics.^{19,46,47} Mitochondrial injury could play a pivotal role in cell death and apoptosis process.^{48,49} Hence, xenobiotics-induced mitochondrial injury could lead to cell injury and hepatotoxicity.

It has been found that thionamide-based antithyroid agents such as methimazole caused mitochondrial injury which was revealed by severe mitochondrial depolarization (decreased $\Delta\Psi_m$).^{20,22} Methimazole reactive metabolites might play a role in its adverse

effects toward hepatocytes mitochondria.^{20,22} PTU is also a thionamide-based antithyroid agent with several cases of severe liver injury.^{44,50-52} Mitochondria seem to be a target for PTU-induced toxicity. Interestingly, megamitochondria (big mitochondria in shape and size) is a pathologic finding in PTU-induced hepatotoxicity.⁵³ Mitochondrial membrane fragmentation and lysis of the mitochondrial matrices has been observed in liver biopsies taken from human cases of PTU-induced hepatotoxicity.⁵³ All these findings might indicate that mitochondrial injury might be involved in the mechanism of PTU-induced liver injury.

Although the exact role of reactive metabolites of PTU in the liver has not been fully investigated, but this drug might convert to hepatotoxic metabolites.¹² The association between PTU reactive metabolite(s) and drug-induced hepatotoxicity is not clear. Further investigation on the role of drug metabolism and the effect of PTU reactive metabolite(s) on vital organelles such as mitochondria might shed some light the mechanisms of PTU-induced liver injury.

Several other thionamide-based structures are known as hepatotoxic agents.^{54,55} Thiourea is the parent chemical for many drugs and industrial agents. Some anti-tuberculosis drugs, anti-HIV reverse transcriptase inhibitors, and centrally acting histamine H₃ antagonists, are among thiourea-containing drugs.^{44,50-51,56,57} Interestingly, the clinical administration of the mentioned drugs is associated with drug-induced liver injury.^{55,58} Mitochondrial dysfunction might play a relevant role in the mechanism of liver injury induced by these drugs.^{55,58}

In conclusion, our data suggest that hepatocytes-mitochondria could be a possible target for PTU-induced liver injury. Hence, this study might shed some light on the current knowledge about the mechanism of PTU-induced liver injury. Moreover, these data might help to develop new preventive and therapeutic options against drugs-induced liver injury. The effect of PTU on targets such as mitochondrial respiratory chain enzymes or tricarboxylic acid cycle could be the subject of future studies.

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Conflict of interests

The authors claim that there is no conflict of interest.

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