The lignan manassantin is a potent and specific inhibitor of mitochondrial complex I and bioenergetic activity in mammals

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Manassantin is a dineolignan first isolated in 1983 by Rao and Alvarez (1) from the herb Saururus cernuus (Saururaceae). The natural compounds have been traditionally used for treatment of edema, gonorrhea, and jaundice in Asian countries, particularly in Korea (2). Cellular and biochemical analyses have identified several potential molecular effectors of manassantin. These include the nuclear factor α-light-chain-enhancer of activated B cells (NF-κB) (3–6) and CCAAT/enhancer-binding protein β transcription factors (7), the mitogen-activated protein kinase (MAPK) (6, 8, 9), interleukin 6–induced signal transducer and activator of transcription 3 (STAT3) (10), and hypoxia-inducible factor 1α (HIF-1α)2 (11–13). Some of these “molecular targets” were observed only in selected cell lines (7, 8, 10). It is unclear whether these biochemically diverse molecules or pathways are direct targets of manassantin or secondary cellular responses. This is particularly true with the inhibitory effect of manassantin on the abundance of HIF-1α. Many intracellular and extracellular factors can impinge on HIF-1α expression, accumulation, or degradation, resulting in a lower protein abundance (14). Moreover, it remains unknown how these effectors contribute to the broad spectrum of medical benefits of manassantin seen in humans. Systematic screening via large-scale protein folding and stability measurements identified a number of hits that did not significantly overlap with those found from random cell-based analyses (3–13, 15–20).

Other physiologically more relevant targets of manassantin may exist. In 2013, Lai et al. (21) reported that manassantin acted as an inhibitor of the mitochondrial electron transport chain (ETC) in zebrafish, causing developmental arrest without necrosis, a phenotype shared by some known ETC inhibitors such as piericidin A and rotenone or the ATP synthase inhibitor oligomycin D. Although biochemical analysis with isolated bovine mitochondria suggested that manassantin inhibited complex I (21), this sole publication falls short of proving that manassantin indeed inhibits complex I in mammalian cells and in live animals. Furthermore, the biological consequences of this new activity of manassantin in mammalian cells or tissues necessarily represent the official views of the National Institutes of Health. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The Abbreviations Used Are: HIF-1α, hypoxia-inducible factor 1α; AMPK, AMP-activated protein kinase; ETC, electron transport chain; MNS, manassantin; OCR, oxygen consumption rate; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; ACC, acetyl-CoA carboxylase; MeOH, methanol.

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remain uncharacterized. Because dysfunction of the mitochondrial complex I is implicated in multiple medical conditions (22), discovery of novel and non-toxic inhibitors of complex I to manipulate its activity remains to be of significant interest to scientists in the field (23, 24).

In this study, we report that manassantin potently and specifically inhibited the mitochondrial complex I–mediated respiration with no effect on complex II or complex IV activity in mammalian cells. Treatment with nanomolar concentrations of manassantin B (MNS-B) caused dose-dependent inhibition of mitochondrial oxygen consumption in cultured cells. Consistent with being a potent and specific inhibitor of complex I, MNS-B decreased cellular ATP and stimulated AMP-activated protein kinase (AMPK) and AMPK-dependent up-regulation of glycolysis. These profound effects suggest that manassantin is a prominent modulator of oxygen, energy, and glucose metabolism in mammals.

Results and discussion

Manassantin inhibits oxygen consumption rate (OCR) in mammalian cells

We examined whether manassantin acts as an effective inhibitor of mitochondrial respiration in intact mammalian cells. Because MNS-B and MNS-A are structurally similar and MNS-B was 3 times more abundant than MNS-A in *Saururus chinensis* extracts, we focused on MNS-B for this and the following experiments. HepG2, a well differentiated hepatocellular carcinoma cell line, and H9C2, a normal rat cardiomyocyte line, were incubated with increasing concentrations of MNS-B (0, 12.5, 25, 50, and 100 nM). Both cell lines are known to display active mitochondrial respiration and have been commonly used for studies of mitochondrial ETC complex functions (25–28). As shown in Fig. 1, MNS-B dose-dependently inhibited OCR (pmol/min/well) in these cells as determined using the Seahorse XF24 Analyzer. In HepG2, marked inhibitions of basal mitochondrial respiration (basal OCR), ATP-linked OCR, maximal respiratory capacity, and reserve capacity were observed at 12.5 nM MNS-B. These were completely blocked by 100 nM MNS-B (Fig. 1, A and B). However, it was less potent in H9C2 cells. The ID_{50} value for MNS-B to inhibit basal OCR was between 25 and 50 nM in H9C2 cells. Complete inhibition of basal OCR, ATP-linked OCR, maximal respiratory capacity, and reserve capacity required more than 100 nM MNS-B in H9C2 cells (Fig. 1, C and D).

Figure 1. Manassantin inhibits OCR in mammalian cells. HepG2 and H9C2 cells were plated in the Seahorse 24-well microplate (3–5 × 10^4 cells/well) and cultured overnight with complete medium. The cells were then refed the assay medium containing the indicated concentrations of MNS-B and incubated in a non-CO_{2} incubator at 37 °C for 1.5 h before transferring to the Seahorse XF24 Extracellular Flux Analyzer for measurement of OCR as detailed under “Experimental Procedures.” The specific ATP synthase inhibitor (oligomycin), ETC inhibitors (antimycin A/rotenone), or protonophore uncoupler (FCCP) was added during measurement. The background in cell-free wells was subtracted from readings of all other wells. The results presented are real-time OCRs (pmol/min; mean ± S.D. of triplicate assays) in HepG2 (A) and H9C2 (C) over the course of measurement. In lower panels, basal mitochondrial respiration (Basal), ATP-linked respiration (ATP-linked), maximal respiratory capacity (Maximum), and reserve capacity in HepG2 (B) and H9C2 (D) were calculated and statistically analyzed to show the effects of MNS-B. Error bars represent S.D. Statistical analysis was done using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
similar to those of rotenone and piericidin A as shown in supplemental Fig. S2.

**Manassantin specifically inhibits complex I**

To explore the mechanism for manassantin-mediated inhibition of OCR, mitochondria were freshly isolated from mouse livers and resuspended in respiration buffer containing substrates for complex I (glutamate/malate), complex II (succinate + rotenone), or complex IV (N,N,N,N-tetramethyl-p-phenylenediamine (TMPD)/ascorbate + rotenone) (29). As shown in Table 1, MNS-B at both 5 and 25 nM significantly inhibited complex I–mediated respiration as indicated by dose-dependent decreases in state 3 respiration without effect on state 4 rate, indicating that manassantin directly interacts with the mitochondrial complex I to exert its effect without involvement of cell surface or cytosolic mediators.

When the complex II substrate succinate was used, MNS-B did not inhibit either state 3 or state 4 rate. Additionally, MNS-B did not interfere with the TMPD/ascorbate (complex IV)–mediated respiration (Table 1). The specific inhibitory effect of MNS-B on complex I–dependent respiration was further confirmed in digitonin-permeabilized H9C2 cells incubated with complex I or complex II substrates. The cellular oxygen consumption was strongly inhibited by 25 nM MNS-B when complex I substrate but not complex II substrate was used (data not shown).

Furthermore, spectrophotometric analysis of complex I enzyme activity revealed that MNS-B inhibited NADH–ubiquinone reductase activity but not NADH–ferricyanide reductase activity (30) (Table 1), suggesting that the putative MNS-B target is distal to the flavin protein domain function of complex I.

**Manassantin causes bioenergetic loss and activation of AMPK**

Because ETC complex I constitutes a rate-limiting step of oxidative phosphorylation and ATP production in mitochondria (30), a functional consequence of manassantin treatment in parallel with suppression of oxygen consumption would be inhibition of ATP production. Indeed, treatment of various cell lines including glycolytic tumor cell lines SKOV3 and MDAMB-468 with MNS-B caused dramatic reduction in steady-state levels of cellular ATP (Fig. 3A). In contrast to ATP, AMP levels were significantly augmented by MNS-B treatment (Fig. 3B). Consistent with these changes in AMP and ATP levels, MNS-B induced activation of AMPKα as reflected by enhanced

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**Table 1**

| Parameters                  | MNS-B 0 nm | MNS-B 5 nM | MNS-B 25 nM |
|-----------------------------|------------|------------|-------------|
| Complex I (glutamate and malate) |            |            |             |
| State 3                     | 131 ± 7    | 97 ± 4**   | 62 ± 27*    |
| State 4                     | 18 ± 3     | 19 ± 3     | 16 ± 4      |
| Complex II (succinate)      |            |            |             |
| State 3                     | 324 ± 33   | 307 ± 21   | 324 ± 36    |
| State 4                     | 69 ± 6     | 75 ± 9     | 78 ± 5      |
| Complex IV (TMPD and ascorbate) |          |            |             |
| State 3 maximum (azide-sensitive) | 743 ± 62  | 781 ± 7    | 727 ± 54    |
| Complex I enzyme activity   |            |            |             |
| NADH–ubiquinone reductase    | 112 ± 7    | 89 ± 9*    | 58 ± 17**   |
| NADH–ferricyanide reductase  | 1992 ± 80  | 2125 ± 161 | 1997 ± 188  |

**Figure 2. MNS-A and MNS-B exhibit similar effects on OCR.** OCR in HepG2 and H9C2 cells in the presence or absence of 25 nM MNS-A or MNS-B was measured with the Seahorse XF24 Extracellular Flux Analyzer as described in Fig. 1. The results presented are real-time OCRs (pmol/min; mean ± S.D. of triplicate assays) in HepG2 (A) and H9C2 (C) over the course of measurement. In lower panels, basal mitochondrial respiration (Basal), ATP-linked respiration (ATP-linked), maximal respiratory capacity (Maximum), and reserve capacity in HepG2 (B) and H9C2 (D) were calculated as in Fig. 1. Error bars represent S.D. Statistical analysis was done using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
phosphorylation of AMPK at Thr-172 in all cell lines we examined (Fig. 4). These results confirmed that manassantin negatively regulates oxygen consumption and energy production in these cells.

**Manassantin induces activation of glycolysis as an adaptive response to bioenergetic deficiency**

The above data suggest that exposure of mammalian cells to manassantin results in bioenergetic deficiency. This new biological effect probably represents a primary cellular function of manassantin. The next question is how cells react to manassantin-induced down-regulation of bioenergetic activity. We thus examined whether MNS-B treatment triggers glucose utilization through glycolysis as an adaptive response to ATP loss. Although limited in ATP generation through direct substrate phosphorylation, glycolysis provides a significant pool of ATP (glycolytic ATP) in highly proliferating cells or in response to energetic stress (31). Activation of glycolysis could partially offset the ATP loss from MNS-B inhibition of complex I. During glycolysis, one molecule of water is released when 2-phosphoglycerate is converted to phosphoenolpyruvate. By labeling cells with [5-3H]glucose, we were able to quantitate the glycolytic rate by measuring generation of [3H]water (32, 33). As demonstrated in Fig. 5A, MNS-B treatment strongly enhanced glycolysis in H9C2, HepG2, SKOV3, and MDA-MB-468 cells. Consistent with the increased glycolysis, culture supernatants of these cells treated with MNS-B contained dramatically higher levels of lactate (Fig. 5B).

**Manassantin drives glycolysis via an AMPK-dependent but HIF-1α–independent mechanism**

The role of manassantin in activation of the glycolytic pathway has not been described. Several groups have previously reported that manassantin inhibits expression of HIF-1α (11–13). Because HIF-1α is a master regulator of a myriad of glycolytic enzymes (32, 34), one may anticipate that manassantin will down-regulate glycolysis as a result of its inhibition of HIF-1α. To clarify the roles of manassantin in regulation of HIF-1α and in glycolysis, we revisited the effect of MNS-B on HIF-1α. At the same range of concentrations that turned on glycolysis, MNS-B decreased basal HIF-1α expression in most cell lines we examined with an exception of SKOV3 (Fig. 4, A and B). Apparently, MNS-B stimulated glycolysis while diminishing HIF-1α in most cells, suggesting that an HIF-1α–independent mechanism is involved in the glycolytic activation in MNS-B–treated cells. We then examined the effect of the specific AMPK inhibitor Compound C (35) on MNS-B–induced glycolysis. Coincubation of mammalian cell lines with MNS-B and Compound C prevented up-regulation of glycolysis in response to MNS-B in HepG2, SKOV3, and MDA-MB-468 cells (Fig. 6A). A similar analysis was not possible in H9C2 cells as Compound C exerted a strong toxic effect, particularly when used in combination with MNS-B. The efficient inhibition of AMPK by Compound C in HepG2, SKOV3, and MDA-MB-468 cells was confirmed by decreased phosphorylation of acetyl-CoA carboxylase (ACC) 1 at Ser-79 (Fig. 6B), a well characterized downstream target of AMPK (36). Therefore, the enhanced activity of AMPK is required for the stimulatory effect of MNS-B on glycolytic metabolism.

**Manassantin is an inhibitor of bioenergetic activity in vivo**

We next examined whether manassantin acts as an effective inhibitor of mitochondrial respiration and ATP generation in live animals. Young adult mice were treated with MNS-B (1.5 mg/kg) or vehicle for 14–16 h before the mice were sacrificed, and liver tissues were collected for analysis of phosphorylation status of AMPKα and abundance of ATP. As shown in Fig. 7, MNS-B treatment led to consistent increases in phosphorylation of AMPKα at Thr-172 in all cell lines we examined (Fig. 4). These results confirmed that manassantin negatively regulates oxygen consumption and energy production in these cells.

**Figure 3. Manassantin reduces ATP but increases AMP in mammalian cells.** The cell lines were cultured with the indicated concentrations of MNS-B for 24 h. The cells were lysed as described under “Experimental Procedures.” A, the concentrations of ATP were quantified with an ATP bioluminescence assay kit, and the results are presented as nmol of ATP/10^6 cells (mean ± S.D. of triplicate assays). In B, AMP concentrations in cells treated with or without 25 nM MNS-B were quantified with an AMP-Glo assay kit. The results are presented as cellular AMP levels (nmol of AMP/10^6 cells) or as fold changes relative to control cells (mean ± S.D. of triplicate assays). Error bars represent S.D. Statistical analysis was done using Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
tion of AMPK in all treated mice. ATP levels in livers of MNS-B–treated mice were reduced by an average of 23% compared with the vehicle group. Despite this level of decrease in ATP, MNS-B–treated mice looked healthy with no obvious decrease in activity or signs of weakness or distress. However, when higher doses (2.5–5.0 mg/kg) of MNS-B were applied, the mice showed significant loss of activity, drowsiness, and subsequent death, precluding further assessment of the effects of greater doses of MNS-B on levels of AMPK phosphorylation and ATP in physiologically healthy conditions. The higher dose of manassantin is toxic to mice most likely due to it acting as a mitochondrial poison.

Bioenergetic homeostasis plays a central role in regulation of many biochemical, physiological, and pathophysiological processes (30, 34, 36). In this report, we have shown that manassantin is a potent inhibitor of mitochondrial complex I and energy anabolism in mammalian systems. We showed that anti-complex I activity of manassantin is sufficient to cause significant loss of ATP and activation of AMPK. The energy deficiency subsequently led to up-regulation of glycolysis as a result of AMPK activation. AMPK plays a crucial role in the control of cellular metabolic states by shutting down energy-consuming anabolic processes and activating energy-yielding catabolic processes (37). It is conceivable that sustained AMPK activity could...
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trigger a multitude of metabolic changes beyond glucose such as inhibition of de novo fatty acid synthesis via phosphorylation and repression of ACC (36), the rate-limiting enzyme of lipogenesis. Thus, manassantin could dramatically alter metabolic patterns of cells. Therapeutic activation of AMPK by low doses of manassantin could potentially promote glucose and fatty acid catabolism, increase insulin sensitivity, and prevent obesity.

It is remarkable that manassantin as a natural product that has been used in humans inhibits complex I activity and attenuates ATP production at nanomolar concentrations in mammalian cells. An interesting question yet to be answered is the mechanism of manassantin’s action on complex I. The universal effect from zebrafish to rodent and human cells suggests that manassantin binds to an evolutionally conserved structure/component of complex I. The interaction details will facilitate future translational and clinical application of manassantin or its derivatives as clinically safe inhibitors of complex I or modulators of cell metabolism.

Experimental procedures

Cell culture and reagents

The cell lines HepG2, H9C2, SKOV3, and MDA-MB-468 were originally obtained from ATCC (Manassas, VA). HepG2 cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum (FBS). SKOV3 and MDA-MB-468 cells were cultured in RPMI 1640 medium + 10% FBS. Early passages of H9C2 cells were maintained in high-glucose DMEM + 10% FBS. Cell culture media, glucose, pyruvate, and glutamine were obtained from Invitrogen. FBS was from Atlanta Biologicals (Atlanta, GA). D-[5-3H]glucose was obtained from PerkinElmer Life Sciences. Oligomycin, carbonyl cyanide p-trifluoromethoxy-phenyl-hydrazone (FCCP), dinitrophenol, rotenone, antimycin A, TMPD, and ascorbate were purchased from Sigma-Aldrich. Compound C was purchased from Selleck Chemicals (Houston, TX).

Purification of MNS-A and MNS-B

The air-dried, aerial part of S. chinensis was cut into pieces and extracted with methanol (MeOH) and ethyl acetate. The ethyl acetate extract was fractionated by flash silica gel column chromatography, subjecting to silica gel (6.5 x 45 cm) with n-hexane/acetone (hexane/ethyl acetate, 2:1, 1:1, 1:2, and 1:4) and eluting into four fractions (F1–F4). F1 was subjected to Sephadex LH-20 column chromatography and an octadecyl silica Sep-Pak cartridge and eluted with MeOH, resulting in MNS-A by HPLC and 1H and 13C NMR spectral data analyses. The purity of MNS-A or MNS-B was confirmed to be higher than 95% by HPLC analysis.
Measurement of OCR in cultured cells
OCR (pmol/min) in each well of cultured cells was measured using the Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, Santa Clara, CA) (22). Approximately 30,000–50,000 cells/well in 100 μl of complete culture medium were seeded in the XF24 cell culture microplates. After attachment, 200 μl of additional medium was added gently. The cells were cultured overnight. Prior to the OCR measurement, the cells were washed once with 1 ml of the Seahorse XF assay medium (basal medium supplemented with 2 mM glutamine, 1 mM pyruvate, and 10 mM glucose, pH 7.4). The cells were then incubated with the assay medium (575 μl) containing MNS-B or other ETC inhibitors at the indicated concentrations. The cells were kept in a 37 °C non-CO2 incubator for about 1.5 h prior to the assay. The Seahorse Analyzer was programmed to sequentially add oligomycin (ATP synthase inhibitor), FCCP (protonophore uncoupling oxygen consumption from ATP production), and a combination of antimycin A (complex III inhibitor) and rotenone (complex I inhibitor) according to the instructions of the Seahorse XF Cell Mito Stress Test. Basal mitochondrial respiration, ATP-linked respiration, maximal respiratory capacity, and reserve capacity were calculated as described previously (38, 39). Specifically, basal OCR and ATP-linked OCR were derived by subtracting non-mitochondrial respiration and the oligomycin rate from baseline cellular OCR, respectively. Maximal respiratory capacity was derived by subtracting non-mitochondrial respiration from the FCCP rate. Mitochondrial reserve capacity was calculated by subtracting basal respiration from the maximal respiratory capacity.

Measurement of oxygen consumption in isolated mitochondria
Mouse liver mitochondria were isolated by differential centrifugation as described previously (40). The rate of oxygen consumption in isolated mitochondria was measured using a Clark-type oxygen electrode at 30 °C (29). Briefly, mitochondria from mouse livers were resuspended in a buffer containing 80 mM KCl, 50 mM MOPS, pH 7.4, 1 mM EGTA, 5 mM KH2PO4, and 1 mg/ml BSA. Glutamate/malate (20 mM/5 mM; complex I substrates), succinate (20 mM; complex II substrate) plus rotenone (5 μM), and TMPD/ascorbate (1 mM/10 mM; complex IV substrate) plus rotenone (5 μM) were used as electron donors. The state 3 rate (after addition of 0.2 mM ADP; ADP-stimulated), state 4 rate (following depletion of ADP; ADP-limited), and respiratory control ratio (state 3/state 4) were measured as described previously (29).

Measurement of complex I enzyme activities
Activity of NADH–ubiquinone reductase or NADH–ferricyanide reductase of complex I was measured in detergent-solubilized, frozen-thawed mitochondria at 37 °C as described previously (29, 41).

Glycolysis
Glycolysis in cultured cells was measured as we described (32, 33). Briefly, cells were plated in 12-well plates and treated with vehicle or MNS-B for 16 h. [5-3H]Glucose was added to a concentration of 2.5 μCi/ml and incubated for an additional 6 h. Hydrochloric acid was then added to the medium at a final concentration of 0.2 N to terminate all biological reactions. The acidified medium (0.6 ml) was collected into 15-ml high-clarity polypropylene conical tubes (Falcon). A 0.5-ml microcentrifuge tube containing 0.25 ml of distilled water was uncapped and inserted into the 15-ml tube. The 15-ml tubes were tightly capped to allow diffusion between two liquid phases for 3 days. Radioactivities in water and medium were determined by liquid scintillation counting. The glycolytic rate was calculated with the formula 3.4a/(a + b) where a is radioactivity present in water and b is activity present in the medium (33).

Quantification of lactate
Lactate levels in culture supernatants were measured with a colorimetric lactate quantification kit according to the manufacturer (Eton Bioscience Inc., San Diego, CA) (29, 41). The lactate contents were normalized on cell numbers.

Quantification of ATP and AMP
After washing twice with PBS, cells were lysed in ice-cold H2O containing 0.75% Nonidet P-40, vortexed at full speed for 10 s, and then incubated on ice for 10 min. After centrifugation (16,000 × g, 3 min), the supernatants were collected and diluted 50–100 times with H2O for ATP measurement with an ATP bioluminescence assay kit (Sigma-Aldrich or Invitrogen) (42). For AMP measurement, cells were lysed in radioimmune precipitation assay buffer. After centrifugation, the supernatants were harvested and diluted 100 times with radioimmune precipitation assay buffer for analysis of AMP levels with an AMP-Glo™ assay kit according to the manufacturer’s instructions (Promega, Madison, WI). Both ATP and AMP concentrations were calculated from the standard curve and presented as nmol/106 cells. To measure ATP levels in liver tissues of manassantin-treated or control mice, liver tissues (15 mg) were homogenized in 1 ml of 2 N perchloric acid and then incubated on ice for 45 min. After centrifugation, the supernatants were neutralized with ice-cold 2 N KOH to adjust pH to ~7.0 (43). After centrifuging again, the supernatants were diluted 50–100 times with H2O for ATP measurement as described above.

Western blot analysis
Lysates from cultured cells or mouse tissues were prepared in lysis buffer supplemented with protease/phosphatase inhibitor mixture (Roche Diagnostics), and proteins were resolved by SDS-PAGE and analyzed by immunoblotting as described previously (44). The antibodies for β-actin and phospho-AMPKα were purchased from Cell Signaling Technology (Danvers, MA). The antibody for HIF-1α was from BD Biosciences. Immunocomplexes were visualized by an enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL) by using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology).

Animal studies
All animal experiments were conducted in compliance with the policies and regulations of Virginia Commonwealth University Institutional Animal Care and Use Committee. Ten-
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week-old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were i.p. injected with 1.5 mg/kg MNS-B in 0.1 ml of 0.5% methyl cellulose. The mice were sacrificed 14–16 h postinjection. Liver tissues were collected and homogenized in lysis buffer for immunoblotting analysis or in perchloric acid for measuring ATP as described earlier. In addition, mitochondria were isolated from livers of three mice for assessment of the effects of MNS-B on mitochondrial respiration and activities of complex I enzymes.

Statistics

All numerical data were presented as mean ± S.D. of triplicate assays. The statistical significances were determined using Student’s unpaired or paired two-tail t test where $p < 0.05$ was considered statistically significant. In all figures, the statistical significances are indicated with * if $p < 0.05$, ** if $p < 0.01$, or *** if $p < 0.001$.

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