

Adverse effects of anti-tuberculosis drugs on HepG2 cell bioenergetics

Journal:	<i>Human and Experimental Toxicology</i>
Manuscript ID	HET-15-0482.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Keyword:	Drug toxicology, Hepatic toxicology, Clinical toxicology, Oxidative stress
Abstract:	Tuberculosis (TB) is an intractable chronic infection. Disease treatment with anti-TB drugs remains challenging due to drug-induced hepatotoxicity. The toxicity of the anti-TB drugs rifampicin (RIF), isoniazid (INH), and pyrazinamide (PZA) either alone or in combination was investigated in HepG2 cells. Assays of intracellular ATP levels at 4, 24, and 48 hour post-exposure to gradient concentrations of RIF, INH, and PZA were conducted. Drug-induced effects on mitochondrial membrane potential (MMP), mitochondrial complex I & complex III activity, NAD ⁺ levels, and cellular lactate production were assessed. Decreased ATP levels were dose-dependent and correlated with drug exposure duration. Approximate 24 hour IC50s were 0.5 mM, 70 mM, and 84 mM for RIF, INH, and PZA, respectively. Twenty-four hours post-drug treatment, reductions of MMP ($p = 0.0005$), mitochondrial complex I & III activities ($p = 0.0001$ & $p = 0.0003$, respectively), NAD ⁺ levels ($p = 0.0057$), and increased lactate production ($p < 0.0001$) were observed. Drug combinations used to mimic cumulative drug treatments induced a synergistic inhibition of mitochondrial complex I activity. An assessment of cellular ultrastructure using transmission electron microscopy indicated drug-induced mitophagy. Collectively, our study suggests that hepatotoxicity of commonly employed anti-TB drugs is mediated by their curtailment of mitochondrial function.

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Adverse effects of anti-tuberculosis drugs on HepG2 cell bioenergetics

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Abstract

Tuberculosis (TB) is an intractable chronic infection. Disease treatment with anti-TB drugs remains challenging due to drug-induced hepatotoxicity. The toxicity of the anti-TB drugs rifampicin (RIF), isoniazid (INH), and pyrazinamide (PZA) either alone or in combination was investigated in HepG2 cells. Assays of intracellular ATP levels at 4, 24, and 48 hour post-exposure to gradient concentrations of RIF, INH, and PZA were conducted. Drug-induced effects on mitochondrial membrane potential (MMP), mitochondrial complex I & complex III activity, **NAD⁺ levels**, and cellular lactate production were assessed. Decreased ATP levels were dose-dependent and correlated with drug exposure duration. Approximate 24 hour IC₅₀s were 0.5 mM, 70 mM, and 84 mM for RIF, INH, and PZA, respectively. Twenty-four hours post-drug treatment, reductions of MMP ($p = 0.0005$), mitochondrial complex I & III activities ($p = 0.0001$ & $p = 0.0003$, respectively), **NAD⁺ levels ($p = 0.0057$)**, and increased lactate production

($p < 0.0001$) were observed. Drug combinations used to mimic cumulative drug treatments induced a synergistic inhibition of mitochondrial complex I activity. An assessment of cellular ultrastructure using transmission electron microscopy indicated drug-induced mitophagy. Collectively, our study suggests that hepatotoxicity of commonly employed anti-TB drugs is mediated by their curtailment of mitochondrial function.

Keywords

Anti-TB drugs, drug-induced hepatotoxicity, mitochondrial complex I & complex III activity, mitochondrial membrane potential, mitophagy.

Introduction

Tuberculosis (TB) is an airborne infectious disease. Treatment of TB remains one of the major public health challenges in the world today. In 2013, 9 million people developed TB, and 1.5 million died from the disease.¹ Rifampicin (RIF), isoniazid (INH), and pyrazinamide (PZA) are basic (first-line) anti-TB drugs.² The treatment regimen currently used in some developing countries is a fixed-dose, single-tablet combination of four drugs: RIF, INH, PZA, and ethambutol (EMB) for 2 months, followed by 4 months of INH-RIF and/or EMB. The multi-drug combination is employed at the intensive phase of treatment in an attempt to reduce primary INH-RIF combination drug resistance.² Additionally, the use of fixed dose combination tablets can improve patient adherence to treatment.³ However, adverse drug reactions during TB treatment persist for which there are risk factors that include gender, age, malnutrition, co-infection with HIV, and liver functionality.⁴

Specifically, anti-TB drug-induced hepatotoxicity during standard multidrug TB treatment has been reported, with incidence influenced by a similar set of risk factors.⁴⁻⁷ However, patients with anti-TB drug-induced elevation of liver transaminase levels may still be

1 asymptomatic.^{6,8} Of the anti-TB drugs prescribed: RIF, INH, and PZA are potentially
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3 hepatotoxic⁹⁻¹⁴, whereas no hepatotoxicity has been described for ethambutol.²
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6 Although the induction of anti-TB drug-induced hepatotoxicity has been documented, the
7
8 mechanism by which individual or combinatorial anti-TB drugs influence cell viability and
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10 mitochondrial bioenergetics has not been extensively studied. Herein we have investigated the
11
12 potential hepatotoxicity of commonly employed anti-TB drugs, and provide a mechanistic
13
14 insight into individual drug or dual-drug combination contributions to drug-induced
15
16 hepatotoxicity.
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20 **Materials and Methods**

21 **Chemical agents**

22
23 The antibiotic, Rifampicin (5,6,9,17,19,21-Hexahydroxy-23-methoxy-2,4,12,16,18,20,22-
24
25 heptamethyl-8-[*N*-(4-methyl-1-piperazinyl)formimidoyl]-2,7-
26
27 (epoxypentadeca[1,11,13]trienimino)-naphtho[2,1-*b*]furan-1,11(2*H*)-dione 21-acetate),
28
29 (C₄₃H₅₈N₄O₁₂; MW = 822.94 g/mol); antibacterial agents Isoniazid (pyridine-4-carbohydrazide)
30
31 (C₆H₇N₃O), MW 137.13 g/mol), and Pyrazinamide (pyrazine-2-carboxamide) (C₅H₅N₃O, MW =
32
33 123.11 g/mol) were all purchased from Sigma (St Louis, MO, USA). For assays, drugs were
34
35 dissolved in DMSO, and diluted into serum-free media when applied to cells. Additions of
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37 vehicle (DMSO) only at identical dilutions to that of assay samples were used to generate control
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39 readings for all assays. All other chemicals and media components were also purchased from
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Sigma unless specified.

50 **Cell culture**

51 Human hepatocellular carcinoma cells (HepG2 cells) were grown in serum-free PC-1 medium
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53 (Cambrex) supplemented with 2 mM L-glutamine. Cells were incubated at 37 °C in a humidified
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55 atmosphere with 5% CO₂.
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Intracellular ATP content determination

Cells were seeded in 96-well plates at 1×10^4 cells/well. At confluence cells were treated with the anti-TB drugs at concentrations of 0.1, 1, 10, and 100 mM for INH or PZA, and concentrations of 1 μ M, 10 μ M, 0.1 mM, and 1 mM for RIF. After 4, 24, and 48 hrs cells were harvested and intracellular ATP content determined according to the manufacturer's protocol (Abcam, Cambridge, MA, USA). Briefly, 50 μ l of the supplied cell lysis buffer was added to each well, and the plates were shaken for 5 minutes. Then 50 μ l of the reconstituted substrate (D-Luciferin) was added to each well. Plates were placed on an orbital shaker for 5 minutes and then kept in the dark for further 10 minutes. The microplate scintillation counter 'TopCount' (Perkin Elmer) was used to determine intracellular ATP levels. Basal values in medium were subtracted from each **test** value. Intracellular ATP levels are represented as a percentage of vehicle controls. Experiments were conducted in triplicates.

Mitochondrial membrane potential (MMP) measurements

HepG2 cells were seeded in 24 well plates at a density of 3×10^4 cells/well. At confluence cells were treated for 24 hours with the anti-TB drugs: RIF at 0.1 and 0.5 mM, INH at 10 and 70 mM, and PZA at 10 and 84 mM. The media was removed and the Mitotracker green assay performed according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Mitotracker green staining solution was added and the plates incubated at 37 °C for 30 minutes. A weak hydrophobic acid, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a protonophoric uncoupler of oxidative phosphorylation in mitochondria, was used as a positive control. After staining, fluorescence was read in fresh phosphate buffer saline (PBS) using a Dyne MRX microplate reader (Dyne technologies, VA, USA) using excitation/emission filters of 490/451nm, respectively.

Mitochondrial complex I & III activity assays

1 HepG2 cells were treated with RIF (0.1 and 0.5 mM), INH (10 and 70 mM), and PZA (10 and 84
2 mM) for 24 hrs. Complex I and complex III activities were assayed. For Complex I assays, a
3 mitochondrial-enriched fraction was used, prepared according to the procedure of Spinazzi et
4 al.¹⁵ in 10 mM ice-cold hypotonic Tris buffer (pH 7.6) containing 1.5 M sucrose. 2,6-
5 Dichloroindophenol (DCIP) sodium salt hydrate was used as the terminal electron acceptor.¹⁶
6 Complex I buffer was composed of 25 mM K-phosphate pH 7.6, 0.12 mM DCIP, 70 μ M
7 decylubiquinone (DUB), and 1 μ M antimycin A. Fatty acid free BSA (35 mg) was added, and the
8 reaction started by the addition of NADH (10 mM). Complex I activity was quantified by
9 monitoring the reduction of DCIP at 620 nm.
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11

12 Complex III activity was measured according to the procedure of Spinazzi et al.¹⁵; measuring the
13 ability of the cell lysate to reduce Cytochrome C monitored by a change in absorbance at 550
14 nm. Specific complex III activity was calculated as the difference between activities measured in
15 the absence and presence of 2 mM antimycin A.
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17

18 To study the effect of combinations of anti-TB drugs upon complex I activity, cells were
19 pretreated with RIF, INH, and PZA at concentrations of 30 μ M, 3 mM, and 3 mM, respectively
20 for 48 hours before initiation of the activity assays. At these drug concentrations ATP levels
21 were not significantly reduced. After removal of media, cells were washed with PBS and then
22 exposed to RIF (0.1 or 0.5 mM), INH (10 or 70 mM), or PZA (10 or 84 mM). After 24 hours,
23 Complex I and III activity measurements were taken.
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26 **Cellular NAD⁺/NADH measurements**

27 Cellular NAD⁺/NADH levels were determined using a commercial kit (product 600480, Cayman
28 Chemical, USA), according to the manufacturer's protocol. HepG2 cells were seeded at 0.1 x
29 10⁴ cells per well in 96 well plates, and grown until confluent. Once confluent, culture media
30 was removed and cells treated with the anti-TB drugs at their IC₅₀ concentrations. After 24 hours,
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1 120 µl of assay buffer was added and then the plates centrifuged at 500 x g for 5 minutes. Assay
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3 buffer was removed and then 110 µl of permeabilisation buffer added to each well. Plates were
4
5 shook for 30 minutes at room temperature and then centrifuged at 1000 x g for 5 minutes at 4 °C.
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7 One hundred µl of the supernatant was removed to a fresh plate, followed by 100 µl of reaction
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9 buffer. Plates were shook for 90 minutes before absorbance readings taken at 450 nm. Reagent
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11 only blanks were subtracted from the absorbance of all wells. The absorbance measurements of
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13 drug-treated samples were represented relative to vehicle controls. Experiments were performed
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15 in triplicates.
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20 **Lactate production assays**

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22 HepG2 cells were seeded in 24-well plates at 5×10^4 cells/well. At confluence cells were treated
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24 for 24 hours with RIF (0.01 and 0.5 mM), INH (10 and 70 mM), and PZA (10 and 84 mM).
25
26 After trypsinization, cells were counted, and cell supernatant media removed and assayed
27
28 immediately for lactate levels using a lactate assay kit (Biovision, USA) according to the
29
30 manufacturer's protocol. Lactate production was normalized to cell number and expressed as a
31
32 percentage of lactate production from control cells. Experimental data points were performed in
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34 triplicates.
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40 **Transmission Electron Microscopy**

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42 HepG2 cells were grown to near confluence in EMEM with 2 mM Glutamine, 1 % non-essential
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44 amino acids, and 10 % Foetal Bovine Serum in 175 cm² flasks. Media was removed and replaced
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46 with media containing low serum (2 %) and cells grown for 24 hours. Cells were then incubated
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48 with media containing the anti-TB drugs RIF, INH, or PZA at concentrations of 0.5, 70, & 84
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50 mM, respectively. After 24 hours of drug treatment, media was removed and cells washed with
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52 media containing fixative (3 % glutaraldehyde in 0.1 M cacodylate buffer). The 1:1 (v/v)
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54 media:fixative solution was replaced with fixative alone, and cells fixed in the cell incubator for
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1 1 hour at 37 °C. Flasks were removed and cells scraped into the fixative. Cells were collected
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3 by centrifugation at 1500 rpm for 5 minutes at 4 °C, and then further fixed for 1 hour at 4 °C.
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5 Cells were washed in 0.1 M cacodylate buffer, transferred to flat-bed embedding capsules, and
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7 then incubated for 1 hour with 1 % osmium tetroxide in 0.1 M cacodylate buffer. Cells were
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9 washed with water and then dehydrated with a graded ethanol series of 50, 70, 90, & 100 %
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11 ethanol, and a transitional solution, 100 % propylene oxide (propox). Cells were infiltrated with
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13 an epoxy resin:propox mix (1:1) overnight, and then infiltrated with epoxy resin 3 times for 2
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15 hours each the following day. Samples were then embedded and polymerized in an oven at 60
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17 °C for 48 hours. Ultra-thins of the cells (80 nm) were sectioned with a diamond knife on a Leica
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19 EM UC6 ultra microtome, collected, and placed on 200 mesh copper grids. Sections were
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21 analysed using a Tecnai G2 BioTWIN transmission electron microscope (TEM) (FEI company,
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23 Eindhoven, The Netherlands), which was run at an accelerated voltage of 100 kV. For each cell
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25 treatment up to 19 fields of view were analysed, with random unbiased selection. Images were
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27 captured using a MegaView SIS camera, with representative images included in Figures.
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34 **Statistical Analysis**

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36 All statistical procedures were performed using PRISM 5 (GraphPad Software Inc., San Diego,
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38 CA). For the 50 % inhibitory concentration (IC₅₀), curve fitting was performed using single use
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40 Log (antagonist) versus response (variable slopes). A one way analysis of variance (ANOVA)
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42 test was performed with Dunnett's multiple comparisons post-test. Unpaired Student's t-tests
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44 were performed for two group comparisons. Data points or histograms in Figures represent
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46 means ± SEMs, with differences compared to control values set at 100 % of activity. Statistical
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48 significance was defined as $p < 0.05$. For Figures, significance is represented as *** for $p <$
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50 0.001 , ** for $p < 0.01$, and * for $p < 0.05$.
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56 **Results**

Hepatotoxicity of anti-TB drugs

To assess the influence of the anti-TB drugs RIF, INH, and PZA on cellular ATP levels, HepG2 cells were incubated with each drug over a broad concentration range for up to 48 hours (Figure 1). Collectively, all drugs reduced ATP levels in a concentration- and exposure duration-dependent manner; albeit with similar drug profiles for 24 & 48 hour incubations (Figure 1). RIF was the most potent drug as it significantly reduced ATP levels ($p = 0.0021$) 4 hours post-treatment at a concentration of 0.1 mM. A summary of the approximate IC_{50} values for each drug are listed in Table 1.

Drug-induced uncoupling of mitochondrial bioenergetics

A mitotracker green assay was performed to quantify the effect of the tested anti-TB drugs on mitochondrial membrane potential (MMP). Drugs were applied at their $\sim IC_{50}$ values as determined by the ATP assay and also at lower concentrations of 0.1 mM, 10 mM, & 10 mM for RIF, INH, & PZA, respectively. All drugs at their IC_{50} concentrations significantly decreased MMP by $\sim 40\%$ 24 hours post-exposure ($p = 0.0005$). At the lower tested concentrations all drugs reduced MMP by $\sim 12-15\%$ but this did not reach significance (Figure 2).

These drug concentrations were then assessed for inhibitory activity toward mitochondrial complex-I (MC-I) activity. At their IC_{50} concentrations RIF, INH, and PZA significantly inhibited MC-I activity ($p = 0.0001$) by approximately 40, 43, and 33%, respectively (Figure 3, upper panel). Additionally at a concentration of 0.1 mM RIF also significantly reduced MC-I activity by $\sim 20\%$ (Figure 3, upper panel). Mitochondrial complex-III (MC-III) activity was less sensitive to drug inhibition, and was inhibited by RIF and INH only at their $\sim IC_{50}$ concentrations ($\sim 30\%$ inhibition, $p = 0.0003$), whereas PZA at an IC_{50} concentration did not significantly reduce MC-III activity (Figure 3, lower panel).

1 To further verify the uncoupling of mitochondrial activity we also quantified cellular
2 NAD⁺ levels. Incubation of cells at IC₅₀ concentrations for RIF & INH significantly reduced
3 cellular NAD⁺ levels by 41 % and 39 %, respectively ($p = 0.0057$). A 21 % reduction of NAD⁺
4 levels at the IC₅₀ concentration for PZA was observed, but this did not reach significance (Figure
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12 As combinations of these anti-TB drugs are medically prescribed for patients suffering
13 from TB, we also investigated the effects of drug combinations on MC-I activity. Cells were pre-
14 incubated with anti-TB drugs at concentrations of 30 μM, 3 mM, and 3 mM for RIF, INH, and
15 PZA, respectively, for 48 hours. At these drug concentrations ATP levels were not significantly
16 reduced (Figure 1). Cells were subsequently treated with RIF at 0.5 mM (24 hour IC₅₀
17 concentration) and MC-I activity quantified (Figure 5A). At this RIF concentration, MC-I
18 activity was significantly reduced to ~60 % of control values, similar to a single drug incubation
19 (as observed in Figure 2). Incubations of cells with RIF at 0.5 mM and addition of either INH (3
20 mM) or PZA (3 mM) reduced MC-I activity a further 1-10 %, but this was not significant (Figure
21 5A). By contrast, incubation of cells with RIF at a lower concentration of 0.1 mM reduced MC-I
22 activity to ~82 % of controls (similar to Figure 2), but the combination of RIF (0.1 mM) with
23 INH (3 mM) significantly reduced MC-I activity by a further 21 % ($p = 0.0417$) (Figure 5B).
24 Cells incubated with RIF (0.1 mM) and PZA (3 mM) evoked a 2 % non-significant reduction of
25 MC-I activity.
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45 Cell incubation with INH at 70 mM significantly reduced MC-I activity to ~56 % of
46 controls (similar to Figure 2), and this was also further reduced by 10 & 3 % with additions of
47 either RIF (3 μM) or PZA (3 mM), respectively, but these reductions were not significant (Figure
48 5C). Incubation of cells with INH at 10 mM produced an ~15 % fall of MC-I activity (similar to
49 Figure 2), but notably a further significant ~16% reduction of MC-I activity ($p = 0.0466$) was
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1 observed with the INH and RIF (3 μ M) drug combination (Figure 5D). Incubation with INH (10
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3 mM) and PZA (3 mM) produced a 4 % non-significant further reduction of MC-I activity (Figure
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6 5D).

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8 Incubation of cells with 84 mM PZA produced a 36 % reduction of MC-I activity, in
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10 keeping with Figure 2, and although this was further reduced by 4 and 14 % with additions of
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12 RIF (3 μ M) and INH (3 mM), respectively, these changes were not significant (Figure 5E).
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14 Incubation with 10 mM PZA reduced MC-I activity by 10 % (as seen in Figure 2), and this was
15
16 further reduced by co-incubation with 3 μ M RIF (12 %, non-significant), and INH at 3 mM (~21
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18 % significant, $p = 0.0078$) (Figure 5F).
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23 To study the bioenergetic shift of HepG2 cells to anaerobic metabolism due to the
24
25 influence of anti-TB drugs, cellular production of lactate was measured. RIF, INH, and PZA at
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27 their IC₅₀ concentrations all significantly increased cellular lactate production ($p < 0.0001$) by
28
29 approximately 41, 37, and 16 %, respectively (Figure 6). However, lower drug concentrations
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31 (0.1, 10, and 10 mM for RIF, INH, and PZA, respectively) did not induce a significant change of
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33 lactate levels (Figure 6).
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36 37 **Drug-induced cellular damage**

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40 To assess cellular and mitochondrial damage as a consequence of anti-TB drug treatment, cells
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42 were incubated with anti-TB drugs for 24 hours at their IC₅₀ concentrations and then fixed for
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44 TEM. Control cells were relatively rich in rod-shaped mitochondria, with well-defined cristae,
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46 and displayed relatively few vacuoles (Figure 7A). By contrast, cells treated with RIF displayed
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48 spherical mitochondria, abundant vacuoles indicative of removal of damaged mitochondria by
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50 mitophagy, and vacuoles thought to contain degrading mitochondria (mitophagolysosomes)
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52 (Figure 7B). For cells treated with INH or PZA, rod-shaped mitochondria similar to control cells
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1 were observed, but also spherical mitochondria and presumed mitophagic vacuoles were present
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3 (Figures 7C & 7D).
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5 Discussion

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8 Anti-TB drug-induced liver insult is a leading cause of drug-induced acute liver injury and
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10 failure in the developing world.¹⁷ However, the correlation between serum anti-TB drugs levels
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12 and drug-induced hepatotoxicity remains unclear.⁷ Collectively, there are toxicity concerns
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14 regarding the use of anti-TB drugs as either dual-drug combinations or as a four drug fixed-dose
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16 combination, with subjects ranging from asymptomatic elevation of liver enzymes to displaying
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18 severe hepatitis.^{4-14,18}
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21 We show here that the anti-TB drugs RIF, INH, & PZA significantly reduce ATP levels in
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23 HepG2 cells in a concentration- and exposure duration- dependent manner. The antibiotic RIF
24
25 with the lowest estimated IC₅₀ was the most potent inhibitor. At these IC₅₀ concentrations a
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27 concurrent and significant decrease of MMP, inhibition of MC-I & MC-III activities, decrease of
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29 NAD⁺ levels, and increased cellular lactate production were also observed. To establish a drug
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31 dose and effect relationship, drug concentrations below IC₅₀ values were also examined. At
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33 these lower drug concentrations reduced MMP, MC-I, & MC-III activities were still evident, but
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35 they did not reach significance except for RIF inhibition of MC-I activity at 0.1 mM.
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42 Mitochondrial protein Complexes I & III are components of the electron transport chain
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44 (ETC) that is crucial for cellular respiration and the generation of ATP. MC-I (NADH:
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46 ubiquinone oxidoreductase) oxidizes NADH produced predominantly from the tricarboxylic acid
47
48 (TCA) cycle, but also from β -oxidation of fatty acids. Two electrons are produced from NADH
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50 oxidation, and these are used to reduce ubiquinone to ubiquinol in the inner mitochondrial
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52 membrane, and initiate the supply of electrons to be passed through the ETC to reduce oxygen to
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54 water. This MC-I redox reaction also drives proton transport across the inner mitochondrial
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1 membrane. Similarly, electron transport is coupled to proton translocation in MC-III and IV, and
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3 this proton motive force supports ATP synthesis in complex V.^{19,20} Hence drug-induced
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5 inhibition or dysfunction of MC-I, and/or MC-III will limit the transfer of electrons along the
6
7 ETC, driving the loss of the MMP, reduced NADH oxidation (NAD⁺ production), and ultimately
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9 a breakdown of cellular ATP production (Figures 1-4), and increased anaerobic metabolism and
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11 lactate production (Figure 6).
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16 In support of our results, drugs or oxidants that induce mitochondrial damage can
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18 provoke a progressive loss of cellular energy (ATP) resource, mitochondria degeneration, and
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20 ultimately cell death.²¹⁻²⁴ Indeed mitochondrial dysfunction is suggested to play a crucial role in
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22 the etiology of drug-induced toxicities. Medication-induced mitochondrial dysfunction may arise
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24 through several mechanisms including direct inhibition of mitochondrial DNA transcription of
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26 electron transport chain (ETC) complexes, and the inhibition of the enzymes required for
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28 glycolysis and β -oxidation.²⁵⁻²⁷ In addition, inhibition of mitochondrial complexes, particularly
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30 MC-I, can increase the production of reactive oxygen species (ROS).²⁸ ROS can damage cellular
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32 components including lipids, proteins, and DNA. Hence once mitochondria are damaged there
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34 will be a disruption of cellular bioenergetics.²⁷ In support of this work, agents with anti-
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36 oxidative activity have been shown to exhibit hepato-protective effects, able to prevent anti-TB
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38 drug-induced hepatotoxicity.^{29,30}
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45 Our strategy of drug pre-treatment followed by subsequent cellular dosing provides an *in*
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47 *vitro* model to mimic cumulative drug treatment *in vivo*.³⁰ Our study has highlighted that a
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49 combination of anti-TB drugs may significantly increase their adverse effect on MC-I activity;
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51 presumably leading to exacerbated drug toxicity. A further reduction of MC-I activity was
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53 registered even with drugs employed at their IC₅₀ concentrations (Figures 5A,C,E), but
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55 moreover, with drug concentrations that produced a 10-20 % reduction of MC-I activity, a
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1 further dual-drug treatment of RIF + INH, or PZA + INH combinations were able to further
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3 significantly reduce MC-I activity (Figures 5B,D,F). The concentration of drugs used for pre-
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5 treatments reflected relatively high therapeutic dose levels, but for which no detectable depletion
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7 of ATP levels were evidenced. For RIF, a literature review has suggested that the current
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9 recommended 600 mg daily could be further increased to be more clinically efficacious without
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11 induction of toxicity.³¹ Ultimately, this synergistic effect of inhibiting MC-I activity at high
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13 therapeutic dose levels could be a contributing factor to patient hepatotoxicity experienced by
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15 dual- or multi- drug combinations.
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20 Damaged or excessive mitochondria are targeted for degradation and elimination by an
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22 autophagosome pathway. Autophagosomes fuse with lysosomes to form mitophagolysosomes
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24 (autolysosomes) in which the enveloped contents are degraded. This process of mitophagy can
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26 be cytoprotective and triggered in response to mitochondrial damaging agents that disrupt the
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28 MMP, generate ROS, and deplete cellular ATP levels.³²⁻³⁴ With TEM we were able to detect the
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30 presence of spherical mitochondria, extensive vacuolization, and the presence of vacuoles
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32 thought to contain degrading mitochondria (mitophagolysosomes) in response to drug
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34 incubations (Figure 7). These mitochondrial changes are similar to those observed in liver cells
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36 as a response to acute toxicological insult from ethanol³⁴. Changes to mitochondrial morphology
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38 and evidence of mitophagy was most apparent for RIF-treated cells, consistent with this drug's
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40 relatively higher mitochondrial toxicity as determined by a reduction of MMP, mitochondrial
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42 complex I & III activities, NAD⁺ levels, and increased lactate production (Figures 2-4,6).
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49 In summary, our results suggest that anti-TB drugs provoke hepatotoxicity by inducing
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51 deficiencies in the functions of mitochondrial ETC proteins. This study also highlights toxicity
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53 concerns regarding multi-drug combinatorial usage, and the importance of pre-clinical *in-vitro*
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1 testing of newly discovered anti-TB drug combinations on cellular bioenergetics, as this
2
3 approach may provide a useful predictive index of hepatotoxic potential.
4

5 **Acknowledgements:** The authors gratefully acknowledge financial support from Mansoura
6 University, Egypt, and the University of Nottingham, UK. The authors are also grateful to Mr
7
8 Ian Ward (University of Nottingham, UK) for technical assistance with resin embedding for
9
10 TEM. The authors would also like to acknowledge the helpful suggestions of the manuscript
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12 reviewers.
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17 **Conflict of interest:** The authors declare no conflict of interest associated with the production of
18 this manuscript.
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20 **Funding:** This research received no specific grant from any funding agency in the public,
21 commercial, or not-for-profit sectors.
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17 18 19 20 **Table 1: Hepatotoxicity of anti-TB drugs.**

21
22 Hep G2 cells were treated with the anti-TB drugs RIF, INH, & PZA, for 4, 24, & 48 hours and
23
24 the drug concentration producing 50 % inhibition (IC₅₀ values) of cellular ATP production
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26 determined.
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28 29 30 **Figure Legends:**

31 32 **Figure 1: Hepatotoxicity of anti-TB drugs.**

33
34 HepG2 cells were treated with the anti-TB drugs RIF, INH, & PZA, and cellular ATP levels
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36 measured after 4 hours (red circles), 24 hours (orange squares), & 48 hours (green triangles).
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38
39 **Graphs depict mean values relative to vehicle control values of 100 %, with significant changes**
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41 **from controls marked with asterisks. For significance: *** = $p < 0.001$, ** = $p < 0.01$, and * = p**
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43 **< 0.05.**
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45 46 47 **Figure 2: Effect of anti-TB drugs on HepG2 cell mitochondrial membrane potential.**

48
49 HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then
50
51 the mitochondrial membrane potential measured using a mitotracker green assay. **Histograms**
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53 **are displayed relative to vehicle control values of 100 %, with significant changes from controls**
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55 **marked with asterisks. For significance: ** = $p < 0.01$, and * = $p < 0.05$.**
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1 **Figure 3: Effect of anti-TB drugs on HepG2 cell mitochondrial Complex I and Complex III**
2 **activities.**

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5 HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then
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mitochondrial complex I and complex III activities were measured. Histograms are displayed
relative to vehicle control values of 100 %, with significant changes from controls marked with
asterisks. For significance: *** = $p < 0.001$, and * = $p < 0.05$.

Figure 4: Effect of anti-TB drugs on HepG2 cell NAD⁺ levels.

HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then
NAD⁺ levels measured. Histograms are displayed relative to vehicle control values of 100 %, with significant changes from controls marked with asterisks. For significance: * = $p < 0.05$.

Figure 5: Effect of combinations of anti-TB drugs on HepG2 cell mitochondrial Complex I activity.

HepG2 cells were pre-incubated with RIF, INH, & PZA at 30 μ M, 3 mM, & 3 mM, respectively for 48 hours. Cells were subsequently treated with anti-TB drugs at the concentrations detailed for 24 hours and then mitochondrial complex I activity measured. Significant changes from dual-drug vs single drug incubations are marked with asterisks. For significance: ** = $p < 0.01$, and * = $p < 0.05$.

Figure 6: Effect of anti-TB drugs on HepG2 cellular lactate production.

HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then the level of lactate produced was measured. Histograms are displayed relative to vehicle control values of 100 %, with significant changes from controls marked with asterisks. For significance: *** = $p < 0.001$, and * = $p < 0.05$.

Figure 7: Effect of anti-TB drugs on HepG2 cell ultrastructure.

1 HepG2 cells were incubated with (A) Vehicle control, (B) RIF at 0.5 mM, (C) INH at 70 mM,
2
3 (D) PZA at 84 mM for 24 hours and then cellular ultrastructure assessed by TEM. Electron
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5 micrographs depict at least one nucleus. A control rod-like mitochondrion is marked with an
6
7 asterisk (panel A), smaller, spherical mitochondrion marked with an arrowhead (panels B,C,D),
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9 and presumed mitophagolysosomes marked with a long arrow (panel B). White bar denotes
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11 5000 nm.
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For Peer Review

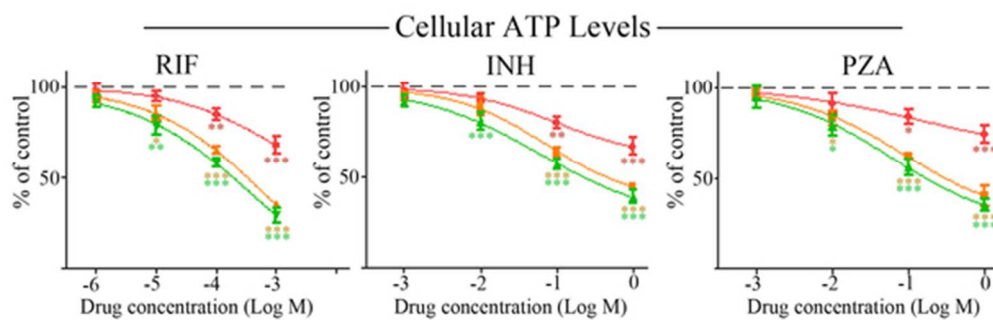
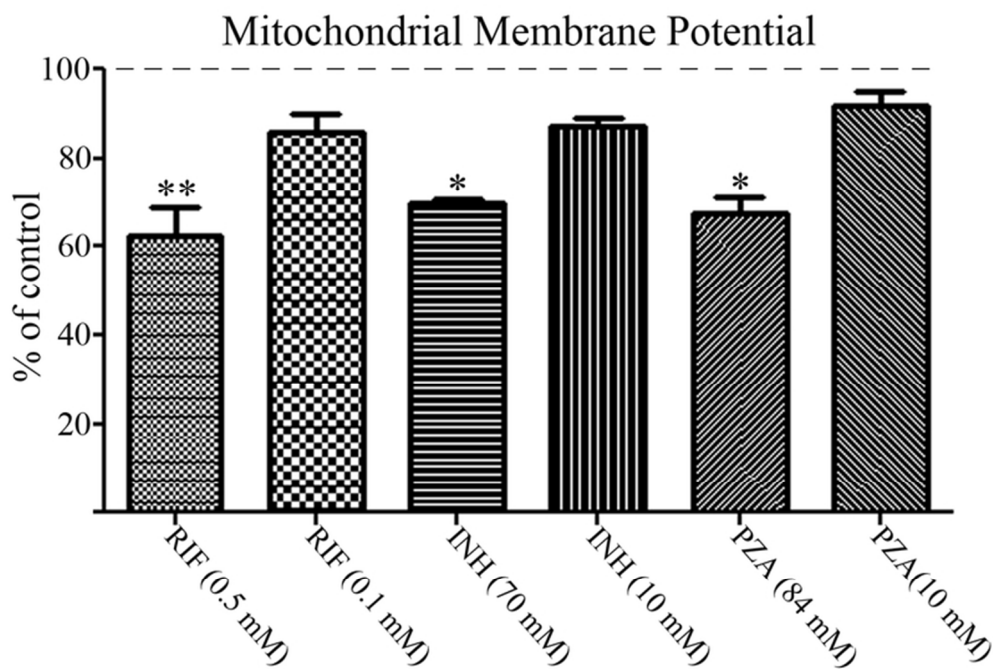


Figure 1: Hepatotoxicity of anti-TB drugs. HepG2 cells were treated with the anti-TB drugs RIF, INH, & PZA, and cellular ATP levels measured after 4 hours (red circles), 24 hours (orange squares), & 48 hours (green triangles). Graphs depict mean values relative to vehicle control values of 100 %, with significant changes from controls marked with asterisks. For significance: *** = $p < 0.001$, ** = $p < 0.01$, and * = $p < 0.05$.

51x16mm (300 x 300 DPI)



30 Figure 2: Effect of anti-TB drugs on HepG2 cell mitochondrial membrane potential.
31 HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then the
32 mitochondrial membrane potential measured using a mitotracker green assay. Histograms are displayed
33 relative to vehicle control values of 100 %, with significant changes from controls marked with
34 asterisks. For significance: ** = $p < 0.01$, and * = $p < 0.05$.

35 68x46mm (300 x 300 DPI)

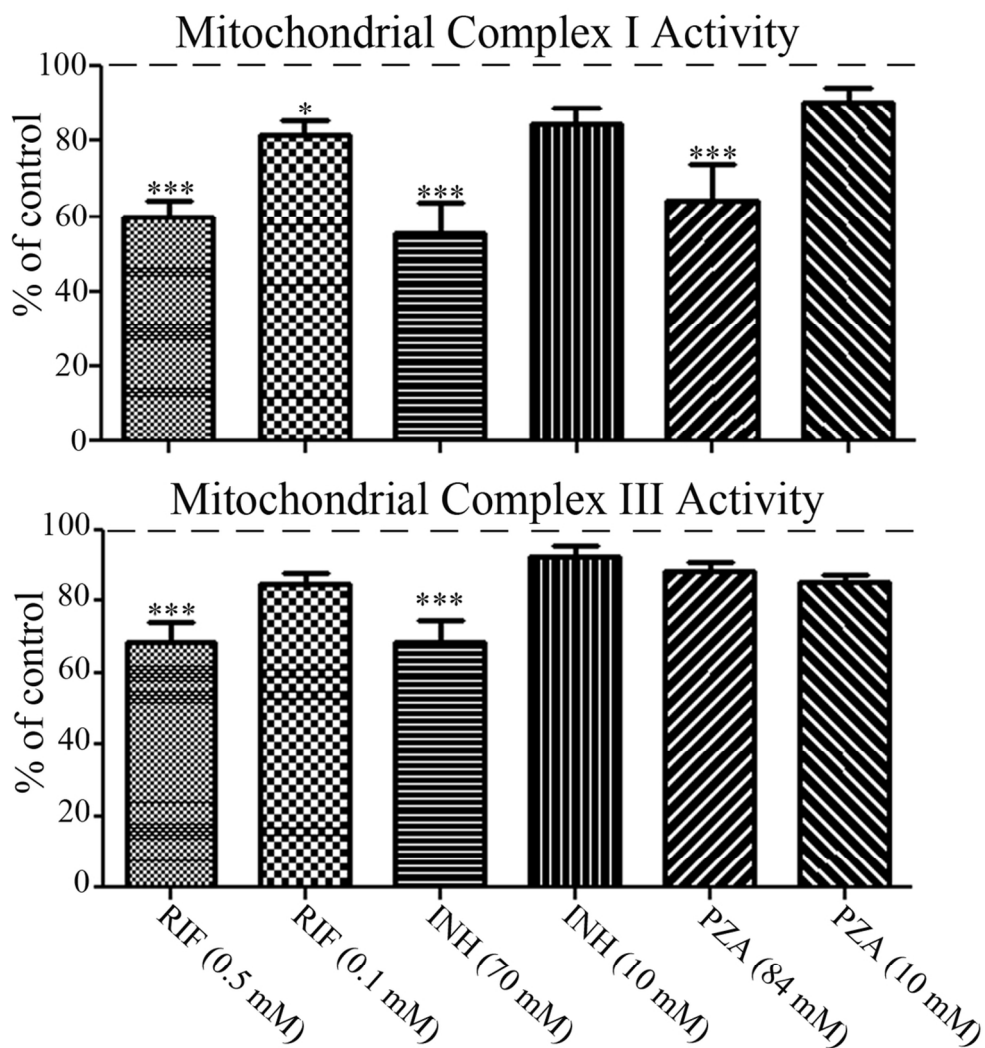


Figure 3: Effect of anti-TB drugs on HepG2 cell mitochondrial Complex I and Complex III activities. HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then mitochondrial complex I and complex III activities were measured. Histograms are displayed relative to vehicle control values of 100 %, with significant changes from controls marked with asterisks. For significance: *** = $p < 0.001$, and * = $p < 0.05$.

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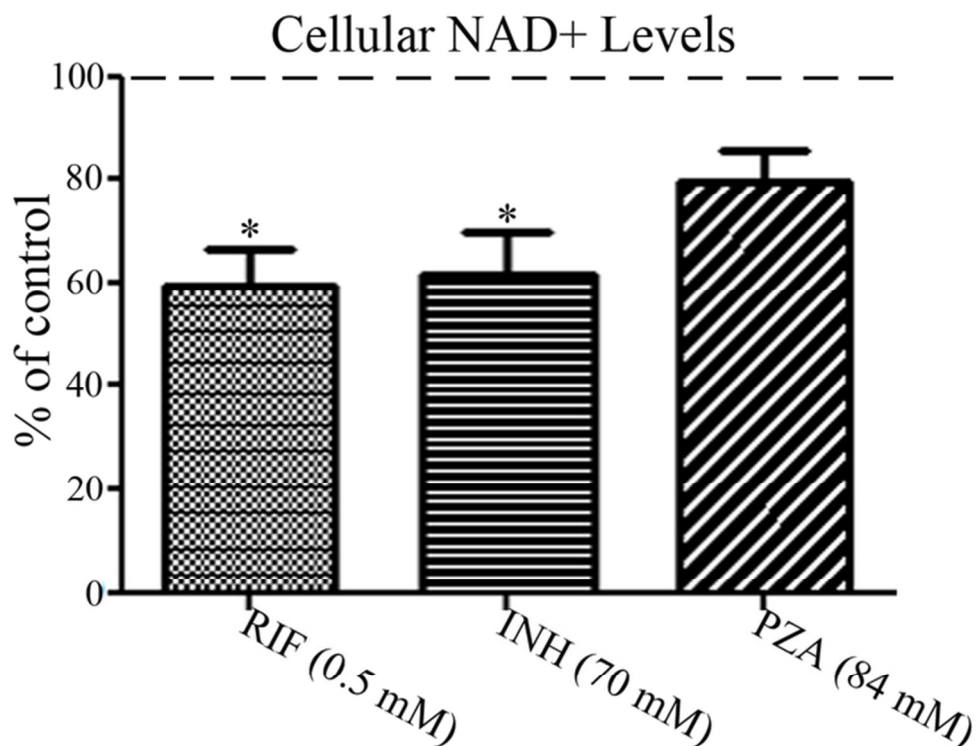


Figure 4: Effect of anti-TB drugs on HepG2 cell NAD⁺ levels. HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then NAD⁺ levels measured. Histograms are displayed relative to vehicle control values of 100 %, with significant changes from controls marked with asterisks. For significance: * = $p < 0.05$.

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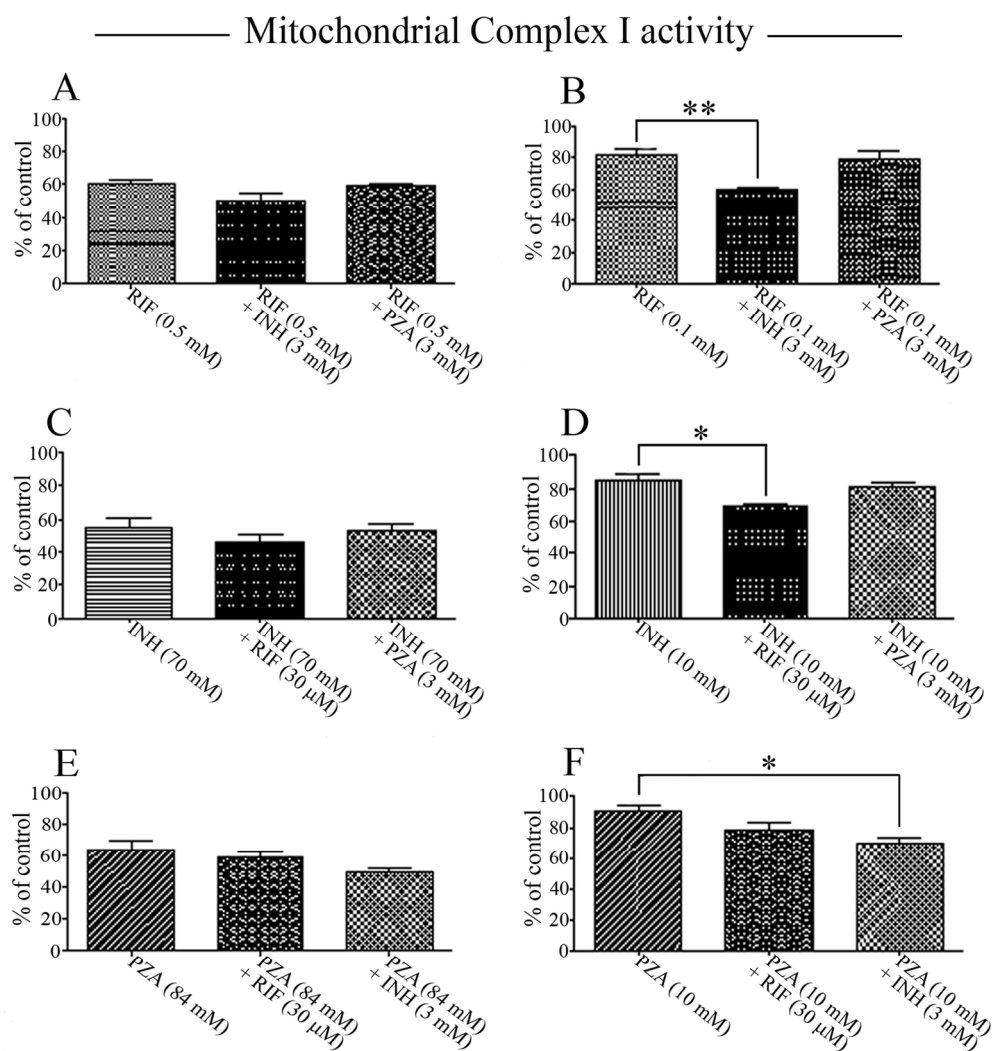


Figure 5: Effect of combinations of anti-TB drugs on HepG2 cell mitochondrial Complex I activity. HepG2 cells were pre-incubated with RIF, INH, & PZA at 30 μM, 3 mM, & 3 mM, respectively for 48 hours. Cells were subsequently treated with anti-TB drugs at the concentrations detailed for 24 hours and then mitochondrial complex I activity measured. Significant changes from dual-drug vs single drug incubations are marked with asterisks. For significance: ** = $p < 0.01$, and * = $p < 0.05$.

149x160mm (300 x 300 DPI)

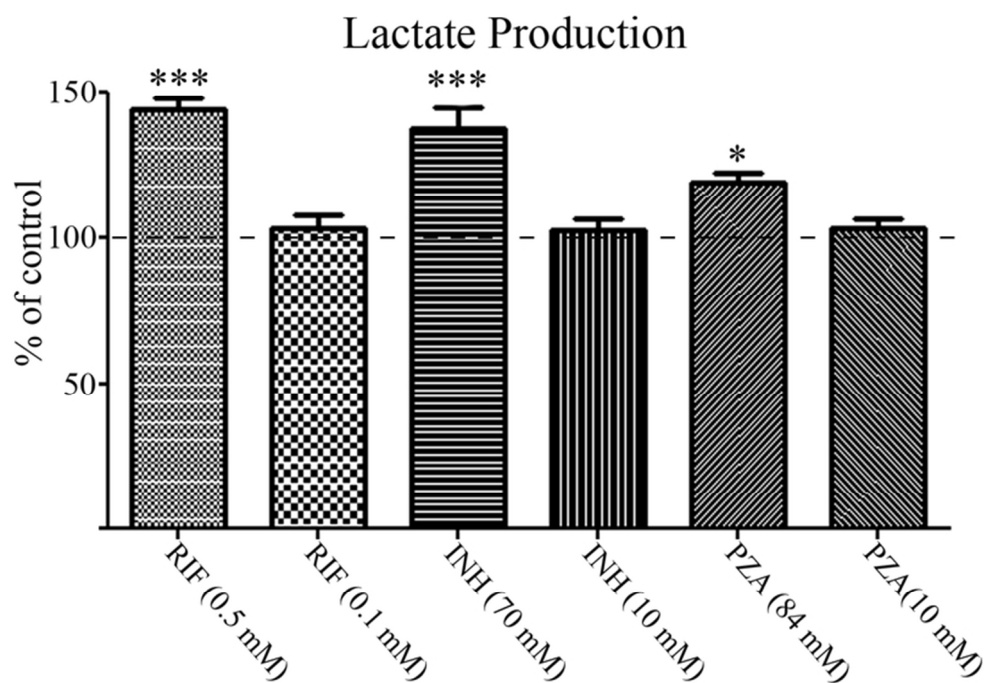


Figure 6: Effect of anti-TB drugs on HepG2 cellular lactate production. HepG2 cells were incubated with anti-TB drugs at the concentrations listed for 24 hours and then the level of lactate produced was measured. Histograms are displayed relative to vehicle control values of 100 %, with significant changes from controls marked with asterisks. For significance: *** = $p < 0.001$, and * = $p < 0.05$.

69x48mm (300 x 300 DPI)

TEM Ultrastructure

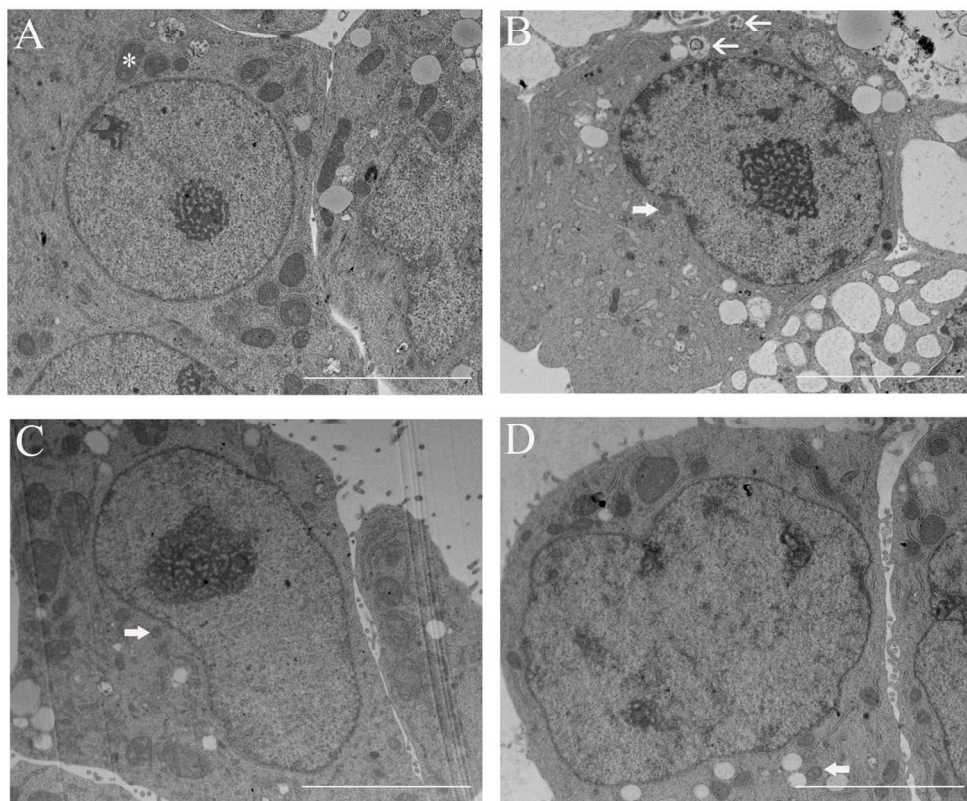
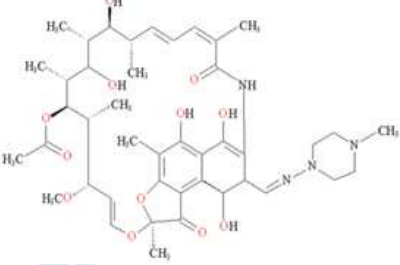
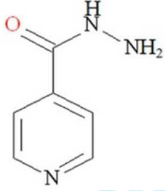
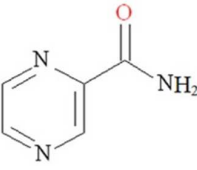


Figure 7: Effect of anti-TB drugs on HepG2 cell ultrastructure.

HepG2 cells were incubated with (A) Vehicle control, (B) RIF at 0.5 mM, (C) INH at 70 mM, (D) PZA at 84 mM for 24 hours and then cellular ultrastructure assessed by TEM. Electron micrographs depict at least one nucleus. A control rod-like mitochondrion is marked with an asterisk (panel A), smaller, spherical mitochondrion marked with an arrowhead (panels B,C,D), and presumed mitophagolysosomes marked with a long arrow (panel B). White bar denotes 5000 nm.

123x109mm (300 x 300 DPI)

Table 1: Hepatotoxicity of anti-TB drugs.

Drug	Chemical structure	IC ₅₀ concentration (mM)		
		4 hr	24 hr	48 hr
Rifampicin (RIF)		0.8	0.5	0.5
Isoniazid (INH)		94	70	57
Pyrazinamide (PZA)		135	84	57