Supporting Information

Thiourea derivatives rejuvenate dysfunctional mitochondria in Chronic Hepatitis B infection

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1. Untargeted Proteomics analysis using UHPLC coupled with High Resolution-MS/MS:

Untargeted proteomic analysis of cell culture samples was performed using ultra high-performance liquid chromatography (UHPLC) coupled with high resolution tandem mass spectrometry (HRMS/MS). In six-well plates, 100,000 HepG2.2.15 cells were seeded. Next day, they were treated with 10µM concentration of thiourea derivatives and ETV. Followed by incubation for 24h then trypsinized cultured cells. The trypsinized cells collected by centrifugation at 5000 RPM. The cells pallet resuspended in RIPA lysis buffer for the Isolation of total proteins from cultured cells. For digestion, 50µg equivalent protein was reconstituted in 200µl ABC buffer (ammonium bicarbonate buffer), 20 µl of 10 mM DTT was added followed by incubation at 60°C for 1 hour in a water bath to reduce proteins. Post-reduction, 15 µl of 10 mM IAA (Iodoacetamide) was added and incubated for 30 min in the dark for alkylation of proteins, then 10 µl of modified sequencing grade trypsin (0.1 µg/µl), was added followed by incubation at 37°C for 20–24 h in a water bath. To inhibit the trypsin activity in reaction mixture, add 5µl of conc. formic acid.

A. C18 column mediated de-saltation of peptides: Sample cleanup was performed using the C18 spin column using the following steps: (1) column washing. (2) Equilibration of the column. (3) Binding of peptides to the column. (4) Elution. Eluted samples were subjected to lyophilisation at 4°C. The lyophilized samples were reconstituted in 40 µl of 0.1% (v/v) formic

acid which was followed by centrifugation at 15,000 g and samples were run on UHPLC-HRMS/MS.

B. Untargeted proteomics by UHPLC-HRMS/MS: The peptides were ionized by nano-electrospray and subsequent tandem mass spectrometry (MS/MS) on a Q-Exactive™ Plus (Thermo Fisher Scientific, San Jose, CA, United States). The fractions were enriched on a trap column (75 µm x 2 cm, 3 µm, 100Å, nano Viper 2Pk C18 Acclaim PepMap™ 100), at a flow rate of 8µl/min and then resolved on an analytical column (75 µm × 25 cm, 2 µm, 100Å, nano Viper C18, Acclaim PepMap™ RSLC. The peptides were eluted by a 3–95% gradient of buffer B (aqueous 80% acetonitrile in 0.1% formic acid) with a continuous flow rate of 800 nL/min for about 70 min on a 25-cm analytical C18 column (C18, 3 mm, 100 A). The peptides were analyzed using a mass spectrometer with the collision-induced dissociation mode with the electrospray voltage of 2.3 kV. Analysis on the orbitrap was performed with full scan MS spectra with a resolution of 70,000 from m/z 350 to 1800. Mascot algorithm (Mascot 2.4, Matrix Science). Significant proteins were identified at ($p < 0.05$) and q values ($p < 0.05$). The threshold of false discovery rate was kept at 0.012.

C. Mass spectrometry proteomics: The isolated proteins were reduced, alkylated, and digested using trypsin followed by mass spectrometry analysis similar to that stated in proteome analysis section. The MS/MS data was acquired and analyzed by Proteome Discoverer (version 2.3, Thermo Fisher Scientific, Waltham, MA, United States) using the human sequence of Uniprot. This was cross validated using Mascot algorithm (Mascot 2.4, Matrix Science) specifically for all possible human peptides. In brief, significant peptide groups were identified at ($p<0.05$) and q values ($p<0.05$) and the false discovery rate at 0.01. Only rank-1 peptides with Peptides Sequence Match (PSMs)>3. Peptides mapping to human database were segregated and were subjected to statistical, and functional analysis.

2. MTR and MTG Staining of HepG2.2.15:

HepG2.2.15 cells which is the well-known chronic HBV infection model (Xu et al., 2021), were seeded in six-well plates. The next day, they were treated with 10µM concentration of thiourea derivatives and ETV, as well as 1mM of Ascorbic Acid (Vitamin C). Followed by incubation for 24h then trypsinized cultured cells, and made into a single cell suspension in PBS containing 1% BSA and 2.5 mM EDTA (FACS Buffer). The complete cells were labelled with

25 nM, 100 nM, or both MitoTracker™ Red and MitoTracker® Deep Red (MTR and MTG; Life Technologies) dyes. Following mitochondrial labelling, cell suspension samples were fixed for 5 min at room temperature in 2% paraformaldehyde before being washed for 5 min at room temperature with PBS. Following daily QC checks using BD CaliBRITE™ beads (BD Biosciences), 50,000 events of each sample were collected using a FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA). For the purposes of measuring fluorescence, green and red fluorescence photomultipliers were used. The percentages of cells that stained positively for MTG and MTR were noted. The FlowJo™ Software (Becton, Dickinson and Company; Ashland, OR 97520 USA) was used for all data analysis.

3. Statistical and Correlation Analysis:

Results are shown as mean and S.D. unless indicated otherwise. Statistical analysis was performed using Graph Pad Prism, version 8 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com), and SPSS, version 20; p values < 0.05 using Benjamini-Hochberg correction (FDR< 0.05) were considered significant. Unpaired (two-tailed) Student t-test and the Mann-Whitney U test were performed to compare the two groups. A one-way analysis of variance and the Kruskal-Wallis test was performed for comparison among more than two groups. All correlations were performed using Spearman correlation analysis, and $R^2>0.5$ and $p<0.05$ were considered statistically significant. All statistical analyses were performed using an unpaired two-sided t test. A difference was considered to be statistically significant at *p$<$ 0.05 and **p$<$ 0.01, and not significant (ns).