Thiourea derivatives rejuvenate dysfunctional mitochondria in Chronic Hepatitis B infection

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Abstract: Chronic hepatitis B (CHB) infection and the Hepatitis B virus X protein (HBx) are major risk factors associated with hepatocellular carcinoma (HCC). In CHB infection, HBx induces mitochondrial dysfunction, exhaustion and impaired function in hepatocytes. Restoring hepatocyte health along with reduction in virus replication could be an ideal treatment for CHB. Thiourea derivatives are well known for their antiviral property though their effect on mitochondrial and/ or hepatocyte health remains obscure. This study focus on the repurposing of thiourea derivatives (DSA-00, DSA-02, and DSA-09) on hepatocyte replenishment. HepG2.2.15 cells were treated with thiourea derivatives, alongside Entecavir (ETV). The proteomics analysis showed both DSA-00 and ETV were enriched with proteins associated with antiviral responses. In addition, DSA-00 additionally showed increase in proteins linked to mitochondrial response. Whereas DSA-02 exhibited association with innate immune system and citric acid cycle and DSA-09 displayed pathways similar to DSA-00 and ETV. Treated groups exhibited enhanced bio-energetic and antiviral response as compared to the untreated group. FACS analysis revealed the restoration of exhausted hepatocytes by thiourea derivatives through targeting mitochondria. Our findings suggest that thiourea derivatives hold potential as a novel therapeutic agent that seems to restore mitochondrial health along with anti-viral response in CHB.

Keywords: Antiviral; Thiourea derivatives; Hepatitis B Virus; Chronic hepatitis B infection; Mitochondria dysfunction; Exhausted hepatocytes

1. Introduction

Chronic hepatitis B (CHB) infection is caused by Hepatitis B Virus (HBV) that lasts for more than six months and leading to long-term liver disease. Currently, 257 million people around the world had a CHB infection [1, 2]. CHB often leads to major problems with the liver, such as cirrhosis, liver failure, and liver cancer. As the illness persists longer, there is more liver inflammation and damage, with likelihood of aggravation of these issues [3]. CHB can also alter mitochondrial and endoplasmic reticulum (ER) functions, which can contribute to liver damage and disease progression [4, 5]. Patients with CHB show higher levels of mitochondrial DNA (mtDNA) in their serum than healthy, indicating increased mitochondrial dysfunction and damage [6]. Alterations in mitochondrial morphology, and their membrane potential, metabolism, and biogenesis have been reported in the HBV-infected hepatocytes [7, 8]. CHB has also been linked to ER stress and its response was found to be activated in liver biopsies from patients with persistent HBV infection, and this was linked to increased liver fibrosis and inflammation[9, 10]. ER stress and dysfunction of mitochondria are associated to the progression to cirrhosis and end stage Hepatocellular carcinoma (HCC) [11]. Several factors have been implicated in the development of HBV infection, including the HBx oncoproteins, immune-mediated death of HBV-infected hepatocytes, and liver regeneration [12]. HBx regulates viral replication, viral and host genes transcription, various cell signaling activities, ubiquitin-proteasome machinery, cell cycle progression, and programmed cell deaths etc [13].

The regulatory protein HBx, which plays an essential role in the viral life cycle, is encoded by the 0.7 kb RNA of HBV[14]. HBV is a member of the Hepadnaviridae family and has a partially double-stranded DNA genome of 3.2 kb. Its genome contains four open

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reading frames (ORFs), which encode 3.5, 2.4, 2.1, and 0.7 kb RNAs and are translated into seven different proteins. The 3.5 kb RNA is responsible for encoding the core protein (HBcAg) and the pre-core protein (HBeAg), while the 2.4 and 2.1 kb RNAs encode the small (S), medium (M), and large (L) surface proteins. Previous research suggests that the regulatory protein HBx may be responsible for various effects attributed to HBV [13]. HBx induced aberrant aggregation of mitochondrial components near the nucleus periphery triggered by HBx-driven p38 mitogen-activated protein kinase (MAPK), which may eventually lead to cell deaths [15]. HBx damage mitochondria via interaction with voltage-dependent anion channel (HVDAC3), which is known as mitochondrial porins and forms pores in mitochondrial outer membranes[16] and interaction alters mitochondrial transmembrane potential, resulting in the formation of reactive oxygen species (ROS) and cellular ATP/ADP ratio [17]. Furthermore, HBx can suppress the expression of nucleus-encoded genes involved in mitochondrial beta-oxidation of fatty acids, resulting in a low level of cellular ATP due to energy source deficit. HBx-induced Raf-1 kinases pathway or the apoptosis regulator bcl-2-associated X protein (BAX) are translocated to mitochondria by HBx, resulting in hepatic cell proliferation or apoptosis, respectively [6]. HBV-infected cells synthesize a lot of surface proteins in the endoplasmic reticulum (ER), disrupting ER homeostasis and causing ER stress [10]. CHB patient's biopsies reported with enlarge ER due to surface proteins (preS1 and preS2). ER stress or HBx can trigger autophagy [18]. These findings imply that HBx-induced mitochondrial dysfunction and ER stress could be contributed to hepatocellular carcinoma pathogenesis, including proliferation, metastasis, and other features of carcinogenesis.

Biological networks have been used as a powerful approach for identifying novel dysregulated pathways driving molecular pathology. Weighted protein co-expression network analysis (WPCNA) is a multi-protein study that identifies groupings of proteins that are frequently altered and orchestrates them into modules without relying on a priori specified proteins sets or pathways. WPCNA is a useful technique for determining how networks of proteins and phenotypes are related [19].

Thiourea and its derivatives have been used therapeutically as antioxidants[20], anti-inflammatory drugs, anti-cancer [21], fungicides [22], as well as anti-viral [23, 24]. Recently, a novel thiourea compound DSA-00 (IR-415) was identified by high-throughput screening of the Maybridge library that suppresses HBV replication like current antivirals [23].

The present study is focused on the analysis of proteome alteration in cell (HepG2.2.15) under the influence of currently used anti-viral drugs in comparison to thiourea derivatives in order to identify key molecular changes linked to mitochondrial dysfunction and antiviral activity. We validated the proteomic finding of mitochondrial health using flow cytometry data with the ultimate goal of identifying whether repurposed thiourea derivatives could be improved mitochondrial and hepatocyte health.

2. Results

2.1. Alteration in protein profile following treatment with antiviral drug and thiourea derivatives

To investigate the alterations in protein profile during antiviral therapy in CHB infection, HepG2.2.15 cells were treated with standard antiviral drug Entecavir (ETV), which is a standard drug for treating CHB patients [25]. A wide-scale comparison of the proteome ETV-treated and untreated cells showed complete separation of the samples based on their similarity (Figure 1B). These was significant changes in the proteome of the ETV-treated group compared to the untreated group, with 607 proteins (274 UP and 246 Down-regulated) found to be significant (Figure 1C, D). According to KEGG analysis [26] the upregulated proteins were primarily linked to the ribosome, glycolysis, HIF-1 signaling, pyruvate metabolism, phagosome, pentose phosphate, necroptosis, tight junction, ferroptosis, peroxisome, fatty acid degradation, IL-17 signaling, mitophagy, and pathways related to the cell cycle ($p<0.05$) (Figure 1E). On the other hand, the downregulated proteins were mostly linked to mRNA surveillance, TCA cycle, protein processing in the ER, oxidative phosphorylation, insulin signaling, glucagon signaling, and RNA transport

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(p<0.05) (Figure 1F). Besides, a large proportion of the proteins that were significantly altered were host antiviral proteins, whereas the proteins involved in bioenergetics were downregulated. These findings suggest that novel approaches are needed to restore the bioenergetics function and modulate the host RNAi-mediated antiviral defense system.

Figure 1

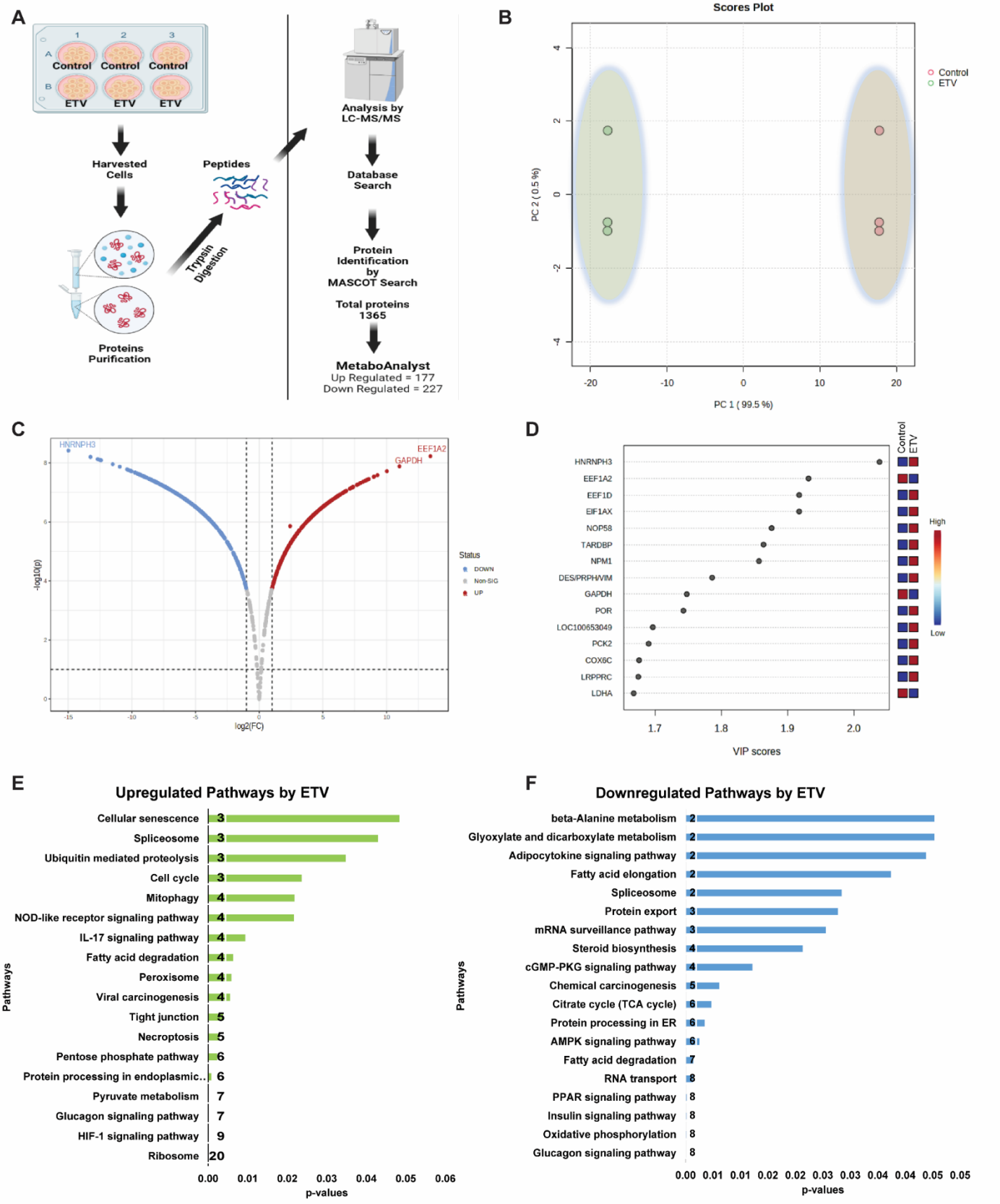

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Figure 1. Alterations in the cellular proteome following treatment of HepG2.2.15 cells with Entecavir (ETV). (A) Study design. (B) Principal coordinate analysis of proteome of untreated and ETV-treated and groups. (C) Volcano plot showing differentially expressed proteins between untreated and Entecavir-treated groups. y-axis corresponds to –log10 p values and x-axis corresponds to -log1 fold changes. (D) Variable importance in projection (VIP) plot derived from the Partial Least-Squares Discriminant Analysis, displaying the discriminant features in the ETV-treated and control groups. KEGG pathway enrichment analysis of up-regulated (E) and down-regulated (F) pathways following ETV-treatment.

2.2. Thiourea derivatives induce specific protein modules to alter biological pathways

Though ETV and thiourea derivatives belonged to different classes of antivirals with distinct modes of action for suppressing HBV replication. ETV is known to have minimal impact on cellular energetics [27] whereas, thiourea derivatives have shown potential in inhibiting HBV replication [23] (Singh et al., 2023; Kumar et al., 2023 in communication). We used weighted protein co-expression network analysis (WPCNA) approach [28] to identify proteins modules specific to DSA-00, DSA-02, DSA-09, ETV, and untreated cells for correlation patterns among proteins that may all work together in persistence of HBV infection (Figure 2A). WPCNA revealed 10 clusters (modules), corresponding to a total of 636 strongly linked proteins in DSA-00, DSA-02, DSA-09, ETV, and untreated groups (Figure 2B).

We got interested in modules whose expression pattern was similar to ETV and had some unique modules. DSA-00 has a similarity of approximately 70 percent with ETV (Black, Magenta, Red, Pink, Green, and Yellow) and also has unique module (Grey), but DSA-09 has two common modules with ETV and DSA-00 (Black and Magenta). The ETV, DSA-00, and DSA-09 shared modules with proteins involved in Ribosome, Proteasome, Fatty acid biosynthesis, PPAR signaling, and RNA transport pathways (Figure 2C). DSA-02 showed unique Brown module proteins that were associated with biological pathways such as Glucagon signaling, tight junction, PPAR signaling, Fatty acid degradation, and Spliceosome (Figure 2D). DSA-09 induced modules were common with DSA-00 and ETV modules. DSA-00 has a specific grey module other than common modules with ETV, DSA-02 and DSA-09. The grey module proteins were involved in protein processing in ER, Oxidative phosphorylation, Apoptosis, Mitochondria Biogenesis, and Protein folding pathways (Figure 2E). Additionally, the untreated group has unique modules (Blue and Turquoise), and the proteins in these modules were linked with Regulation of actin cytoskeleton, Tight junction, HIF-1 signaling, Glycolysis/Gluconeogenesis, and protein processing in ER pathways (Figure 2F). Based on these findings we conclude that, proteomic changes induced by DSA-00 are 70% similar to that seen under the treatment with ETV. In addition, our results highlight that thiourea derivatives modulate unique protein modules majorly linked to mitochondrial and ER activity suggesting that the thiourea derivatives not on provide anti-viral support but also rejuvenates the mitochondria and hepatocyte health in CHB.

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Figure 2

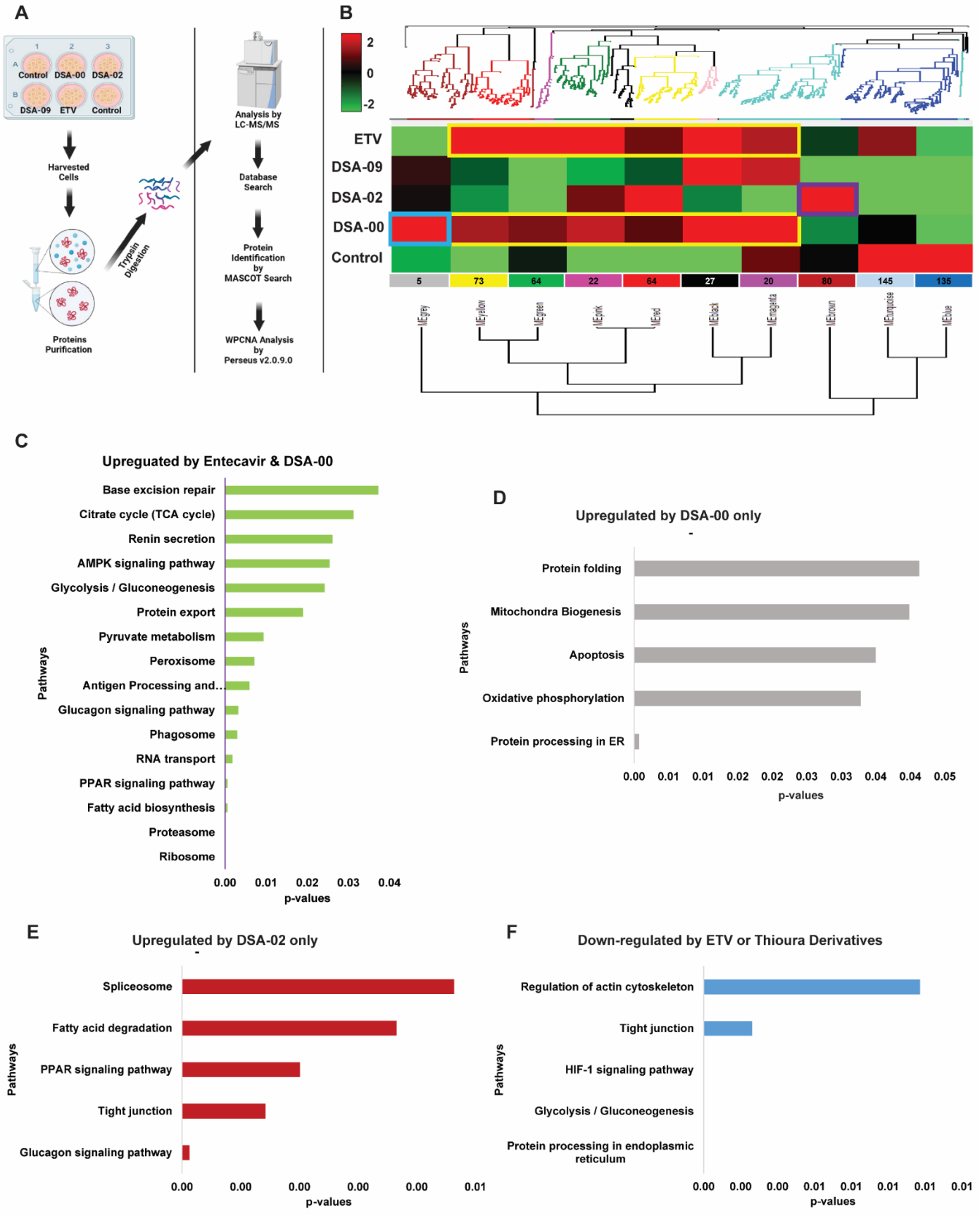

Figure 2. Identification of proteins associated with antiviral response and mitochondrial dysfunction. (A) Scheme of identification of antiviral response proteins by BlastP with antiviral response database. (B) Antiviral response proteins and their expression in DSA-02 specific module (Brown)

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and (C) DSA-00-related modules. (D) Induction of proteins associated with mitochondrial functions by DSA-00. (E) PANTHER analysis of biological processes associated with antiviral response.

2.3. Thiourea derivatives restore bioenergetics of exhausted hepatocytes

It is well established that HBx causes mitochondrial dysfunction and inflammation/injury in hepatocytes in CHB infection by disrupting mitochondrial respiration and membrane potential [29]. Thiourea derivatives, on the other hands, have been shown antioxidant activity [30] as well as bind to HBx and block the HBV-induced functions (Kumar et al., 2023 in communication). Based on these findings, we hypothesized that thiourea derivatives may have the potential to restore mitochondrial dysfunction in HBV-related liver diseases. To investigate this hypothesis, HepG2.2.15 cells were treated with DSA-00, DSA-02, DSA-09, and ETV. The proteome profile was subjected to WPCNA that led to the identification of several modules of proteins that were differentially expressed between the groups. Pathway analysis of these modules showed that 31 in DSA-00 and 8 proteins in DSA-02 were linked with the antiviral response (Figure 3A). The brown module of DSA-02 contained eight proteins that were associated with enhanced host immunity and responsiveness to chronic infection (FIGURE 3B), whereas DSA-00 had twenty proteins providing negative regulation and ten associated with positive regulation to the HBV infection (Figure 3C).

Furthermore, we observed that DSA-00 induced a unique grey module that contained only five proteins associated with mitochondrial functions like oxidative phosphorylation, membrane potential, and apoptosis (Figure 3D). All antiviral response proteins that were up-regulated by DSA-00 (31 proteins), DSA-02 (8 proteins), and DSA-00 (5 proteins) were analyzed for their biological processes using the PANTHER classification system (v.14.0). This analysis revealed that nearly 50% of the proteins belonged to metabolic and cellular processes, suggesting that thiourea derivatives can restore bioenergetics in exhausted hepatocytes by targeting dysfunctional mitochondria (Figure 3E).

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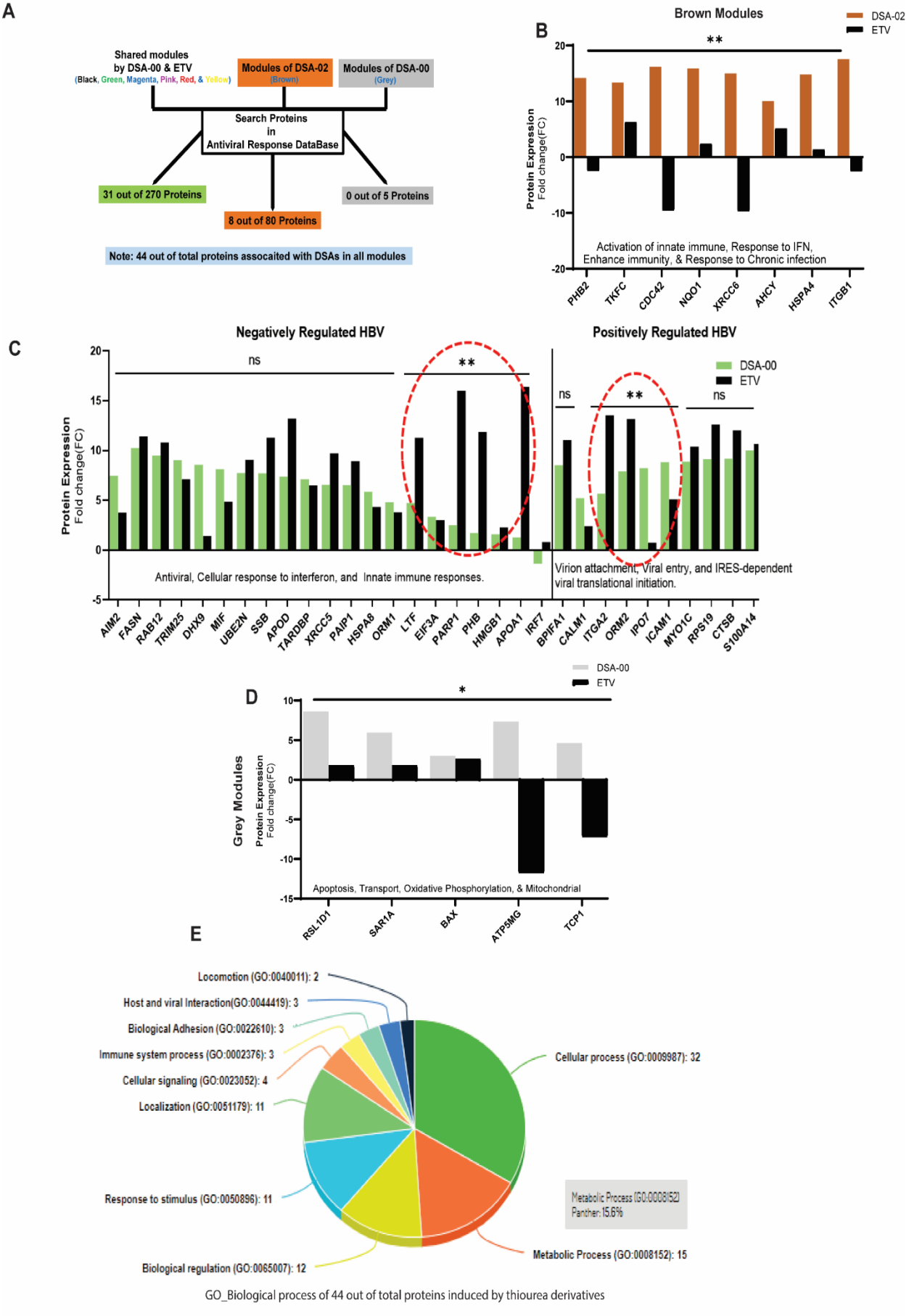
Figure 3



GO_Biological process of 44 out of total proteins induced by thiourea derivatives

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Figure 3. Identification of proteins associated with antiviral response and mitochondrial dysfunction. (A) Scheme of identification of antiviral response proteins by BlastP with antiviral response database. (B) Antiviral response proteins and their expression in DSA-02 specific module (Brown) and (C) DSA-00-related modules. (D) Induction of proteins associated with mitochondrial functions by DSA-00. (E) PANTHER analysis of biological processes associated with antiviral response.

2.4. Thiourea derivatives restore mitochondrial functions

After analyzing the metabolic and cellular process associated proteins, we found that most of the proteins were linked to the mitochondria (Figure 4A). Based on these results, we hypothesized that thiourea derivatives may have a role in mitochondrial-related functions. To validate these findings, we further blasted the proteins with the MitoProteome database [31] and found nine proteins (BAX, LMNA, HSPA5, HSPA4, PHB2, CTSB, MYO1C, PHB, and FASN) were directly linked to mitochondria and its functions (Figure 4B, C). These results suggested that thiourea derivatives may improving mitochondrial health and functionality. To validate our hypothesis, HepG2.2.15 cells were treated with thiourea derivatives. Vitamin C was used as a positive control whereas ETV was used as a control for HBV infection. FACS analysis revealed that thiourea derivatives had the potential to increase mitochondrial mass (Figure 4D) and restore the membrane potential of mitochondria (Figure 4E). Therefore, the ratio of mass and membrane potential of mitochondria represents the functionality of the mitochondrial pool. Moreover, these thiourea derivatives were able to restore and maintain the pool of functional mitochondria in chronic HBV infected hepatocytes (Figure 4F). These observations clearly outline unique role of the thiourea derivative improving mitochondrial health not described earlier. Thus, the use of thiourea derivative in CHB care is could be complementary to the currently used antivirals.

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Figure 4

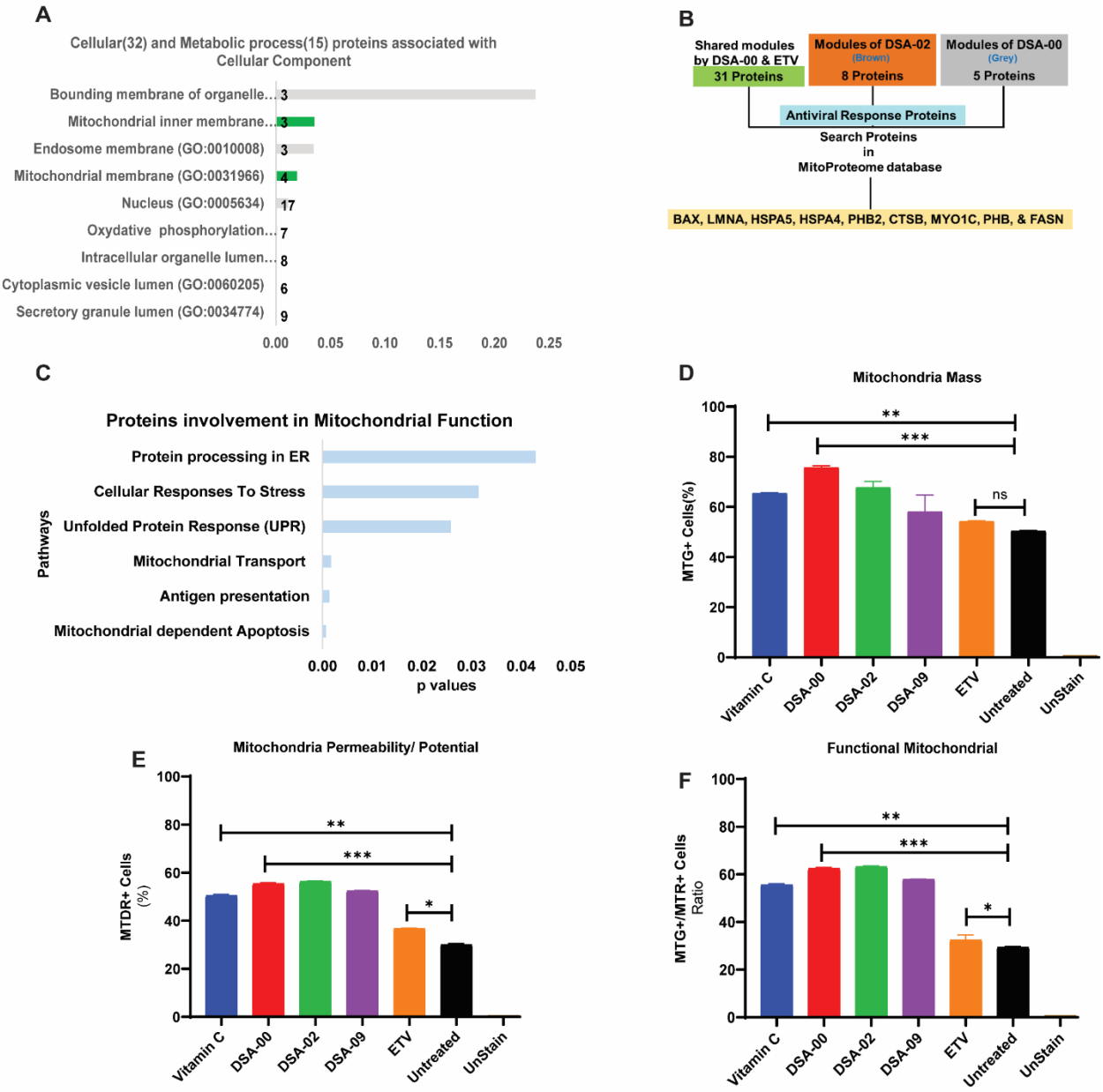

Figure 4. Restoration of mitochondrial functions by thiourea derivatives. (A) PANTHER classified proteins involved with cellular and mitochondrial functions. (B) strategy to identify proteins linked to mitochondria and its functions. (C) KEGG analysis of proteins involved in mitochondrial functions. Analysis of mitochondrial health and functionality in the presence of thiourea derivatives (DSA-00, DSA-02 and DSA-09): (D) mitochondrial mass, (E) membrane potential of mitochondria, and (F) functional pool of mitochondria. Vitamin C was used as an anti-oxidative control whereas ETV was used as a control for HBV infection. Each experiment was performed thrice and in triplicate. Statistical significance was calculated by student 't' test. *, $p< 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; ns=non-significant.

3. Discussion

CHB is a major risk to the progression to HCC, the sustained presence of the virus in the liver causes liver inflammation and exhaustion of hepatocytes[7]. Previous studies have shown that HBV replication and the expression of the non-structural protein HBx lead to mitochondrial dysfunction, including alterations in mitochondrial unfolded protein response, biogenesis, mitophagy, and increased production of reactive oxygen species (ROS) such as superoxide and peroxynitrite [7, 32]. HBx has been shown to have diverse

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roles in regulatory HBV replication, cellular transcription and signal transduction pathways, proteasome activity, and cell cycle progression, and has also been linked to the progression of HBV-associated HCC [33]. Furthermore, a portion of cytosolic HBx may localize to mitochondria of hepatocytes, where it can modulate mitochondrial membrane potential and alter cytosolic calcium levels via the mitochondrial permeability transition pore (mPTP) [16, 34]. The current study has focused on HBx as a potential therapeutic target to block HBV replication, restore mitochondrial function, and reduce liver inflammation in CHB patients. One promising candidate for this approach is compound DSA-00 and its derivatives, that have been found to regulate HBx-induce activities, including HBV replication, HBx-induced kinase activity, and HBx-modulated host RNAi machinery [23] (Kumar et al., 2023 communicated).

Now, using a proteomic approach, we have identified the proteomic perturbation associated with the decrease in HBV viral replication and improvement of mitochondrial functions and exhaustion of hepatocytes during CHB infection. To achieve this, proteome profile of cell culture-based model of chronically infected hepatocytes was compared to chronically infected hepatocytes treated with commonly used anti-viral ETV or different thiourea derivatives. Instead of performing conventional differential expression analysis based on predetermined protein sets or pathways, we conducted a modular analysis that used an unsupervised hierarchical clustering method to examine the link between groups of co-expressed proteins. We used the WPCNA method which enables the identification of protein co-expression and the determines of how well a particular protein is connected to other proteins pathways that are jointly involved in a biological process. The protein co-expression networks of intricate biological processes are segmented into modules by WPCNA, which identified 10 different modules (Figure 2), with Black, Magenta, Red, Pink, Green, and Yellow modules most shared modules between DSA-00 and ETV treatment. According to enrichment analysis, these modules were mostly correlated with ribosome, proteasome, fatty acid biosynthesis, PPAR signaling, RNA transport pathways, Brown module was unique for the DSA-02 treatment, with proteins significantly associated with biological processes such as Glucagon signaling, tight junction, PPAR signaling, fatty acid degradation, spliceosome, etc. The Grey modules had only five proteins, which were upregulated by the treatment of DSA-00 (Figure 2E). KEGG analysis of these proteins revealed that they were significantly involved in mitochondrial biogenesis, oxidative phosphorylation, and unfolded protein response.

Furthermore, we looked at proteins involved in the antiviral response by blasting with the antiviral response database, and found 44 proteins in Black, Magenta, Red, Pink, Green, Yellow, Brown, and Grey modules (Figure 3D). Analysis of the correlation between the changed biological functions in DSA-00, DSA-02, and ETV groups and found that antiviral response proteins were associated with positively regulated and negatively regulated HBV in DSA-00+ETV groups. DSA-02 induced proteins that supported the antiviral response. Additionally, DSA-00 had a unique Grey module, which had five proteins associated with mitochondrial functions.

The aim of our study was to repurpose drugs that have the potential to reduce HBV infection and restore the exhausted hepatocytes that are notably exhausted during CHB infection. Restoration of dysfunctional mitochondria has been correlated with the improvement of cell health in the past, although not being previously linked to thiourea derivatives efficacy. Moreover, the blast hits with mitoproteome database [31] revealed that thiourea derivatives are suitable candidates that have the efficacy to restore dysfunctional mitochondria as well as have the potential to reduce HBV infection. Our study provides valuable insights into the potential of thiourea derivatives for the treatment of CHB and restoration of exhausted hepatocytes.

Limitations of the investigation include the fact that it is a pilot study and that just one type of in-vitro CHB model (HepG2.2.15) was employed. Instead of measuring mitochondrial respiration, oxygen consumption rate (OCR), and ATP production in the validation research, just test the mitochondrial health using flow cytometry.

4. Material and Methods

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4.1. Cell culture and reagents

HepG2.2.15 cells, which have integrated two copies of HBV genome, used in this study, were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco of Invitrogen), GlutaMax, 10 mM HEPES, 100 unit/ml penicillin, 100 µg/ml streptomycin, and 10% FBS at 37°C in a humidified atmosphere containing 5% CO2.

DSA-00, DSA-02, DSA-09, Entecavir (ETV, Gilead Sciences), and Ascorbic Acid (Vitamin C, Sigma-Aldrich). All compounds were dissolved in dimethyl sulfoxide and stored at -20°C or -80°C until used.

4.2. Proteomics Analysis

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)[35]. HepG2.2.15 cells (10^5), were seeded in six well plates. Following day, cells were treated with 10µM of thiourea derivatives, ETV and Vitamin C. After 24 h, cells were trypsinized and collected by centrifugation at 5000 RPM. The cells pellet was then resuspended in RIPA lysis buffer to isolate total proteins. The scheme of proteomics analysis is shown in (Figure 1A, and 2A). The detailed protocol for the untargeted proteomics analysis are provided in the supporting information (SI).

4.3. MTR and MTG HepG2.2.15 cell staining

MTR CM-H2XROS and MTG staining were done according to the manufacturer's instructions (Molecular Probes). Detailed methods are provided in the supporting information (SI).

4.4. Statistical and correlation analysis

The results are presented as mean ± S.D. Statistical analysis was performed using Graph Pad Prism, version 8 (GraphPad Software, La Jolla, CA, USA; www.graphpad.com. A difference was considered to be statistically significant at *P< 0.05 and **P< 0.01, and not significant (NS).

5. Conclusions

Our research concentrated on CHB, which causes mitochondrial dysfunction, HBV reactivation, and HBV replication. Thiourea derivatives known for the antiviral activity and restoration of host RNA interference mediated defense machinery. Here, we repurposed thiourea derivatives, they have the potential to restoration of the dysfunctional mitochondria. Mitochondrion-targeted antioxidants considerably enhanced mitochondrial and antiviral hepatocytes functions, revealing a crucial role for reactive oxygen species (ROS) in cell exhaustion. our study provides valuable insights into the potential of thiourea derivatives for the treatment of CHB and restoration of exhausted hepatocytes. Further experimental validation of our findings is necessary to confirm their potential clinical application.

Author Contributions: Conceptualization, J.K., PT, and J.S.M.; methodology, J.K., and J.S.M.; software, J.K.; validation, J.K. and P.T.; formal analysis, J.K.; investigation, J.K..; resources, J.K., and AKS; data curation, J.K.; writing-original draft preparation, J.K.,; writing-review and editing, J.S.M., and V.K.; visualization, J.K.; supervision, V.K.; project administration, DS; funding acquisition, V.K. All authors have read and agreed to the published version of the manuscript.

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Supporting Information

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.

- 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.
- 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.
- 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®

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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).