

Supporting Information

Thiourea derivatives rejuvenate mitochondria and provide anti-viral activity against Hepatitis B infection

Authors: Jitendra Kumar^{1#}, Purnima Tyagi¹, Akhilesh K Saini¹, Deepti Sharma², Jaswinder Singh Maras¹, Vijay Kumar¹

Affiliations:

¹Departments of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences, New Delhi, India

²Department of Chemistry, Sri Venkateswara College, University of Delhi, New Delhi, India

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 10 μ M 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, 50 μ g equivalent protein was reconstituted in 200 μ l ABC buffer (ammonium bicarbonate buffer), 20 μ l of 10 mM Dithiothreitol (DTT) was added followed by incubation at 60°C for 1 hour in a water bath to reduce proteins. Post-reduction, 15 μ l of 10 mM IAA (Iodoacetamide) was added and incubated for 30 min in the dark for alkylation of proteins, then 10 μ l of modified sequencing grade trypsin (0.1 μ g/ μ l), 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 5 μ l 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 μ l

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 Å). 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 MitoTrackerTM 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 CaliBRITETM beads (BD Biosciences), 50,000 events of each sample were collected using a FACSCaliburTM 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 FlowJoTM Software (Becton, Dickinson and Company; Ashland, OR 97520 USA) was used for all data analysis.

3. Statistical and Correlation Analysis:

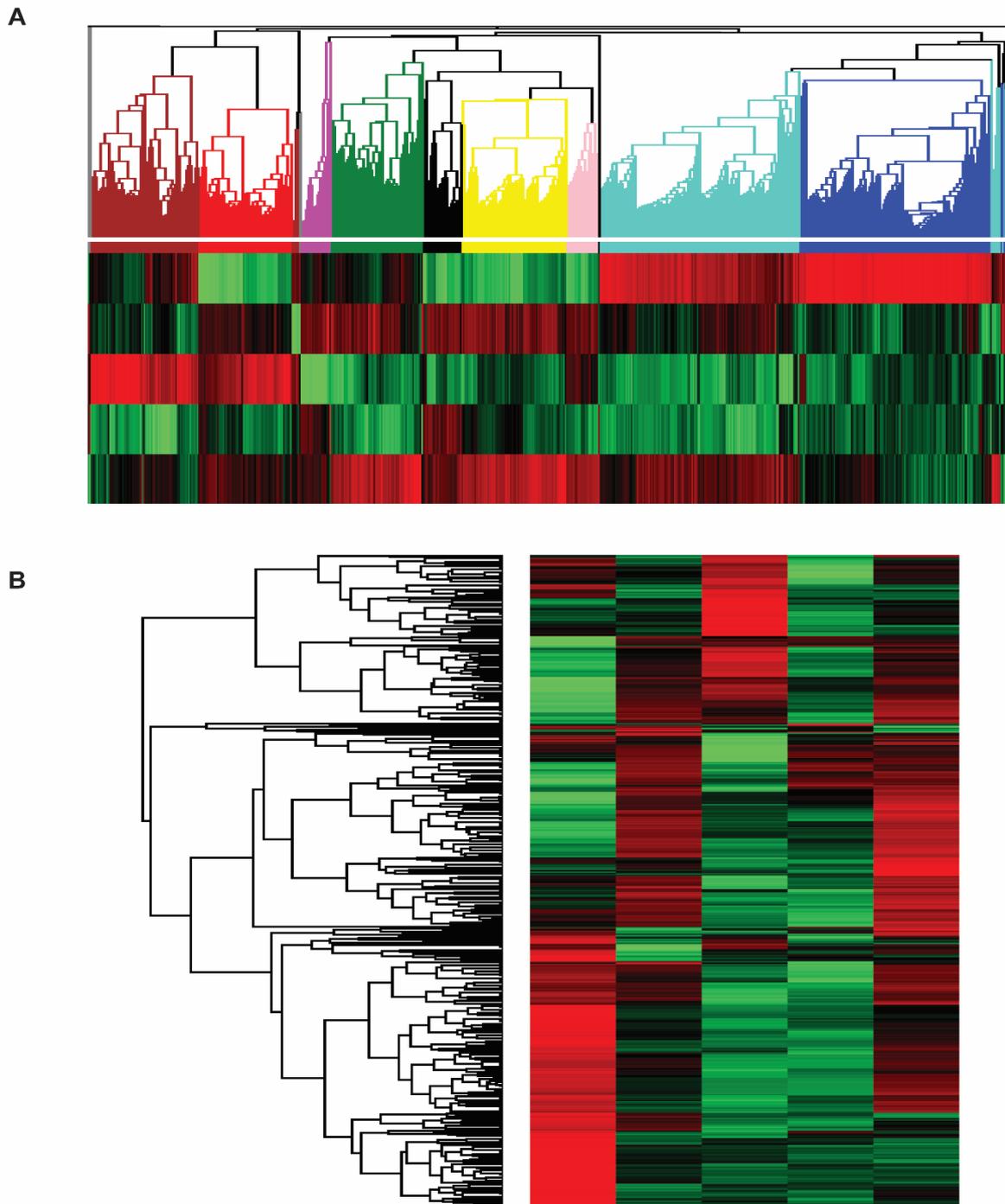
Results are shown as mean and S.D. unless indicated otherwise. Statistical analysis was performed using Graph Pad Prism, version 8 ([GraphPad Software, La Jolla, CA, USA; www.graphpad.com](http://www.graphpad.com)), and SPSS, version 20; p values < 0.05 using Benjamini-Hochberg correction (FDR < 0.05) 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).

Supplementary Table S1. Expression and fold change of proteins in each module.

Module	No. of Proteins	Control vs				ETV vs		
		ETV	DSA-00	DSA-02	DSA-09	DSA-00	DSA-02	DSA-09
Turquoise	145	-0.43	-0.94	-1.85	-1.79	7.98	-2.47	-2.38
Blue	135	-1.17	-1.22	-1.31	-1.30	-0.23	0.78	0.72
Brown	80	-1.52	-3.54	6.63	-6.57	-0.80	-15.68	9.72
Yellow	72	-2.10	-1.70	-0.49	-0.71	0.58	-1.46	-1.26
Green	64	70.57	35.11	-52.92	-57.76	0.98	-1.73	-1.79
Red	64	-1.30	-1.23	-1.83	-0.64	0.31	1.79	-2.21
Black	27	-1.52	-1.51	-0.41	-1.55	-0.02	-2.13	0.06
Pink	22	-1.82	-1.56	-1.30	-0.33	0.47	-0.64	-1.82
Magenta	20	5.33	13.80	-32.06	7.92	-0.57	-5.90	0.41
Grey	5	2.27	-1.59	-1.68	-4.01	-6.57	-1.21	-1.92

Supplementary Table S2. Expression and fold change of HBV replication regulatory proteins in thiourea derivatives treated groups

Negatively Regulated HBV	Proteins involve Antiviral, Cellular response to interferon, and Innate immune responses.	AIM2	7.47747	-16.8159	6.77347	3.74659
		APOA1	1.26487	-4.14615	-10.0838	16.3689
		APOD	7.37899	-8.47426	-9.71152	13.1825
		DHX9	8.57202	10.3011	-7.59447	1.40089
		EIF3A	3.33901	13.3401	-11.8845	2.99511
		FASN	10.2436	-8.27332	-8.5329	11.4139
		HMGB1	1.58206	14.3897	-6.04495	2.26926
		HSPA8	5.86643	-17.8233	4.00811	4.33432
		IRF7	-1.36765	16.2356	-5.5076	0.814994
		LTF	4.71953	-1.70679	1.4077	11.2631
		MIF	8.10967	-9.66454	8.6396	4.85676
		ORM1	4.81002	9.65477	-1.94231	3.77544
		PAIP1	6.51535	5.29141	-6.16652	8.91912
		PARP1	2.50745	-10.1027	-4.48135	16.0043
		PHB	1.72028	-11.9935	-8.24333	11.8864
		RAB12	9.48733	-8.50353	-10.9762	10.7962
		SSB	7.70609	1.36024	-11.6504	11.2844
		TARDBP	7.10869	-3.94993	6.24677	6.49122
		TRIM25	9.03867	5.54106	-9.7862	7.09903
		UBE2N	7.73645	-7.60742	-13.4806	9.06477
XRCC5	6.55691	3.18305	-4.08623	9.707		
Positively Regulated HBV	Proteins involve in Virion attachment, Viral entry, and IRES-dependent viral translational initiation.	BP1FA1	8.5239	-2.60431	-3.31076	11.0689
		RPS19	9.13875	-7.32855	-7.23231	12.6047
		ORM2	7.89449	-7.38319	-3.86301	13.135
		MYO1C	8.87218	-3.82182	-1.40306	10.3841
		ITGA2	5.65928	-7.32604	-0.392149	13.52
		S100A14	10.0127	-3.52999	-4.88998	10.6514
		CTSB	9.17912	-3.97093	-6.60433	11.9981
		IPO7	8.23029	11.2442	-10.8287	0.736544
		CALM1	5.20756	5.65249	4.48218	2.40729
		ICAM1	8.8161	-7.68788	7.33142	5.08792



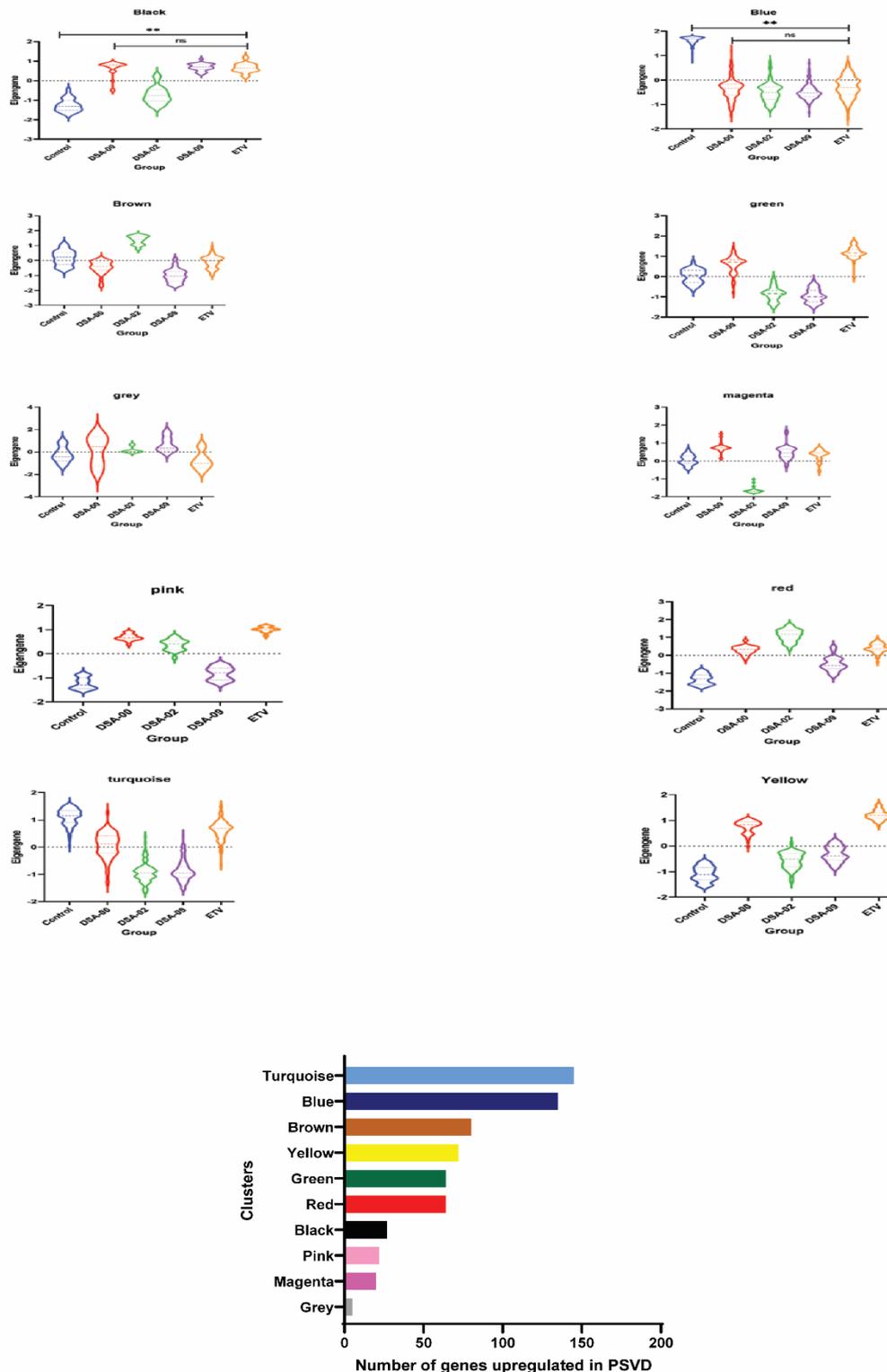


Figure S2. The expression levels of each sample proteins. The median proteins expression in the thiourea derivatives and ETV treated samples for each module. Student's t test was used to analyse group differences. P value is 0.05. (C-D) Bar plots illustrating the number of significantly altered proteins in thiourea derivatives compared to the sum of ETV and control in each of the 10 modules identified by WPCNA.

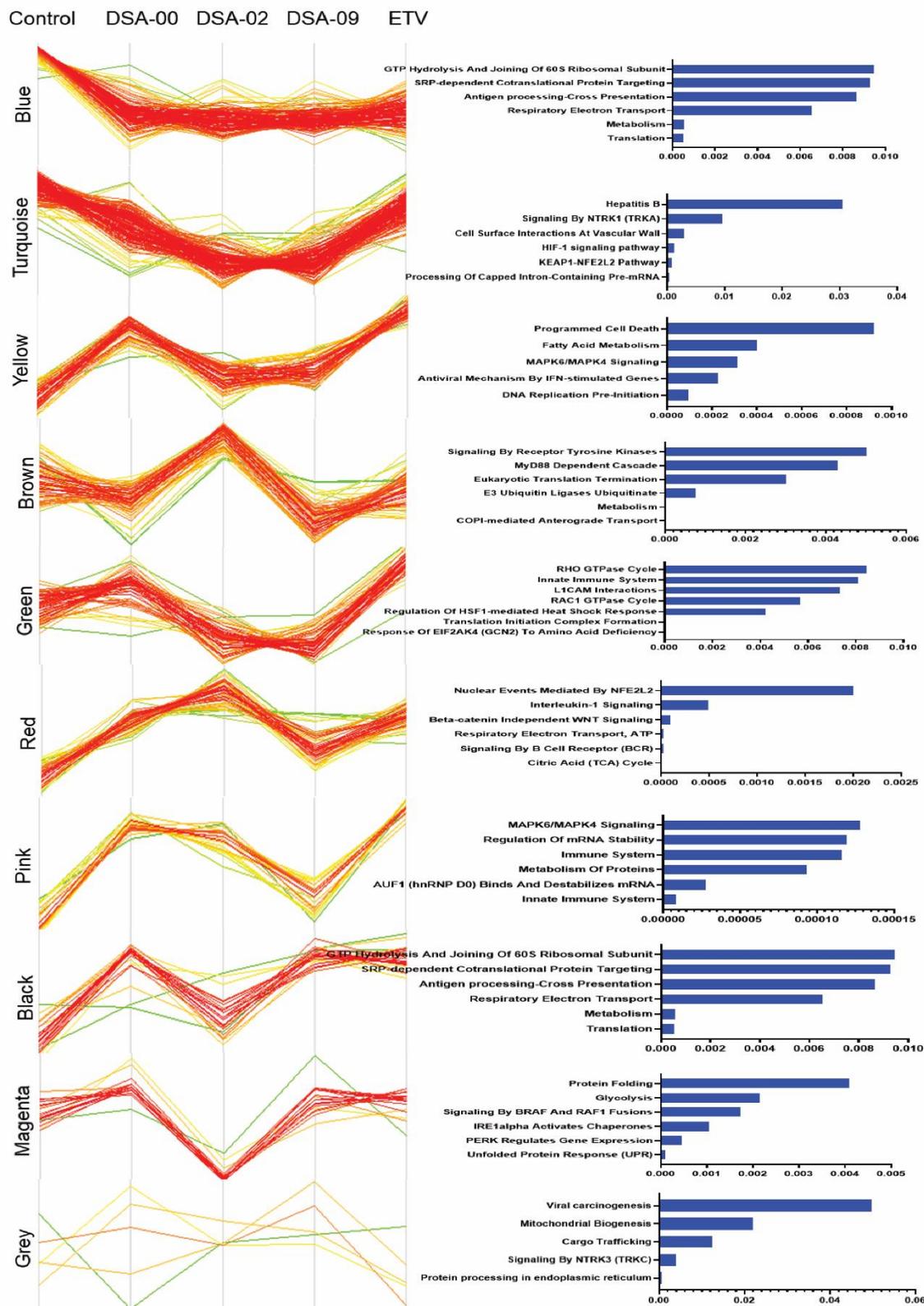


Figure S3. Module's proteins associated with KEGG pathways with real time Expression. The median proteins expression in the thiourea derivatives and ETV treated samples for each module compared with control associated with upregulated KEGG pathways of proteins.