Characterization of immune cell phenotypes in patient derived malignant pleural effusion treated with thiostrepton

Abstract #4144
Poster #6

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Background

Tumor cells generate elevated levels of reactive oxygen species (ROS) and therefore exhibit increased expression, activity, and reliance on critical ROS scavenging pathways, including the mitochondrial peroxiredoxin 3 (PRX3) system. PRX3 is a critical reactive oxygen species (ROS) scavenging enzyme localized to the mitochondrial matrix. Our group has characterized the mechanism of action of the PRX3 inhibitor and pro-oxidant therapeutic thiostrepton (TS). TS covalently crosslinks the peroxidatic and resolving cysteines of PRX3, leading to increased levels of mitochondrial ROS, resulting in tumor cell death. We have characterized the cellular and chemical composition of malignant pleural effusion (MPE) from patients with advanced cancer and evaluated cellular responses to TS. TS targets PRX3 in both adherent tumor cells and in non-adherent immune cell populations in a dose-dependent manner. We further characterized MPE-infiltrated immune cells and cytokines-released following TS-treatment. Together, these data provide the first evidence for the activity of TS in MPE and resulting changes to immune cell profiles. TS is the active pharmaceutical ingredient of RSO-021, a new covalent inhibitor of PRX3 currently being tested in the MITOPE phase 1/2 clinical trial in patients with malignant pleural effusion (MPE) due to advanced solid tumors or malignant mesothelioma (**NCT05278975**).

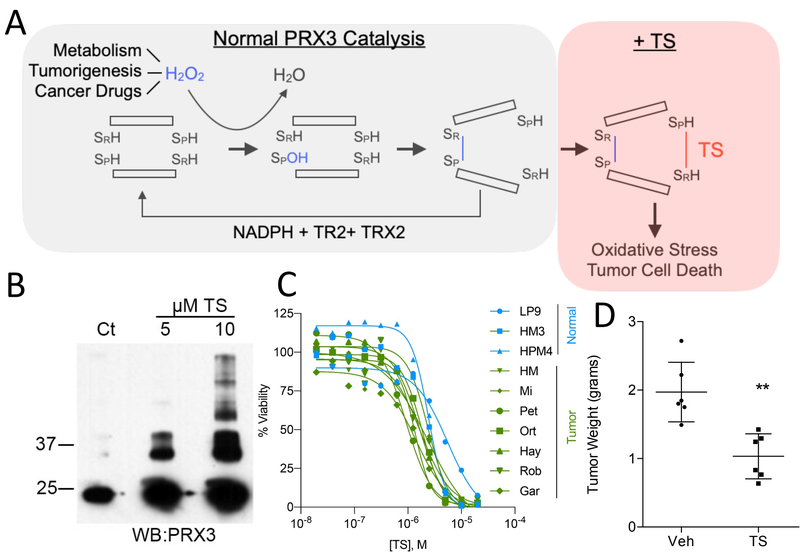


Figure 1: Thiostrepton (TS) is a covalent inhibitor of mitochondrial Peroxiredoxin 3 (PRX3). A) The proposed mechanism of action (MOA) of TS. PRX3 is the primary mitochondrial peroxidase required for H_2O_2 clearance from the mitochondria induced by metabolic, tumorigenic, and drug treatment inputs. During the metabolism of H_2O_2, PRX3 forms an intramolecular disulfide bond that orients the second active site for TS-dependent covalent crosslinking, inactivating the protein leading to increased oxidative stress and tumor cell death. **B**) Western blot of protein from Malignant Mesothelioma (MM) cells (HMESO, cell line) treated with 5 or 10 μM TS for 24 hours. TS induces a covalent modification to PRX3 (antibody reactive band at ~37 kD, PRX3-TS-PRX3) which is the dimeric species of PRX3. **C)** Dose-response curves of normal (blue) and MM (green) cell lines to TS. **D**) Weight of residual tumors resected from mice harboring MM xenografts in the peritoneal cavity following 4 weeks of treatment with 20 mg/ml TS 2x weekly.

Methods

MPE samples were collected, with Institutional Review Board approval, from 11 patients at the University of Vermont Medical Center via thoracentesis. Cellular material from MPE was phenotyped by flow cytometry for the identification of resident cell populations. Cytokine profiles were evaluated using ELISA plates. The ability of TS to inhibit its primary molecular target, mitochondrial PRX3, was evaluated in cells derived from MPE samples. The amount of PRX3 inhibition and cell viability was quantified following treatment of MPE samples with increasing concentrations of TS. We thank Dr. Geoff Scriver and Anthony Kopecky for collection of MPE.

Results

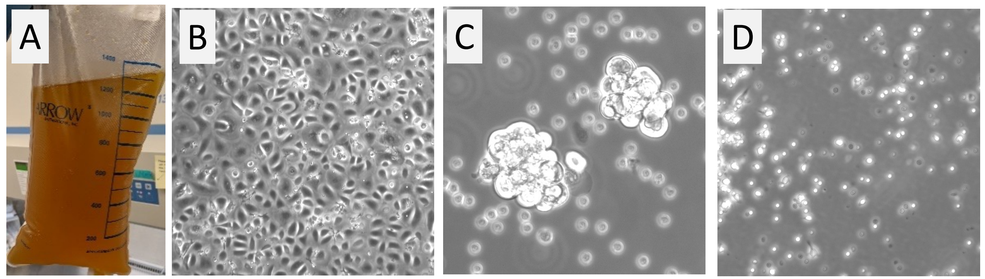


Figure 2: Collection and processing of malignant pleural effusions (MPE). A) 0.2 – 1.4 Liter of MPE was collected by thoracentesis and immediately transported to the lab for processing. MPE samples were centrifuged at 1000xG for 10 minutes to separate MPE cellular content from the supernatant. MPE cells were cultured in MPE supernatant for 24 hours +/- TS prior to analysis of cellular material by flow cytometry, cytokine levels by ELISA (**Figure 3**), PRX3 crosslinking, and cell viability (**Figure 4**). Tumor cells grew either as adherent monolayers (**B**) or tumor spheroids (**C**). Immune cells were present as non-adherent cells (**D**).

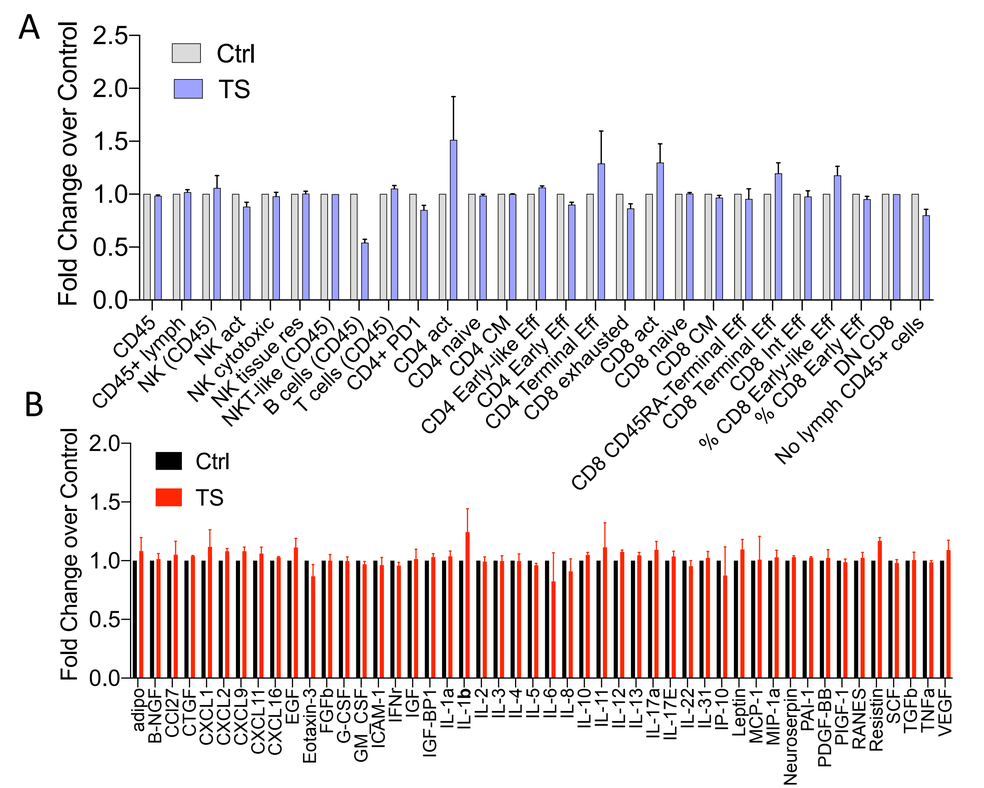
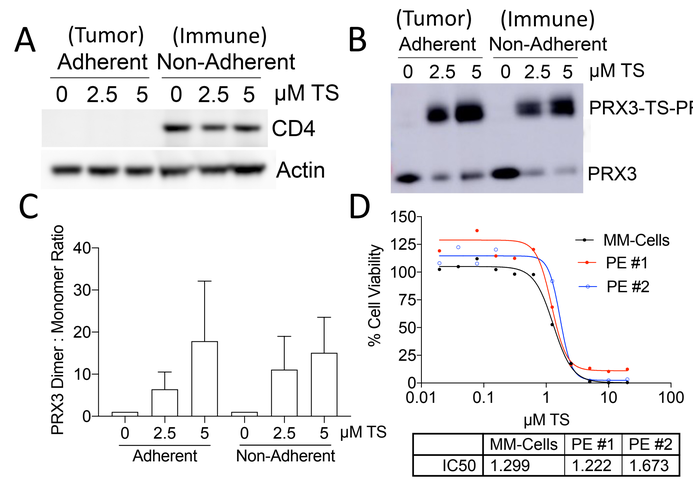


Figure 3: Primary immune cells and cytokines identified in MPE +/- TS treatment. MPE cells were incubated with 5μM TS for 16 hours before analysis. **A)** Antibody cocktail was prepared in 50 ul of Brilliant Stain Buffer and added to the sample. Samples were incubated with the antibody cocktail in the dark for 30 min at 4°C, then washed twice with FC buffer. All samples were acquired on Cytek Aurora using SpectroFlo 2.1 version. Subsequently, flow cytometric analysis was performed using FlowJo. **B)** Cytokine levels in harvested PE-fluid from MPE cells was evaluated using Signosis ELISA cytokine profiling plates (EA-4015) following the manufacturer's instructions. Absorbance at 450nm using a Biotek Synergy HTX plate reader was used to read absorbance at 450 nm to evaluate the fold change of a panel of 48 cytokines in TS-treated over non-treated MPE samples.



	MM-Cells	PE #1	PE #2
IC50	1.299	1.222	1.673

Figure 4: Thiostrepton retains target activity and cytotoxicity against adherent (tumor) and non-adherent (immune) MPE cells. A) Adherent and non-adherent MPE cells, cultured in decellularized MPE supernatant were collected after 24 hours incubation with indicated concentrations of TS. Protein lysates were generated in RIPA buffer and subjected to SDS-PAGE. The CD4 T-cell co-receptor was only detected in the non-adherent cell population. **B**) TS retains activity in MPE cells cultured in MPE fluid. The TS-dependent PRX3-TS-PRX3 covalent crosslink is present in adherent and non-adherent cell populations. **C**) Densitometry quantification of PRX3-TS-PRX3 (dimer) to PRX3 (monomer) ratio (n = 5 samples). **D**) Cell viability assay of human MM cells (HMESO cell line) and cells derived from PE #1 and PE #2 treated with increasing concentrations of TS. The IC_{50} values (concentration of TS required to kill 50% of cells) are similar between MM cells in culture and MPE-derived cells.

MITOPE Trial Status

The presented strategies and findings are being further explored using patient samples from the ongoing MITOPE trial. The MITOPE phase 1/2 clinical trial is designed to assess the safety, tolerability, and activity of RSO-021 (TS as API) in patients with malignant pleural effusion (MPE) arising from metastatic disease or malignant mesothelioma (MM). The MITOPE study initiated first patient treatment in March 2022 and is open for recruitment of patients at the following UK sites:

- Dr. James Spicer - Guy's Hospital, London
- Dr. Dean Fennell – Leicester
- Dr. Simon Lord – Oxford
- Dr. Fiona Thistlethwaite - The Christie, Manchester

Clinicians are encouraged to refer any eligible patients to the open sites. MITOPE trial is supported by Mesothelioma UK (www.mesothelioma.uk.com), NIHR (www.nihr.ac.uk) and clinicaltrials.gov: ***NCT05278975***

For more information scan the QR code or contact:
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Funding and Financial Disclosures

This work was funded by RS Oncology, LLC through a Sponsored Research Contract to The University of Vermont. T.M., B.C and G.N.N are paid consultants and B.C. and G.N.N. are equity holders in RS Oncology, LLC. The MITOPE study is sponsored by RS Oncology LLC.