RSL3 Induces Ferroptosis via GPX4 Inhibition in Papillary Thyroid Cancer

**Background:** Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy and demonstrates significantly reduced patient survival with tumor progression. Tumor evasive behavior and progression are influenced by metabolism, including antioxidant Glutathione (GSH). Glutathione peroxidase 4 (GPX4) regulates GSH oxidation to prevent damaging lipid peroxidation of cell membranes during increased oxidative stress, and regulates the ferroptosis cell death pathway in tumor cells. This study aims to determine whether ferroptosis is a critical pathway in thyroid cancer cells to abrogate GSH cytoprotective and chemo-resistant behaviors.

**Methods:** To target the ferroptosis pathway, we treated human thyroid cancer cell lines (K1, MDA-T68, MDA-T32) and control human thyroid fibroblasts (HThF) with RSL3, a pharmacologic inhibitor of GPX4. We examined the effect of RSL3 on ferroptosis activation, tumor cell survival, oxidative stress, and activation of signaling pathways.

**Results:** RSL3 treatment induced inhibition of GPX4, activation of ferroptosis, rapid rise in reactive oxygen species (ROS) and arrested migration of thyroid cancer cells *in vitro*. RSL3 effect was rescued by treatment with ferrostatin-1. Mechanism of RSL3-induced ferroptosis appears to be through suppression of mTOR signaling pathway and subsequent activation of autophagy. Moreover, we have made a novel observation that RSL3 treatment suppresses phosphorylation of nucleophosmin 1 (NPM1), which is critical for DNA damage response.

**Conclusion:** Effective inhibition of GPX4 with RSL3 induced a robust activation of ferroptosis in thyroid tumor cells *in vitro*. Our study identified novel mechanism of action of GPX4 inhibitor, RSL3, that can be further exploited for therapeutic benefit in advanced therapy-resistant thyroid cancers.

**Figure 1. RSL3-induced Activation of Ferroptosis in Thyroid Cancer *in vitro*.**

A. Thyroid cancer cells (K1, MDA-T32, MDA-T68) and control human thyroid fibroblast cells (HThF) cells were treated with various concentrations of RSL3 and control (DMSO) for 48 hr and assessed for viability using a CellTiter-Glo Luminescent method. B. K1 thyroid cancer cells and control HThF cells were treated with RSL3 and control (DMSO) for 18 hrs and H$_2$O$_2$ levels were measured. C. K1 and HThF cells were treated with RSL3 (3 µM) or DMSO for 2 or 4 hrs and protein levels for mTOR pathway (p70S6K), ferroptosis marker (TfR1) and GAPDH were analyzed by Western blot. D. K1 cells were treated with ferrostatin-1 (5 µM) 30 min prior to RSL3 (3 µM) or DMSO treatment for 4 hrs and levels of p70S6K, pS6, phosphorylated NPM1, TfR1 and GAPDH were analyzed by Western blot.