Title: Targeting Glutamine Metabolism to Enhance Radiosensitivity in Ewing Sarcoma

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Significance & Hypothesis: Ewing sarcoma (ES) is a highly aggressive bone and soft tissue malignancy that primarily affects children and young adults. It is the second most common pediatric sarcoma and accounts for 1% of all pediatric cancers in children under the age of 15. Despite being characterized by a specific 11:22 translocation and its associated fusion protein, there have been few improvements in ES treatment for decades, and the prognosis remains relatively poor. However, ES is highly dependent on glutamine metabolism, which supports tumor growth and DNA damage repair (DDR) while maintaining redox balance. Moreover, the glutathione synthesis in ES is driven by the EWS: FLI1 fusion protein, making glutaminase inhibition a particularly promising therapeutic strategy specifically in this disease. Our *goal* was to evaluate *in vitro* a specific glutaminase inhibitor, CB-839 (Telaglenastat), alone and as a potential radiosensitizer in the treatment of ES. We *hypothesized that glutaminase inhibition in combination with radiotherapy in Ewing Sarcoma has a synergistic effect resulting in cytotoxicity by interfering with DNA damage repair*.

Innovation: This novel treatment approach targets the EWS-FLI1 fusion protein, which drives the inherent glutamine addiction on which Ewing sarcoma depends, by specific inhibition using a selective glutaminase inhibitor.

Approach (Materials and Methods): Two ES cell lines, A673 and SK-ES-1, both EWS: FLI1 positive, were selected as SK-ES-1 has the Type 1 fusion and is considered more treatment sensitive, while A673 has the Type 2 fusion and is more treatment resistant. After initial clonogenic dose response studies, single fraction radiation doses of 2 or 4 Gy radiation were selected as radiation treatments for both cell lines. Based on MTT assays, CB-839 was administered at 2 μM for the more sensitive SK-ES-1 cell line and 4 μM for the more resistant A673 cell line for 24 hours, and the cells were left undisturbed for 10-15 days. Treatment groups included untreated controls, vehicle controls (DMSO only), radiation alone, CB-839 alone, or a combination of CB-839 with radiation. Outcome measures included clonogenic survival assays to evaluate cytotoxicity, γH2AX levels as a marker of DNA-DSB, immunofluorescence for γH2AXfoci formation to confirm presence of DNA-DSB at the cellular level, oxygen consumption rate (OCR) to assess effects on mitochondrial function, and extracellular acidification rate (ECAR) to measure ATP respiration.

Results: The glutaminase inhibitor CB-839 caused cytotoxicity in both cell lines associated with increased DNA-DSB, decreased mitochondrial function and decreased ATP respiration. Both RT doses caused effects in the same directions to varying degrees. The effects of both CB-839 and RT doses individually were more substantial for the more drug sensitive SK-ES-1 cell line compared to the A673 line. The combination of CB-839 and either RT of 2 Gy or 4 Gy resulted in synergistic enhancement ratios of 38-fold and 136-fold in SK-ES-1 cells (Table 1) and 10-fold in the A673 cells (Table 2).

Discussion & Impact: These findings suggest that targeting glutamine metabolism through this combined approach may offer an effective strategy for improving therapeutic response in ES, specifically for tumors where surgery is not an option. Further investigation is needed to determine if radio sensitization also occurs *in vivo*, further delineate the mechanism behind this effect, evaluate similar chemosensitization effects, and understand the differences in glutamine metabolism and susceptibility to glutaminase inhibition between ES tumor subtypes, including those that lack the EWS: FLI1 specific fusion protein.

Fig. 1: SK-ES-1 cell line	**CB-839 alone (2 μM)**	**2 Gy RT alone**	**4 Gy RT alone**	**CB-839 + 2 Gy RT**	**CB-839 + 4 Gy RT**
Decreased colony formation (% of unRx ctrl)	3.9%	18.8%	12.2%	0.49%	0.09%
γH2AX expression (vs ctrl =1.0)	1.91x	2.22x			3.32x
γH2AX foci formation (foci/nucleus) (vs 0.044 in ctrl)	3.56	8.89x			10.66
Oxygen consumption rate (OCR) at 30 min (vs ctrl)	16.5%		43.9%		-8.9%
OCR at 24 hrs (vs ctrl)					23%
Extracellular acidification rate (ECR)(ATP respiration) vs ctrl at 30 min	37.7%		27.5		6.9%
Extracellular acidification rate (ECR)(ATP respiration) vs ctrl at 24 hrs					7%

Fig. 2: A673 cell line	**CB-839 alone (4 μM)**	**2 Gy RT alone**	**4 Gy RT alone**	**CB-839 + 2 Gy RT**	**CB-839 + 4 Gy RT**
Decreased colony formation (% of unRx ctrl)	60%	69%	29%	7%	5%
γH2AX expression (DNA-DSB)	0.78x (NS)		4.0x		4.29
γH2AX foci formation (foci/nucleus) (vs 0.34 in ctrl)	0.21		8.56		10.92
Oxygen consumption rate (OCR) vs ctrl at 30 min	67.4%		50.6%		19.5%
OCR vs ctrl at 24 hrs	66.9%		48.3%		29%
Extracellular acidification rate (ECR)(ATP respiration) at 30 min	80%		53.9%		26%
Extracellular acidification rate (ECR)(ATP respiration) at 24 hrs	45.1%		100.5%		40.8%