Impact and Therapeutic Exploitation of Hypoxia for Rhabdomyosarcomas

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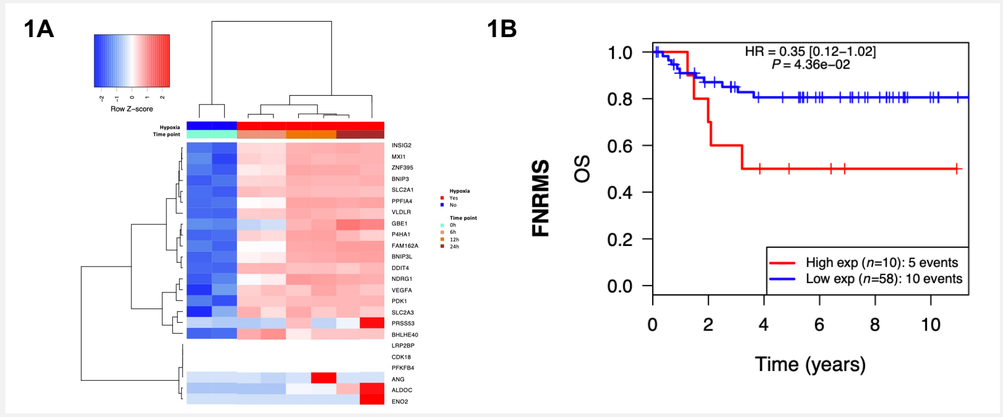
Introduction

- **Rhabdomyosarcomas (RMS)** are the most common soft tissue sarcoma in **children** and a significant contributor to cancer morbidity and mortality. There are two main histological subtypes, **embryonal** and **alveolar** RMS. Approximately 80% of alveolar RMS patients have an oncogenic mutation, the PAX3-FOXO1 or PAX7-FOXO1 fusion gene. Fusion positive (FP)-RMS patients have significantly *worse* outcomes than fusion negative (FN)-RMS patients.
- Hypoxia, resulting from an imbalance between oxygen delivery and oxygen consumption, is prevalent in cancer. **Tumour hypoxia** is associated with **resistance** to radiotherapy and chemotherapy, and is an important **negative** factor in overall **prognosis** and in predicting treatment efficacy.
- **Atovaquone** is a safe, FDA-approved, antimalarial that has been *repurposed* to sensitise tumours to chemoradiotherapy.[1] Through inhibition of the mitochondrial complex III, atovaquone **reduces the oxygen consumption rate** in perihypoxic areas, increasing the availability of oxygen to hypoxic areas.

Project aim: To investigate altering hypoxia to increase sensitivity to chemotherapy in RMS and improve outcomes at relapse setting.

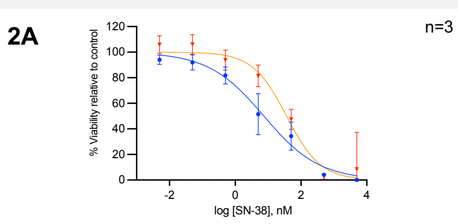
1. Hypoxia correlates with worse overall survival in FN-RMS.

- Yang *et al.* generated a sarcoma-specific **hypoxia-gene signature** that predicted worse outcomes in adult soft tissue sarcomas.[2]
- We tested a publicly available dataset of RH30 cells cultured in hypoxia for 24h[3] and observed that the expression of the signature was significantly higher in hypoxia compared to normoxia, increasing over time (**1A**).
- The signature was then tested on RMS gene expression datasets[4,5] and a significant correlation with worse OS was observed in FN-RMS (**1B**).



2. Sensitivity to irinotecan (SN-38) is significantly reduced in hypoxia.

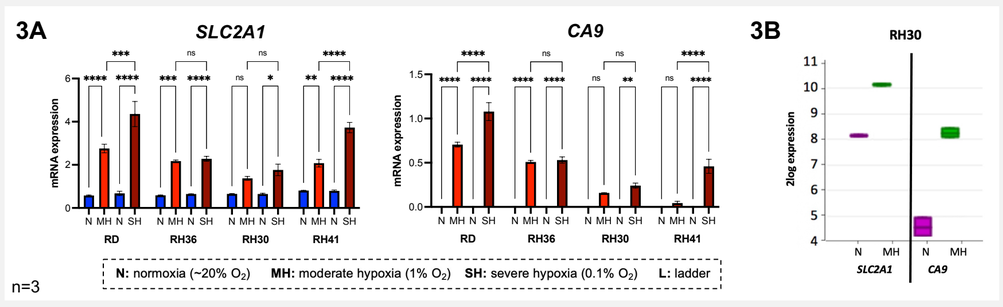
- RMS cell lines (RD, RH36, RH30 and RH41) were cultured in 2D in the presence or absence of hypoxia for 24h prior to 72h treatment with SN-38 (irinotecan's active metabolite). An MTS assay was used to determine cell viability.
- Fig **2A** shows dose-response curves in normoxia and hypoxia for RD.



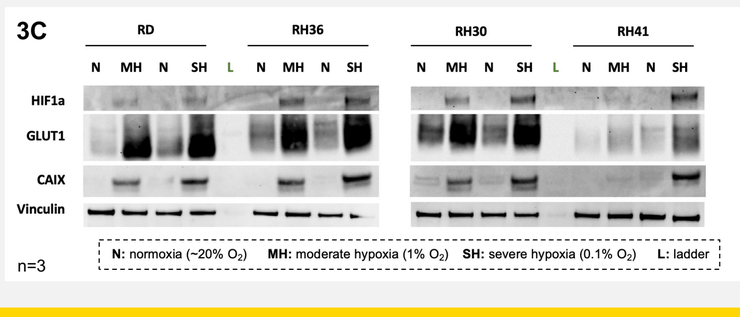
	Normoxia	Severe hypoxia
GI_{50} mean (nM)	8.1 (±4.2)	36.4 (±12.0)
Cytotoxicity ratio	4.5	
P-value	0.02 (*)	

3. GLUT1 and CAIX are robust hypoxia markers for RMS.

- The hypoxia inducible factor (**HIF**) **pathway** is activated in hypoxic conditions. Glucose transporter 1 (**GLUT1**) and carbonic anhydrase IX (**CAIX**) are HIF downstream targets, which regulate glucose uptake and pH, respectively.
- RMS cell lines (RD, RH36, RH30 and RH41) were cultured in 2D in the presence or absence of hypoxia for 24h in a ***Whitley H35 Hypoxystation***.
- RT-qPCR showed that *SLC2A1* (gene encoding GLUT1) and *CA9* (gene encoding CAIX) mRNA expression was significantly increased after 24h in hypoxia (**3A**). These same genes were overexpressed in an RH30 gene expression dataset[3] (**3B**).

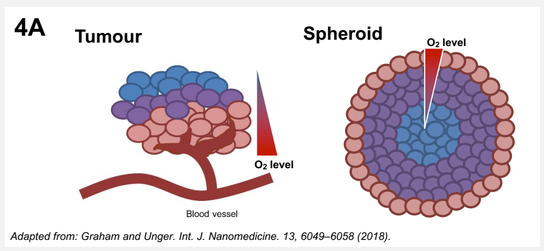


- Protein levels of hypoxia markers HIF-1α, GLUT1 and CAIX were also consistently overexpressed in hypoxic cells, shown by western blotting (**3C**).



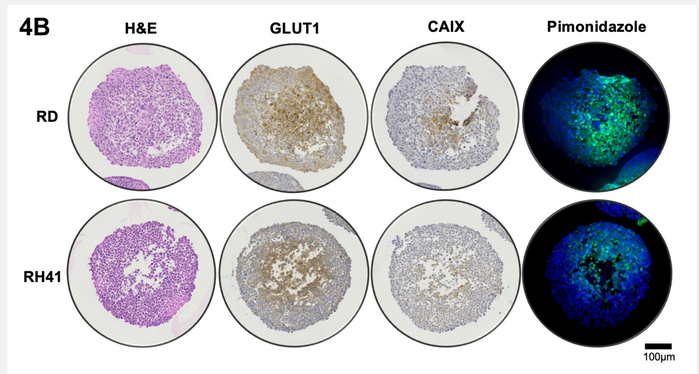
4. RMS spheroids provide a good model for tumour hypoxia.

- Similarly to a tumour in a patient, 3D tumour spheroids cultured *in vitro*, have a hypoxia and nutrient gradient (**4A**).
- RD and RH41 spheroids were generated in ultra-low attachment plates, collected, fixed and stained.



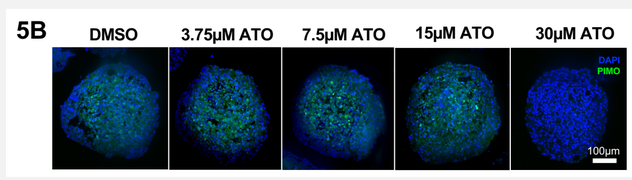
Adapted from: Graham and Unger. Int. J. Nanomedicine. 13, 6049–6058 (2018).

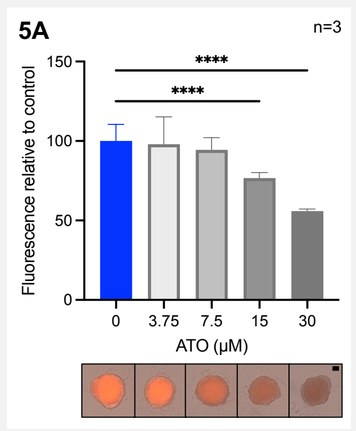
- GLUT1 and CAIX staining in the **hypoxic core** is consistent with **pimonidazole** staining, a standard exogenous hypoxia marker (**4B**).



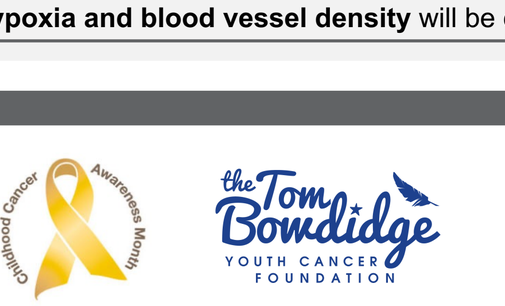
5. Atovaquone significantly reduces hypoxia in RMS spheroids.

- RD and RH41 spheroids were treated with atovaquone for 24h. RD is shown as an example.
- A real-time dye Image-iT™ Red Hypoxia Reagent was used to asses hypoxia after treatment (**5A**). Spheroids were also fixed and stained for pimonidazole (**5B**).





6. Atovaquone pretreatment may sensitise spheroids to irinotecan.



- Preliminary results show that in RH41 spheroids, ***pretreatment*** w/ atovaquone for 24h followed by an 8 day treatment with SN-38, caused a significant reduction in spheroid viability, measured using CellTiter-Glo, compared to single agent treatment and simultaneous treatment. (**6A**)

Key findings and future work

- Tumour hypoxia negatively impacts outcome in patients with FN-RMS.
- A consistent upregulation of HIF downstream-targets GLUT1 and CAIX, as well as reduced sensitivity to irinotecan in hypoxia, was demonstrated in cell line models.
- Hypoxia in RMS spheroids was alleviated by atovaquone and preliminary data suggests that atovaquone sensitises RMS spheroids to irinotecan.
- Atovaquone provides a potential novel hypoxia-targeted strategy to improve oxygenation in tumours and increase sensitivity to current treatment options.

Future work: We will further investigate the combination of atovaquone with irinotecan to provide robust evidence for atovaquone's sensitising effect. This combination will then be taken into ***in vivo* studies** with cutting-edge **oxygen-enhanced (OE)-MRI** technology to monitor oxygen levels in tumours. 40 RMS diagnostic **patient samples** have been double-stained for GLUT1 or CAIX and a blood vessel marker. **Digital quantification of hypoxia and blood vessel density** will be correlated with patient outcomes.

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LinkedIn QR code

References

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