REGORAFENIB IN GLIOBLASTOMA: *in vitro* CHARACTERIZATION OF TUMOR CELLS RESPONSE

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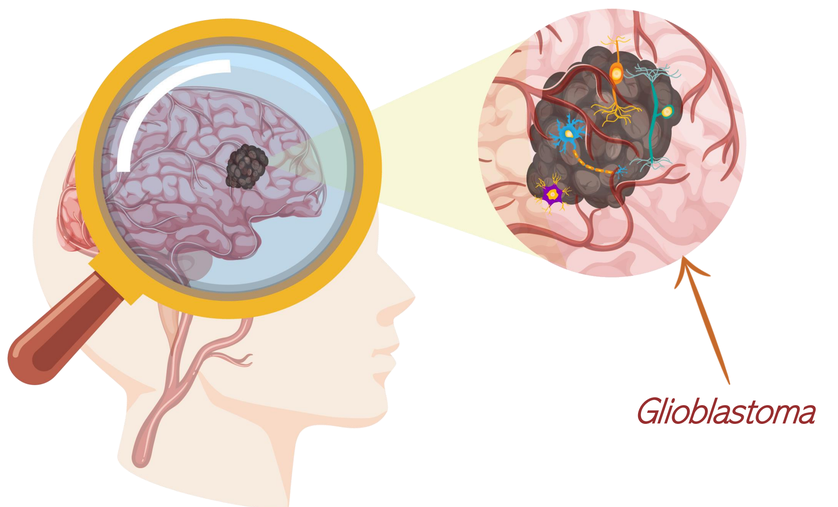
Introduction

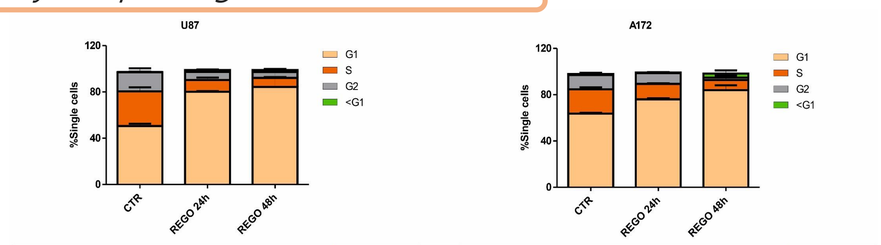
Glioblastoma IDH wild-type (GBM), is the most malignant primary brain tumor in adults. A phase II clinical trial (REGOMA)[1] demonstrated that the multikinase inhibitor regorafenib significantly increased the median overall survival of GBM patients when compared to lomustine-treated patients. On this basis, the National Comprehensive Cancer Network (NCCN) 2020 Guidelines included ***Regorafenib*** as a preferred regimen in relapsed GBM treatment. However, very little is known about the mechanisms governing GBM cells response to regorafenib.

Here we report an in vitro characterization of GBM tumor cells' response to regorafenib, performed both on cell lines and on patient-derived glioma stem cells (GSCs)[2].



Results and Discussion

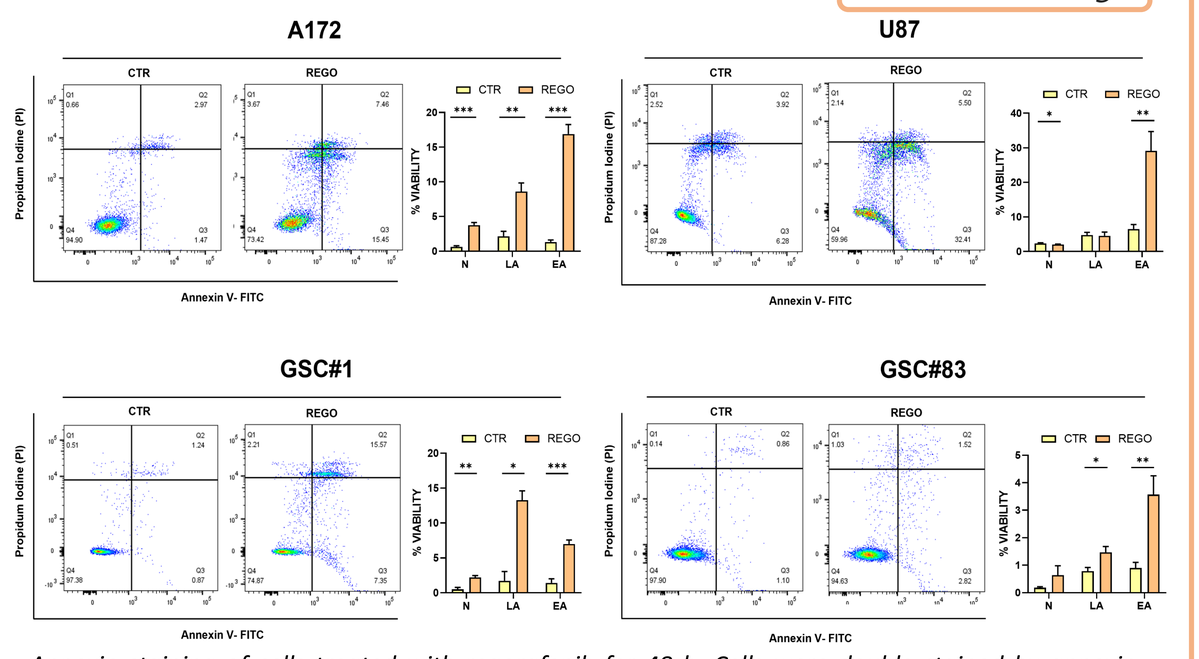
Cell cycle upon regorafenib treatment



		Phase G1 (%)	Phase S (%)	Phase G2 (%)	Phase <G1 (%)
U87	CTR	50.53 ± 2.0	29.93 ± 3.5	16.53 ± 3.5	0.34 ± 0.1
	REGO 24h	80.17 ± 0.5 ***	10.14 ± 2.1**	7.07 ± 1.6*	1.46 ± 0.6*
	REGO 48h	84.23 ± 8.6 **	7.96 ± 7.5*	4.95 ± 0.5**	1.71 ± 1.0
A172	CTR	63.63 ± 0.7	21.07 ± 1.7	12.4 ± 1.8	0.7 ± 0.1
	REGO 24h	76.03 ± 0.9***	13.33 ±0.3**	9.26 ± 0.4*	0.49 ± 0.4
	REGO 48h	83.9 ± 4.3**	8.89 ± 3.0**	1.95 ± 1.7**	3.49 ± 2.8
GSC1	CTR	57.77 ± 2.3	24.83 ± 1.7	12.63 ± 2.0	5.04 ± 1.8
	REGO 24h	60.83 ± 2.5	19.8 ± 1.0*	9.14 ± 1.3	10.42 ± 0.6**
	REGO 48h	67.13 ± 4.3*	22.4 ± 4.4	6.95 ± 4.1	5.06 ± 3.7
GSC83	CTR	61.17 ± 0.9	18.2 ± 2.1	17.63 ± 1.0	1.22 ± 0.7
	REGO 24h	51.43 ± 3.9*	22.3 ± 2.4	19.17 ± 2.9	3.78 ± 2.0
	REGO 48h	59.1 ± 7.8	24.4 ± 6.1	10.67 ± 0.3***	4.14 ± 2.7

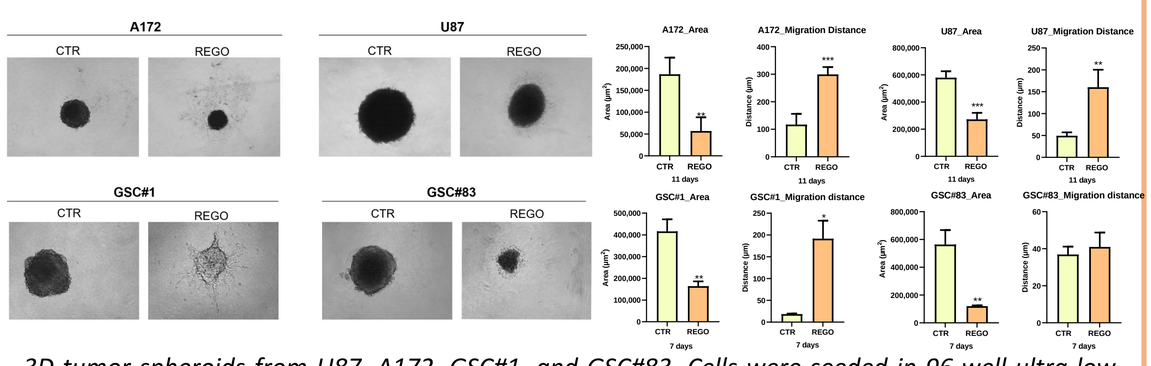
Cell lines were treated with 7.5 μM regorafenib or with vehicle for 24-48 h before fixation and propidium iodide staining.

Annexin staining



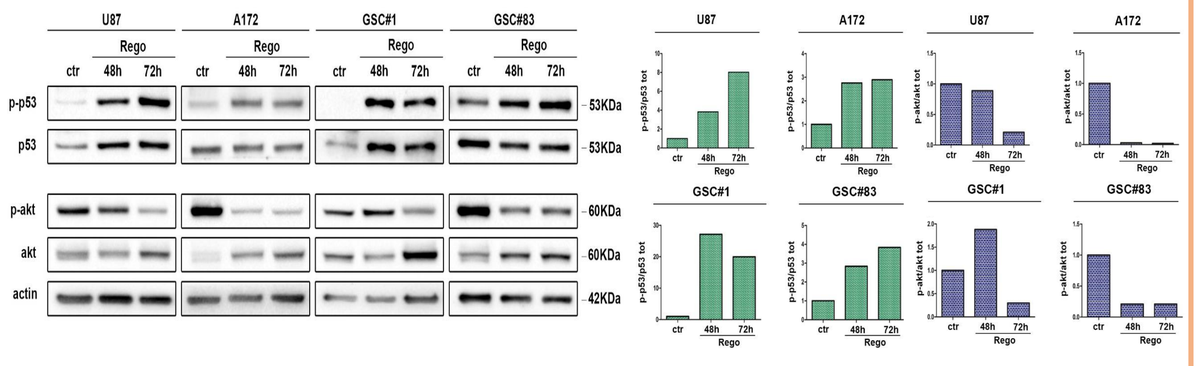
Annexin staining of cells treated with regorafenib for 48 h. Cells were double stained by annexin V/propidium iodide, for apoptotic and dead-cell labeling.

Regorafenib effect on 3D spheroid cultures



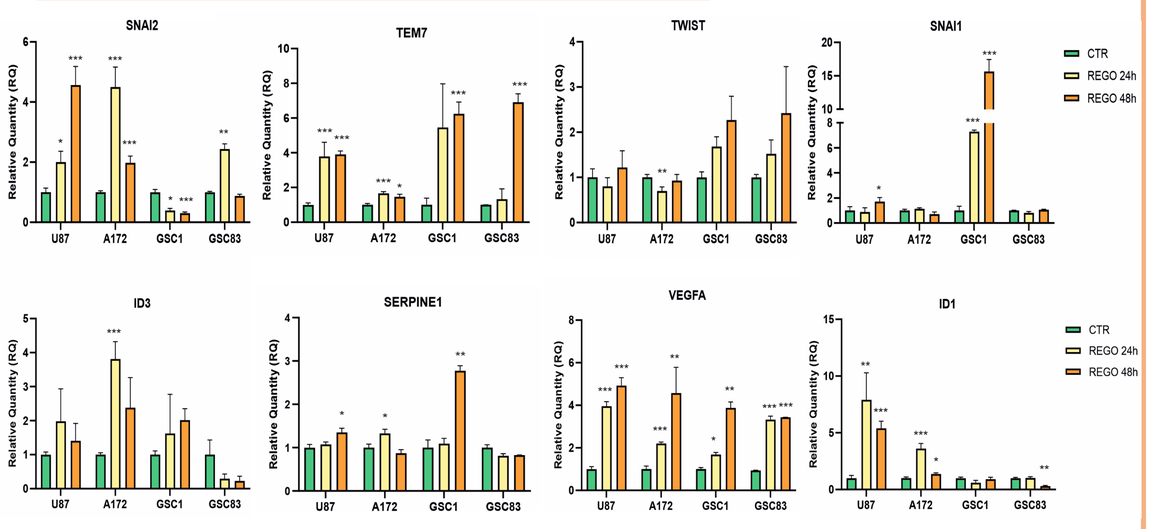
3D tumor spheroids from U87, A172, GSC#1, and GSC#83. Cells were seeded in 96-well ultra-low attachment (ULA) plates; spheroid formation was induced by plating cells in 20% methylcellulose in culture medium. We allowed spheroids to grow for 48 h before starting regorafenib treatment.

Expression of apoptosis-related proteins



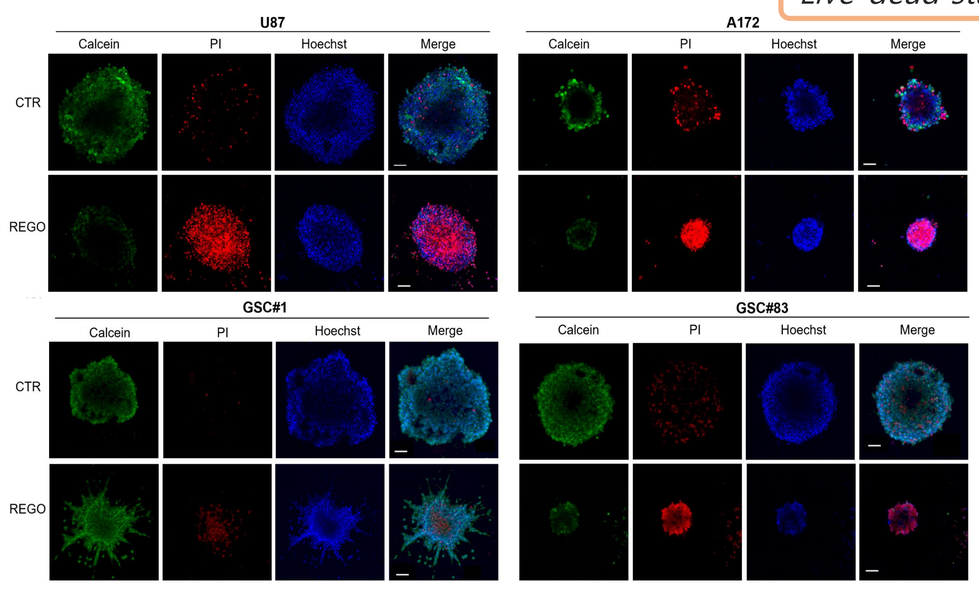
Western blots analysis of apoptosis-related proteins in regorafenib-treated GBM cells. U87, A172, GSC#1, and GSC#83 were treated with regorafenib for 48 and 72 h.

Real-time PCR analysis of EMT-related genes



Real-time PCR analysis of a pool of EMT-related genes, performed on both GBM cell lines and patient-derived GSCs. Cells were treated with 7.5 uM regorafenib for 24-48 h. Relative quantities were calculated normalizing for TBP and are given relative to control.

Live-dead staining



Combined fluorescence images of 3D tumor spheroids, stained with calcein-propidium-hoechst, for staining metabolically active cells, dead cells, and cell nuclei and analyzed by confocal microscopy.

Conclusion

Treatment with regorafenib affects cancer cells viability and proliferation in all the cellular models tested, both in 2D cell cultures and in 3D spheroids. Regorafenib administration is accompanied by regulation of genes involved in mesenchymal to epithelial transition (EMT) and by an increased migration ability, documented in tumor spheroids as the presence of irregular spheroids edges, with extended thin branches invading the surrounding matrix. **Our study shows that, *in vitro*, regorafenib limits tumor cells viability but promotes epithelial to mesenchymal transition (EMT) and tumor cells' migratory ability**. So, to the best of our knowledge, our data are the first to suggest the idea that regorafenib might elicit a pro-invasive phenotype in GBM.

NEXT STEP: perform a genome-wide *CRISPR/Cas9 screen* on glioma stem cells (GSCs) to identify genes modulating regorafenib responsiveness.

References

1. *Indraccolo, S.; et al., REGOMA Trial Biomarker Analysis Clin. Cancer Res. 2020, 26, 4478–4484;*
2. ***Mongiardi, M.P.; et al., Cancers 2022, 14, 6193.***

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