**SUPPLEMENTARY RESULTS : Sensitivity to Regorafenib in 2D and 3D commercial cell lines.**

1. **Analysis of Regorafenib efficacy in 2D and 3D GB cell lines**

Before using GB patients-derived organoids, we tested Regorafenib (REGO) in U118, U87 and T98G glioblastoma cell lines 2D and 3D models, firstly using standard viability and proliferation analysis methods and then FLIM metabolic-imaging approach.

Figures S1a and S1b show the viability assay performed in 2D and 3D cell lines 72 h post-treatment with 10 µM, 50µM and 100µM of REGO. All cells showed a percentage of viability less than 50% with dosages higher than 50 µM of REGO.

As an indicator of cell proliferation, we also measured the Ki67 mRNA level at 72 h in REGO-treated 3D cell lines model by ddPCR: the expression of Ki67 was reduced by about 50% using 50 µM of REGO treatment (Fig. S1c) for all the 3 cell lines used.

H&E and Ki67 immunohistochemistry of 3D cell lines model confirmed a visible reduction in nuclei % and in Ki67 protein expression in 100 µM REGO treated cells (Fig.S1d).

***Figure S1******Viability and proliferation effects of Regorafenib on 2D and 3D cell lines models, T98G, U87 and U118.***

***a-b*** *Cell viability performed on 2D cell lines (a) using WST1 assay and on 3D spheres (b) using CellTiterGlo assay.* ***c*** *Ki76 mRNA expression level analysis using ddPCR in 3D cell lines model.* ***d-e*** *Hematoxylin and Eosin staining (d) and Ki67 immunohistochemistry (e) in T98G-3D spheres. All Rego treatments were conducted for 72hrs.*

1. **FLIM metabolic imaging to test Regorafenib sensitivity in 3D GB cell lines**

After spheroids formation using hanging drop method (Fig.S2a), 3D cell lines were treated with 10 µM, 50µM and 100µM of REGO (Fig.S2b). FLIM image data were recorded from 15 fields of view for each slide of all cell lines 72hr post treatment (Fig.S2c). Data were analyzed using phasor approach targeting the autofluorescence of the intracellular metabolic cofactor NAD(P)H15 (Fig.S2d). A fractional NAD(P)H mean distribution curve of free and bound NAD(P)H molecules for each image was obtained (see Materials and Methods) and from these a mean distribution curve for treated and one for control 3D cell lines, that identifies a metabolic signature that goes from an oxidative phosphorylation phenotype with low free/bound NAD(P)H fractions to a glycolytic phenotype with high free/bound NAD(P)H. Then, from the comparison of the mean curves we can obtained a % of drug response (see Materials and Methods) (Fig.S2e). Figure 2f shows representative images of 3D-T98G cells treated with different REGO concentration, including brightfield images (top row) and phasor-FLIM NAD(P)H lifetime maps (on the bottom), colored in accordance with the color bar defined on the side. The color bar defines the metabolic pathway from NAD(P)H in the bound state (red/magenta) to NAD(P)H in the free state (green/white). In Figure S2g we report the fractional NAD(P)H mean distribution curves of the control and REGO -treated 3D-T98G cells: in 10 M Rego treated spheroids no significant shift of treated curve was observed respect to controls, that means %DR=0; using REGO concentrations of 50 M and 100M a 74% and 91% of drug response was detected, respectively. For 3D-U87 and 3D-U118 cell lines similar results were obtained: 29, 32, 77 %DR in 3D-U87 and 3, 46,75%DR in 3D-U118 after 10 M ,50 M and 100 M, respectively (data not shown).

Considering the comprehensive results from viability, proliferation, and metabolic analyses in both 2D and 3D cell line models, a REGO concentration of 50 µM was selected for explants treatment.

***Figure S2. FLIM analysis on 3D-T98G cell line using different REGO concentrations****.*

***a-e*** *Spheroids were formed using hanging drop technique (a, top). An example of brightfield sphere image is shown (a bottom). 3D cells were treated with 10M, 50M and 100M or with DMSO (representing the controls) (b) and then FLIM images were acquired (c) and analyzed using phasor method (d), from which we obtained a mean NAD(P)H fractional distribution curves, one for treated spheroids and another for the controls. Comparing these 2 curves we calculated a percentage of drug response (%DR).* ***f*** *examples of brightfield images (top) and phasor maps (bottom) of 3D-T98G after 72 hrs of REGO treatment using different drug concentrations.* ***g*** *Comparison of mean NAD(P)H fractional distribution curves for controls and REGO -treated 3D-T98G spheroids at different REGO concentrations. 0, 74 and 91 %DR was detected for 10M, 50M and 100M of REGO -treatment, respectively. The green area under the treated-curve represents the statistically significant area by which %DR is calculated.*