New approaches to influence 5-ALA-induced Protoporphyrin IX (PpIX) fluorescence and accumulation in GBM cell lines with different epidermal growth factor (EGFR) expression

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Background

Glioblastoma (GBM) is the most frequent and devastating brain tumor, it is classified as a Type IV glioma indeed it is the highest-grade infiltrative astrocytoma. Invasive cancer cells that escape surgical resection remain the main source of disease recurrence that generally occurs between 6 and 9 months. Approximately 50% of GBM expresses epidermal growth factor receptor (EGFR) and from these another 50% expresses the mutated form EGFR variant III (EGFRvIII), this is an important factor in driving tumor progression and invasion in GBM tumors.
One of the most innovative techniques that came to the forefront in 1992 to improve the local control rate in GBM surgery was the use of 5-aminolevulinic acid (5-ALA)-induced fluorescence to guide surgery (FIGR). 5-ALA is an heme precursor that is administered to the patient 4h before surgery and induces fluorescence due to accumulation of protoporphyrin IX (PpIX) in the cells of GBM leading to intraoperative biological tagging of these cells. It has been shown in GBM cell lines that the EGFR expression status influences the 5-ALA-induced fluorescence by inducing the rate limiting enzyme Heme Oxygenase-1 (HO-1). The major problem is that the intensity of 5-ALA-induced fluorescence has been described to vary in confirmed glioma and the detection method is currently subjective and non-quantitative.

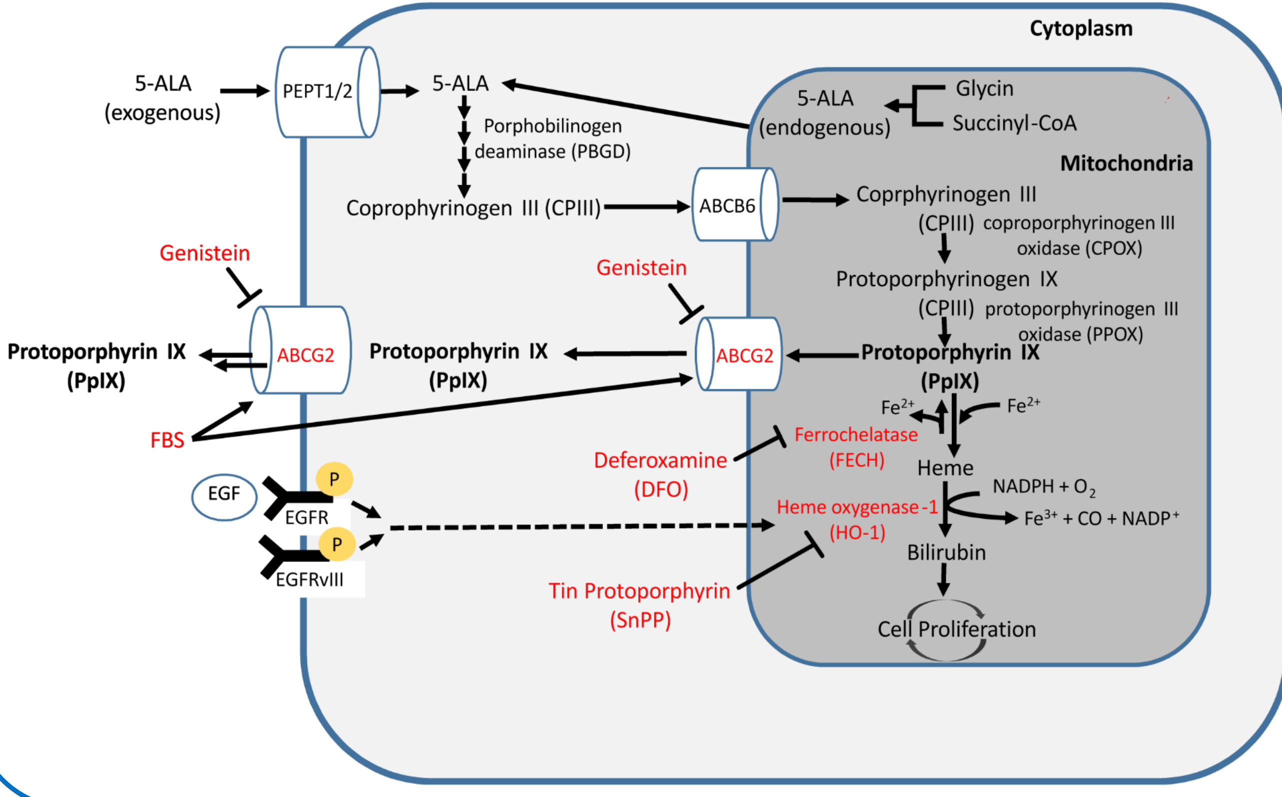


Figure 1. PpIX metabolism, efflux and degradation. Uptake and conversion of exogenous and endogenous 5-ALA into PpIX inside the mitochondria and degradation of PpIX into heme catalyzed by FECH enzyme and accelerated by HO-1 enzyme, which could be induced by EGFR activation.
Inductors or inhibitors of the conversion and efflux of PpIX and the proteins blocked are represented in red.
The dotted arrow represents putative pathway and interaction of EGFR with HO-1.

Aim

- ✓ The extent of 5-ALA guided tumor resection has a decisive impact in glioma surgery. Our translational research point to improve the resection and particularly the visualization of the tumor during surgery.
- ✓ We hypothesize that Protoporphyrin IX (PpIX) accumulation results from an interplay between active metabolism of PpIX through expression of the enzyme HO-1 and ferrochelatase (FECH) and PpIX efflux through the ATP-binding cassette subfamily G member 2 transporter (ABCG2).
- ✓ To date our goal is to increase PpIX fluorescence and to obtain the maximal accumulation in all the cell lines using different drugs able to block HO-1 and FECH enzymes like tin protoporphyrin (SnPP) that block HO-1 and Deferoxamine mesylate (DFO) that chelate (Fe^{2+}) iron ions inhibiting FECH, and at the same time use genistein to block ABCG2.
- ✓ This strategy could be applied to facilitate the tracing of tumor cells in-vivo, possibly improving FIGR.

Methods

The human GBM cell lines namely U87MG (low EGFR expression), U87wtEGFR (EGFR overexpression), U87vIII (EGFR expression/EGFRvIII+) were used for the analysis of PpIX accumulation. EGFR/EGFRvIII expression was confirmed by western blot (WB).
To analyze PpIX kinetic cells were incubated for 24 hours with 5-ALA at 1 mM and a time course was performed to analyze the PpIX accumulation. Fluorescence of PpIX was measured in adherent cells (by flow cytometry) and in conditioned media (CM) (by microplate reader (TECAN)) separately with a standard excitation of 408 nm and a fixed emission of 635 nm. After 24 hours of continuous 5-ALA treatment the CM were removed and replaced with fresh media to analyze the washout of PpIX.
Moreover, cell confluency and presence or absence of fetal bovine serum (FBS) were analyzed to assess a possible influence on PpIX fluorescence. U87 cells were cultured both in FBS-free medium or medium added with 10% FBS; after 24h cells were treated for additional 8h with 5-ALA at 1 mM, then analyzed by flow cytometry to assess the mean PpIX fluorescence.
Finally, effects of direct pharmacological inhibition by SnPP or DFO and effect of impaired PpIX efflux by genistein were analyzed in respect to 5-ALA-induced fluorescence. Cells were incubated with exogenous 5-ALA at 1mM, alone or in combination with DFO, SnPP and Genistein. After 8 hours of single or combined treatments, PpIX fluorescence was analyzed by PpIX extraction, by flow cytometry and/or confocal microscopy.

Results

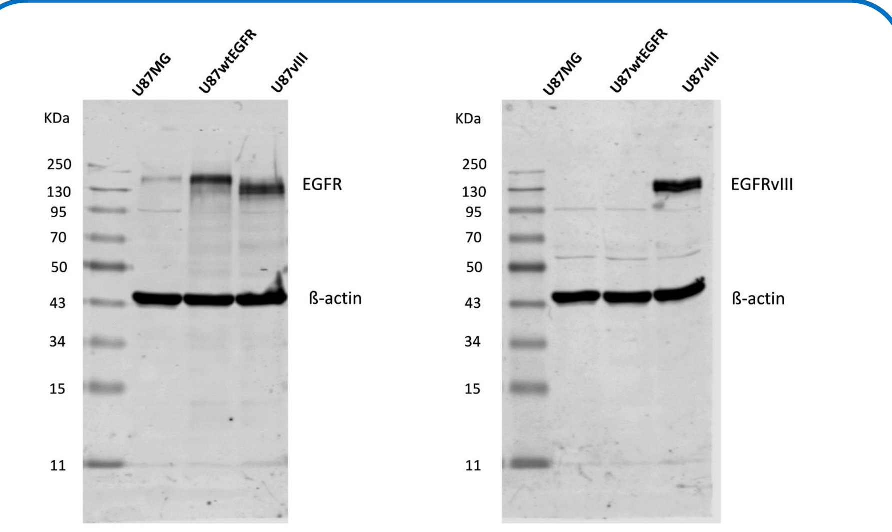


Figure 2. Protein expression of EGFR and EGFR version III (EGFRvIII) in U87MG, U87wtEGFR and U87vIII cells

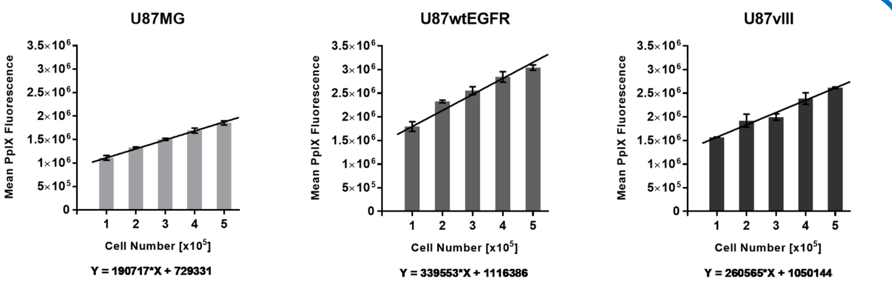


Figure 4. PpIX accumulation dependency on cellular confluence after 24h of serum starvation and treatment with 5-ALA at 1 mM for 8h.
In serum-free condition, cell density has a positive effect on PpIX accumulation as shown by the linear regression obtained at different cell plating densities. This confluency-dependent effect was strongest in U87wtEGFR cells followed by U87MG and U87vIII cells.

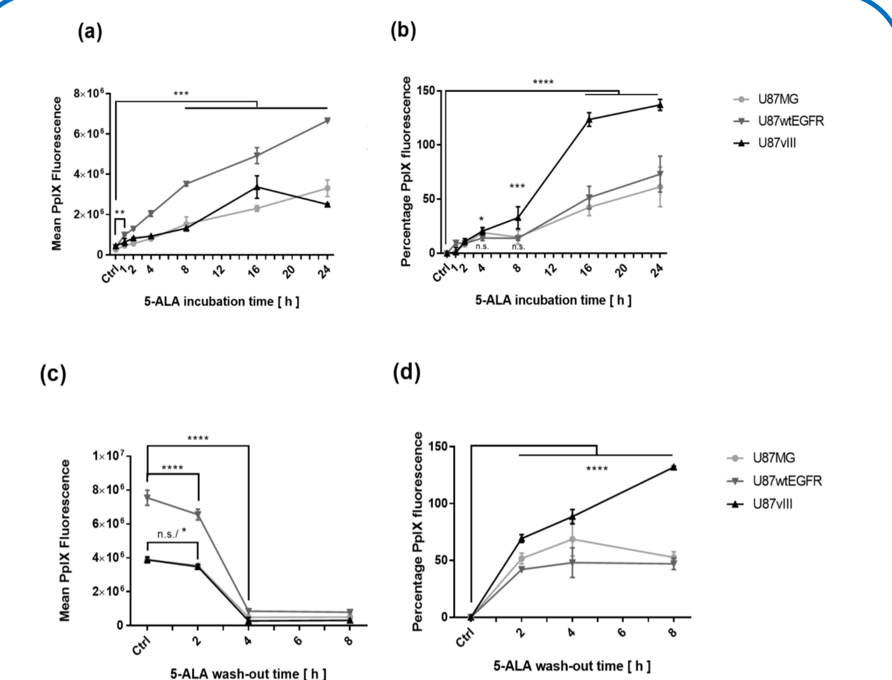


Figure 5. PpIX metabolism is different in cells with different EGFR expression. (a) PpIX fluorescence was determined by flow cytometry at the indicated time points to assess the PpIX accumulation in cells. **(b)** PpIX release and accumulation in CM were analyzed by multiplate reader **(c, d)**. PpIX clearance within cells **(panel c)** or PpIX release from cells **(panel d)** was analyzed at the indicated time points. The graphs represent mean ± SD of an independent experiment performed in triplicate (two-way ANOVA, Dunnet's multiple comparison test).
The greatest increase in cellular PpIX fluorescence is found between 4 and 16h in the presence of 5-ALA at 1mM.

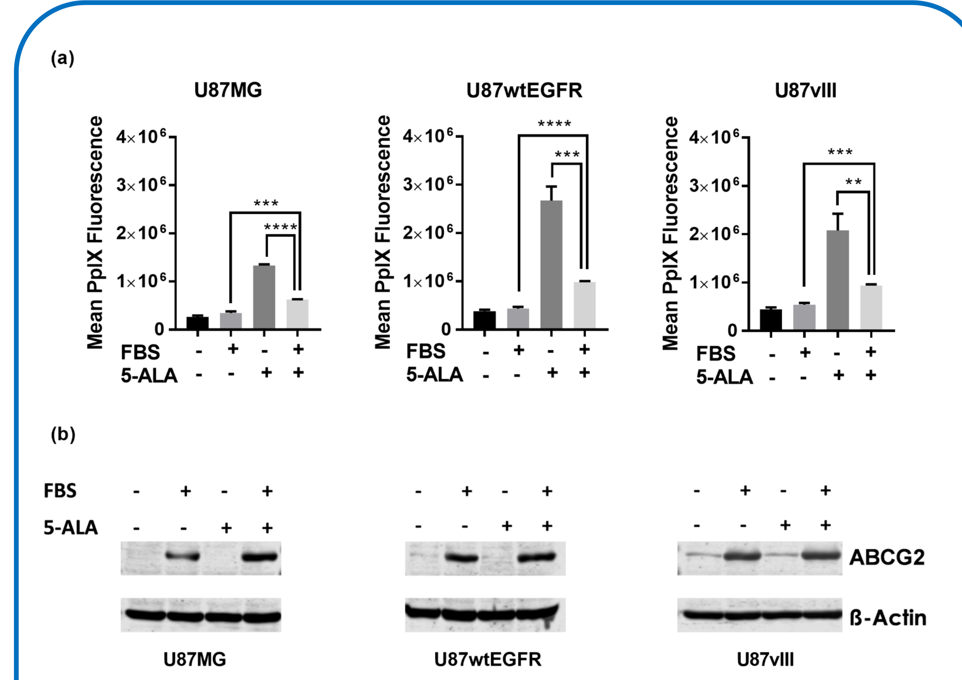


Figure 3. Influence of FBS on PpIX accumulation. (a) The graphs represent the mean ± SD (unpaired t-test). **(b)** WB analysis of ABCG2 protein expression.
Notably, cells incubated with 5-ALA in the presence of FBS significantly reduces PpIX accumulation when compared to FBS depleted cells also incubated with 5-ALA. Western blot analysis of the PpIX-efflux transporter ABCG2 reveals a robust FBS-dependent induction of this protein.

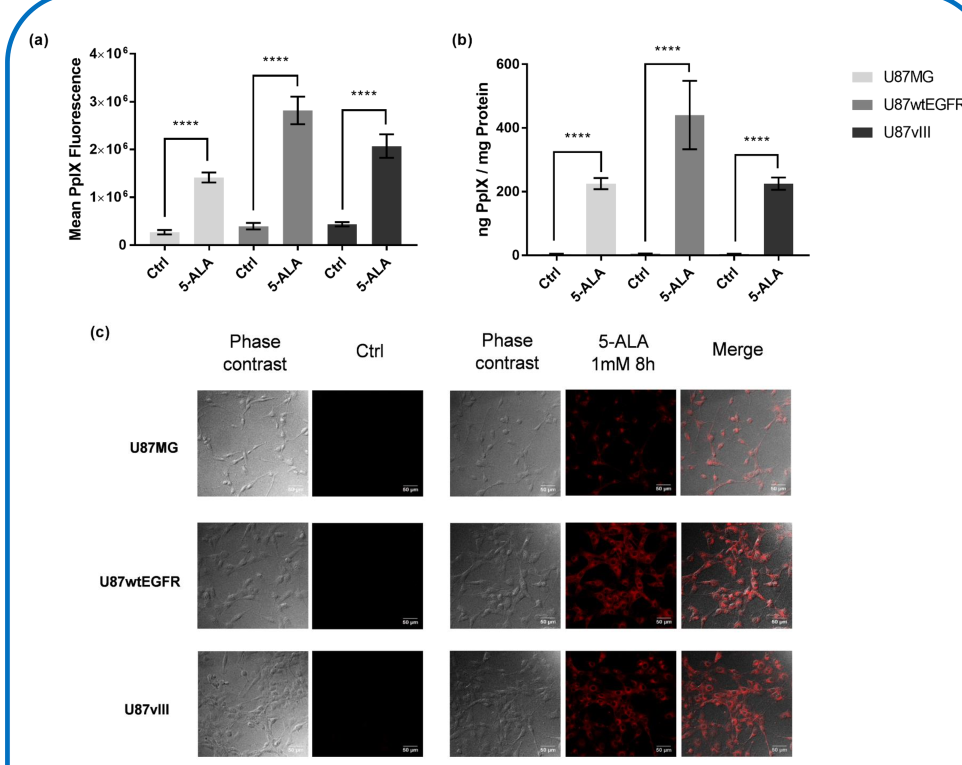


Figure 6. Quantification of PpIX accumulation after 5-ALA treatment at 1mM for 8h. (a) Cellular mean PpIX fluorescence analyzed by flow cytometry. **(b)** Cell lysates were performed with 0.2% (vol/vol) of Triton X-100 for the BCA analysis then PpIX was extracted from samples with 5.6% (vol/vol) perchloric acid in 50% (vol/vol) methanol. **(c)** Confocal images of control (untreated) U87 cells and cells treated with 5-ALA (excitation 405 nm, emission 620-650 nm). Scale bar represent 50 µM. The graph represents the mean ± SD (one-way ANOVA, Sidak's multiple comparison test).
Based on the validation with independent analytical procedures, we concluded that quantification of cellular PpIX fluorescence by flow cytometry analysis is an appropriate method for our experiments

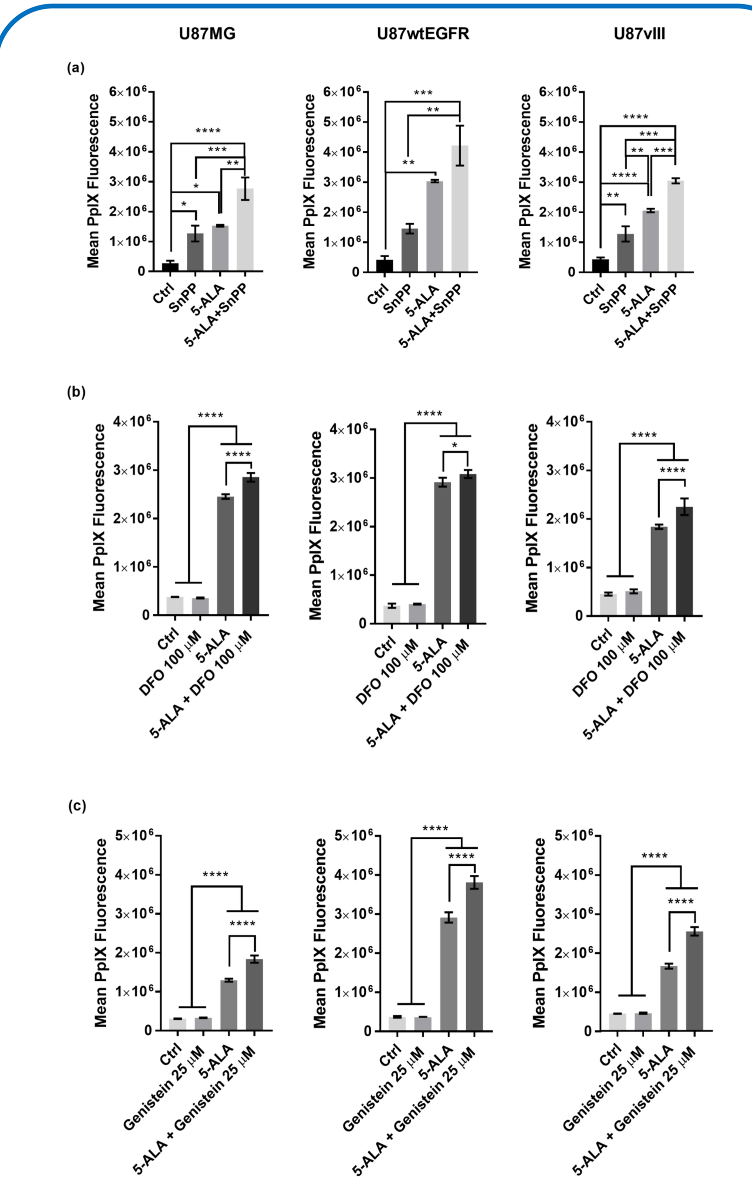
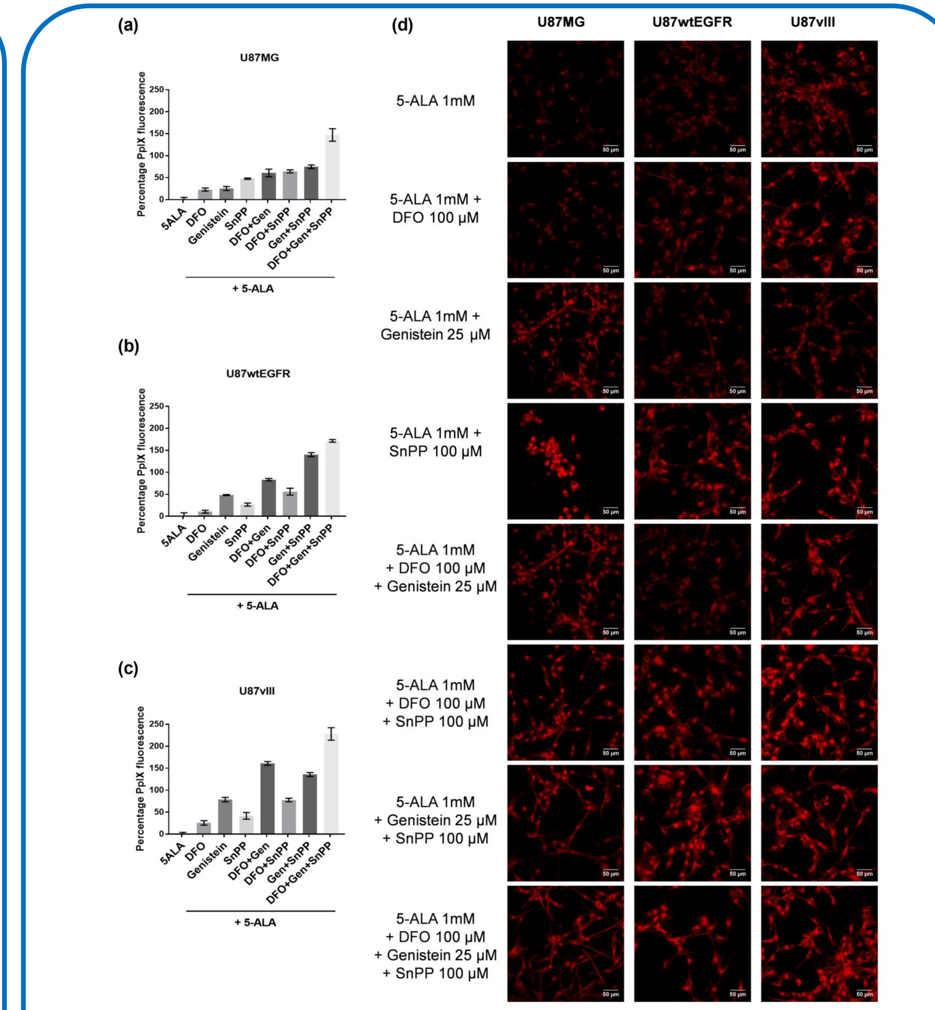


Figure 7. Evaluation of PpIX fluorescence increment after drugs treatments. U87 cells after 24h serum starvation were treated for 8h with either 5-ALA at 1 mM or SnPP 100 µM **(a)**, DFO 100 µM **(b)**, genistein 25 µM **(c)** or a combination of both treatments. The graphs represent the mean ± SD, (one-way ANOVA, Sidak's multiple comparisons test).
The HO-1 inhibitor SnPP is able to significantly improve 5-ALA-induced PpIX fluorescence in U87MG and U87vIII, alone or in combination with 5-ALA. In the presence of exogenous 5-ALA, DFO significantly improved 5-ALA-induced PpIX fluorescence in all the three cell lines. Combined treatment with genistein and 5-ALA compared to 5-ALA alone significantly increased PpIX fluorescence in all the three cell lines.



Cell line	5-ALA	DFO + 5-ALA	Genistein + 5-ALA	SnPP + 5-ALA	DFO + Genistein + 5-ALA	DFO + SnPP + 5-ALA	Genistein + SnPP + 5-ALA	DFO + Genistein + SnPP + 5-ALA
U87MG	0 %	23 %	25 %	48 %	61 %	64 %	75 %	147 %
U87wtEGFR	0%	11%	48%	27%	83%	56%	140%	172%
U87vIII	0 %	26 %	79 %	42 %	161 %	78 %	136 %	228 %

Figure 8. PpIX accumulation after combined treatments. Flow cytometric analysis of U87MG **(a)**, U87wtEGFR **(b)** and U87vIII cells **(c)** treated with 5-ALA alone or 5-ALA combined respectively with DFO, genistein or SnPP and combination of these drugs. Results are expressed as mean ± SD from two representative experiment performed in triplicate and they are represented as a percentage in respect to 5-ALA that represents the 0%. The table summarize the increment of PpIX fluorescence obtained by flow cytometry in percentage. **(d)** Confocal analysis of U87 cells treated with 5-ALA alone or in combination with DFO, genistein or SnPP respectively and combination of them. Scale bar = 50 µM.
The three cell lines respond differently to the single treatments and to combination of them, obtaining the greatest PpIX accumulation in the combined treatments with three or four compounds.

Conclusions

- ✓ SnPP treatment was able to inhibit HO-1 activity and restore the PpIX fluorescence in the three GBM cell lines, independently of EGFR quantitative and qualitative expression.
- ✓ Co-treatment with 5-ALA and DFO and co-treatment with 5-ALA and Genistein of U87 cells lead to a significant increase in PpIX fluorescence.
- ✓ We demonstrated that the treatment that led to a greater PpIX accumulation was the combination of the three drugs for all the three U87 cell lines.
- ✓ The drugs combinations tested allows to act on PpIX accumulation regardless of the EGFR expression status. These findings will be further investigated in animal models.
- ✓ Enhancement of PpIX fluorescence can be helpful during intraoperative imaging of GBM and surgical resection of the tumor.

Future Perspectives:

- ✓ Based on the promising data obtained *in-vitro* we would like to test these drugs in combination with 5-ALA also *in-vivo.*
- ✓ Another important goal is the quantification of the accumulated 5-ALA *in-vivo.*
- ✓ Moreover, we will examine another potent and selective inhibitor of HO-1, OB-24, *in-vitro.*
- ✓ A second important compound that we would like to test in order to study more in detail the relation between EGFR pathway and PpIX fluorescence is cetuximab. This antibody is able to inhibit both EGFR and EGFRvIII and could be useful to investigate its effect on HO-1 and Akt/PIK3 pathway and/or on ABCG2 and PpIX efflux.

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