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Sulforaphane potentiates gemcitabine mediated cytotoxicity towards pancreatic cancer stem-like cells

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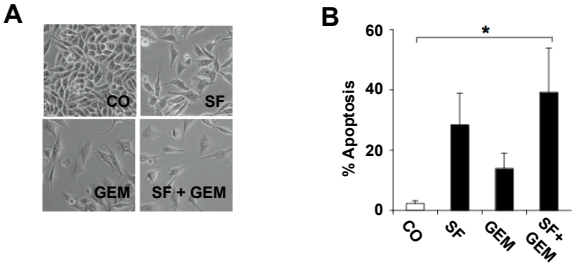
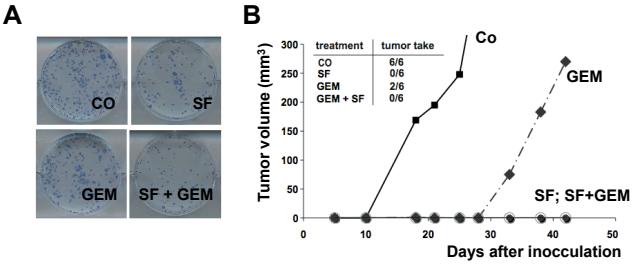
Introduction: Despite intense efforts to develop high effective treatments against pancreatic cancer, agents that cure this disease are still not available. Cancer stem cells may be responsible for high resistance and early metastasis of pancreatic cancer.Considerable attention has focused on broccoli compound sulforaphane, which is suggested for combination therapy targeting pancreatic cancer stem cells. Since increasing evidence suggests that chemotherapeutic treatment alone results in an enrichment of cancer stem cells, we investigated whether the natural compound sulforaphane can increase drug-mediated cytotoxicity in the pancreatic cancer cell line MIA-PaCa2, which is highly enriched in cancer stem cell characteristics (CSC^{high} cell line).
Methods: The effect of single and combined treatment was examined by MTT-test , Annexin V staining and colony formation assay. To investigate cytotoxicity towards CSCs we performed spheroid formation assays and evaluated ALDH activity and protein expression in CSC^{high} MIA-PaCa2 cells. Moreover we studied tumor growth following single or combined treatment in nude mice.

Combined treatment with sulforaphane and gemcitabine reduced tumor cell viability



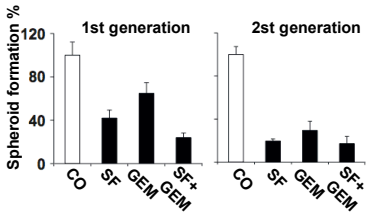
A: CSC^{high} MIA-PaCa2 pancreatic cancer cells were left untreated (CO), or were treated with sulforaphane (SF) or gemcitabine (GEM) alone or in a combination (SF+GEM) for 72 h and representative pictures were shown. **B:** CSC^{high} MIA-PaCa2 cells were seeded in 6-well tissue culture plates and treated as indicated. Seventy-two hours later apoptosis induction was evaluated by annexin V staining and flow cytometry and is presented as percentage of annexin-V positive cells.
Decreased cell viability and high levels of apoptosis induction after combined treatment adverts increased cytotoxic effects.

Sulforaphane and gemcitabine together strongly inhibited long term survival and tumor growth *in vivo*



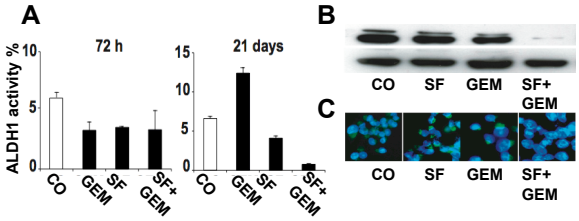
A: Long term effects of combined treatment were assessed by colony forming assays. Following 72 h treatment with SF and GEM alone or in combination (SF+GEM), CSC^{high} MIA--PaCa2 cells were reseeded and cultured in absence of treatment. The quantification of colonies revealed that SF alone and in combination with GEM strongly inhibited colony formation.
B: CSC^{high} MIA-PaCa2 cells were treated for 72 hours with SF, GEM and SF +GEM. Following treatment, alife cells were injected subcutaneously into nude mice. Tumor growth was measured and tumor take was evaluated. Treatment with GEM delayed the tumor growth whereas SF and combination therapy abrogated tumor growth.

Combined treatment inhibited self-renewal properties



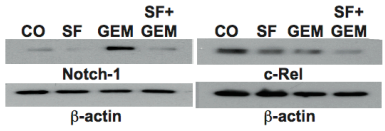
Spheroid assays were performed to investigate whether treatment affects self-renewal of CSCs. MIA-PaCa2 cells were treated with SF, GEM or SF+ GEM for 24 h. Afterwards cells were seeded at clonal density in low adhesion plates. Spheroid formation was evaluated in the first and second generation. Combination treatment shows strong inhibition of self-renewal in CSC^{high} pancreatic cancer cells.

Long term treatment reduced ALDH1 activity



A: Following long term (21 d) or short term treatment (72 h), ALDH1 activity was analyzed by flow cytometry and the percentage of ALDH1-positive cells is presented. **B:** After long term treatment (21 d), expression of ALDH1 protein was analyzed by Western blot. Expression of β-actin served as loading control. **C:** Following long term treatment (21 d) cells were subjected to immunofluorescence for ALDH1.
The reduction of the stem cell marker ALDH1 suggest that combined treatment reduced the number of CSCs within the population.

Combined treatment targeted pathways involved in resistance and self renewal



To investigate the SF mediated re-sensitivation of cells, Notch-1 and NF-kappa B expression were studied. CSC^{high} pancreatic cancer cells were left untreated (CO), or were treated with SF or GEM alone or in a combination (SF+GEM) for 48 h. Protein expression of Notch-1 and NF-kappa B was analyzed by Western blot. Results indicate a down regulation of CSC related proteins following combination treatment.

Conclusion: In this study we demonstrate that SF potentiates cytotoxicity of GEM towards pancreatic CSCs *in vitro* and *in vivo*. Moreover, combined treatment with SF and GEM targeted CSC characteristics such as clonogenic potential, self renewal and ALDH1 activity. CSC sensitizing mechanism of SF might be a down regulation of proteins which are involved in resistance and self renewal. SF has no side effects on normal cells and combination treatment with GEM might be a promising therapeutic strategy in patients.

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