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Marine sponge *Crambe crambe* for efficient elimination of pancreatic cancer stem cells

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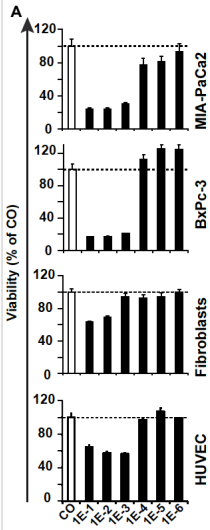
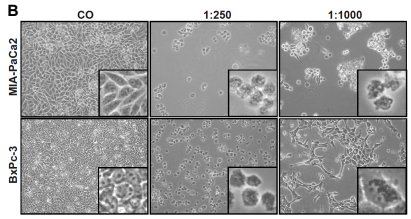
Marine organisms are known to produce a variety of secondary metabolites, from which the most are non characterized so far. Some of the characterized sponge metabolites exhibit bioactivity toward cancer cells and are therefore suggested for development of new anticancer therapeutics. We screened methanolic extracts from ten different marine sponges and one freshwater sponge for anticarcinogenic activity. Marine sponge *Crambe crambe* (CR) showed highest activity towards pancreatic cell lines MIA-PaCa2 and BxPc-3, with high (CSC^{high}) or low (CSC^{low}) content in cancer stem cell (CSC) markers, respectively. In contrast, nonmalignant cells like fibroblasts or HUVEC were not affected. CR induced efficiently apoptosis as measured by Annexin-V staining and detection of active Caspase 3, 7, 8 & 9. We could also show that CR treatment overcomes intrinsic and acquired resistance towards chemotherapy in CSC-enriched highly resistant pancreatic tumor cell lines. CR extract efficiently and specifically inhibits proliferation and induces cell cycle arrest. A long term effect on CSCs was observed in colony formation and spheroid assays. The side population defined for its CSC characteristics and multi-drug-resistance was reduced by CR treatment. In xenograft studies CR avoided growth *in vivo* indicating that CSC are eliminated.

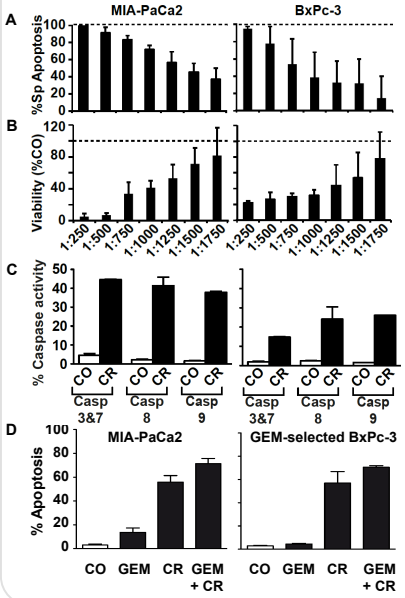
CR reduces viability of pancreatic CSCs

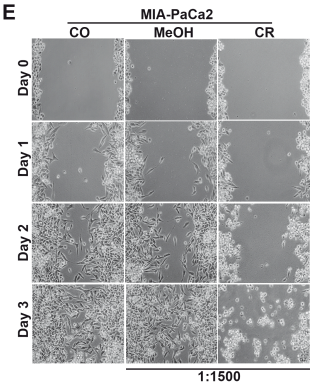


Viability Assay 72h after sponge treatment. **A)** Cells were treated with dilutions of CR extract. MIA-PaCa2, BxPc-3 and, as a control, Fibroblasts and Human Umbilical Vein Endothelial Cells (HUVEC) were examined. CR significantly inhibited viability at dilutions of 1:1000. Interestingly, non-malignant human cell lines were much more resistant towards CR compared to cancer cell lines. While 1:1000 diluted CR targeted approximately 80% of pancreatic cell lines MIA-PaCa2 and BxPc-3, it failed to reduce viability of human fibroblasts. HUVEC showed reduced viability of about 60%. **B)** Light microscopy of cancer cells. Pictures of MIA-PaCa2 and BxPc-3 are shown in 100x magnification 72h after treatment with different dilutions of *Crambe crambe*.



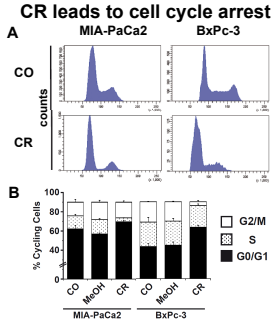
CR overcomes apoptosis resistance of pancreatic CSCs





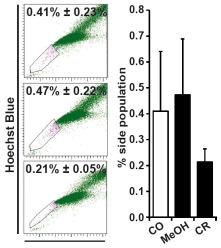
Induction of apoptosis **A)** Apoptosis assay. Cells were treated 72h with increasing concentrations of CR extract. Apoptosis was assessed by flow cytometry after Annexin-V staining. Values represent specific apoptosis of Annexin-V positive cells at each dilution. **B)** Viability measured by MTT assay 72h after treatment **C)** Caspase substrate assay measured by flow cytometry. **D)** CR increases apoptosis induction in MIA-PaCa2 cells and in Gemcitabine-resistant cell line BxPc-3 cells. **E)** CR inhibits hypoxia-induced migration measured by Scratch-assay.

CR leads to cell cycle arrest



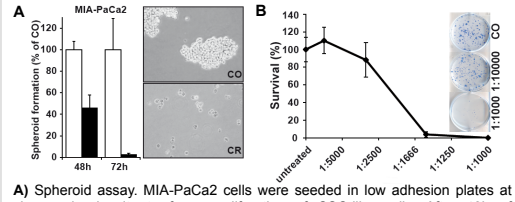
Cells were treated with a 1:1000 dilution of CR, methanol or were left untreated for 24h. Cell cycle analysis was performed after PI staining by flow cytometry. **A)** Representative graphs are shown. **B)** CR caused a significant G0/G1 arrest in MIA-PaCa2 and in BxPc-3 cell line. CR leads to a nearly complete breakdown of G2/M phase in BxPc3 cell line. Methanol treated cells did not show any effect on cell cycle compared to untreated cells.

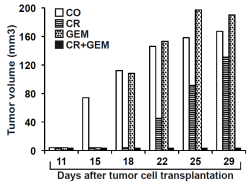
Decrease of hoechst SP by CR



The side population (SP) phenotype is defined by ABC-transporter activity, which are known to efflux chemotherapeutics and are associated with multi drug resistance (MDR). The SP also shows CSC characteristics. MIA-PaCa2 was stained with hoechst 33342 dye in presence or absence of Verapamil. The SP was gated and is shown as percentage of viable cell population. After 24h treatment with CR, SP was reduced significantly. Treatment with vehicle control MeOH has no significant change to SP.

CR targets CSC characteristics and re-sensitizes to chemotherapy





A) Spheroid assay. MIA-PaCa2 cells were seeded in low adhesion plates at clonogenic density to favor proliferation of CSC-like cells. After 48h of treatment with 1:1000 dilution of CR the number of spheroids decreased about 50%. After 72h all spheroids were abrogated. **B)** Survival assay. MIA-PaCa2 were treated for 72h with increasing concentrations of CR extract. Cells were harvested, plated at a density of 100 cells per well in a 6well plate and incubated for 2 weeks. Colonies were fixed, stained and counted. Surviving fractions were calculated as the ratio of the absolute number of colonies arising from surviving-treated cells over the number of colonies arising from surviving-untreated control cells. Survival curve is shown. **C)** MIA-PaCa2 cells were pre-treated 72h with CR (1:1500), GEM (25nM) or both together. Control cells were left untreated. Seventy-two hours later an equal amount of $6x10^3$ surviving cells were transplated s.c. with 50% Matrigel into the flank of 5-6 weeks old NMRI-nu (nu/nu) female mice, 6 per treatment group. External tumor size was measured using a caliper at time points indicated. The number of tumors, which start to regrow in each group, is shown. Data are presented as mean of 6 animals in the control group, as mean of 3 animals in the GEM-treated group and as mean of 2 animals in the CR-treated group.

Number of tumors grown per treatment group

Days	0	7	11	15	18	22	25	29
CO	0	0	0	3	4	6	6	6
CR	0	0	0	0	0	1	2	2
GEM	0	0	0	0	3	3	3	3
CR+GEM	0	0	0	0	0	0	0	0

Conclusion

In the present work we screened crude methanol extracts from 11 different sponges and identified the species *Crambe crambe* as most effective. CR extract efficiently and specifically inhibits proliferation, cell cycle arrest and clonogenicity and induces apoptosis in pancreatic cancer cell lines with low or high content of CSC markers. In contrast normal cells were less affected. Furthermore, for the first time we demonstrate, that extracts from marine sponges potentially target stem cell characteristics in cancer stem cell-like cells, including self-renewal potential and hoechst-efflux population. CR also re-sensitzes Gemcitabine-selected highly resistant cancer cells to chemotherapy. Our study suggests *Crambe crambe* as a promising source for isolation of new therapeutics for elimination of pancreatic tumors enriched in CSCs.

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