Mechanisms of Betulinic acid-induced cell death

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Betulinic acid induces a rapid form of cell death in colon cancer stem cells

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Door: Why it’s simply impassible!
Alice: Why? Don’t you mean impossible?
Door: No, I do mean impassible (chuckles) Nothing’s impossible!

Lewis Carroll, Alice in Wonderland
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Abstract

Cancer stem cells (CSCs) are considered to be the origin of cancer and it is suggested that they are resistant to chemotherapy. Current therapies fail to eradicate the CSCs and therefore select a resistant cell subset that is able to induce tumor recurrence. Betulinic acid (BetA) is a broad acting natural compound, shown to induce cell death via inhibition of the stearoyl-CoA- desaturase (SCD-1). This enzyme converts saturated fatty acids into unsaturated fatty acids and is over-expressed in tumor cells. Here we show that BetA induces rapid cell death in all colon CSCs tested and is able to affect the CSCs directly as shown via the loss of clonogenic capacity. Similar results were observed with inhibition of SCD-1, suggesting that SCD-1 activity is indeed a vulnerable link in colon CSCs and can be considered an ideal target for therapy in colon cancer.
Introduction

Colorectal cancer (CRC) is one of the major causes of death worldwide\(^{(1)}\) and the third leading cause of cancer death in the western world (WHO 2014, World Cancer Report 2014). Despite different and intensive treatments, current therapies are inadequate in eradicating the disease due to the occurrence of resistance. It has been suggested that such resistance depends on the intra-tumoral heterogeneity, in which the so-called cancer stem cells (CSCs) play a fundamental role.\(^{(2)}\) This cell subset is characterized by self-renewal and differentiation capacity\(^{(3, 4)}\) and it is regulated by a coordination of several pathways and signals responsible for the maintenance of such phenotype, as WNT\(^{(5-8)}\), BMP\(^{(9-12)}\) and Notch.\(^{(13-15)}\) CSCs presence was confirmed by several studies in different solid tumors, including colon cancer.\(^{(16-19)}\)

The resistance of colon CSCs to conventional therapy\(^{(17, 20-22)}\) makes these cells an obvious target to optimize current therapies. Strategies to identify and target specific cell surface markers are currently under investigation, with a particular focus on a functional characterization of the CSCs.\(^{(3, 23-26)}\) Despite more than two decades of research on the genetics of CRC\(^{(27)}\), it appears that we have only uncovered the tip of the iceberg.\(^{(28)}\) The extensive inter- and intra-tumor heterogeneity both genetically and therapeutically still forms an enormous challenge. We are only starting to understand the role and nature of genetic and epigenetic changes, the tumor microenvironment and the effects of different cell subsets within the tumor mass.\(^{(29-32)}\)

Current therapeutic approaches in colon cancer mainly utilize the proliferative capacity of tumor cells as a means to target the cancer. As cells can escape this type of therapy through multiple mechanisms, novel broad-acting compounds that target a distinct, but essential feature of the cancer cells are required. Such a therapy is able to target all the cells belonging to the tumor bulk, including the CSCs. A promising compound is represented by Betulinic Acid (BetA), which is a plant-derived compound that shows selective cytotoxicity for tumor cells sparing healthy cells.\(^{(33-35)}\) We have previously shown that BetA is very potent and effective in a wide range of tumor cells.\(^{(24, 34, 36, 37)}\) More importantly, we recently showed that BetA induces cell death via the inhibition of stearoyl-CoA desaturase (SCD-1) activity, an enzymatic activity that appears crucial to cancer but not normal cells.\(^{(38)}\) This enzyme converts saturated fatty acids derived from the \textit{de novo} fatty acid synthesis pathway into monounsaturated fatty acids and as such provides building blocks for new lipid biomass. The saturation grade of fatty acids in cells is crucial to maintain optimal fluidity of cellular membranes and it has been shown that inhibition of SCD-1 induces cell death in a variety of cells including lung CSCs.\(^{(39-41)}\) Due to its broad and potent activity we investigated the role of BetA in colon CSCs and also evaluated the effect of SCD-1 inhibition on these cells. Here we show that BetA and SCD-1 inhibition both induces cell death and loss of clonogenicity in colon CSCs. BetA induces a more rapid cell death in these cells.
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compared to SCD-1 inhibitor, thus suggesting that BetA is a very potent compound for cancer therapy.

**Results**

**BetA induces a rapid cell death in colon cancer stem cells.**

BetA has been shown to be very potent in killing several cancer cells after 48h with 60-90 % cell death in a p53 and BAX/BAK-independent manner\(^{36, 38}\) (see Table 1 for mutation analysis of colon CSCs). To determine whether BetA was also effective against colon CSCs, we used a panel of CSC cultures that were characterized for the most common stemness markers CD166, CD133\(^{16}\) and CD44v6\(^{3}\), and CK20, a colonic differentiation marker\(^{42}\) (Fig. 1A). We treated these spheroid cultures with BetA and observed a very rapid and potent induction of cell death. Within 2 h about 60 % of cell death was detected in BetA-treated GTG7 cells, while this resulted in 30 % and 40 % cell death in CC09 and Co147 cells respectively. Colon CSCs death induction is concentration- and time-dependent with cell death increasing to 60-80 % cell death after 8 h using the highest BetA concentration (Fig. 1B-G). Importantly, the rapid cell death is not dependent on culture conditions, but seemed to be selective for these spheroid cultured CSC cultures as normal colon cancer cell lines, such as RKO cells, only showed around 20% cell death at these early time points (Supplementary Fig. 1A-B). According with previous findings\(^{43}\), we observed BetA-induced cell death in both p53 mutant and p53 wild-type cell lines (Table 1).

**Table 1. Mutation status of colon CSCs**

<table>
<thead>
<tr>
<th>PI3KCA</th>
<th>APC</th>
<th>BRAF</th>
<th>SMAD4</th>
<th>KRAS</th>
<th>P53</th>
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<tr>
<td>GTG7</td>
<td>c.1258T&gt;C</td>
<td>c.2413C&gt;T</td>
<td>c.1607T&gt;G</td>
<td>c.38G&gt;A</td>
<td>wt</td>
</tr>
<tr>
<td>CC09</td>
<td>c.344G&gt;A</td>
<td>c.2557G&gt;T</td>
<td>c.931C&gt;T</td>
<td>wt</td>
<td>c.818G&gt;A</td>
</tr>
<tr>
<td>CO147</td>
<td>c.1090G&gt;C</td>
<td>c.2839T&gt;C</td>
<td>c.1799T&gt;A</td>
<td>wt</td>
<td>c.733G&gt;A</td>
</tr>
</tbody>
</table>

**Colon cancer stem cells lose their clonogenic capacity upon BetA treatment**

One of the hallmarks of CSCs is represented by their resistance to therapy. Most conventional therapies induce cell death in differentiated tumor cells, but not in CSCs.\(^{44}\) As our spheroid cultures represent a mixture of CSCs and more differentiated tumor cells, we set out to directly analyze the effect of BetA on CSCs using WNT signaling activity as a read-out for stemness.\(^{5}\) Spheroid cells were therefore sorted for 10% low GFP, representing the more differentiated tumor cells, 10% high GFP, representing CSCs, and the total population. In all three cell populations BetA induced a rapid cell death suggesting that BetA targets both the more differentiated cancer cells as well as the CSCs (Figure 2A-D).
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To confirm the cytotoxic effect on CSCs we looked into the functional impact of BetA treatment. One of the important features of CSCs is their ability to self-renew and to give arise to a heterogeneous population of CSCs and more differentiated cells. In other words, a single CSC is capable of forming a new colony with all cell types present.\(^{(15)}\) This capacity is present in the TOP-GFP high cells within the spheroids and defines the true CSCs. We therefore analyzed three distinct colon CSC cultures for their clonogenic capacity after treatment with different concentrations of BetA. BetA pre-treatment (2 h, single hit) clearly showed a strong reduction on stem cell frequency (60-80 % of reduction) in all the colon CSC cultures, in a dose-dependent manner (Figure 2E-G). In contrast, these short treatments did not affect the clonogenicity of RKO cells, confirming that BetA indeed targeted colon CSCs very effectively. (Supplementary Fig. 2) A separate measure of stemness is to determine the effect of therapy at later time points. We therefore analyzed the long-term effect of conventional chemotherapy (oxaliplatin) in comparison with BetA treatment on survival and regrowth of colon CSCs, using a CellTiter-Blue\(^{®}\) cell viability assay over a longer period. Treatment of two independent colon CSC cultures with oxaliplatin showed that despite an initial decrease in cell number, cultures eventually overcame the negative impact of chemotherapy and expanded over time, indicating that CSCs did not succumb to therapy even when higher doses of oxaliplatin were used (Figure 2H-I and Supplementary Fig. 3A-D). In contrast, treatment with increasing doses of BetA for only two hours revealed that long term expansion was completely annihilated at the highest concentrations of BetA (Figure 2H-I). Differential sensitivity was observed between the two CSC cultures, similar to the short-term toxicity assays with GTG7 being the most BetA sensitive cell line tested. In these cultures BetA concentrations of 5 µg/ml completely prevented outgrowth of cells, whereas in CC09, which were less sensitive to BetA, only the highest concentration of BetA (10 µg/ml) prevented outgrowth completely (Figure 2H-I and Supplementary Fig. 3 E-F). These data suggest that BetA affects colon CSCs viability and consequently long-term clonogenicity, while conventional therapy, such as oxaliplatin, gave rise to CSC-based resistance phenomena and subsequent recurrences in terms of growth.

Inhibition of stearoyl CoA desaturase leads to cell death and loss of clonogenicity in colon CSCs

We recently showed that BetA functions through inhibition of the enzymatic activity of SCD-1.\(^{(38)}\) In agreement, BetA treatment, SCD-1 inhibition or genetic knock down of SCD-1 in cancer cells, affect the saturation grade of fatty acids and specifically cardiolipin, resulting in impairment of the mitochondrial structure with consequent cell death.\(^{(38)}\) To validate whether BetA-induced death of colon CSCs is due to a similar SCD-1-dependent mechanism,
Figure 1. BetA-induced cell death in colon cancer stem cells. Flow cytometry analysis (A) of CD166, CD166, CD44v6 and CK20 in GTG7, CC09 and CO147 colon CSC lines (black histograms represent isotype control). GTG7 (B, C), CC09 (D, E) and CO147 (F, G) colon CSCs were treated for 2 h, 4 h or 8 h with indicated concentrations of BetA after which cell death was assessed via PI exclusion. In B, D, F representative FACS plots of GTG7, CC09 and CO147 colon CSCs are shown which were treated for 8 hours with DMSO or 10 µg/ml BetA. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**); P < 0.001 (***)
cells were treated with a commercially available SCD-1 inhibitor. Interestingly, SCD-1 inhibition led to a time- and concentration-dependent cell death in both GTG7 and CC09. The timing of cell death was different in these cells, as GTG7 reaches a maximum cell death at 72 h, while this appears to take longer in CC09 (Figure 3A-B). In agreement with the effect observed with BetA treatment, SCD-1 inhibition dramatically reduced clonogenic potential of GTG7 and CC09 cells (Figure 3C-D). Henceforth, we conclude that BetA is an effective compound for the targeting of colon CSCs and this can be explained at least in part by the effect of BetA on SCD-1 activity in CSCs.

Discussion

CSCs are known to be more resistant to conventional treatment due to their (acquired) resistance mechanisms, such as high levels of ATP-binding cassette (ABC) transporters and anti-apoptotic molecules, active DNA-repair, in some cases reduced proliferation and the production of growth factors that confer refractoriness to anti-neoplastic treatments. The escape mechanisms from standard therapies were shown both in vitro and in vivo. In particular chemotherapy treatment of colon cancer results in an increase in CD133+ (oxaliplatin treatment), or ESA+/CD44+/CD166+ (irinotecan treatment) cell populations, suggesting that these markers identify the CSC population, which are more resistant than their differentiated counterpart. CSC resistance mechanisms are frequently suggested to provide an explanation for minimal residual disease in which an initial response is followed by recurrences due to survival and subsequent expansion of colon CSC. Here we show that BetA and SCD-1 inhibition can both circumvent this CSC resistance and induced cell death and loss of clonogenicity in colon CSCs. Our current study provides evidence for a vulnerability in colon CSCs in the SCD-1 pathway. Previously, we have also shown that this BetA-induced killing depends on the mitochondria, but occurs in a BCL-2 family-independent manner. This may be essential for its capacity to kill CSCs as we and others recently provided evidence that CSCs protect themselves from classical chemotherapy using BCLXL-dependent mechanisms. As BetA kills cells in a BCL-2 family independent manner it is expected to also target these CSCs. BetA has previously been shown to be tumor specific and not targeting healthy cells. No toxicity to BetA was observed in mice treated with BetA as well as vehicle liposomes, as both had the same amount of proliferating cells in the small intestine. On top of this, we showed that non-transformed fibroblasts are insensitive to BetA as well as SCD-1 inhibition. Cancer cells have reprogrammed their metabolism with increased glycolysis and lipogenesis (de novo fatty acid synthesis) to maintain the need for new biomaterials. This reprogramming needs to already be present in the tumor initiating cell, either pushing the cell fate toward transformation or creating an appropriate “metabolic state” required for
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A

B

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Figure 2. BetA-induced loss of clonogenicity in colon cancer stem cells. (A, B) GTG7 colon CSCs were sorted into three fractions, high GFP (indicating the stem cells), low GFP (indicating more differentiated cells) and total cells. Overnight adhered cells were treated with vehicle (DMSO) or 10 µg/ml BetA for 2 h, after which cell death was measured via PI exclusion. Representative FACS plots are shown in A. Mean ± SD of 3 experiments are shown. P < 0.001 (**). (C, D) CC09 colon CSCs were sorted into three fractions, high GFP (indicating the stem cells), low GFP (indicating more differentiated cells) and total cells. Overnight adhered cells were treated with vehicle (DMSO) or 10 µg/ml BetA for 2 h, after which cell death was measured via PI exclusion. Representative FACS plots are shown in C. Mean ± SD of 3 experiments are shown. P < 0.001 (**). Limiting dilutions experiments were performed and stem cell frequency was calculated in GTG7 (E), CC09 (F) and CO147 (G) colon CSCs that were pre-treated for 2 h with indicated concentrations BetA. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**); P < 0.001 (***). GTG7 (H) and CC09 (I) colon CSCs were treated with indicated concentrations BetA for 2 h or with 0.5 µM oxaliplatin for 24 h after which cell numbers were measured at different time points using cell titer blue.

Figure 3. SCD-1 inhibitor-induced cell death and loss of clonogenicity in colon cancer stem cells. GTG7 (A) and CC09 (B) colon CSCs were treated for 24 h, 48 h, 72 h or 96 h with indicated concentrations of SCD-1 inhibitor after which cell death was assessed via PI exclusion. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**); P < 0.001 (***). Limiting dilutions experiments were performed and stem cell frequency was calculated in GTG7 (C) and CC09 (D) colon CSCs that were pre-treated for 48 h (GTG7) or 72 h (CC09) with 1µM SCD-1 inhibitor. Mean ± SD of 3 experiments are shown. P < 0.001 (**).
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Tumorigenesis.\(^{(48)}\) Although there is no direct proof, a growing body of evidence suggests that metabolic reprogramming is at the core of cancer and it may provide a vulnerability of cancer cells.\(^{(48-50)}\) To maintain a pool of healthy fatty acids needed for new biomaterials, tumor cells start to depend on de novo fatty acid synthesis, while normal cells hardly utilize this pathway as they acquire unsaturated fatty acids from their surroundings. Newly generated fatty acids are fully saturated and are relatively toxic to the cells necessitating their conversion to unsaturated fatty acids by introduction of a double bond at the \(\omega-9\) position by the enzyme SCD-1.\(^{(40, 51-54)}\) This points out the need for SCD-1 activity in cancer cells, but could also hint to a vulnerability of cancer cells to this pathway. In several types of cancer, including colon cancer, SCD-1 expression and activity are increased \(^{(38, 54, 55)}\), moreover SCD-1 levels were found elevated in \textit{in vivo} models that were genetically predisposed to develop certain cancers.\(^{(56)}\) These observations suggest that SCD-1 activity is needed in cancer cells in order to maintain the levels of unsaturated fatty acids and by doing so protecting tumor cells from a surplus of toxic saturated fatty acids.\(^{(40, 55, 57)}\)

Indeed when inhibiting SCD-1 by small molecule inhibitors or genetic knockdown induction of cell death was observed in cancer cells\(^{(38, 57)}\) and this is a result of a strong switch in the saturation grade of the mitochondrial lipid cardiolipin and as a consequence the induction of mitochondrial fragmentation and induction of cell death.

Here we show that colon CSCs are also sensitive to inhibition of the SCD-1 enzyme. The vulnerability to inhibition of SCD-1 activity was also recently observed in another study where inhibition of SCD-1 in lung CSCs resulted in a reduced stem cell marker expression and induced cell death.\(^{(41)}\) These data are in agreement with what we observed in our colon CSCs model. One striking observation is the velocity of cell death induction, which appears to be much higher as compared to classical colon cancer cell lines. This is specific for BetA and not observed with SCD-1 inhibition, which appears as effective in cancer cells and colon CSC cultures.\(^{(38)}\) We previously have hypothesized that the rapid effect of BetA is due to a combined effect of BetA, on one hand blocking the production of unsaturated fatty acids, but on the other hand increasing turnover of lipids and thereby the incorporation of saturated fatty acids.\(^{(38)}\) This concept therefore may point to an additional effect of BetA on colon CSC cultures that strongly enhances the impact on these cells. At this point we have no definitive proof as to this effect, but it is interesting to note that colon CSCs can be identified by their high level of lipid droplets when compared with differentiated tumor and even more so when compared to cancer cell lines.\(^{(58)}\) Lipid droplets are fat storing organelles, that are suggested to be involved in the storage, transport and metabolism of lipids, in signaling, and as a specialized microenvironment for metabolism.\(^{(59, 60)}\) SCD-1 plays an important role in lipid droplets formation and size. In SCD-1 WT C. elegans, large-sized lipid droplets were observed, while in SCD-1 mutant C. elegans small-sized lipid droplets are found.\(^{(61)}\) Small-sized lipid droplets are also found in patients with Berardinelli-Seip congenital lipodystrophy, which have decreased SCD-1 activity.\(^{(62)}\) These lipid droplets
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appear to be crucial for the CSCs and we hypothesize that BetA might interfere with these lipid droplets and therefore induces a very rapid induction of mitochondrial damage.\(^{(38)}\)

We conclude that SCD-1 is a crucial player in the maintenance of stemness of cancer cells and inhibition is incompatible with long-term survival. Targeting the activity of this enzyme in CSCs provides an effective means to target these cells and can be achieved either with direct chemical inhibitors or even more effectively with BetA.

**Material & Methods**

**Chemicals/antibodies**
Betulinic acid (BioSolution Halle, Germany, >99% purity) was dissolved in DMSO at 4 mg/ml and stored at -80°C. Oxaliplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Stearoyl-CoA desaturase inhibitor (cat# 1716) was from Biovision (Milpitas, CA, USA).

**Isolation, Culture and Characterization of Colon Cancer Cells**
Colon cancer tissues were collected at the Department of Surgical and Oncological Sciences, and CEMM Department, in accordance with the ethical standards of the University of Palermo and AMC institutional committees. Colon spheroid cultures were derived from colorectal cancer patients and maintained in stem cell medium (advanced DMEM/F12 (Gibco) supplemented with N2 Supplement (Gibco), 6 mg/ml glucose, 5 mM HEPES, 2 mM L-glutamine, 4 µg/ml heparin, 50 ng/ml epidermal growth factor and 10 ng/ml basic fibroblast growth factor) as previously described \(^{(16)}\). The colon CSC lines obtained were subjected to genotypic characterization in order to validate each cell line’s individuality and their mutational status (Table 1), and further tested for their ability to generate tumor xenografts that replicated the parental histology. Colon CSCs were characterized by their expression of the putative cancer stem cell markers CD166-PE (3A6; mouse IgG1k; BD Biosciences), CD133-APC (293C3; mouse IgG2bk; Miltenyi) and CD44v6-APC (2F10; mouse IgG1; R&D systems), and CK20 (Ks20.8; mouse; IgG2ak), a colonic differentiation marker, data were acquired by flow cytometry using a BD ACCURI C6 and analyzed using the FlowJo software (Tree Star Inc., Ashland, OR, USA). CK20 was detected by using a goat anti-Mouse IgG (H+L) secondary antibody, Alexa Fluor®-488 conjugate. RKO cells were obtained from ATCC (Manassas, VA, USA) and maintained in IMDM supplemented with 8% FCS, 2 mM L-glutamine, 40 U/ml penicillin and 40 mg/ml streptomycin. GTG7 and CC09 cells bear the TCF Optimal Promoter (TOP)-green fluorescent protein (GFP) construct.\(^{(5)}\) These cells, derived from single-cell cloned TOPGFP cultures, still show a big heterogeneity in Wnt signaling levels, which positively correlates with cancer stemness in colon CSCs.\(^{(63)}\)

**Cell death analysis**
Cell death was determined by PI exclusion. In short, 50 000 single colon CSCs were seeded in a 24-well plate and allowed to attach overnight. Cells were treated for indicated time-
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points with BetA or SCD-1 inhibitor and harvested. The cell pellet was resuspended in 200 µl medium and stained with PI at 0.5 µg/ml just before measuring by flow cytometry (FACSCanto, BD Biosciences). Samples were analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cell Proliferation Assay
For determination of cell viability, 1000 cells per well were plated in repellent surface for low attachment 96-well plates (CELLSTAR® Cell-Repellent Surface, Greiner Bio- One) in medium containing vehicle (DMSO), 0.5 µM oxaliplatin or increasing doses of BetA. Viable cells were determined at indicated time-points using Cell Titer Blue (Promega, Fitchburg, WI, USA) by measurement of absorbance at 540 nm in a Synergy plate reader (Biotek). Cell viability at each concentration was expressed as OD cells – OD background.

Limiting dilution assay
The self-renewal capacity of colon CSCs was assayed by dissociation of colon cancer spheroids. Colon CSCs were cultured in adhesion o/n and pre-treated 2 h with indicated concentrations BetA or pre-treated 48 h or 72 h with SCD-1 inhibitor after which cells were collected and plated at serial dilution (1, 2, 4, 8, 16, 32, 64 and 128 cells/ well) in 96 well microplates with flat bottom and repellent surface for low attachment (CELLSTAR® Cell-Repellent Surface, Greiner Bio- One), using a FACS Aria II. Results were statistically evaluated after 3 weeks by using the Extreme Limiting Dilution Analysis (ELDA) software.\(^{(64)}\)

Statistical analysis.
Results are shown as the mean ± SD for at least three repeated independent experiments for each group. The mean and SD were obtained by analyzing three replicates using Prism 5 (GraphPad Software, La Jolla, CA, USA) applying a 2-tailed unpaired Student t-test. Differences were considered significant when p values P < 0.05 (*); P < 0.01 (**); P< 0.001 (***)
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Conflict of interest

The authors declare no conflict of interest.

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References


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**Supplementary figures**

**Supplementary Figure 1. BetA-induced cell death in colon cancer cells** (A, B) RKO cells were treated for 2 h, 4 h, and 8 h with indicated concentrations of BetA after which cell death was assessed via PI exclusion. Representative FACS plots of RKO cells treated for 8 h with DMSO or 10 µg/ml BetA are shown in A. Mean ± SD of 3 experiments are shown. P < 0.05 (*); P < 0.01 (**); P < 0.001 (***)

**Supplementary Figure 2. Clonogenicity in colon cancer cells** RKO cells were pre-treated for 2 h with indicated concentrations BetA after which limiting dilution experiments were performed and stem cell frequency was calculated.
Supplementary Figure 3. Long term assay on CSCs. (A) GTG7 and CC09 (B) colon CSCs were treated with 0.5 μM oxaliplatin for 24 h or indicated concentrations BetA (μg/ml) for 2 h after which cells were left to grow. Pictures (10x magnification) were taken at day 10 after which the number of cells were determined with cell titer blue. (C) GTG7 and CC09 (D) colon CSCs were treated with indicated concentrations oxaliplatin (μM) for 24 h after which cells were plated and cell numbers were measured at different time points using cell titer blue. (E) GTG7 and CC09 (F) colon CSCs were treated with indicated concentration oxaliplatin (μM) for 24 h after which cells were left to grow. Pictures (4x magnification) were taken at day 15 after which the number of cells were determined with cell titer blue.