RESEARCH ARTICLE

Opposing effects of low versus high concentrations of water soluble vitamins/dietary ingredients Vitamin C and niacin on colon cancer stem cells (CSCs)

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Abstract

Colorectal cancer is one of the global causes of cancer deaths. Cancer stem cells (CSCs) inside the tumour niche responsible for metastasis and relapses, and hence need to be targeted for cancer therapeutics. Although dietary fibre and lifestyle changes have been recommended as measures for colorectal cancer prevention, no such recommendations are available for using water soluble vitamins as prophylaxis measure for colorectal cancers. High dose of Vitamin C has been proven to selectively kill colon cancer cells having BRAF and KRAS mutations by inducing oxidative stress. In this study, we show for the first time the opposing effects of the low and high dose of Vitamin C and vitamin B3 on colon CSCs isolated from HT-29 and HCT-15 colorectal carcinoma cell lines. At small doses, both of these vitamins exerted a cell proliferative effect only on CSCs, while there was no change in the proliferation status of non-stem cancer cells and wild-type (WT) populations. On the other hand, the death effects induced by high doses of Vitamin C and B3 were of the order of 50–60% and ~30% on CSCs from HT-29 and HCT15, respectively. Interestingly, the control fibroblast cell line (NIH3T3) was highly refractory all the tested concentrations of Vitamin C and B3, except for the highest dose ~10,000 μg of Vitamin C that induced only 15% of cell death. Hence, these results indicate the future scope of use of therapeutic doses of Vitamin C and B3 especially in patients with advanced colorectal cancer.

Keywords: cancer stem cells; colorectal cancer; HT-29; non-stem cancer cells; vitamin B3; Vitamin C

Introduction

Amongst all the cancers, the third most common cancer of all types is colorectal cancers which affect both men and women at an equal frequency (Jemal et al. 2009). Moreover, with the sedentary lifestyle and fad dietary habits for food containing high fat and less fibre, colorectal cancer is on the rise on a global scale especially in the developed countries (Marley and Nan, 2016). Hence, dietary fibres and lifestyle changes are recommended measures for preventing colorectal cancers (Hastert and White, 2016).

The patients with advanced colorectal cancers have only 8% 5-year survival rates (Marley and Nan, 2016). Fifty percent amongst all the patients undergoing aggressive tumour resection surgery, in combination with chemotherapy, suffer from a cancer recurrence (Yazilitas et al. 2016). Less than 1% population amongst the colorectal cancer nicher also known as the cancer stem cells (CSCs) or tumour-initiating cells (TICs) are the rogue population of cells that are responsible for cancer relapse (Bagheri et al. 2017). As the CSCs/TICs are the ones which have survived during the initial chemotherapy, such CSCs/TICs tend to acquire chemoresistance and hence, the patient fails to respond to the same chemotherapeutic drugs after the cancer relapses. Moreover, the CSCs have the capacity to regenerate the bulk of cancer. Hence, 90% of the drug failures in metastatic cancer are due to chemoresistance (Longley and Johnston, 2005; Eduati et al. 2017). Such chemoresistance is attributed to various features of CSCs such as presence of ABC transporter proteins for effluxing the drug (Zhou et al. 2001; Lou and Dean, 2007; Hao et al., 2010; Gatti et al., 2011; Shaf and Jabeen, 2017); presence of prosurvival protein BCL2 and associated proteins such as BCL-XL, BCL-W, BCL A1A, MCL-1 (Sinicrope et al. 1995; Abdullah and Chow, 2013;
Carter et al. 2016); altered DNA damage/repair response (Abdullah and Chow, 2013; Yu et al., 2016a; Wang et al., 2016; Wang et al., 2017); and also structural changes in the endocrine receptors (Stender et al., 2017).

Human colorectal cancer cells (CRCs) carrying KRAS or BRAF mutations that are often refractory to targeted therapies are reportedly killed by high doses of Vitamin C in vitro (Yun et al., 2015). The mechanism of killing of cancer cells through the high dose of Vitamin C treatment has been attributed to the increased uptake dehydroascorbate, the oxidised form of Vitamin C which, in turn, results in oxidative stress. The ROS thus generated inhibits the glycerdehyde 3-phosphate dehydrogenase (GAPDH) leading to an energy crisis and cell killing in KRAS and BRAF mutated colon cancer cells (Yun et al., 2015). However, the effect of varying doses of Vitamin C and other dietary ingredients in cancer stem cells has never been elucidated. Here, we have studied the effects of varying doses of dietary ingredients such as Vitamin C and Niacin on colon cancer stem cells isolated from HT-29 and HCT-15 colon cancer cell line. HT-29 cell line has mutations in BRAF, PIK3CA, SMAD4, APC and TP53 while KRAS and PTEN genes are non-mutated in this cell line. On the other hand, HCT-15 cell line has mutations in KRAS, APC and PIK3CA (Ahmed et al., 2013). The details of the mutations of these two colorectal cancer cell lines can also be obtained in the ATCC document (https://www.atcc.org/~/media/PDFs/Culture%20Guides/Cell_Lines_by_Gene_Mutation.ashx).

In this study, we show for the first time that small doses of Vitamin C and Niacin are promoting the proliferation of cancer stem cells isolated from HT-29 and HCT-15 colon cancer cell lines. However, it is only the high doses of these dietary ingredients that have a cell killing effect on the CSCs isolated from the HT-29 colon cancer cell line.

Materials and methods

Cell culture

The colon adenocarcinoma cell lines HT-29 and HCT-15 and the control fibroblast cell line NIH3T3 were procured from the National Centre for Cell Science, Pune, India. The cells were cultured in media containing 10% fetal bovine serum from the National Centre for Cell Science, Pune, India, DMEM and 1% Penstrep and 1%L-Glutamine all from Thermo Fisher Scientific, USA in 5% CO2 and humidified atmosphere. The cells were split by using 0.25% Trypsin-EDTA (Thermo Fisher Scientific, USA) upon reaching 70–80% confluency. The colon carcinoma cell lines needed splitting at a ratio of 1:6 while the control fibroblasts were split at a ratio of 1:3.

Enumeration of the percentage of cancer stem cells using analytical flow cytometry

Adherent cells were washed with DPBS/Ca2+-Mg2+- free (Thermo Fisher Scientific) followed by dislodging the cells using 0.25% Trypsin-EDTA. Three to four million cells were collected, the pellet washed using DPBS. Cells were fixed using 4% paraformaldehyde for 30 min to overnight before performing analytical flow cytometry. 0.5 × 106 cells were stained using Alexa Fluo® 488 conjugated anti-human/mouse CD44 antibody (Biolegend, Catalog No- 103015) for 45 min. 10,000 events were acquired using Guava Easycyte flow cytometer, and the results were analysed using Prosort flow cytometry software. The cell morphologies were photographed using the Zeiss Primovert microscope with the camera attachment.

Isolation of cancer stem cells and non-stem cancer cell populations using flow cytometric cell sorting

All the procedure after cell harvesting was done at 4°C to ensure live cells for sorting. From a growing dish at 70–80% confluency, media was discarded, and the cells were washed with DPBS without Ca2+ and Mg2+. The cells were harvested using 0.25% Trypsin-EDTA. 5.0 million cells were suspended in 500 μL of ice-cold FACS dissociation buffer for 15 min at 4Deg C. FACS dissociation buffer comprised of 3% FBS in PBS with 1% Penstrep and 7.5 mM EDTA. Centrifugation at 1200 rpm at 4°C was carried out for the removal of the FACS dissociation buffer. Up to 5.0 × 106 cells were stained at a concentration of 0.5 μg of antibody in a staining volume of 100 μL in FACS staining/sorting buffer containing 2% FBS in PBS, 5 mM EDTA and 1% Penstrep for 1 h in the dark at 4°C. 0.2 × 106 cells served as the unstained control. Stained cells were washed thrice, resuspended in 1 mL of staining/sorting buffer and sorted using 53e Bio-Rad cell sorter. Cancer stem cells (CD44+), and non-stem cancer cells (CD44−) cells were sorted and collected in FBS containing media followed by cell plating and expansion. CD44 has been taken as a robust single marker to sort colon cancer stem cells as it has been reported to reprogram colon cancer cells into cancer stem cell phenotype (Su et al., 2011a) and is also a proven potent functional marker to isolate colon cancer stem cells (Du et al., 2008).

Population doubling time

WT, CD44+ and CD44− cells were plated into triplicates onto 10 cm dish at a cell number of 0.5 million. Cell harvesting at different time points followed by cell counting using a hemocytometer and tabulated. The doubling time of each of the cell populations was calculated using the online

MTT assay

MTT assay was performed as per the protocol freely available on R and D systems’ website. Briefly, 25,000 cells per well of a 96-well plate were seeded followed by 4 h attachment time. The cells were treated with various doses of Vitamin C (low concentrations – 5, 10, 15, 20, 25 μM; and high concentrations 100, 200, 500, 1,000 and 10,000 μM) for 2 h. Cells were given a media change containing 0.5 mg/mL of MTT [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and incubated for 4 h. The reaction was stopped using DMSO/incubated for 30 min, and absorbance was read at 620 nm using a microplate reader (Fluo Star Omega, BMG Labtech, Germany). The cell growth/inhibition was expressed as the percentage of cell proliferation as compared to untreated controls run with each of the treatments and assigned a value of 100%.

Apoptosis versus live cell detection in live cells (live–dead cell staining)

Live–dead cell staining was performed for corroborating the MTT assay results. Only two concentrations of each vitamin (25 μM – low dose and 1,000 μM – high dose) were tested followed by the live-Hoechst versus dead-Propidium iodide

Figure 1

Analytical flow cytometry showing the gross percentage of cancer stem cells in HT-29 and HCT-15 colorectal cancer cell lines. Scatter plots of (a) HT-29 and (b) HCT-15 colorectal cell lines showing the cell size (forward scatter-FSC-Area) versus cell granularity (side scatter-SSC-Area). Unstained control cells from (b) HT-29 and (e) HCT-15 cell line in quadrant R2. CD44 stained cells/cancer stem cells from (c) HT-29 cell line and (f) HCT-15 cell line in quadrant R3.
Figure 2  Flow sorting of CD44+ cancer stem cells and CD44− non-stem cancer cells from HT-29 and HCT-15 colorectal cancer cell lines. Scatter plots with forward and side scatter indicating cell size and granularity respectively from (a) HT-29 and (e) HCT-15 cell line. Dot plot with side scatter area (SSC-Area) versus side scatter height (SSC-Height) indicating singlets and doublets in (b) HT-29 and (f) HCT-15 cell line. The singlets have been gated in and included for sorting. Dot plot showing unstained cells (Unstained control) in (c) HT-29 cell line and (g) HCT-15 cell line. CD44 stained cells showing the CD44 positive population in gate R4 and CD44 negative population in gate R3 in (d) HT-29 cell line and (h) HCT-15 cell line. Both the cell fractions CD44 positive and CD44 negative were collected and expanded.
staining of all six cell types (HT29-WT, HT29-CD44+ and HT29-CD44−, HCT15-WT, HCT15-CD44+, HCT15-CD44−) and NIH3T3 in the live cell cultures. Twenty thousand cells per well were plated onto each well of 24-well plate and allowed to grow for 18 h till 70% confluence. Each cell type was treated with two concentrations (25 and 1,000 μM) of each Niacin and Vitamin C and controls (no treatment) in triplicates for 2 h at 37°C under 5% CO2. The cells were then subjected to media change with staining solution containing DMEM+ 1% Penstrep, 5 μg/mL of Hoechst 33342 dye (Sigma, Cat No-B2261) and 20 μg/mL Propidium Iodide (Sigma, Cat No-P4170). After 10 min incubation of the cells in staining solution, a fresh media change with complete media, visualisation and photomicrographs under dark using ZOE cell imager (Bio-Rad) was carried out. The live cell nuclei stained blue (with Hoechst 33342 dye), the apoptotic cells stained light blue with fragmented nuclei (with Hoechst 33342 dye) while the dead cells stained Red with PI. PI-stained cells were enumerated from five different fields for each of the treatments and plotted graphically.

Tumourosphere 3D assay

2D cells of all types (HT29-WT, HT29-CD44+ and HT29-CD44−, HCT15-WT, HCT15-CD44+, HCT15-CD44−) and NIH3T3 control fibroblasts were harvested by trypsinization. Cells were suspended in media in hanging drops/droplets. Hanging drops were allowed to form against gravity on the lid of a 10 cm dish under humidified conditions. We have modified the protocol of hanging drops by Foty (2011), regarding cell number and droplet size so as to obtain an average diameter of the 3D tumourospheres ~200–250 μ for the cell types that successfully formed spheroids by 48 h of seeding. The same cell number for hanging drops were maintained across all the six cell types. The hanging drops were observed and photographed for the formation of 3D spheroids/tumourosphere after four days under ZOE cell imager (Bio-Rad). The spheroids with sharp boundaries were graded as tumourospheres, in contrast to, the cell aggregates that exhibited irregular boundaries.

Live immunostaining of 3D spheroids/tumourosphere for the assessment of CD44+ cancer stem cells

2D cells of all types (HT29-WT, HT29-CD44+ and HT29-CD44−, HCT15-WT, HCT15-CD44+, HCT15-CD44−) and NIH3T3 control fibroblasts were harvested by trypsinization. Cells were suspended in media in hanging drops/droplets. Hanging drops were allowed to form against gravity on the lid of a 10 cm dish under humidified conditions. We have modified the protocol of hanging drops by Foty (2011), regarding cell number and droplet size so as to obtain the average diameter of the 3D tumourospheres ~200–250 μ for the cell types that successfully formed spheroids by 48 h of seeding. The same cell number for hanging drops were maintained across all the six cell types. The hanging drops were observed and photographed for the formation of 3D spheroids/tumourosphere after four days under ZOE cell imager (Bio-Rad). The spheroids with sharp boundaries were graded as tumourospheres, in contrast to, the cell aggregates that exhibited irregular boundaries.

Figure 3 Gene expression analyses of cellular derivatives (WT, CSC-CD44+ and non-CSC-CD44−) from HT-29 and HCT-15 colorectal carcinoma cell lines (a) CD44 expression (ΔCt values with respect to GAPDH). (b) EMT (Snail, Slug and Vimentin) and MET (EpCAM and E-Cadherin) markers in HT-29 cell lines (fold change calculated with respect to WT) (c) EMT (Snail, Slug and Vimentin) and MET (EpCAM and E-Cadherin) markers in HCT-15 cell line (fold change calculated with respect to WT).
contrast to, the cell aggregates that exhibited irregular borders.

**Gene expression analysis**

All the cell types (HT29-WT, HT29-CD44\(^+\) and HT29-CD44\(^-\), HCT15-WT, HCT15-CD44\(^+\), HCT15-CD44\(^-\)), 1 million each were harvested by trypsinization. RNA was isolated using the RNeasy mini kit (Qiagen, Germany, Catalog No-74104) as per manufacturer’s instructions. RNA was quantified using Nanophotometer NP80 (Implen, Germany). One microgram of RNA was used for transcribing c-DNA using iSCRIPT\textsuperscript{TM} c-DNA synthesis kit (Bio-Rad Catalog Number-1708891), and qRT-PCR reactions were performed using SS-Fast\textsuperscript{TM} Eva Green Supermix (Bio-Rad, USA, Catalog No-172-5201) also as per manufacturer’s instructions. Results were expressed as fold change to the WT cells for each of the cell lines (plotted as value 1 in the Y axis). Fold change was calculated by the \(2^{\Delta\Delta Ct}\). The housekeeping gene GAPDH was found to express consistently in all the cell types (Ct value \(~20\)). The individual mRNA expressions of the tested genes of all the cell types (Ct

![Figure 4](image-url)

**Figure 4** Live cell co-staining for CD44\(^-\) AF488 (green); nuclear-Hoechst (blue) of 3D partial tumourospheres + cellular aggregates (WT cells); fully formed tumourospheres (CSCs), cellular aggregates (non-CSC) obtained from cellular derivates (WT, CSC-CD44\(^+\) and non-CSC-CD44\(^-\)) of HT-29 and HCT-15 colorectal carcinoma cell lines. Phase contrast, Hoechst live nuclear stain (blue) and CD44\(^-\) AF488 live cell staining and Phase + CD44\(^-\) AF488 merged image of partially formed tumourosphere + cellular aggregates of (a) WT-HT-29 cells and (b) WT-HCT-15 cells. Phase contrast, Hoechst live nuclear stain (blue) and CD44\(^-\) AF488 live cell staining and Phase + CD44\(^-\) AF488 merged image of fully formed 3D tumourospheres of (b) CD44\(^-\) CSCs from HT-29 cell line and (e) CD44\(^-\) CSCs from HCT-15 cell line. Phase contrast, Hoechst live nuclear stain (blue) and CD44\(^-\) AF488 live cell staining and Phase + CD44\(^-\) AF488 merged image of cellular aggregates of (c) non-CSC/CD44\(^-\) HT-29 cells and (f) non-CSC-CD44\(^-\) HCT-15 cells. The cellular aggregates from CD44\(^-\) /non-CSCs stained negative for CD44\(^-\) AF488 (green). Scale bar – 100 \(\mu\)M.
values) were first normalised with their respective GAPDH values for obtaining the ΔCt values. The absolute expression of CD44 in all the six cell types (HT29-WT, HT29-CD44⁺, and HT29-CD44⁻, HCT15-WT, HCT15-CD44⁺, HCT15-CD44⁻) have been represented as ΔCt values, to ensure a very high expression of this CSC marker in the desired cell types. The primer sequences were designed by the authors and were ordered from Sigma. The details of primers are provided in Supplementary Table S1.

Statistical analysis

All the experiments were performed as three biological replicates using three independently sorted batches of cells...
(CD44\(^+\) and CD44\(^-\)). The results were expressed as Mean ± SEM.

**Results**

Cancer stem cells exhibited a high percentage ~60% in the HT-29 cell line versus HCT-115–35% versus ~35% in HCT-15 cell lines (Figure 1f), respectively.

The colorectal carcinoma cell lines upon subjection to flow cytometry for the assessment of the percentage of cancer stem cells (CSCs) prior sorting, as CD44\(^+\) cells exhibited a high ~62% in HT-29 (Figure 1c) versus ~35% in HCT-15 cell lines (Figure 1f), respectively. The populations gated as R3 (CD44 negative), and R4 (CD44 positive) were sorted and expanded from HT-29 (Figures 2c and 2d for) and HCT-15 (Figures 2g and 2h) colorectal carcinoma cell lines, respectively, for the entire study. Only 2c and 2d for) and HCT-15 (Figures 2g and 2h) colorectal positive) were sorted and expanded from HT-29 (Figures 2f and 2h), while 25% of cells gated as R3 were sorted as HT-29 CD44\(^-\) singlets (Figures 2b and 2d) and expanded for all future experiments. Thirty-one percent of cells gated as R4 were sorted as CD44\(^+\) singlets (Figures 2f and 2h), while 25% of cells gated as R3 were sorted as CD44\(^-\) singlets (Figures 2f and 2h) from the HCT-15 cell line. The sorted cells CD44\(^+\) and CD44\(^-\) were only used within two passages of culturing, so that the cells maintain their distinct characteristics. The respective parent cell lines containing mixed populations of CD44\(^+\) and CD44\(^-\) and other uncharacterised cell types were termed as the wild type (WT).

The sorted CSC populations from both the cell lines maintained a high CSC expression in 2D cultures and exhibited high EMT markers

The sorted cells, CD44\(^+\) and CD44\(^-\), were maintained in 2D cultures for a maximum of two passages. The sorted CSC-CD44\(^+\) cells from both the cell types, HT-29 and HCT-15 maintained a very high expression of cancer stem cell marker CD44 as evident from the gene expression analysis for CD44 gene (Figure 3a). The CD44\(^+\) non-stem cancer cells sorted from both the cell lines HT-29 and HCT-15 exhibited little to nil expression of CD44 as expected (Figure 3a). The CD44\(^+\) cancer stem cells showed high expression of the epithelial to mesenchymal transition (EMT) genes such as Snail, Slug and Vimentin as analysed by qRT-PCR gene expression analysis (Figures 3b and 3c). However, mesenchymal to epithelial markers (MET) such as EpCAM and E-Cadherin were equally expressed in WT, CD44\(^+\) CSCs and non-CSCs from both HT-29 and HCT-15 cell lines probably owing to their origin from epithelial cells of the colon (Figures 3b and 3c). In the case of WT cells, both the cell lines exhibited similar expression levels of all the EMT (Snail, Slug and Vimentin) and MET (EpCAM and E-Cadherin) markers (Figures 3b and 3c).

The sorted CSC populations from both the cell lines formed 3D spheroids/tumourospheres which could be distinguished from the non-spheroid aggregates of non-stem cells and control fibroblasts

All the six cell types (HT-29 WT, HT-29 CD44\(^+\), HT-29 CD44\(^-\), HCT-15 WT, HCT-15 CD44\(^+\) and HCT-15 CD44\(^-\)) were subjected to 3D tumoursphere assay as described in the methods section. Live 3D spheroids and cellular aggregates were stained with CD44 and Hoechst. Only CD44\(^+\) cells obtained from both the cell lines formed tumourosphere with distinct boundaries which also stained strongly positive for CD44 (Figures 4b and 4e). However, the CD44\(^-\) populations from both the cell types did form cellular aggregates and stained negative for CD44 as expected (Figures 4c and 4f). NIH3T3 control fibroblasts also formed cellular aggregates (data not shown). WT HT-29, as well as HCT-15 cells, also formed cellular aggregates and partial spheroids with sparse staining with the CD44 antibody (Figures 4a and 4d). All the 3D tumourosphere and cellular aggregates stained negative for propidium iodide thereby confirming the absence of dead cells (data not shown).

![Image](image_url)
Cancer stem cells, non-stem cancer cells and WT populations exhibited a variable doubling time and morphological behavior

The sorted populations as Cancer stem cells CSCs (CD44⁺), non-stem cancer cells (CD44⁻) from both the colorectal carcinoma cell lines HT-29 and HCT-15 showed distinct morphologies (Figure 5). The WT and CSC populations exhibited a mixed mesenchymal and epithelial morphology (Figures 5a, 5b, 5e and 5f) in contrast to, the epithelial morphology of non-CSC/CD44⁻ cells (Figures 5c and 5g). The CSC populations from the HT-29 cell line exhibited a doubling time of 18 h, in contrast, to the CD44⁻ and WT populations that took around 24 h thereby indicating an influence of slow growing CD44⁻ non-stem cancer cells on the overall growth of WT population (Figure 5d, Table 1). Similarly, the CSC CD44⁺ populations from the HCT-15 cell line grew fastest with a population doubling time of ~16 h.

![Graphical representation for the percentage of cell proliferation of all three populations (WT, CD44⁺ and CD44⁻) and also NIH3T3 control fibroblast cell line with respect to low (5–25 μM) and high (100–10,000 μM) concentration ranges of Vitamin C and Niacin. Percentage cell proliferation with respect to varying concentrations of Vitamin C for all three cellular derivatives (WT, CD44⁺, CD44⁻) of (a) HT-29 cell line (b) HCT-15 cell line. Percentage cell proliferation with respect to varying concentrations of Niacin for all three cellular sorted derivatives (WT, CD44⁺, CD44⁻) of (c) HT-29 cell line (d) HCT-15 cell line. (e) Percentage cell proliferations of NIH3T3 control fibroblast, in response to, varying concentrations of Vitamin C and Niacin. Vitamin C/Niacin concentrations in μM in X-axis are plotted versus percentage of cell proliferation plotted in the Y-axis. An arbitrary value of 100% proliferation has been assigned to the respective untreated control cells. The values for this plot have been taken as a mean ± SE of three independent experiments.](image)
while the non-CSCs (CD44⁻) grew slowest with a population doubling time of 27 h and WT had intermediate cell doubling time of ∼18 h (Figure 5h, Table 1).

Proliferative effects of cancer stem cells, in response to, low concentrations of Vitamin C and vitamin B3/niacin CSC/CD44⁺ populations isolated from HT-29 and HCT-15 cell lines, respectively, when subjected to low concentrations (5, 10, 20, and 25 μM) of Vitamin C (Figures 6a and 6b, Table 2) and that of Niacin (Figures 6c and 6d, Table 3), exhibited an increase in cell proliferation. In the case of the CSCs isolated from the HT-29 cell line, the growth in cell proliferation, in response to the aforementioned small doses of both the vitamins was more pronounced, as compared to the CSCs isolated from HCT-15 cell line (Figures 6a–6d).

In the case of Vitamin C exposure, the CSC/CD44⁺ populations from HT-29 cell line exhibited about 170% increase in cell proliferation as compared to the untreated controls (Figure 6a, Table 2). Also, at all the five low concentrations of Vitamin C from 5 to 25 μM, the CSCs exhibited similar proliferation rates 160–170% in HT-29 CSCs and 107–116% in HCT-15 CSCs cell proliferation as compared to their respective untreated controls (Figures

Table 2 Percentage of cell proliferation upon exposure to low (5–25 μM) and high concentration ranges (100–10,000 μM) of vitamin C/ascorbic acid in various cell populations obtained from HT-29 and HCT-15 colorectal carcinoma cell lines respectively. Table showing the respective percentages of cell proliferation of the cell populations WT, CSCs (CD44⁺) and non-stem cancer cells (CD44⁻) with respect to various concentrations (5–10,000 μM) of Vitamin C/Vitamin C from HT-29 and HCT-15 cell lines. The untreated control cells for each of the cell type WT, CSCs (CD44⁺) and non-stem cancer cells (CD44⁻) have been assigned an arbitrary value of 100% cell proliferation.

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Table 3 Percentage of cell proliferation upon exposure to low (5–25 μM) and high concentration ranges (100–10,000 μM) of Niacin in various cell populations obtained from HT-29 and HCT-15 colorectal cancer cell lines, respectively. Table showing the respective percentages of cell proliferation of the cell populations WT, CSCs (CD44⁺) and non-stem cancer cells (CD44⁻) with respect to various concentrations (5–10,000 μM) of Vitamin B3/Niacin from HT-29 and HCT-15 cell lines. The untreated control cells for each of the cell type WT, CSCs (CD44⁺) and non-stem cancer cells (CD44⁻) have been assigned an arbitrary value of 100% cell proliferation.

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6a and 6b, Table 2). Similarly, low (25 μM) concentrations of Niacin also induced an increased proliferation of the CSC/CD44⁺ cell populations to ~130% in HT-29 CSCs and ~113% in HCT-15 CSCs (Figures 6c and 6d, Table 3). Interestingly, in the Niacin low dose group (5–25 μM), 5–15 μM doses were inhibitory to the growth and 20 and 25 μM induced proliferation of CSC/CD44⁺ populations from the HCT-15 cell line. In the case of, the HT-29 CSC/CD44⁺ cell populations exhibited continued over-proliferation in all the low tested low doses (Figures 6c and 6d, Table 3).

In the case of WT populations of both the cell lines, HT-29 and HCT-15 Vitamin C treatment of 5 μM failed to exhibit any significant difference in the percentage of cell proliferation as compared to the untreated controls (Figures 6a and 6b, Table 2). However, the 25μM concentration of Vitamin C was able to exert a cell killing effect of the order of only ~10 and ~12%, respectively, in the WT populations of HCT-15 and HT-29 cell lines (Figures 6a and 6b, Table 2). Interestingly, the non-stem cancer cells (CD44⁻) from HT-29 and HCT-15 exhibited different behaviour regarding their sensitivities/cell killings with both Niacin and Vitamin C all tested doses from 5 to 10,000 μM. Doses as small as 25 μM of Vitamin C induced a dose-dependent reduction in cell proliferation of CD44⁺ HT-29 cells of the order of ~86% (14% cell killing) (Figures 6a, Table 2) and ~78% (22% cell killing) in the case of Niacin (Figure 6c, Table 3). However, the CD44⁺ cells from HCT-15 cell line were refractory to the low doses (5–15 μM) of Vitamin C and all the small doses of Niacin (5–25 μM) thereby indicating a probable distinct regulation of such cells.

Cell killing effects of cancer stem cells to high concentrations of Vitamin C and Vitamin B3/Niacin

In contrast to the small doses of Vitamin C and Niacin, the high doses of these dietary ingredients from 100 to 1,000 μM were capable of inducing cell killing effects in CSCs. The cell killings caused by the high dose of Vitamin C (1,000 μM) were of the order of ~35 and ~29% (percentage of cell killing is indicated as 100 minus percentage of cell proliferation) in CD44⁺/CSC from HT-29 and HCT-15, respectively (Figures 6a and 6c, Table 2). However, in the case of 1,000 μM of Niacin treatment, we observed a cell killing in CD44⁺/CSC from HT-29 and HCT-15 cell lines of the order of 48 and 30%, respectively (Figures 6c and 6d and Tables 2 and 3). Precisely, there was a dose-dependent reduction in the proliferation of CSC population from HT-29 cell line from ~68 to 61% upon treatment with 100–10,000 μM Vitamin C (Figure 6a, Table 2) as compared to the untreated controls. However, we observed a Vitamin C dose-dependent reduction in HCT-15/CD44⁺ CSC proliferation from ~93 to 69%. One significant observation was the highest proliferation of CD44⁺/CSC from HCT-15, in response to, the highest tested concentration of Vitamin C (10,000 μM). Similarly, the CSC populations also exhibited a Niacin dose (100–10,000 μM) dependent reduction in cell proliferation from ~65 to 56% in HT-29/CD44⁺ and ~77 to 69% in HCT-15/CD44⁺ as compared to the untreated controls (Figure 5, Table 3). However, the CSCs from both the cell types still failed to achieve LD50 at any of the studied doses of both Vitamin C and Niacin.

The WT and non-CSC populations only from the HT-29 cell line exhibited greater sensitivities/higher cell killing effects of both Vitamin C and Niacin. Precisely, doses from 100 to 10,000 μM (Figures 6a–6d and Tables 2 and 3) induced greater cell killings in HT-29 cell line derivatives (WT and CD44⁻) as compared to the WT and non-CSC populations from HCT-15 cell line. The HT-29 WT cell populations exhibited a cell proliferation of the order of ~21 to 27% in case of Vitamin C treatment (100–10,000 μM) (Figure 6a, Table 2). HT-29 WT population also exhibited ~22 to 19% reduction in cell proliferation in case of Niacin treatment (100–10,000 μM) (Figure 6c, Table 3) as compared to the untreated controls. The highest sensitivity towards Vitamin C and Niacin was indeed observed in the case of the HT-29 non-stem cancer cell populations (CD44⁻). The dose-dependent reduced cell proliferation of HT-29 non-CSCs, in response to high doses (100–10,000 μM) Vitamin C was ~14–10% (Figure 6a, Table 2) and to that of Niacin was ~15–11%, respectively (Figure 6c, Table 3) as compared to untreated controls. This dose-dependent reduced cell proliferation of non-CSCs from HT-29 indicates the achievability of a value of more than LD50 in WT and non-CSC cell populations.

The WT and non-CSC populations from HCT-15 cell line failed to exhibit any dose-dependent behaviour in cell proliferation/inhibition through the doses 100–10,000 μM of both Vitamin C and Niacin. Vitamin C doses from 100 to 1,000 μM, however, induced a dose-dependent reduction in cell proliferation from ~89 to ~83% in HCT15-WT and ~96 to ~88% in HCT-15 non-CSC (CD44⁻) cell populations. We, however, observed a high degree of cell proliferation ~119 and ~233% in HCT-15 WT and non-CSC (CD44⁻) populations, respectively, only at the highest concentration (10,000 μM) of Vitamin C thereby indicating an increased cell proliferation after initial dose-dependent decreased proliferation with Vitamin C levels from 100 to 1,000 μM.

In response to Niacin concentrations from 100 to 10,000 μM, HCT-15 WT exhibited a dose-dependent decrease in cell proliferation from ~97 to ~87% (Figure 6d, Table 3). However, the non-CSC population showed an increase in cell proliferation from ~103 to ~113% in response to Niacin doses from 100 to 10,000 μM.
individual doses (5–25 μM) of Vitamin C and Niacin, all six cell types (HT-29-WT, HT-29-CD44+, HT-29-CD44−, HCT-15-WT, HCT-15-CD44+ and HCT-15-CD44−). One single low dose (25 μM) and one single high dose (1000 μM) of each of Vitamin C and Niacin were tested. The maximum dose 10,000 μM was avoided because it had earlier induced ~300 and ~230% cell proliferation in CD44+ CSC and non-CSC (CD44−) cells, respectively, from HCT-15 cells as estimated by MTT assay (Figures 6b and 6d, Table 2). The live cells stained simultaneously with Hoechst and PI upon quantification exhibited the high cell killing (percentage of PI positive cells), upon treatment with 1,000 μM of Vitamin C and Niacin in CD44+.CSC from HT-29 cells, as compared to the CD44+.CSC from HCT-15 cells (Figure 7a). The percentage of cell killings by 1,000 μM of Vitamin C and Niacin in HT-29-CD44+-.CSC were of the order of ~35 and ~50%, respectively (Figure 7a) which corroborates with the MTT-assay results (Figures 6a, 6c and Tables 2 and 3). However, the percentage of cell killings by 1,000 μM of Vitamin C and Niacin in HCT-15 CD44+-.CSC were only of the order of ~30% (Figure 7a). Figures 7b–7d, respectively, represent high cell killing (~80–90% PI positive cells), moderate cell killing (30–50% PI positive cells) and low cell killing (5–10% PI positive cells).

However, with regard to, the low concentration (25 μM) of Vitamin C and Niacin, almost negligible PI positive cells were detected in CD44+.CSC from HT-29 and HCT-15 cell lines, respectively (Figures 7a and 7d). No cell killings, with response to, 25 μM of Vitamin C and Niacin in the CD44+.CSC from HT-29 and HCT-15 cell lines also corroborate well with the MTT data (Figures 6a–6d, Tables 2 and 3) in which we had observed an increase in cell proliferation at a concentration of 25 μM. Amongst all the six cell types, HT-29-WT and HT-29-CD44− (non-CSC) exhibited highest cell killing of the order of ~80–90%, in response to, 1,000 μM of both Vitamin C and Niacin thereby again corroborating with the MTT data (Figures 6a, 6c and Table 3).

Discussion

In all forms of cancer, the first appearance either detected early or at the second stage appears to be less complicated and is well managed with the help of conventional therapies such as surgery, chemotherapy and radiotherapy. However, minimal interventions are available, in response to, possible relapse of the cancers. It is mostly a relapsed cancer that is responsible for the major deaths of the cancer patients. Colorectal cancers have very high rates of relapses (Guerrera et al., 2016). Moreover, it is now known that cancer stem cells-CSGs/tumour initiating cells-TICs are the ones that form the bulk of a relapsed tumour (Yu et al., 2016b). Moreover, the cells that home to the metastatic sites are also likely to be predominated with cancer stem cells. Therefore, it is important to target the cancer stem cells mainly after the first phase of conventional therapy for any cancer patient.

Colorectal cancer stem cells are often isolated as CD44high/CD133high (Zhou et al., 2016). Additional markers such as CD24, CD166 (ALCAM), Oct4, Nanog, Sox2, ALDH1, CD166, CD26, EpCAM are reportedly expressed in colon CSC (Abetov et al., 2015, Wahab et al., 2017). The classical Nature paper had the first time reported the isolation of human colorectal CSC as CD133 positive in 2007 (Ricci-Vitiani et al., 2007). However, CD44 has been proved to be a robust functional marker for colon CSC by the loss of colony forming abilities of CD44+ colon CSC that were knocked down for CD44 in vitro and in vivo in APC/Min+ mice (the Mouse model for colorectal cancers). However, CD133+ colon CSC, despite knockdown of CD133 could still form tumour colonies (Du et al., 2008). Later on, Su et al. (2011a) could directly reprogram colon cancer cells into colon cancer stem cells using a single marker CD44 (Su et al., 2011b). Hence, we decided to isolate the colon cancer stem cells by the unique, robust marker CD44. Also, the CD44+ has been reported to preserve the parental stem cell characteristics in culture, as compared to CD44− cells (Du et al., 2008). However, as we further characterised the flow-sorted CSCs (CD44+) and non-CSCs (CD44−) cells up to two passages of culture, we found high expression of CD44 and stemness markers/EMT markers in...
CSCs versus low expression of CD44 in non-CSCs using qRT-PCR (Figure 3). Most important, the CSCs from both HT-29 and HCT-15 cell lines successfully formed 3D tumourspheres (Figure 4) thereby validating the stemness of CD44⁺ cells isolated in this study. Hence, we confirm the use of bona fide colon CSC (CD44⁺) for all our experiments for studying the effects of Vitamin C and Niacin.

The period between cancer remission and cancer relapse is the time which is largely unmonitored. Hence, it is important to identify individual molecules or dietary ingredients that can target the small populations of cancer stem cells left behind after first round of conventional cancer therapy, surgery, chemotherapy and radiotherapy. Also, various vitamins are used as nutritional and epigenetic approaches for cancer control and prevention (Verma, 2012). Often, the early on and terminal cancer patients are advised to take various forms of dietary supplementation (Cuhls et al., 2016). Vitamin C and Niacin are such common dietary ingredients that have been recommended for consumption by the cancer patients (Starr, 2015; Cuhls et al., 2016). The specific logic of choosing the vitamins niacin and ascorbic acid in this study is because of making a beginning in studying the effects of the vitamins that play a significant role in cellular energetics/metabolism (niacin)

Figure 7 Graphical representation of enumeration for the percentage of cell death (% PI positive cells) from five microscopic fields of live cells (WT, CD44⁺ and CD44⁻) co-stained with Hoechst and PI from HT-29 and HCT-15 cell lines and representative images of microscopic images showing high, medium and low cell death in response to low (25 μM) and high (1,000 μM) concentrations of Vitamin C and Niacin, respectively. (a) Graphical representation of percentage PI positive cells in WT, CD44⁺ and CD44⁻ from HT-29 and HCT-15 cell lines treated with low (25 μM) and high (1,000 μM) concentrations of Vitamin C and Niacin, respectively. Representative phase contrast, PI dead cells and Hoechst live cells showing (b) high (80–90%) PI positive cells, (c) moderate number (30–50%) of PI positive cells and (d) low (5–10%) of PI positive cells. Scale bar – 100 μM.
and oxidative stress reduction (vitamin C) on healthy cells. Moreover, cancer cells have an altered cellular energetics (Uetaki et al., 2015; Yang and Sauve, 2016). Hence, we wanted to study if there is an alteration of cell behaviour upon treatment with niacin and ascorbic acid on colorectal cancer stem cells. In our next study, we intend to further work on cellular energetics upon treatment of colorectal cancer stem cells with niacin and ascorbic acid respectively. Secondly, we had simultaneously found the literature on the effect of high doses of vitamin C on the killing of colorectal cancer cells by inducing oxidative stress. Hence, we were interested in studying the status of colorectal cancer stem cells using high versus low doses of vitamin C.

Reports regarding the usage and research with Vitamin C have been rather exhaustive, as compared to that of Niacin. Vitamin C has been reported to reduce the malignant potential of malignant melanoma cell lines (Fischer and Miles, 2017); reduce cancer in patients when administered in high doses via intravenous route (Padayatty et al., 2006; Sawant et al., 2012; Sharma et al., 2012). However, intravenous administration of palmitoyl ascorbate liposomes proved to be more potent, as compared to, free Vitamin C injection in Balb/c mice model of mammary carcinoma (Sawant et al., 2012). Also, Vitamin C reportedly targets the cancer cells thereby killing them through multiple mechanisms (Du et al., 2012). A recently deciphered mechanism regarding Vitamin C induced selective killing of prostate cancer cells is via enhanced expression of NFκB transcription factor RelB, which in turn decreased the expression of Sirtuin and intracellular antioxidant MnSOD (Wei et al., 2017). However, in normal prostate epithelial cells, Vitamin C-mediated enhanced expression of RelB rather improved antioxidant defences of the cells thereby leading to better health of the cells and enhanced proliferation (Wei et al., 2017). In our context, it would be hence, interesting to decipher the status of RelB in Vitamin C and Niacin treated CSC, non-stem cells and WT cells which form the future scope of this study.

The observations viz. low versus high concentrations of vitamin C and niacin on proliferations and cell death, respectively, in cancer stem cells is being solely elucidated under in vitro culture conditions in this study. However, further corroboration of these findings under in-vivo conditions in tumour xenografts model of CD44+ colon cancer stem cells from HT-29 and HCT-15 colon carcinoma cell lines will be able to provide a clearer picture regarding the effect of such water-soluble vitamins. Secondly, with regard to, the different effects of Vitamin C on the cell types, attributes such as sodium-dependent vitamin C transporter proteins (SVCT) might be responsible. Various levels of absorption of vitamin C has been reported in vitro in MCDK–MRD1 kidney cells (Luo et al., 2008), in response to, different concentrations of sodium in the media, pH, as well as, temperature conditions. However, in our case, since the media compositions and culture conditions of all the cell types was same, the sodium, pH or temperature dependent variation in SVCT activity, and hence, vitamin C absorption is a remote possibility. Nevertheless, it forms the scope of the future study to assess the levels of SVCT in various cell lines cultured in the given media conditions with or without the presence of the tested water-soluble vitamins, vitamin C and niacin. Moreover, the presence of trace amounts of transition metals mostly iron in the synthetic media such as DMEM might also be responsible for inducing variation in the uptake of the tested water soluble vitamins (Yao and Asayama, 2017). However, that is a given situation under in-vitro conditions and corroboration of the experiments in xenografts mouse models for cancer stem cells in the best alternative.

Thirdly, the different response of CSCs towards low versus high concentrations of the tested water soluble vitamins also can be attributed to the differential expression of the cell’s internal antioxidant defence mechanisms. It is possible that elevated levels of these vitamins are inhibitory to the cellular antioxidant defence machinery and is responsible for the reduced expression of catalase and superoxide dismutase. As a result of reduced expression of superoxide dismutase and catalase, the ROS generated might be responsible for the killing of the cells at a high concentration. In other words, homeostatic levels of catalase and superoxide dismutase in a cell prevent the cell from undergoing ROS-mediated apoptosis. Hence, it would be of great importance to understand whether the different effects of cell proliferation versus cell killing, in response to, low versus high concentrations of the tested water soluble vitamins is due to the effect of ROS versus ROS quenching. Accordingly, assessment of ROS and hydrogen peroxide in CSCs treated with varying concentrations of Vitamin C and Niacin makes the future scope of this study.

Fourthly, regarding the mechanisms of action of Niacin, with regard to, cell proliferation versus cell killing, in high and low doses respectively, we speculate a shift in the attenuation of ROS. Niacin at concentrations 100–300 nm reportedly quenches ROS, Nitric oxide (NO) and Nitric oxide synthase 2 (NOS2) in LPS treated human mature macrophages (Montserrat-de la Paz et al., 2017). In our studies, the concentrations of Niacin used were much higher than the ones reported to quench ROS. Precisely, our lower concentrations of Niacin ranged from 5 to 100 μM that is ~50 to 500 times more than the levels reported by Montserrat-de la Paz et al. (2017), that caused proliferation of the CSCs. Also, long-term (8 weeks) administration of very high doses of Niacin (~30 mg/kg body weight) through drinking water to the mice diet resulted in ROS induction, hyperglycemia, reduced glucose-induced insulin release in pancreatic islets (Chen et al., 2015). Hence, we can conclude
that lower concentrations of Niacin up to 100 µM can quench the ROS, while the higher levels (200–10,000 µM) of Niacin might be inducing ROS and resulting in cell killings of CSCs. However, the levels of ROS generated via varying dose of Niacin need further validation. We also propose a mechanism of action of high versus low concentrations of Niacin, in response to, cell proliferation versus cell killing (Figure 8).

Interestingly, cancer cells exhibit higher levels of oxidative stress, as compared to, normal cells, and hence this mechanism can be used as a strategy to induce selective killing of cancer cells (Lee et al., 2017). Hence, increasing the amounts of pro-oxidants in the tumour milieu can induce sensitization of cancer cells. In the context of the current work, it would be interesting to further elucidate the levels of oxidative stress in CSC, non-CSC and wild-type cells and also NIH3T3 control cell line under normal untreated conditions versus Vitamin C and Niacin treatments. Hence, pre-exposure of cancer cells to oxidative agents further increases the oxidative stress of cancer cells, as compared to normal healthy cells. For example, exposure of MCF-7 breast cancer cells with triethylenetetramine (TETA) enhanced the oxidative stress of cancer cells, as compared to normal cells, and hence this sensitization of cancer cells. In the context of the current work, it would be interesting to further elucidate the levels of oxidative stress in CSC, non-CSC and wild-type cells and also NIH3T3 control cell line under normal untreated conditions versus Vitamin C and Niacin treatments. Hence, pre-exposure of cancer cells to oxidative agents further increases the oxidative stress of cancer cells, as compared to normal healthy cells. For example, exposure of MCF-7 breast cancer cells with triethylenetetramine (TETA) enhanced the autooxidation of Vitamin C thereby inducing elevated oxidative stress, and H₂O₂ production, finally leading to cell death (Wang et al., 2017). With particular reference to ascorbic acid, it is known as an antioxidant vitamin and hence, is non-toxic to normal healthy cells. Moreover, as the oxidative stress in cancer cells is already elevated to moderately high levels, the cancer cells remain proliferative. However, a further increase in the oxidative stress in cancer

Figure 8 Proposed scheme for the mechanism of action Niacin towards of cell proliferation versus cell killings, in response to, low and high concentrations respectively. Low levels of Niacin (5–25 µM) leading to the inhibition of PPARγ, thereby resulting in the reduction in ROS production and hence cell proliferation is represented on the left side of the diagram. High concentrations of Niacin (100–10,000 µM) leading to the activation of PPARγ, accumulation of ROS leading to cell death is the proposed mechanism for the action of Niacin on colon CSCs.
the growth of CSC. Furthermore, very high levels of ROS are indeed inductive of apoptosis of cancer cells (Lee et al., 2017).

One interesting observation in this study involved differential killing rates of cancer stem cells, non-stem cancer cells and the mixed populations (WT) cells, in response to a high dose of Vitamin C and Niacin. The non-stem cancer cells from HT-29 cell line (CD44+), that showed a high response to a high dose of Vitamin C and Niacin. The order of cell killings were ~86% and ~89%, respectively, when treated with the highest dose of Vitamin C (14% cell proliferation) and Niacin (11% cell proliferation) compared to a baseline of 100% for untreated controls (Tables 2 and 3). However, the non-stem cancer cells from HCT-15 cell line (CD44+) did exhibit cell killing only up to 1,000 μM of Vitamin C to the order of ~12% (88% cell proliferation) (Figure 6b, Table 2). The highest dose (10,000 μM) of Vitamin C, on the contrary, was conducive to very high proliferation (~233%) in HCT-15/CD44+ (Table 2). Moreover, the standard fibroblast cell line NH3T3 at a 10,000 μM dose of Vitamin C also exhibited a cell killing of the order of ~20% (Figure 6d). Also, the highest dose of Vitamin C (10,000 μM) induced a cell proliferation to the extent of 226% in HCT-15 WT cells (Table 2). Moreover, the maximum dose of Niacin did not produce extremely high levels of cell proliferation in any of the sorted derivatives from HCT-15 cell line (Table 3).

In the HT-29 CSCs moderate cell killing only ~39% cell killing (61.18% cell proliferation) with highest dose of Vitamin C (Figure 6a, Table 2) and ~43% cell killing upon treatment with the maximum dose of Niacin (~56% cell proliferation) was observed (Figure 6c, Table 3). Also, HCT-15 CSCs exhibited comparatively lower cell killings ~30% each with 1,000 μM of Vitamin C and Niacin. Hence, the maximum cell killing in CSC by any dose of Vitamin C or Niacin did not attain an LD50. So, attainment of LD50 values in these CSC provides a scope for optimisation for the combinatorial use of both the vitamins.

Wild-type populations could achieve high cell killings only in HT-29 cell line with the maximum dose of Vitamin C ~73% (Cell proliferation ~27%) (Figure 6a, Table 2) and ~82% with the highest dose of Niacin (Cell proliferation ~19.89%) (Figure 6c, Table 3). On the other hand, the HCT-15-WT cells attained maximum cell death of the order of ~17% (Cell proliferation – 83.12%) with 1,000 μM Vitamin C and ~13% with 1,000 and 10,000 μM Niacin (Cell proliferation 87.12 and 87.06%, respectively) (Figures 6b, 6d and Tables 3 and 4). The cell killings of the order of >60% in HT-29 WT populations are possibly an indicator of a predominance of non-stem cancer cells/cell populations that are sensitive to high doses of Vitamin C and Niacin. However, the WT and non-CSC from HCT-15 cell line were less sensitive as compared to their HT-29 counterparts that again form an interesting observation with regards to, HCT-15 cell line that harbours KRAS mutation. Hence, molecular interactions of mutated KRAS protein of HCT-15 cell line and its cellular derivatives (WT, CSC and non-CSC), in response to various doses of Vitamin C and Niacin forms a new future scope of this study.

This phenomenon of differential cell killings is probably an indicative of different cell survival mechanisms and cellular energetics of non-stem cancer cells and cancer stem cells. Cancer cells mainly rely on aerobic glycolysis, in contrast to oxidative phosphorylation in the case of somatic/ healthy cells (Ooi and Gomperts, 2015; Xu et al., 2015). Moreover, transcriptome analyses of metastatic competent colon cancer cell lines such as CTC-MCC-41 and HT-29 harbouring high percentages of cancer stem cells have indicated differential expression of energy metabolism genes. Examples of energy metabolism genes are peroxisome proliferator-activated receptor γ coactivator 1B (PPARGC1B), peroxisome proliferator-activated receptor γ coactivator 1A (PPARGC1A), fatty acid binding protein 1 (FABP1), DNA repair [BRCA1 interacting protein C-terminal helicase 1 (BRIPI), aldehyde dehydrogenase three family member A1 (ALDH3A1), Fanconi anemia complementation group B and M (FANCB, FANCM), stemness glutaminase 2 (GLS2), cystathionine-beta-synthase (CBS) and cystathionine gamma-lyase (CTH) (Alix-Panabières et al., 2016). Hence, targeting cancer stem metabolism has been discussed as an effective strategy for cancer therapeutics in the review by Deshmukh et al. (2016). Accordingly, the results of the current work, regarding enhanced proliferation and cell killings of CSCs by respectively low and high doses of Vitamin C and Niacin also has also a scope for evaluation regarding various marker genes for energy metabolism. Differences in cellular proliferation/cell death behaviour of CSC, non-stem cancer cells and normal cells can also be explained by probable differences in the intrinsic metabolic reprogramming (Shen et al., 2015) and the presence of certain programmed cell death ligands (Wu et al., 2017) that need further validation.

Mechanisms of targeting colon cancer cells having BRAF and KRAS mutations using high concentrations of Vitamin C involve the inhibition of GAPDH thereby leading to energy crisis has already been worked out by Yun et al. (2015). Moreover, the colorectal cancer cell line HT-29 harbours only BRAF mutation and has a wild-type KRAS gene. On the other hand, comparatively less sensitive cell line HCT-15 has a mutated KRAS and wild-type BRAF. The mechanisms for the differences in sensitivities towards doses of Vitamin C and Niacin in KRAS and BRAF mutated cell lines are rather unknown. However, it is likely to further high cell killing can be achieved by treatment of the high dose of Vitamin C in the colorectal cancer cells harbouring mutations in both KRAS and BRAF. Also, there might be
additional mechanisms involved in cell survival of HCT-15 colorectal cancer stem cells upon treatment with high dose of Vitamin C and Niacin.

Amongst both the vitamins, our results indicate a better cell killing effect of Niacin on HT-19 CSC populations with the highest individual dose of these two vitamins. Indeed, reports regarding cancer inhibitory effects of Niacin are scanty as compared to the reports on Vitamin C. There might be additional mechanisms for greater sensitivity of CSCs to Niacin that needs to be elucidated. Nevertheless, we have hypothesised pathway (Figure 8) involving the mechanism of action of Niacin on colorectal CSC that needs further validation. Moreover, this observation opens the scope for studying the GAPDH pathways of the energy crisis and various genes involved in cellular energetics and cell death upon treatment with concentrations of Niacin as well in the HT-29 and HCT-15 colorectal cancer stem cells.

Finally, small doses of the vitamins, Vitamin C and Niacin are conducive towards cell proliferation of colon cancer stem cells as indicated in this study, in contrast to, not enough literature available regarding the dose-dependent behaviour of cancer/cancer stem cells towards Vitamin C and Niacin. However, dose responsive cellular response namely loss of cardioprotection, an unrelated context, has been reported about the high dose of vitamin A (Csepanyi et al., 2015).

Conclusion

High doses of Vitamin C and Niacin-induced cell killings, in contrast to small doses of Vitamin C and Niacin that induced cell proliferation of colon cancer stem cells isolated from HT-29 and HCT-15 colon cancer cell lines. The detailed mechanism for this different phenomenon needs to be worked out concerning cellular energetics upon low versus the high dose of exposure to Vitamin C and Niacin.

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Authors’ contributions

BB and SS conceptualized and designed the project, performed cell sorting, 3D tumourosphere assay, analysed the data, compiled the manuscript figures using Adobe Illustrator, wrote and approved the manuscript. US helped in cell sorting, maintained the cells, carried out MTT assays with HT-29 and HCT-15 colorectal carcinoma cell lines and its flow-sorted sorted derivatives and NIH3T3 cell line, performed live–dead cell staining experiments and imaging.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information should be found in the online version of this article at the publisher’s website.

Table S1. List and sequence of primers.