Effect of Cinnamic acid and FOLFOX in diminishing side population and downregulating cancer stem cell markers in colon cancer cell line HT-29

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Abstract

Purpose There is a lot of evidence suggesting that a small subset of cancer cells resistant to conventional chemotherapy and radiotherapy and known as cancer stem cells (CSCs) is responsible for promoting metastasis and cancer relapse. Therefore, targeting and eliminating the CSCs could lead to higher survival rates and a better quality of life. In comparison with conventional chemical drugs that may not be effective against CSCs, phytochemicals are strong anti-CSCs agents. The current study examines the effect of 5-fluorouracil plus oxaliplatin (FOLFOX) as a common chemotherapy drug on colorectal cancer as well as the influence of Cinnamic acid (CINN) as a plant-derived phytochemical on colon cancer stem-like cells in HT-29 adenocarcinoma cell line.

Methods The anti-proliferative effect of FOLFOX and CINN was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Flow cytometry analysis was used for the identification of side population (SP), CD44, and CD133 positive cells. The expression of OCT4, NANOG, ABCB1, and ALDH1A was assessed by RT-PCR.

Results The FOLFOX and CINN decreased cell viability in certain drug concentrations: IC50 = 5.40 μM oxaliplatin +220 μM 5-fluorouracil, and 13.50 mM for CINN. The CSC-associated markers (OCT4, NANOG, ABCB1, and ALDH1A) and the proportion of cancer stem-like cells (SP cells, CD44, and CD133 positive cells) were downregulated following the treatment of HT-29 adenocarcinoma cell line with IC50 concentrations of FOLFOX and CINN.

Conclusion Our data suggests that CINN, a naturally occurring component, could be more effective than FOLFOX treatment in reducing the cancer stem-like cells and expression of CSC markers from HT-29 colon cancer cells.

Keywords Colon cancer stem cells · Side population · Cinnamic acid · FOLFOX · Cancer stem cell markers

Introduction

Cancer stem cells (CSCs) persist in tumors as a distinct population of pluripotent cancer cells with tumorigenic ability. Such cells result in the growth of a primary tumor and generate new tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. In addition, it is found that CSCs are resistant to drug treatment due to having several mechanisms that inhibit cell death such as increased activity of detoxifying aldehyde dehydrogenases (ALDH) enzymes, enhanced DNA repair abilities, a slower cell-cycle, and an impressive drug efflux by upregulation of ATP-binding cassette (ABC) transporters [1–7]. The transport activity of ABC can be measured by the ability of side population (SP) cells to efflux fluorescent dyes such as Hoechst 33,342 and Rhodamine 123 (Rh123) [8–13]. There are a number of signaling pathways that play a significant role in maintaining self-renewal, ability of differentiation, and chemoresistance of CSCs, and these include the Wnt, Notch, and Hedgehog signaling pathways [14–21]. According to CSCs characteristics, this subpopulation of cancer cells is considered to be the main cause of treatment failure, relapse, and metastasis, therefore targeting the CSCs could be an effective and promising therapy for preventing recurrence and eliminating cancer [22–24].
Colorectal cancer is the third-most common cancer in men and women and a leading cause of death. It has been reported that cancer recurrence is prevalent in 50% of patients having colon cancer, this indicates that the current strategies of cancer therapy are ineffective [25, 26]. In colorectal cancer, CSCs are resistant to therapy and are also responsible for tumor recurrence. Although the combination chemotherapy of 5-fluorouracil (5-FU) with oxaliplatin (FOLFOX) is one of the most common treatments for colorectal cancer, providing regression in many cases, the relapse of cancer is also associated with the resistance of colorectal cancer cells to FOLFOX [27]. Furthermore, given the side effects of chemotherapy drugs like FOLFOX, researchers have been encouraged to find non-toxic active agents which can target and eliminate CSCs.

Several natural drugs have shown fewer side effects than synthetic chemotherapy drugs, hence many researchers tend to study plant-derived phytochemicals — evaluating their effects alone or together with conventional chemotherapy in eliminating CSCs, and thereby effectively treating cancer [28–32]. Cinnamic acid (CINN) as an organic compound is a phenylalanine deamination product and classified as an unsaturated carboxylic acid [33, 34]. This component and its derivative act as antimicrobial, anti-atherosclerotic, antioxidant, hypcholesterolemic, hepatoprotective, and antidiabetic agents [35–46]. Furthermore, many studies have shown the cytotoxic effects of CINN and its derivatives on different cancer cells, their anticancer potential in the treatment of various cancers has also been presented [47–51].

Until now, to our knowledge, the inhibitory effect of CINN on CSCs has been proven only in the lung adenocarcinoma cell line. Our study compared the effect of CINN (as a plant-derived phytochemical) with FOLFOX (as a common chemotherapy drug) on human colon cancer stem-like cells. For this purpose, the expression of OCT4, NANOG, ABCB1, and ALDH1A1 as CSC markers were quantified after treatment of human colorectal adenocarcinoma cell line HT-29 with FOLFOX and CINN. We also examined the effect of CINN and FOLFOX on the percentage of SP cells, and CD44+ and CD133+ (colon CSC markers) cells in HT-29 cell line.

Oxaliplatin and 5-FU were purchased from Mylan S.A.S, France and Ebewe Pharma, Austria, respectively. CINN (Cat. No. C80857) and Rh123 (Cat. No. R8004) were obtained from Sigma Aldrich, Munich, Germany. FITC Mouse Anti-Human CD44 Clone G44–26 (also known as C26) antibody (Cat. No. 560977) from BD Biosciences, San Jose, CA, U.S.A and Human CD133/2 (clone: 29C3) antibody from Miltenyi Biotec, Bergisch Gladbach, Germany were provided. FITC Mouse IgG1, isotype control clone MOPC-21 (Cat. No. 555748; BD Biosciences, San Jose, CA, USA) and PE Mouse IgG1, isotype control clone MOPC-21 (Cat. No. 554680, BD Biosciences, San Jose, CA, USA) served as the control in flow cytometry analysis. Moreover, staining with a non-vital DNA dye such as 7-aminoactinomycin D (7-AAD) (Cat. No. 559763; BD Biosciences, San Jose, CA, U.S.A) and propidium iodide (PI) (Cat. No. P4170; Sigma Aldrich, Munich, Germany) allows for discrimination of dead cells in flow cytometry.

**Growth inhibition assay**

Inhibition of cell growth in response to FOLFOX or CINN was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Briefly, $9 \times 10^3$ cells were seeded into 96-well culture plates with three replicates. After 24 h of plating, the incubation was continued for another 48 h in the absence (control) or presence of different concentration of 5-FU plus oxaliplatin or CINN as shown in Fig. 1. At the end of the 48 h, the reaction was terminated by adding $20 \mu l$ of 5-mg/ml stock of MTT (Atocel, Austria) to each well. The reaction was allowed to proceed for 3 h at 37 °C. The culture medium was then removed. The formazan crystals were then dissolved by adding 0.10 ml of dimethyl sulfoxide. The intensity of the color, indicating the number of live cells, was measured using a microplate reader (BioTek-ELx800, USA) at a wavelength of 490 nm. The percentage of living cells was calculated by dividing the mean absorbance of treated cells in each well to the mean absorbance of control cells multiplied by 100. All the assays were performed in triplicates. The IC50 values of agents were calculated using Prism 6.0 (GraphPad Software, Inc., San Diego, California, USA).

**Flow cytometry analysis**

The treatment of HT-29 cell line with FOLFOX was carried out with 220 μM of 5-FU and 5.40 μM of oxaliplatin for 48 h. In order to study the effect of CINN, the cells were incubated with a medium containing 13.50 mM of CINN for 48 h. The flow cytometry analysis was carried out on the untreated control and FOOLX or CINN-treated cells.
Rh123 staining

In order to analyze the SP cells, the untreated control and treated cells were suspended at 10^6 cell/ml in PBS containing 2% FBS and then incubated with 0.10 μg/ml Rh123 for 30 min at 37 °C. After washing with ice-cold PBS, the cells were incubated at 37 °C for 40 min in order to allow the cells to efflux the Rh123 dye. Finally, to exclude dead cells, the cells were suspended in PBS/FBS 2% with 1 μg/ml PI. The flow cytometric analysis of Rhodamine fluorescence was carried out with a Partec particle and cell sorting instrument (Munster, Germany). To inhibit the ABC transporters, 100 μm verapamil (Cat. No. V4629; Sigma-Aldrich, Munich, Germany) was added along with Rh123 to each cell suspension.

Antibodies

The untreated control and treated HT-29 colon cancer cells were subjected to direct immunofluorescence staining followed by flow cytometric analysis. Briefly, cells were treated with trypsin and washed with PBS containing 2% FBS. One million cells were suspended in 100 μl of PBS/ 2% FBS followed by the addition of 20 μl of FITC Mouse Anti-Human CD44 or 10 μl of PE Mouse Anti- Human CD CD133/2 and incubated for 30 min in dark at room temperature. The samples were then washed and analyzed using flow cytometry. To detect dead cells, PI and 7-AAD was used with Mouse Anti-Human CD44 and Mouse Anti- Human CD133/2 antibody, respectively. FITC Mouse IgG1 isotype control and PE Mouse IgG1 isotype control served as control in flow cytometry.

Quantitative real-time PCR analysis

The total RNA was isolated from cells using Total RNA isolation kit (DENAzist Asia, Mashhad, Iran). The quantity and quality of RNA were assessed using a Nano drop and agarose gel electrophoresis. To avoid DNA contamination, the extracted RNAs were treated with RNase-free DNase I (Cat. No. EN0521; Thermo Scientific, Wilmington, USA). The cDNA was synthesized using M-MuLV reverse transcriptase (Cat. No. EP0441; Thermo Scientific, Wilmington, USA), according to protocol.

The real-time PCR (RT-PCR) was performed using SYBR Green master mix (Cat. No. PB20.11–01; Biosystems, Barcelona, Spain) in RT PCR System (Analytik Jena, Jena, Germany). To study the expression of OCT4, NANOG, ABCB1, ALDH1A1, and β2M, specific primers were designed using Oligo7 primer analysis software. The sequence of primers and product length are described in Table 1. The thermal cycling conditions involved initial denaturation at 95 °C for 4 min followed by amplification of OCT4, NANOG, and β2M cDNA for 40 cycles (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s), and ALDH1A1 and ABCB1 cDNA for 40 cycles (95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s). To derive the melting curves, the temperature was increased by 1 °C for 10 s from 60 to 95 °C. The analysis of melting curves clearly indicated that each of the primer pairs, described in Table 1, amplified only the expected product and the accuracy of amplification reaction was validated by gel electrophoresis (Fig. 1). The PCR efficiencies (E) were calculated for all used primers from the given slopes of standard curves, generated from serial dilutions of positive controls. The qPCR readings were performed in triplicate for each sample and the mean value of each triplicate was used for the calculation of the mRNA expression levels. To compare the level of gene expression in FOLFOX or CINN treated cells with the untreated control cells, β2M transcripts were used as an internal control and the fold-change in the target gene of treated cell relative to the untreated control sample was calculated according to the following equation: Fold change = 2^(-ΔΔCT).
Statistical analysis

All data was compiled from a minimum of three replicate experiments. Data for statistical analysis is expressed as the mean ± standard error. The comparison of results from treated versus control cells was done using the student’s t-test with SPSS Version 16 Software. A P-value less than 0.05 was considered statistically significant.

Results

Proliferative inhibition of FOFOX and CINN on HT-29 cell line

The cytotoxic effect of different concentrations of FOFOX on HT-29 cells for 48 h was assessed by MTT assay (Fig. 2-a). The cell survival analysis showed that the FOFOX has an inhibitory effect on the growth of HT-29 cells. The half maximal inhibitory concentration (IC50) values of FOFOX was oxaliplatin: 5,40 μM and 5-FU: 220 μM after 48 h of treatment. In order to determine the IC50 value of CINN as a plant-derived phytochemical in HT-29 cells, they were treated with varying concentrations of CINN (0,40–51,20 mM) for 48 h. The results showed that CINN inhibited the HT-29 cells with IC50: 13,50 mM using MTT (Fig. 2-b).

FOLFOX and CINN decreased the percentage of SP cells, and CD44+ and CD133+ cells in human colon cancer cell line HT-29

The flow cytometry analysis showed that the percentage of SP cells in the HT-29 cell line was about 2,88% of the total cells when stained with Rh123 alone and reduced significantly in the presence of verapamil (0,02%) (Figs. 3-a, b). This is because P-gp /ABCB1 is responsible for the efflux of Rh123 out of the cells. Therefore, the reduced Rh123 staining with verapamil as a P-gp inhibitor showed that the presence of SP cells is not due to the decreased initial dye uptake but is because of the role of P-gp /ABCB1 in the rapid efflux of dye out of cells [52–55]. We found that the proportion of SP cells in FOFOX-treated cells and CINN-treated cells was about 4,50 and 18 folds lower than the control group, respectively (Figs. 3c, d).

CSCs are identified by specific surface epitopes. Some of the important surface markers in colon CSCs are CD44, CD166, and CD133 [56, 57]. Here, untreated control and FOFOX or CINN- treated HT-29 cells were analyzed for colon cancer stem-like cells by tagging them separately with CD44 and CD133 antibodies, and then subsequently, they were tested by flow cytometry. The results revealed that the percentage of CD44+ and CD133+ cells in untreated control cells was about 34–38% of total cells. However, the
FOLFOX treatment diminished the percentage of positive CD44 and CD133 cells to around 19 and 13%, respectively. After CINN treatment, the expression of CD133 and CD44 expression was reflected in 7.90% and 4.30% cells, respectively, suggesting that CINN might be more effective than FOLFOX in inhibiting colon CSCs in HT-29 cell line (Figs. 4 and 5).

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**Fig. 2** Anti-proliferative activity of FOLFOX (a) and Cinnamic acid (b) on HT-29 cell line. HT-29 cells were treated with increasing concentrations of FOLFOX and Cinnamic acid for 48 h. All points represent results of cell viability percentage from three independent experiments performed in triplicate. Data is expressed as mean ± SD

**Fig. 3** FOLFOX and Cinnamic acid diminished SP cells in HT-29 cells. Untreated HT-29 cells in absence (a) and presence of verapamil (b), FOLFOX (220 μM 5-FU/5.4 μM Oxaliplatin) treated (c) and Cinnamic acid (13.5 Mm) treated HT-29 cells (d) were stained with Rh123 and propidium iodide dyes and analyzed using flow cytometry. The cell population that excludes Pi and Rh123 are representative of SP cells and were counted in the left low quadrants. The data represents the mean (± standard deviation, SD) of three independent experiments and the difference in fraction of SP cells between control and treated cells was significant according to student’s t-test (*P < 0.05)
Downregulation of stem cell-associated genes by FOLFOX and CIINN

The expression of stem cell-associated genes including OCT4, NANOG, ABCB1, and ALDH1A1 were analyzed by RT after treating the HT-29 cell line with FOLFOX and CINN. The results indicated that after treatment, OCT4, NANOG, ABCB1, and ALDH1A1 were downregulated. FOLFOX decreased the fold-change of OCT4 and NANOG to 0.50 ± 0.01 and 0.60 ± 0.05 whereas CINN decreased the fold-change of OCT4 and NANOG to 0.26 ± 0.20 and 0.33 ± 0.10, respectively (Fig. 6). In addition, the expression of ABCB1 and ALDH1A1 also significantly decreased after CINN (fold-change of 0.09 ± 0.07 and 0.08 ± 0.03) and FOLFOX treatment (fold-change of 0.20 ± 0.05 and 0.40 ± 0.00) (Fig. 7).

Discussion

According to the CSC theory, CSCs constitute a small proportion of tumor cells responsible for cancer initiation, invasion, metastasis, and recurrence [58, 59]. Therefore, identifying therapeutic agents that can target CSCs is considered more effective for tumor destruction as well as for reducing the risk of recurrence. The CSCs resistance to various chemotherapy drugs is attributed to the increased expression of ABC transporters and elevated activity of ALDH, which are a superfami

Fig. 4 FOLFOX and Cinnamic acid decreased the proportion of CD133+ cells in HT-29 cell line. Untreated HT-29 (a), HT-29 cells which were incubated with FOLFOX (220 μM 5-FU/5.4 μM Oxaliplatin) (b), and in medium containing 13.5 Mm Cinnamic acid (c) for 48 h were analyzed after incubation with PE anti-human CD133 Antibody+7-AAD with flow cytometry. The results obtained are from the mean ± SD of three independent experiments. Each CD133/7-AAD dot plot represents one of the three independent experiments. *P < 0.05 compared to the control as tested by the student’s t-test.

Fig. 5 FOLFOX and Cinnamic acid decreased the percentage of CD44+ cells in HT-29 cell line. Untreated HT-29 (a), HT-29 cells which were incubated with FOLFOX (220 μM 5-FU/5.4 μM Oxaliplatin) (b), and in medium containing 13.5 Mm Cinnamic acid (c) for 48 h were analyzed after incubation with FITC anti-human CD44 Antibody+ PI with flow cytometry. The results are obtained from the mean ± SD of three independent experiments. Each CD44/PI dot plot represents one of the three independent experiments. *P < 0.05 compared to the control as tested by the Student’s t-test.
measured by flow cytometry [66–68]. This population of negatively stained cells is known as SP cells [8–13, 68, 69]. The SP cells isolated from various cancer cell lines and tumors possess CSC properties such as self-renewal capabilities, ability to differentiate into heterogeneous cells, high proliferation, and high colony forming potential [8–13, 70–72]. Therefore, in this study we used SP cell analysis as a tool to evaluate the effect of CINN and FOLFOX on elimination of CSCs. The fact that CSCs constitute a small proportion of cancer cells has been demonstrated in our study which showed that only 2–3% of the total colon cancer HT-29 cells are SP cells. Furthermore, the present study indicated that CINN reduced the proportion of SP cells more effectively than FOLFOX. In addition to the SP phenotype, the CSCs carry lineage-specific surface markers. Several cell surface biomarkers have been detected to identify and isolate CSCs in various types of cancers [73–80]. In colon CSCs, multiple cell surface markers including CD133, CD166, CD44, CD24, beta1 integrin-CD29, Lgr5, EpCAM (ESA), ALDH-1, Msi-1, DCAMLK1, or EphB receptors have been identified. Among these markers, CD133, CD166, and CD44 are the three main markers [25, 56, 57, 81–85]. The detection of colorectal CSC markers—CD44 and CD133—in this study showed that after incubation of the HT-29 cell line with CINN and FOLFOX, these CSC markers reduced significantly in the CINN-treated cells compared to the FOLFOX-treated cells. Consequently, the flow cytometry results showed that CINN has a better inhibitory effect on size of the cancer stem-like cells including SP cells, and CD 44 and CD133 positive cells. The CSCs and normal stem cells share some markers such as OCT4, NANOG, and SOX2 which are key factors in maintaining pluripotency and self-renewal of stem cells [68, 86–88]. Therefore, added support for the effect of FOLFOX and CINN on colon CSCs can be derived from the expression analysis of two stem cell-associated factors including OCT4 and NANOG as well as ALDH1A1 and ABCB1 as CSC markers using RT-PCR. The results showed that CINN is effective in the downregulation of OCT4, NANOG, ABCB1, and ALDH1A1 compared to FOLFOX.

CINN is a plant-derived component displaying a wide range of biological activities including cytotoxic effects on cancer cells [47–51]. The anti-cancer activity of CINN has been demonstrated only in the lung adenocarcinoma cell line, wherein the CSC-like abilities were diminished by decreasing the proliferation, invasive abilities, and in vivo tumorigenicity of sphere-derived stem cells. Furthermore, the CINN improved the sensitivity of CSCs to chemotherapeutic drugs via apoptosis induction [89]. In order to support the antitumor effect of CINN, we investigated the effect of CINN on colon CSCs and showed that the incubation of colon cancer cell line with CINN led to the reduction of colon cancer stem-like cells and the
downregulation of CSC markers. Accordingly, CINN was demonstrated to be a more effective chemotherapeutic agent than FOLFOX to eliminate HT-29 CSCs.

Similar to our results, the comparison of chemotherapy drugs and plant-derived phytochemicals in other studies showed that chemotherapy drugs are less effective in CSCs eradication than plant-derived phytochemicals. For instance, while the exposure of HCT-116 colon cancer cell line to FOLFOX led to the enrichment of CSCs phenotype, treatment with curcumin alone or together with FOLFOX or dasatinib could target the CSC subpopulation [90–94]. Several studies have shown that breast cancer cell treatment with Berberine as an isoquinoline alkaloid, isolated from medicinal herbs, leads to decreased expression of ABCG2, stem cell-associated genes, and SP fraction [31, 95, 96]. However, no statistically significant decrease was detected at the percentage of SP cells and expression level of ABCG2 after exposure of MCF-7 breast cancer cells to chemotherapy drugs such as doxorubicin and docetaxel, or mitoxantrone [31]. Likewise, oxymatrine is a plant alkaloid that reduced the percentage of SP cells and downregulated the activity of Wnt/b-catenin signaling in MCF-7, whereas cisplatin treatment was not able to do so [30]. Resveratrol and quercetin are naturally occurring polyphenolic compounds affecting CSCs by inducing apoptosis, inhibiting self-renewal capacity, reducing ALDH1 activity, and downregulating pluripotency [97–99]. In addition, it was proven that apigenin and baicalin, which are plant-derived flavones, and sulforaphane, a major glucosinolate in broccoli/broccoli sprouts, suppresses self-renewal capacity, cell growth, clonogenicity, and migration of CSCs in various cancers [32, 63, 96, 100–106]. To sum up, many reports indicate a low efficacy of common anticancer drugs compared to plant-derived compounds in combating CSCs. In addition, the adverse effects of synthetic drugs and the lower toxicity of plant-derived components make the latter a better option for cancer treatment [107–111]. Similarly, we have demonstrated that CINN as a natural agent not only inhibits the growth of HT-29 cells in cell-culture, but also leads to a dramatic decrease in colon cancer stem-like cell population. Furthermore, CINN has stronger anti-colon CSCs properties, which indicates that CINN either alone or together with FOLFOX could be more effective in eliminating colorectal cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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