The Antitumor Molecule TPEN Inhibits the Self-Renewal Potential of 5-Fluorouracil Resistant Colorectal Cancer Stem Cells

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Abstract

Background: The major causes of treatment failure and mortality in patients with colorectal cancer are the high recurrence rates and the drug resistance to the conventional chemotherapeutic agent 5-fluorouracil (5-FU). This resistance is greatly associated with a chemoresistant population of self-renewing cancer stem cells (CSCs). The antitumor molecule TPEN has been found to be effective against several cancers including colon cancer *in vitro* and *in vivo*. However, its effect on CSCs has not been evaluated yet. Here, we investigated the ability of TPEN to overcome chemoresistance and prevent cancer recurrence by targeting the self-renewal potential of colorectal CSCs.

Methods: MTT assay was used to assess TPEN effect on the viability of 5-FU resistant colorectal cancer cells cultured in 2D monolayers. Sphere-formation and propagation assays were performed to assess the efficacy of TPEN on CSCs enriched from 5-FU sensitive and resistant colon cancer cell lines in 3D cultures over several generations.

Results: Our data showed that TPEN dose-dependently reduced the viability of 5-FU resistant colon cancer cells in 2D cell cultures. In 3D cultures, results showed that TPEN reduced the self-renewal capacity of CSCs derived from both cell lines over five generations. In addition, TPEN reduced the expression level of stem cell marker CD44 in colonospheres derived from both cell lines at generation 1.

Conclusion: Our results suggest that TPEN could be a potent therapeutic agent for eradicating and preventing the re-emergence of colorectal tumors.

Introduction

Colorectal cancer (CRC) is the third most frequent cancer and the second leading cause of cancer- related mortality in men and women worldwide. Around two million new cases and nearly one million deaths are estimated globally in 2020 [1]. Most of the CRC-related deaths are due to metastasis mainly to the liver and lungs [2]. Around 25% of CRC patients have metastasis at time of diagnosis, and 50% of patients diagnosed with CRC develop metastasis [3]. Chemotherapy with the conventional agent 5-fluorouracil (5-FU) is the mainstay therapeutic modality for metastatic CRC [4]. However, less than 25% of patients with advanced CRC respond to 5-FU. The therapeutic inefficacy of 5-FU is mainly due to drug resistance and tumor relapse [5]. Indeed, there is increasing evidence supporting the key role of the intratumoral heterogeneity, a defining feature of cancer, in drug resistance and tumor recurrence [6,7]. Cancer stem cells (CSCs) have been proposed to be the major contributor to intratumoral heterogeneity and chemotherapy resistance [8].

Colorectal cancer stem cells are a subpopulation of tumour cells that can self-renew, initiate a tumor, and produce liver and lung metastases when implanted in immunocompromised mice [9,10]. In addition, these colorectal CSCs were found to be resistant to the conventional chemotherapeutic agent 5-FU [11,12] and have been implicated in post-treatment tumor recurrence. Thus, targeting CSCs by a drug that simultaneously eliminates the 5-FU

sensitive and resistant CSCs as well as the highly proliferating cells that they generate is of significance for clinical treatment and for effective and complete eradication of CRCs.

TPEN (N,N,N,N-Tetrakis (2-pyridylmethyl)-ethylenedi-amine), a lipid-soluble metal chelator, has been shown to be effective in depleting zinc and inducing apoptosis in several cancer cells both *in vitro* and *in vivo* [13-18]. In our laboratory, TPEN was found to selectively induce apoptosis in HCT116 human colorectal cancer cells without affecting their normal counterpart *in vitro* and *in vivo* [19]. We have also elucidated the mechanism of TPEN-induced apoptosis [20]. Our findings revealed that TPEN caused DNA damage by chelating accumulated copper in colon cancer cells, engaging redox cycling reactions and inducing the generation of reactive oxygen species (ROS) [20].

In light of the promising potential of TPEN in selectively targeting 5-FU sensitive HCT116 cells, here we sought to investigate TPEN efficacy on targeting the self-renewal capacity of colorectal CSCs derived from the parental 5-FU sensitive and resistant HCT116 cell lines. This was done using the advanced three-dimensional (3D) culture sphere-formation and propagation assays over several generations. We found that TPEN has a promising inhibitory effect on colorectal CSCs population reflected by the reduced sphere forming ability over five generations and the diminished expression level of stem cell marker CD44 at generation 1 (G1) in colonospheres derived from both cell lines.

Methods

Cell Culture and Treatment

5-FU sensitive HCT116 human colorectal cancer cell line was purchased from ATCC and 5-FU resistant HCT116 human colorectal cancer cell line was provided from the laboratory of Prof. Nadine Darwiche (American University of Beirut, Lebanon). Both cell lines were cultured in RPMI 1640 (Sigma- Aldrich, Germany) with 20 mM HEPES and L-Glutamine supplemented with 1% Penicillin- Streptomycin (100 U/ml) and 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Germany) in a humidified incubator (95% air, 5% CO_2) at 37°C. TPEN (Sigma-Aldrich) was prepared in dimethylsulfoxide (DMSO). TPEN concentrations were prepared by serial dilutions in the cell culture media. The final DMSO concentration used on cells was <0.3%.

MTT Cell Viability Assay

The effect of TPEN on cancer cell viability was evaluated using the MTT ([3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide]) assay that is based on the ability of metabolically active cells to reduce the MTT dye to purple formazan. 5-FU resistant HCT116 colorectal cancer cells were seeded overnight in 96-well tissue culture plates at a density of 12,000 cells/well

and then treated in sextuplicates with different concentrations of TPEN for 24, 48, and 72h. After treatment, 10μL of MTT reagent (5 mg/mL in PBS) was added to each well and incubated at 37°C for 2h. To solubilize the obtained formazan product, 100μL of iso-propanol was added to each well and incubated at 37°C for 30 min. The optical density (O.D.) of the reduced MTT was then recorded by an enzyme linked immuno-sorbent assay (ELISA) microplate reader at 595 nm. Cell viability was presented as a percentage of the control calculated using the following formula.

% viability = [mean O.D. treatment/mean O.D. control] × 100

Sphere - Formation Assay

The effect of TPEN on the self- renewal capacity of HCT116 colorectal CSCs was evaluated by testing their sphere forming efficiency when cultured in 3D over several generations. HCT116 colorectal cancer cells were suspended at a density of 2000 cells/well in 50μL of a mixture of cold Matrigel™/ serum-free medium (1:1) and then seeded at the bottom rim of the well of a 24-well tissue culture plate. The Matrigel was allowed to solidify by incubation at 37°C for 45 min. Subsequently, 500μL media with 5% FBS with or without TPEN was added gently in the middle of the well. Colonospheres were replenished with media or treatment every other day. They were counted and imaged at day 9 and 13 of sphere culture for the 5-FU sensitive and resistant cell lines, respectively.

Propagation Assay

Colonospheres were propagated over five generations. At the end of each generation, the medium was discarded and the colonospheres were dissociated from the Matrigel by digesting the latter with 500μL of dispase solution (Invitrogen, Carlsbad, CA) for 1h at 37°C. Colonospheres were collected by centrifugation and then incubated in 500μL trypsin/EDTA at 37°C for 1-2 min. The single cells obtained from the dissociated colonospheres were re-seeded as described above.

Immunofluorescence and 3D Imaging of Colonospheres

The effect of TPEN on the expression of stem cell marker CD44 was evaluated by immunofluorescence. After treatment, colonospheres of G1 were collected with cold RPMI media and centrifuged in order to remove all Matrigel debris. Subsequently, colonospheres were fixed by formalin for 20 min, and then washed with PBS thrice. They were permeabilized with 0.5% Triton X-100 for 30 min and blocked with blocking buffer for 2h at room temperature. The blocking buffer consists of 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20, and 10% normal goat serum in PBS. Colonospheres were then washed and incubated with primary mouse anti- CD44 (1:50 dilution) antibody with blocking solution overnight at 4°C. Colonospheres were then washed with PBS and incubated with secondary Alexa fluoro 488 goat anti-

mouse antibody (dilution 1:200) for 1h at room temperature. Subsequently, colonospheres were washed, counterstained with DAPI, and mounted with the anti-fade Fluorogel II. Colonospheres were then examined and imaged with Carl Zeiss LSM 710 laser scanning confocal microscope. Finally, the acquired images were analyzed using the Carl Zeiss ZEN 2012 image software.

Statistical Analysis

Data are presented as means ±SD or means ±SEM. Two-tailed Student's t-test and one-way ANOVA with Tukey's post-hoc tests were used to analyze the statistical significance of the difference between treatment groups and control groups. These tests were performed using Microsoft Excel 2013 software and IBM SPSS Statistics 25. Statistical significance was set at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

Results

TPEN Reduces the Viability of 5-FU Resistant HCT116 Human Colorectal Cancer Cell Line

We have previously evaluated TPEN's effect on the viability of 5-FU sensitive HCT116 colorectal cancer cells as well as on NCM460 non-tumorigenic colon cells and FHS74Int intestinal cells cultured in conventional 2D monolayers. We have found that 5μM of TPEN selectively killed 5-FU sensitive HCT116 colorectal cancer cells 24h post-treatment without being toxic to non-tumorigenic cells [19]. Therefore, we assessed the effect of TPEN on the viability of 5-FU resistant HCT116 colorectal cancer cells cultured in 2D. Cells were treated with TPEN at concentrations ranging between 1 and 10μM for 24, 48 or 72h after which cell viability was determined by MTT assay. The significant inhibitory

effect of TPEN was achieved at 5 and 10μM for the 3 time points. The percentages of reduction in viability of 5-FU resistant cells induced by 5 and 10μM of TPEN were respectively 66 and 71% at 24h, 73 and 77% at 48h, 86 and 84% at 72h. The IC_{50} of TPEN at 48h was 4.5μM (Figure 1).

TPEN Targets an Enriched Population of 5-FU Sensitive and Resistant HCT116 Human Colorectal Cancer Stem-Like Cells

Self-renewal capacity is one of the major hallmarks of stem cells that allows them to form and propagate spheres when cultured within Matrigel in 3D. We investigated TPEN efficacy on targeting self-renewal potential of colorectal CSCs derived from 5-FU sensitive and resistant cell lines by assessing their sphere forming ability over five generations. This was performed in 3D using sphere-formation and propagation assays. Colonospheres of G1 untreated or treated with TPEN were propagated by dissociating them into single cells and re-plating the resulting cells at the same density of 2000 cells/well. The re-seeded cells formed the colonospheres of the second generation (G2). This assay was repeated until the fifth generation (G5) in presence or absence of treatment.

Our data showed that the sphere forming unit of 5-FU sensitive and resistant HCT116 cells at each generation was lower in treated cells compared with that of the untreated control in a dose-dependent manner. Successive propagation and treatment of both cell lines over five generations with 1μM TPEN significantly reduced the sphere forming unit of 5-FU sensitive HCT116 cells by 66% (Figure 2A) and decreased that of 5-FU resistant HCT116 cells by remarkable 99% (Figure 2B).
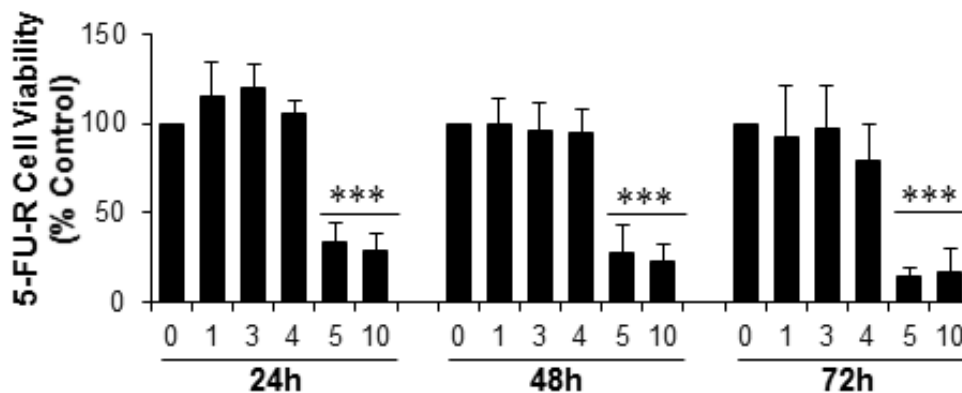

Figure 1: The effect of TPEN on the viability of 5-FU resistant (5-FU-R) HCT116 human colorectal cancer cells cultured in 2D. After treatment of cells with or without TPEN for 24, 48 and 72h, cell viability was determined using MTT assay. The values are expressed as percentage of viable cells relative to untreated control. Each value represents the mean ± SD of n=2 experiments performed in sextuplicates. *** p < 0.001 are significantly different from untreated control using two-tailed Student's t-test.
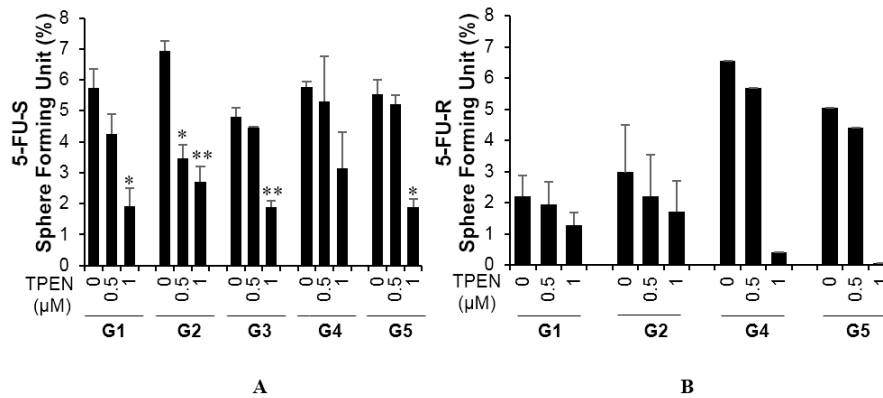
Figure 2: The effect of TPEN on the sphere forming ability of 5-FU sensitive (5-FU-S) and resistant (5-FU-R) HCT116 colorectal cancer stem cells. Sphere forming unit (SFU) over five generations for 5-FU-S (A) and four generations for 5-FU-R (B) HCT116 cells is shown in untreated or TPEN-treated (0.5 and 1µM) cells. SFU values are calculated according to the following formula: SFU = (number of colonospheres counted / number of input cells)×100. Colonospheres were generated by propagating cells in Matrigel enriched culture plates in the presence or absence of TPEN. Data are represented as mean ±SEM of n = 2 for (A) 5-FU-S and as mean ±SEM of n=3 for G1, n=2 for G2 and as mean of n=1 for G4 and G5 for (B) 5-FU-R HCT116 colonospheres. All experiments were performed in monoplicates. *p < 0.05 and **p <0.01 are significantly different from untreated control using one-way ANOVA with Tukey's post-hoc tests.

Interestingly, while 5µM of TPEN was needed to significantly reduce the viability of 5-FU sensitive and resistant HCT116 colorectal cancer cells grown in 2D, a dose of 1µM of TPEN was effective in decreasing the sphere forming unit of both cell lines.

We also investigated the effect of TPEN on the size of 5-FU sensitive and resistant HCT116 colonospheres. The diameter of the colonospheres was measured at each generation with or without treatment. Successive propagation and treatment of both cell lines over five generations with 1µM TPEN significantly decreased the diameter of 5-FU sensitive HCT116 colonospheres by 26% (Figure 3A) and diminished that of 5-FU resistant HCT116 colonospheres by 16% (Figure 3B).
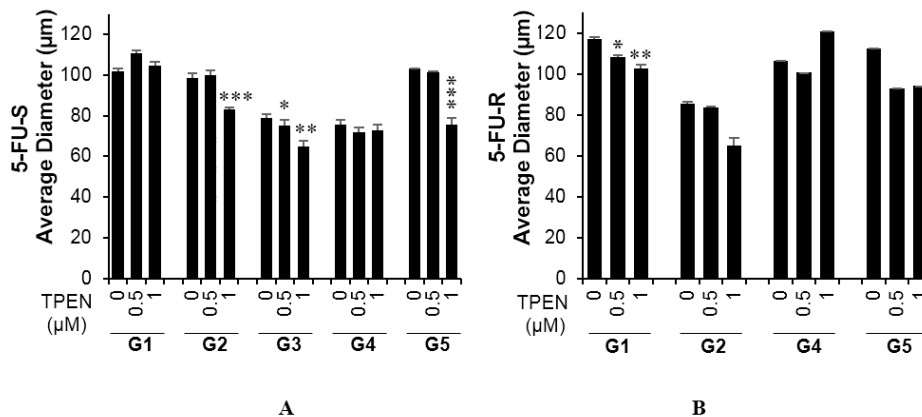

Figure 3: The effect of TPEN on the size of 5-FU sensitive (5-FU-S) and resistant (5-FU-R) HCT116 colonospheres. The diameter of colonospheres over five generations for 5-FU-S (A) and four generations for 5-FU-R (B) HCT116 colonospheres is shown in untreated or TPEN-treated (0.5 and 1µM) cells. Colonospheres were generated by propagating cells in Matrigel enriched culture plates in the presence or absence of TPEN. Data are represented as mean ±SEM of n = 2 for (A) 5-FU-S and as mean ±SEM of n=3 for G1, n=2 for G2 and as mean of n=1 for G4 and G5 for (B) 5-FU-R HCT116 colonospheres. All experiments were performed in monoplicates. *p < 0.05, **p <0.01 and ***p <0.001 are significantly different from untreated control using one-way ANOVA with Tukey's post-hoc tests.

TPEN Reduces the Expression of CD44 in 5-FU Sensitive and Resistant HCT116 Colonospheres

To further investigate TPEN's effect on enriched HCT116 CSCs population, we evaluated the expression of the stem cell marker CD44. This marker was reported to play an essential role in maintaining the stemness of colon CSCs and in initiating colon cancer [21]. 5-FU sensitive and resistant HCT116 G1 colonospheres treated with 1μM TPEN were collected, immunostained for CD44 and counterstained with DAPI. Immunofluorescence staining showed that TPEN decreased the expression of CD44 in treated spheres derived from both cell lines compared to the untreated control (Figures 4A and 4B).
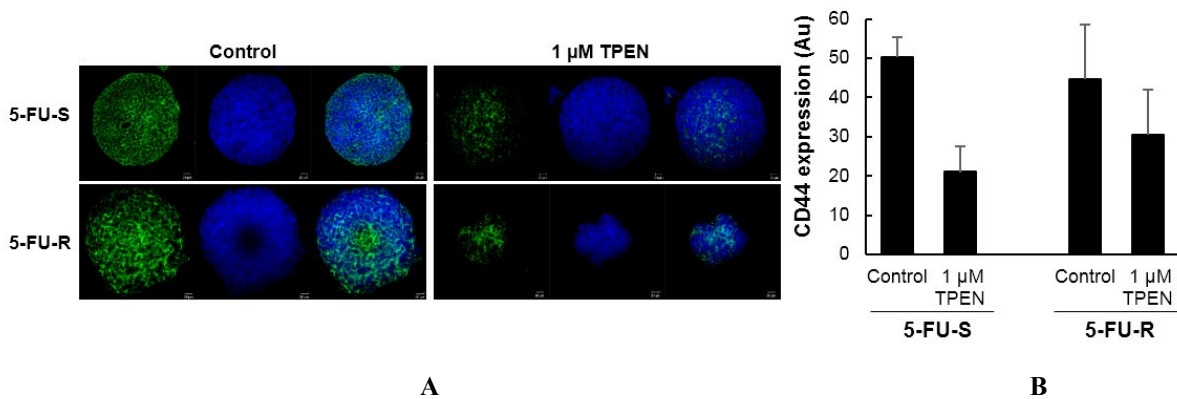

Figure 4: The effect of TPEN on the expression of CD44 in generation 1 colonospheres derived from 5-FU sensitive (5-FU-S) and resistant (5-FU-R) HCT116 cells. Colonospheres derived from 5-FU-S and 5-FU-R HCT116 cell line were untreated or treated with 1 μM of TPEN. The generated spheres were collected, fixed and immunostained for CD44. Representative images were obtained using confocal microscopy. Quantification of the intensity of CD44 staining in control and TPEN treated cells was performed using Carl Zeiss Zen 2012 software. The intensity of CD44 staining was normalized to the size of the spheres. Scale bar 20μm.

Discussion

The standard therapy for metastatic CRC is the conventional agent 5-FU which acts by inhibiting the activity of thymidylate synthase enzyme leading to imbalances in the nucleotide pool. This results in the disruption of DNA replication, transcription, and repair which causes the death of cancer cells [22]. However, the therapeutic application of 5-FU has been restricted due to drug resistance and cancer recurrence leading to a 5-year survival rate of 13% in distant metastatic CRCs [5,23]. Numerous factors have been implicated in 5-FU resistance such as epigenetic alterations, disruption in cellular functions including apoptosis, autophagy, glucose metabolism and mitochondrial respiration in addition to cell cycle perturbation [24]. Recent studies have demonstrated that a population of cells with stem-like properties play an important role in 5-FU resistance [11,12,25]. Although 5-FU targets the rapidly proliferating cells in the tumor bulk, the quiescent or slowly cycling CSCs are resistant to 5-FU treatment [25]. The fact that 5-FU resistant HCT116 colonospheres are enriched with CSCs proved that 5-FU based chemotherapy failed to eradicate CSCs [26]. These dormant CSCs can enter the cell cycle and generate highly proliferating cancer cells resulting in tumor re-emergence. A recent report has shown that CRC cells enter stemness and quiescence in order to escape apoptosis induced by 5-FU [27].

Achieving a successful and complete course of CRC treatment requires selectively targeting the heterogeneous population of the colorectal tumor including the 5-FU sensitive and resistant cells as well as their derived CSCs while sparing the viable normal cells. Here, we have identified that the metal chelator TPEN has the potential to target the diverse population of the tumor and overcome 5-FU induced resistance in CRC without affecting normal cells. Although we and others have demonstrated TPEN's selective anti-cancer activity [17-20], no studies have assessed its effect on colorectal CSCs. In order to evaluate the inhibitory effect of TPEN to target the whole tumor including its stem cell population, we have used 5-FU sensitive as well as 5-FU resistant HCT116 colorectal cancer cell lines generated by exposure to increasing doses of 5-FU. HCT116 is an excellent model to use for assessing drug effects on CSCs and for studying the anti-tumor properties of TPEN. Colonospheres generated from HCT116 cell lines in 3D were found to be highly enriched with cells having stem-like properties and expressing the majority of known colorectal CSC markers [28,29]. Furthermore, TPEN was found to reduce the viability of 5-FU resistant HCT116 cell line at IC_{50} concentrations of less than 5μM.

To assess TPEN effect on CSCs derived from both cell lines, we performed the sphere-formation and propagation assays. Our

results showed that 1µM TPEN exerted a strong inhibitory effect on the stem cell population as revealed by a remarkable decrease in the sphere forming unit of both the sensitive and the resistant cell lines accompanied by a decrease in the size of colonospheres as well as in the expression of CSC marker CD44. Of note, CD44 was reported to be a CSC marker of high importance essential in maintaining stem-like properties in HCT116 cells [30]. CD44 knockdown decreased proliferation of these cancer cells and prevented tumor initiation in a mouse xenograft model [21]. In addition, CD44 expression was reported to be more pronounced in 5-FU resistant HCT116 colonospheres when compared to 5-FU sensitive reflecting its role in resistance to 5-FU [26].

TPEN has been shown to induce apoptosis in many cancer cell lines through various mechanisms dependent or not on its chelating activity. It was reported to induce the generation of ROS, the up- regulation of apoptotic mediators, the damage of DNA in addition to the inhibition of epithelial to mesenchymal transition (EMT) [13,14,19,31]. In colon cancer cells, TPEN was found to form a complex with copper and engage in redox cycling leading to the production of ROS. This results in double strands breaks (DSBs) in DNA and therefore activating DNA damage pathways ATM/ATR and DNA-PK [19,20]. Collectively, this data implies that TPEN acts by inducing cell death rather than by blocking cell proliferation.

Thus, it is reasonable to speculate that the decrease in self-renewal capacity of colorectal CSCs enriched from 5-FU sensitive and resistant cell lines is likely to be in large part due to apoptosis induction. In addition, the reduction in HCT116 sphere forming unit and in colonospheres sizes induced by TPEN also suggest the involvement of apoptosis in eliminating colorectal CSCs. However, further mechanistic studies are needed to decipher the detailed molecular mechanisms for TPEN to eradicate colorectal CSCs.

In conclusion, our study demonstrated that TPEN is effective in depleting the CSC population enriched from 5-FU sensitive and resistant HCT116 colorectal cancer cell lines. Additional studies need to be conducted to provide insights into its mechanism of action and to further evaluate its effect on CSCs in animal models of CRC in order to advance it into clinical translation.

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Disclosure Statement

The authors declare no conflicts of interest.

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