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Research Article

Effect of Curcumin on SMCT-1 Expression and Dichloroacetate Toxicity in HCT116 Colon Cancer Cells

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ABSTRACT

Background: Colorectal cancer is a common cause of cancer-related deaths Epigenetic regulation of the influx sodium dependent monocarboxylate transporter-1 (SMCT1), a tumor suppressor, was recognized in colorectal cancer. In this study, effects of Curcumin (Cur), on SMCT1 gene expression was determined. A low SMCT1 expression, HCT116, cell line was used to test an *in vitro* effect of Cur on epigenetic regulation of SMCT1 expression via DNA methylation and its function. It was hypothesized that Cur can induce SMCT1 expression in the cells via hypomethylation effect. Measurement of increase in SMCT1 function was performed using dichloroacetate (DCA), a cytotoxic substrate of SMCT1.

Methods: The effect of 5′Azacytidine (Aza), a hypomethylating agent, and Cur on SMCT1 expression and function was determined. Cells were treated with Aza and various concentrations of Cur for 72 h. After that SMCT1 expression was determined by real time PCR and Western blotting. To evaluate the SMCT1 function, DCA was used in MTT assay.

Results: After treatment with 40 µM Cur, SMCT1 mRNA was significantly increased ($p < 0.05$). This was correlated with SMCT1 protein expression. Cells treated with 40 µM of Cur showed significant increase of cytotoxicity at DCA concentrations of 25 ($p < 0.001$) and 12.5 mM ($p < 0.01$), respectively.

Conclusion: Cur was shown to significantly induce the SMCT1 mRNA and protein expression in HCT116 cells. The induction of the SMCT1 protein increased DCA cytotoxicity, presumably through an increase of DCA transport into the cells. The mechanism underlying of SMCT1 induction by Cur may result from not only hypomethylation but other epigenetics.

Introduction

ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters are the two largest superfamilies concerning the transport of many substances of the cells. These two superfamilies are the most interesting in the medical and pharmaceutical sciences because their functions are involved in many diseases and therapeutics particularly in pharmacokinetics and drug disposition.[1,2] Their roles in cancer are increasing in recent years. Sodium dependent monocarboxylate transporter-1 (SMCT1) is the eighth member of solute carrier family 5 (SLC5A8). SMCT1 is encoded by *SLC5A8* gene. SMCT1 is localized to the apical membrane of epithelial cells in many tissues such as thyroid gland, kidney, adrenal gland, salivary gland, colon and prostate gland.[3-5] The major function of SMCT1 is electrogenic sodium-solute symporters.[6] SMCT1 functions in the influx transport of short chain fatty acids (SCFA) such as acetate, propionate, butyrate, and other monocarboxylate compounds including pyruvate, lactate and nicotinate.[7] In the colon and small intestine, SMCT1 is involved in the absorption of SCFA, products of dietary fiber fermentation by colonic bacteria. Propionate and butyrate possess histone deacetylase (HDAC) inhibition activity. This provides a mechanism for the tumor-suppressive function of SMCT1.[8] Moreover, higher SMCT1 expression may be a favorable indicator of colorectal cancer prognosis and correlated with longer disease-free survival.[9] SLC5A8 promoter region is frequently methylated in primary colon cancers, colon adenomas, and aberrant crypt foci.[10] Increased expression of SMCT1 in breast cancer

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cells induces changes in the location and levels of surviving which result in cell-cycle arrest and induction of apoptosis and enhancement in chemosensitivity of SLC5A8-expressing cells exposed to two cytotoxic drugs (cisplatin and 5-fluorouracil).[11] In patients with hepatocellular carcinoma (HCC), low SMCT1 expression was correlated with the clinicopathological features, and a shorter overall survival. The authors concluded that SMCT1 has a tumor-suppressive function that acts by interfering with Wnt/ß-catenin signaling in HCC.[12]

The regulation of SMCT1 expression has been studied by many groups of researchers.[13-16] The suppression of SMCT1 expression has been documented in cancers of a wide variety of tissues such as colon,[9,10,17] thyroid,[18-20] and gastric cancer.[21] The hypermethylation at the CpG island in promoter region in many cancers is the most frequently reported.[20-25]

Cancer is the major cause of deaths in many developed and developing countries including Thailand.[26] Conventional chemotherapy has a limitation by their toxicity, side effects or the resistance of cancers and relapses of the diseases. The development of targeted drugs is nowadays widely accepted as a promising strategy against cancers.[27-30] However, new drug development is also focused on the many different pathways of cancer development including epigenetics.[31,32]

Today, there has been a great interest in the roles of natural products. Constituents from many plants or herbs have been developed for use in many diseases including cancer. Some of these substances play their roles through epigenetic mechanisms.[33] Many ingredients from plant extracts were studied for cancer prevention and treatment. Several mechanisms including epigenetic pathways of plant extracts in cancer inhibition have been reported and reviewed.[34-37]

Curcumin (Cur), a principle ingredient from *Curcuma longa* Linn., has high antioxidant property and safety .The turmeric of *Curcuma* has been used in traditional dishes and, nowadays, the curcuminoids from the turmeric extract have also been used in many forms of cosmetics and drugs. Cur has been reported for the anticancer activities in many pathways.[38] From the literature, Cur is demonstrated to have hypomethylation property that can induce the expression of proteins.[36,39-41] Cur can induce global DNA hypomethylation in a leukemia cell line,[39] re-expression of nuclear factor erythroid 2–related factor 2 (Nrf2) in prostate cancer,[40] Neurogenin 1 (Neurog1) gene in prostate LNCaP cells,[41] and reactivation of retinoic acid receptor β (RARβ), a tumor suppressor, expression in lung cancer and other solid tumors.[42] In this study, we were interested in investigating whether Cur would have an effect on tumor suppressor transporter SMCT1 expression. The aim of this study is to determine the effect of Cur on the SMCT1 mRNA and protein expression in HCT116 colon cancer cells compared to the effect of 5′Azacytidine (Aza), a potent hypomethylating agent, and to study the effect of Cur on SMCT1 transport function using dichloroacetate (DCA), a known substrate of SMCT1.

Materials and Methods

Materials

Cur, Aza and DCA (>98% purity) were purchased from Sigma-Aldrich®, St. Louise, USA. DMEM, L-glutamine, penicillin-streptomycin, and fetal bovine serum were purchased from Invitrogen, Paisley, UK. Bisulfite conversion kit, Rabbit anti-SMCT1 polyclonal antibody and goat anti-rabbit IgG polyclonal antibody were purchased from Abcam, Cambridge, UK. High pure RNA isolation kit, High fidelity cDNA synthesis kit and Fast start Essential DNA green master were purchased from Roche Diagnostics GmbH, Germany. EZlys™ Tissue protein extraction reagent was purchased from Biovision Incorporated, USA. Pierce™ BCA Protein Assay Kit was purchased from Thermo Fisher Scientific, Germany. 50 mM of Cur in DMSO was prepared as stock solution. The aliquot stock solution of Cur was kept at -20°C. Aza was diluted in DMSO at 10 mM as stock solution. The aliquot stock solution of Aza was kept at -80 °C.

Culture of HCT116 cells

HCT116 cell line was cultured according to standard protocol in DMEM 4.5 g/l glucose supplemented with 10% heat–inactivated fetal bovine serum, 1% glutamine and 150 U/ml penicillin, 5 µg/ml streptomycin. The HCT116 culture flask was incubated in the humidified 37 °C, 5% CO_2 incubator.

Cur cytotoxicity on HCT116 cell line

Three independent passages of HCT116 were seeded at density of 40,000 cells/well in 100 µl medium in a 96-well plate. The 50 mM Cur stock solution was added to the culture medium to obtain different final concentrations from 25 to 200 µM. 48 hours after seeding, at 90% confluence, the culture medium was changed to 100 µl Cur-containing medium at different concentrations. 1% DMSO medium was used as 100% viability control. After 72 hours of the treatment, the viability of each well was evaluated by adding 10 µl WST1 and further incubated at 37 °C for 25 minutes. The absorbance was read by a microplate reader at 450 nm. The 50% inhibitory concentrations (IC_{50}) were determined.

Table 1. Primers used in the Real time-PCR for SMCT1 and reference RNA.

Target	Forward primer	Reverse primer
SMCT1	5' CAGACCAGCTCATGCCTTATT 3'	5' CTGTGCTTAATGTCCCACTGTA 3'
18S rRNA	5' CTGAGAAACGGCTACCACATC 3'	5' GCCTCGAAAGAGTCCTGTATTG 3'

Effects of Cur on SMCT1 expression

Three different independent passages of the HCT116 cells were seeded in the 6-well plates in the quantity of $1x10^6$ cells/well.

After 48 hours of seeding the cultured medium was changed to the 40, 25 and 10 µM Cur containing medium. The cultured cells were grown for another 72 hours in 37 °C, 5% CO_2 incubator. 0.1 % DMSO in cultured medium was used as negative control. 5 µM of Aza was used as potent hypomethylating control. The culture medium both of the test and the control were changed to the new one every 24 hours. The DNA, mRNA and protein of the cells were collected and tested.

SMCT1 mRNA expression

Total RNA of the cultured cells was extracted by high pure RNA isolation kit following the kit instruction. Briefly, the cultured cells were broken by lysis buffer. RNA was trapped by the resin fiber column of the kit. The unwanted DNA was eliminated by DNase-I enzyme provided. RNA was then eluted, quantified by UV-spectrophotometer at 260 nm and store at -80 °C until tested. One microgram of RNA was reversely transcribed to obtain 20 µl cDNA by Transcriptor High Fidelity cDNA Synthesis Kit according to the kit proto col. The 1 µl of cDNA was determined for SMCT1 expression in triplicate by Sybr green based quantitative real-time PCR in LightCycler 480® using FastStart Essential DNA Green Master kit. After normalization with reference 18S rRNA expression, the SMCT1 expressions of Cur treatments were compared to that of the negative control (0.1% DMSO-treated) HCT116 cells. Forward and reverse primer sequences used in real time PCR method were designed by NCBI primer-blast website using NCBI reference sequence NR_003286.2 and NM_145913.3 for reference (18S) and SMCT1 genes, respectively. The sequences of forward and reverse primers were shown in Table 1. Real time PCR condition for both SMCT1 and reference (18S) gene amplification were 95 °C for 10 minutes followed by 45 cycles of amplification at 95 °C for 20 seconds, and 60 °C for 20 seconds and 72 °C for another 20 seconds.

SMCT1 protein expression

The effect of Cur on SMCT1 protein expression was carried out by Western blot analysis. Total protein of treated cells was collected after breaking the cells with lysis buffer. The protein was quantified by a commercial assay kit (Pierce™BCA Protein Assay Kit, ThermoScientific) compared to a set of standard protein concentration according to the assay protocol. Thirty micrograms of total protein from each treatment was used for analyzing SMCT1 protein content compared to the GAPDH protein as loading control. SMCT1 rabbit anti-mouse polyclonal antibody and secondary goat anti-rabbit IgG antibody were used for detection the SMCT1 protein. SMCT1 protein obtained from the experiment was compared to the GAPDH protein expression. GelQuant.NET software provided by Biochemlab Solutions Co. was used to quantify the band intensity.[43]

Effect of Cur on dichloroacetate (DCA) toxicity

Three independent passages of HCT116 were used in the experiment. HCT116 cells at the concentration of $1.75x10^4$ cells in 100 µl culturing medium were seeded in 96 well plates. 48 hours after seeding, the culturing medium was replaced by 100 µl medium containing 10, 25, 40 µM of Cur, or 5 µM Aza. DMSO at 0.1% was used as the negative control. After pre-treatment of 72 hours, the culturing medium of each concentration of the treatment was replaced by 100 µl of 0, 6.25, 12.5 and 25 mM of sodium dichloroacetate (DCA) for 48 hours. At the end of DCA exposure, 20 µl of 5 mg/ml of MTT solution was added into each well and incubated further for 2.5 hours at 37 °C in a dark place. After incubation, the culture medium was carefully removed and 100 µl of DMSO was added. The absorbance at 570 nm was measured by a microplate reader .The cell viability was then calculated using 0 µM of DCA as 100% viability control. To confirm that DCA was transported into the cell by SMCT1, ibuprofen, a specific inhibitor of SMCT1 was used to inhibit the SMCT1 transport function. Ibuprofen was added into the culture medium together with DCA solution obtaining the final concentration of 200 µM.

Statistics

Data from the experiments were analyzed for means, standard deviation and standard error by Microsoft Excel. The significant statistic values were determined by Prism 5 GraphPad software.

Results

Cur cytotoxicity on HCT116 cells

The viabilities of HCT116 cells by WST1 assay after the treatment of Cur in different concentrations were presented in the Figure 1. The 72 hour IC_{50} of Cur obtained from the test was

about 70 µM. The concentrations of 40, 25 and 10 µM of Cur were then selected for the further studies.

Effect of Cur on SMCT1 expression

Cur at the final concentrations of 40, 25 and 10 µM was used to determine SMCT1 mRNA and protein expression in HCT116 cell line. Aza, a potent hypomethylating agent, at 5 µM was used as positive control. 0.1% DMSO was used as negative control.

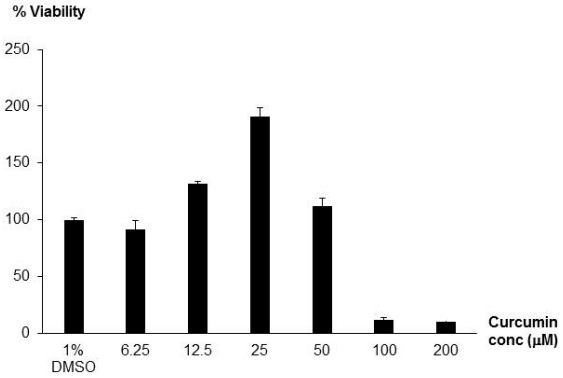

Figure 1. The viability after 72-h Cur treatment in HCT116 cells with different concentrations. The percentage viability was compared to control (1% DMSO). Data are means ± standard error of three independent experiments.

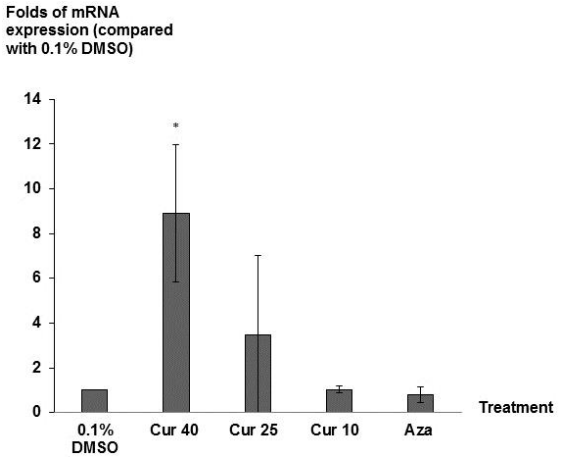

Figure 2. Effect of Cur on SMCT1 mRNA expression. The mRNA expressions of 72-h treatments of Aza (positive control) and Cur on HCT116 cells, normalized with 18S rRNA. Data are means ± standard error of three independent experiments. *p<0.05, significantly different from the negative control as treatment with 0.1 % of DMSO.

Effect of Cur on SMCT1 mRNA expression

The SMCT1 mRNA expressions of each treatment after normalization were shown in Figure 2. Aza did not significantly affect the SMCT1 mRNA expression in HCT116 cell line. Compared to negative control, Cur at concentrations of 40 and 25 µM induced SMCT1 mRNA expression by 8.91 and 5.98 folds, respectively. Statistically, 40 µM of Cur significantly induced SMCT1 expression compared to negative control ($p < 0.05$). However, Cur at concentrations of 25 and 10 µM, as well as 5 µM of Aza increased the SMCT1 mRNA expression insignificantly.

Effect of Cur on SMCT1 protein expression

SMCT1 protein expressions after Cur treatment detected by Western blotting were shown in Figure 3 (a). HCT116 cells treated with Cur (40 µM, 25 µM and 10 µM) showed higher protein expression than cells treated with 0.1 %DMSO and 5 µM Aza. SMCT1protein intensity was quantified by GelQuant. Net software compared to GAPDH loading control as shown in Figure 3 (b). The results indicated that higher concentration of Cur increased expression of SMCT1 protein significantly.

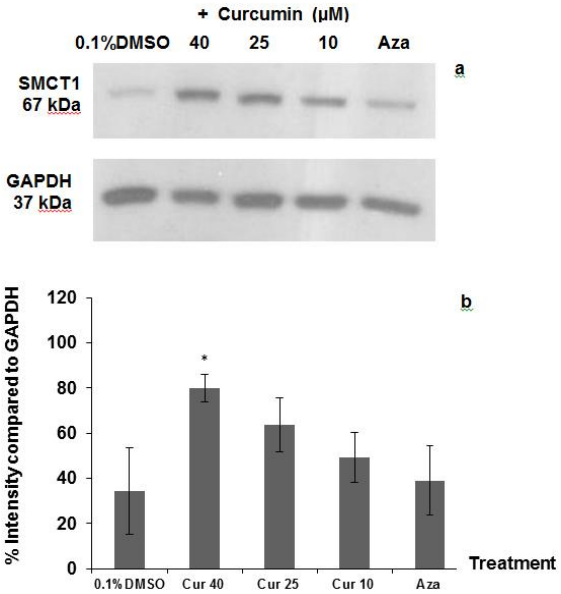

Figure 3. (a) Western immunoblotting of SMCT1 using GAPDH protein as loading control after 72-h treatment of Cur at different concentrations. (b) The %intensity of SMCT1 protein from Cur and Aza treatment samples were compared with GAPDH protein by GelQuant.NET software. Each value is the mean ± standard error. Data are representative of three experiments. *p<0.05, significantly different compared to negative control (0.1% of DMSO).

Effect of Cur on DCA toxicity

The function of SMCT1 can be monitored by determining the transport of its substrates. Decrease or increase of SMCT1 function can affect the substrate transport. To this end, the effect of Cur on SMCT1 transport function was evaluated by indirect measurement of cytotoxicity of dichloroacetate (DCA), a known cytotoxic substrate of SMCT1. DCA kills cancer cells by its ability to inhibit pyruvate dehydrogenase kinase (PDK) and to activate pyruvate dehydrogenase complex (PDC). With increased SMCT1 protein, it was hypothesized that addition of non-toxic concentrations of DCA would induce toxicity, and

thus decrease viability of the cells. Preliminary experiment of DCA toxicity was also tested to determine the non-toxic concentration to be used for SMCT1 functional test. The result showed that the IC_{50} of DCA on HCT116 cells was more than 100 mM (data not shown). The highest concentration of DCA that did not affect the cell viability was 25 mM. The relative viability of HCT116 cells after pre-treatment with Cur for 72 hours followed by 48-hours exposure to DCA was shown in Figure 4. Cur at the concentration of 40 µM statistically increased the cytotoxicity caused by DCA treatment at the concentrations of 25 and 12.5 mM ($p<0.001$ and 0.01, respectively). 10 µM of Cur and 5 µM of Aza were not able to change the DCA toxicity. Furthermore, the DCA toxicity mediated by Cur was reduced by adding 200 µM of ibuprofen, an inhibitor of SMCT1 (Figure 5).

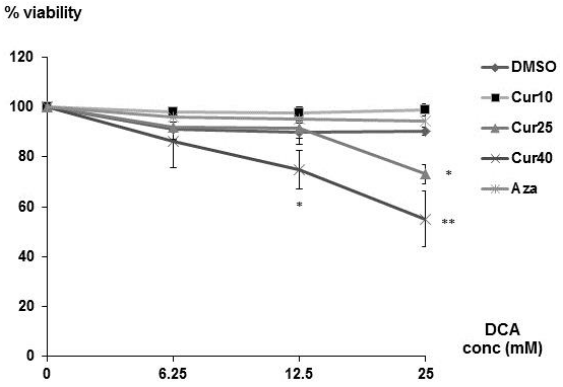

Figure 4. An increase of DCA cytotoxicity in HCT116 measured by MTT assay. HCT116 cells were treated with various concentrations of Cur or Aza (5 µM) as a positive control, followed by dichloroacetate (DCA). Each % relative viability value is the mean ± standard error of triplicate of cultures. The % cell viability of 25 and 40 µM Cur pre-treated cells statistically differed from 0.1%DMSO-treated cells for the DCA at the concentration of 12.5 and 25 µM (** $p < 0.001$, * $p < 0.01$).

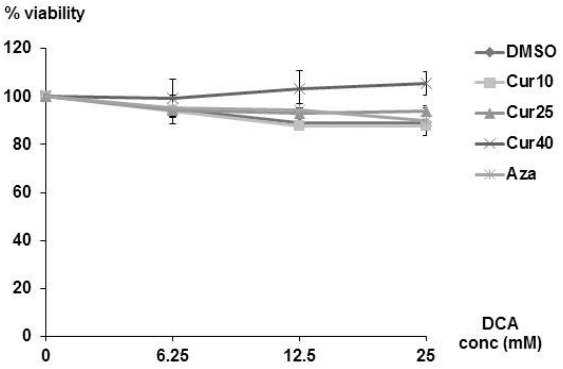

Figure 5. Effect of ibuprofen, SMCT1 inhibitor, on DCA cytotoxicity in HCT116 cells by MTT assay. Relative viability of HCT116 cells after DCA toxicity tested in the presence of 200 µM ibuprofen. Each value is the mean ± SD of triplicate of cultures. The % cell viability after DCA treatment in the presence of ibuprofen was not significantly different compared with 1% DMSO treated cells ($p > 0.05$).

Discussion

Cur has been on the spotlight of herbal remedy of many diseases including cancers both alone and as an adjunct to chemotherapy. Here we showed that non-toxic concentration of Cur but high enough level could induce *SLC5A8* gene and SMCT1 protein expression. Non-toxic concentrations of Cur were first determined by incubation of Cur at various concentrations with HCT116 colorectal cancer cells. After 72 hours of Cur treatment, low concentrations (6.25, 12.5, 25 and 50 µM) increased cell proliferation, whereas high concentrations (≥100 µM) caused a decrease in cell proliferation. This was not an unexpected result since curcumin has been shown to regulate proliferation of other cell types, including neural cells,[44] Schwann cells,[45] astrocytes,[46] whereas high concentrations are often toxic to various cancer cells and cell lines.[47-49] This protective effect of Cur was probably due to anti-oxidative effect[50] and transient activation of ERK and p38 kinase pathways at low concentrations.[51] However, higher concentration of Cur exhibits antiproliferative effect by controlling many signaling molecules such as p53, NF-κB, Akt, MAPK.[52]

At 40 µM, Cur statistically increased SMCT1 expression. As SMCT1 was recognized as a tumor suppressor, the induction of SMCT1 whose function is mainly associated with the inhibition of histone deacetylase enzyme may be useful in cancer therapeutics. It has been suggested that SMCT1 expression is suppressed by DNA hypermethylation in the promoter region of *SLC5A8* gene[25] and/or histone modification.[23] SMCT1 protein was almost absent in HCT116 cell line and inducible by Aza, a potent hypomethylating agent.[17,53] SMCT1 protein expression of HCT116 cell, under our culture conditions, was low (Figure 3). Interestingly, incubation of the hypomethylating agent Aza for 72 h had a negligible effect on SMCT1 mRNA and protein expression in our study. The reason for this has not yet to be determined and may come from differences in the incubation time and conditions of culturing system. However, our study showed that Cur could effectively induce SMCT1 expression soon within 72 hours. Cur was reported to inhibit some cancers such as colon and genitourinary cancer due to its hypomethylation effect.[39,54-56] Moreover, Cur also has other epigenetic activities rather than hypomethylating effect such as histone modification[54-58] and miRNA modulation.[54,58-59] Therefore, it may be possible that other epigenetic effects of Cur may play a role in SMCT1 gene induction. Since SMCT1 was shown to transport dichloroacetate (DCA), an orphan drug frequently used as an investigational treatment for cancers, in a Na^+-coupled manner; and was required in DCA-

induced apoptosis in colon cancer cell lines, we then tested induced SMCT1 could enhance antitumor activity when DCA was added into cells pre-treated with Cur.[53] As expected, Cur at 25 and 40 µM statistically and significantly enhanced anti-cancer activity of DCA at concentrations (when used alone) that exhibited minimal cytotoxic effects. Viability of DCA-treated cells was markedly decreased, and this effect was almost completely reversible when 200 µM of ibuprofen, a specific and proven inhibitor of SMCT1, was added. This implied that induction of SMCT1 by Cur enhanced DCA transport, and Cur, therefore, may become promising cancer-fighting treatment or alternative when used in combination with other chemotherapeutic drugs.

Conclusion

In this study, the expression of SMCT1 mRNA and protein in HCT116 cells are induced by Cur. Apart from hypomethylation, Cur also possesses other epigenetic activities such as histone modification and miRNA modulation. The SMCT1 induction by Cur enhances the transport function of its substrate such as DCA. SMCT1 induction by Cur may be useful for increased transport of tumor suppressive drugs which are SMCT1 substrates. Thus, the usefulness of Cur in the cancer prevention, not only for the colon cancer but also for the other types of cancers, may result from the up-regulation of SMCT1. Epigenetic mechanism regulation of SMCT1, the hypermethylation of CpG island of the gene and histone modification mechanism, such as HDAC inhibition, should deserve further attention in future study. The combination effect of Cur on SMCT1 induction and effect on DCA toxicity should be confirmed *in vivo*.

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Conflict of interests

The authors claim that there is no conflict of interest.

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