Xanthone from *Swertia chirata* exerts chemotherapeutic potential against colon carcinoma

Atish Barua[1], Pritha Choudhury[1], Niraj Nag[2], Anirban Nath[3], Sabyasachi Kundagrami[3], Amit Pal[2], Chinmay Kumar Panda[4] and Prosenjit Saha[1,*]

[1]Department of Cancer Chemoprevention, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700 026, India
[2]Division of Pathophysiology, National Institute of Cholera and Enteric Diseases, P-33, CIT Road, Scheme-XM, Beliaghata, Kolkata 700 010, India
[3]Department of Genetics and Plant Breeding, University of Calcutta, Kolkata 700 073, India
[4]Department of Oncogene Regulation, Chittaranjan National Cancer Institute, 37, S.P. Mukherjee Road, Kolkata 700 026, India

The present study examines whether 1,5,8-tri-hydroxy-3-methoxy xanthone (TMX) isolated from *Swertia chirata* could restrict colon cancer cells by downregulating proliferation and inducing apoptosis. The chemotherapeutic activity of TMX was evaluated in several colon cancer and normal cell lines using *in vitro* assays like MTT assay, cell-cycle analysis, caspase-3 activity assay, annexin V/PI staining, JC10 assay, intracellular reactive oxygen species (ROS) level determination by dicholorofluorescein di-acetate (DCFH-DA). The present study revealed that TMX from *S. chirata* could effectively inhibit proliferation of metastatic colon cancer cell lines. The chemotherapeutic potential of TMX against metastatic colon cancer cell lines was achieved by downregulating several critical regulatory genes enabling the suppression of the proliferative potential of colon cancer cells and driving them towards apoptosis in a ROS dependent manner. In addition, TMX showed chemosensitization potential in colon cancer cell lines.

Keywords: Apoptosis, chemosensitization, colon cancer, reactive oxygen species, *Swertia chirata*.

IN India, the annual incidence rate (AIR) for colon cancer in men is 4.4 per 100,000 and in women is 3.9 per 100,000. Colon cancer ranks eighth among men and ninth among women. Survival chances are 50% for every patient for five years. However, depending on the time of diagnosis and staging, it varies as follows: for stage 1 colon cancer, the survival chances are 90% for five years, stage 2 80–83%, stage 3 60% and stage 4 11% for five years[1]. In the developed countries, where modern diagnostic infrastructure is readily available, early diagnosis and treatment significantly increase a patient's chances of overall survival. However, since the cost of cancer treatment is unaffordable to most people in the developing countries, traditional medicine provides the much affordable alternative for the poor population[2]. Majority of the conventional medicines depend on natural products of plant origin and in the vast majority of cases, purified plant products have shown promising results. One of the main advantages of the drugs of plant origin is that since most of the products come from plants, they are non-toxic to normal cells[3]. Since the current treatments show toxic side effects by killing both normal, healthy, dividing cells and cancerous ones, plant extracts can shed new light on the treatment of colon cancer by lowering the side effects and increasing the efficacy of the chemotherapeutic drugs and decreasing their toxic side effects[4]. The major hurdle in establishing the small molecules of herbal origin as successful drug candidates is their low bioavailability.

In a recent study, a small molecule was proven to have bio-availability in *in vivo* studies and chemotherapeutic potential against cancer cells, while being non-toxic to normal cells[5]. *Swertia chirata* is one of the oldest known medicinal plants; it forms a reservoir for a large number of small, bioactive molecules possessing various therapeutic activities. Among the large number of small bioactive molecules, our laboratory had previously established 1,5,8-tri-hydroxy-3-methoxy xanthone (TMX) to have chemotherapeutic potential against metastatic breast and skin cancer while being non-toxic to normal cells, and also to be bio-available in normal Swiss albino mice[6,7]. We have also shown TMX to be more efficacious compared to other chemotherapeutic drugs[6,8]. Here we examine the efficacy of TMX against colon cancer cell lines and also evaluate its chemosensitizing potential by calculating the combination index along with a chemotherapeutic drug, 5 fluoro uracil (5FU).

Materials and methods

Plant material

The whole plant of *S. chirata* was obtained from a regional plant supplier from Kolkata, India, and validated by

*For correspondence. (e-mail: prosenjit_cnci@yahoo.co.in)

Dr S. R. Das (Central Research Institute (Ayurveda), Kolkata). The collected *S. chirata* specimen has been preserved in the Herbarium of the Central Research Institute (Ayurveda), Kolkata.

Isolation and purification of TMX from S. chirata

Extraction, purification and characterization of TMX from *S. chirata* were done at the National Research Institute for Ayurvedic Drug Development, Kolkata. The isolation process has been patented, the details of which are as follows: Indian Patent no. 191129, dated 26.03.2002. Mandal, S., Das, P. C., Das, A., Das, S. and Saha, P. (2002).

Cell line and cell culture conditions

Cell lines CaCo2, HCT-116, HT-29, SW480, SW837 and CCD-18Co were obtained from the National Centre for Cell Sciences, Pune, India. CCD-18Co and CaCo2 were maintained in Eagle's minimum essential medium, HCT-116 and HT-29 in McCoy's 5A medium, and SW837 in Leibovitz's L-15 medium with 10% foetal bovine serum, 2 mM glutamine, 100 U penicillin and 100 μg/ml streptomycin. Cells were grown at 37°C in a humidified CO_2 incubator with 5% CO_2. Refeeding with fresh growth medium and subculturing (using 0.05% trypsin–EDTA) of the cells was done as required[9].

Cytotoxicity analysis

Cytotoxic effects of TMX on CaCo2, HCT-116, HT-29, SW480, SW837 and CCD-18Co cells were assessed using the MTT assay according to the manufacturer's protocol (HiMedia, India). Briefly, the cells (6×10^4) were seeded into 96-well plates. After 24 h, they were treated with different concentrations of TMX (1–10 μM for cancer cells, and 1–100 μM for normal cells) and 5FU (1–10 μM for all the cells) for 48 h. Then cell viability test was performed and the dose–response curve was plotted[10].

Cell-cycle analysis

Cell-cycle analysis was done to evaluate the effect of TMX and 5FU on normal and cancerous colon cell lines. The pattern of cell-cycle phase distribution of all the cells was analysed using FACS (Calibur, Becton Dickinson, USA) assay with 50 μg/ml propidium iodide (PI) staining, and data were analysed utilizing cell Quest Pro Software[11].

Mitochondrial membrane potential by JC10 staining

JC10 assay was performed in normal and cancerous colon cell lines using JC10 kit from Abcam, according to the manufacturer's protocol. Data were obtained using 96-well plates and a plate reader (Cary Varian, Germany); fluorescence intensities were monitored at exitation/emission = 490/525 nm (cut-off at 515 nm) and 540/590 nm (cut-off at 570 nm) for ratio analysis[12].

Caspase-3 activity assay

Caspase-3 enzyme activity assay was performed to evaluate whether TMX was effectively induced in colon cancer cells. According to the manufacturer's protocol, the assay was performed with the caspase-3 colorimetric assay kit (Abcam kit no. ab39401) to detect the caspase-3 enzyme activity. Briefly, after 24 h of different treatments, the supernatant was removed and the cells were trypsinized. Then they were collected and centrifuged at 14,000 rpm for 5 min. Next, cell-lysis buffer which was supplied with the kit was added and the cells were retained on ice and centrifuged. Finally, protein concentration was calculated using Bradford assay[13].

Flowcytometric analysis of apoptosis

Validation of apoptosis was done by quantitating through dual staining of annexin V/PI staining. Cells were treated with IC-50 dosage of TMX, 5FU and a combination of TMX and 5FU. Cells were washed with PBS and incubated with PI and Annexin-V at 37°C for 15 min. Then they were analysed by FACS Aria II using Cell Quest software[14].

Evaluation of cellular damage by quantitating intracellular ROS generation

Cancer cells have higher ROS content than normal cells as they are much more metabolically active to facilitate cancer cell proliferation, survival and adaptation. Therefore, if ROS is further increased, it leads to the death of cancer cells through apoptosis[15]. The relative level of ROS was measured using DCFH-DA dye and quantitated using spectrofluorimetric (Cary Varian) analysis of both cancer and normal cells, following the method previously used in our laboratory[16].

Is apoptosis mediated by TMX was ROS-dependent

NAC (*N*-acetyl-L-cysteine) is commonly used to identify and test ROS inducers and to inhibit ROS. Previous studies revealed that NAC was able to scavenge intracellular ROS of cells at a concentration of 3 μM (ref. 17). So this dose was chosen in the present study along with the IC-50 dosage of TMX to validate whether TMX-induced apoptosis is ROS-dependent.

Evaluation of chemosensitization potential of TMX

The effect (synergistic, additive or antagonastic) TMX exerts in combination with chemotherapeutic drug 5FU is calculated by combination index (CI).

$$CI = (D)1/(D\chi)1 + (D)2/(D\chi)2,$$

where $(D\chi)1$ and $(D\chi)2$ are the concentration of each drug alone to exert χ% effect, while $(D)1$ and (D)2 are the concentration of the drugs in combination to elicit the same effect[18].

Quantitation of mRNA of different genes

RNA was isolated from both normal and cancer colon cells using Roche high-pure RNA isolation kit, according to the Roche life science kit no. 11828665001 protocol. cDNA synthesis was done and real-time analysis was performed using cDNA Synthesis Kit and FastStart Essential DNA Green Master (Roche Life Science) respectively, according to the manufacturer's protocol. All the data were normalized using GAPDH as an internal standard. The data were acquired in the light cycle (Roche). Table 1 provides the list of primers used for qRT-PCR.

Statistical analysis

Statistical analysis was performed to find any significance in the present study. A *t*-test was used for the analysis, where $P < 0.005$ was considered to be statistically significant. Data were represented as mean with standard deviation (SD) of at least three different experiments.

Results

Cellular cytotoxicity analysis upon treatment with TMX

The cytotoxic effect of TMX on colon cell lines, both normal and cancerous, was measured using MTT assay. This revealed that TMX exerted selective cytotoxicity, i.e. it was cytotoxic to cancer cells as evident by their low IC-50 values, such as 5 μM for CACO2, 3 μM for HCT116, 3.8 μM for HT29, 3.9 μM for SW480 and 3.5 μM for SW837, whereas it remained non-toxic to CCD-18Co cell line up to a concentration of 60 μM. This dose was much higher than the IC-50 dosage for cancerous cell lines. Therefore, for normal cell lines 60 μM and for cancer cells, their respective IC-50 dosages were taken as the dose of TMX for further downstream analysis. Here 5FU, a conventional chemotherapeutic drug, was used as the positive control. 5FU showed IC-50 which is comparable with that of TMX against colon cancer cell lines. But unlike TMX, 5FU showed toxicity to normal cell line CCD-18Co with IC-50 of 9 μM (Figure 1 *a* and *b*).

Cell-cycle analysis upon treatment with TMX

Cell-cycle analysis by FACS using PI staining was performed on both normal and cancer colon cell lines. This validated the results obtained in the MTT assay, which showed TMX effectively induced apoptosis to colon cancer cells as evidenced by 40% SUBG0 population in CACO2 cells, 45% SUBG0 population in HCT-116, 41% SUBG0 population in HT29, 47% SUBG0 population in SW480, 43% SUBG0 population in SW837, and which significantly decreased to 2% SUBG0 population in normal cell line CCD-18Co (Figure 1 *c* and *d*).

Effect of TMX treatment on intracellular ROS generation

TMX treatment significantly increased intracellular ROS generation in cancer cells, while no such modulation was observed in normal colon cells. The increased intracellular ROS upon TMX treatment in cancer cells indicated cellular cytotoxicity, which may drive the cells towards apoptosis (Figure 1 *e*).

Effect of TMX treatment on mitochondrial membrane potential

JC-10 is capable of selectively entering the mitochondria and reversibly changing its colour from green to orange as the membrane potential increases. This property is due to the reversible formation of JC-10 aggregates upon membrane polarization that causes shifts in emitted light from 520 nm (i.e. emission of JC-10 monomeric form) to 570 nm (i.e. emission of J-aggregate form). When excited at 490 nm, the colour of JC-10 changes reversibly from green to greenish-orange as the mitochondrial membrane

Table 1. List of primers used for qRT-PCR

Gene	Primer
PCNA	F.P. CTGCAGATGTACCCCTTGGT
	R.P. ACAACTGAAAGACAGGAAGATGGT
Cyclin D1	F.P. GTGCTGTCTGGGAAGATGTCC
	R.P. ACCCATTCTGGAATACCGGC
P21	F.P. GACATGTGCACGGAAGGACT
	R.P. GGGCAGGGTGACAAGAATGT
BCL2	F.P. CAGGCAATGGAAAAGGGCAA
	R.P. AGGTTAATGAGACCGGGGGA
BAX	F.P. TTGGTGATGTGAGTCTGGGC
	R.P. AACGCTTTGTCCAGAGGAGG
P53	F.P. CTTCTTTGGCTGGGGAGAGG
	R.P. CTTCTTTGGCTGGGGAGAGG

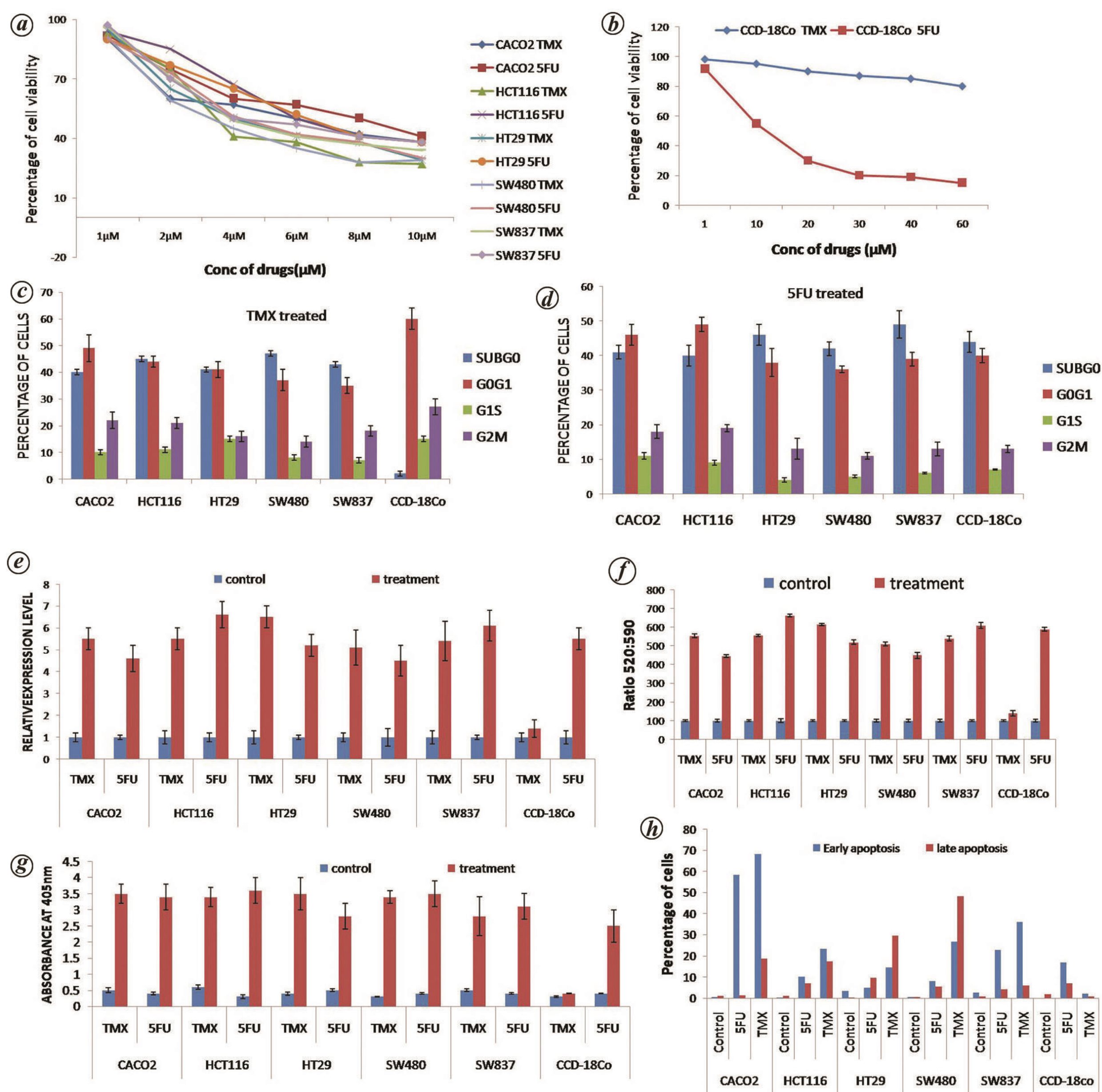

Figure 1. ***a***, Cellular viability assay using MTT analysis (cytotoxic effects) of different dosages of TMX and 5FU after 48 h of treatment against CACO2, HCT116, HT29, SW480 and SW837. Data are presented as mean ± SD. Each experiment was done in triplicate and repeated at least three times. ***b***, Cellular viability assay by MTT analysis (cytotoxic effects) of different dosages of TMX and 5FU after 48 h of treatment against normal colon CCD-18Co. Data are presented as mean ± SD. Each experiment was done in triplicate and repeated at least three times. ***c***, Cell-cycle phase distribution upon treatment with IC-50 dosage of TMX and 5FU against colon cancer cells determined by propidium iodide staining in FACS. Data are represented as ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***d***, Cell-cycle phase distribution upon treatment with the highest non-toxic dosage of TMX and IC-50 dosage of 5FU against normal colon cells determined by propidium iodide staining in FACS. Data are represented as ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***e***, Intracellular ROS generation determination in the CACO2, HCT116, HT29, SW480, SW837 and CCD-18Co cells by DCFH-DA staining after treatment with TMX at IC-50 dosage for cancer cell lines and the highest dosage of 60 μM for normal cell lines. Data were taken 48 h after TMX treatment. Each value shows mean ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***f***, Mitochondrial membrane potential measured in the CACO2, HCT116, HT29, SW480, SW837 and CCD-18Co cells by JC1 staining after treatment with TMX at IC-50 dosage for cancer cell lines and the highest dosage of 60 μM for the normal cell line. Data were taken 48 h after TMX treatment. Each value shows mean ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***g***, Activity of caspase-3 measured in the CACO2, HCT116, HT29, SW480, SW837 and CCD-18Co cells by caspase-3 activation assay after treatment with TMX at IC-50 dosage for cancer cell lines and the highest dosage of 60 μM for the normal cell line. Data were taken 48 h after TMX treatment. Each value shows the mean ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***h***, Induction of apoptosis upon treatment with TMX analysed in colon cell lines by annexin V/PI staining. The respective IC-50 dosage was used for colon cancer cell lines and a high dose 60 μM was used for the normal colon cell line. Data were taken 48 h after TMX treatment. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times.

becomes more polarized. Increased green fluorescence upon TMX treatment indicated a higher number of monomeric forms followed by a lower number of J-aggregates. An increase in the number of monomeric forms of JC-10 upon TMX treatment is indicative of apoptosis (Figure 1 *f*).

Effect of TMX treatment on caspase-3 activation assay

Caspase-3 is a critical indicator of apoptosis, as it is responsible for the proteolytic cleavage of many essential proteins. TMX treatment selectively induced apoptosis in cancer cells while eliciting no effect in normal colon cells, while 5FU indiscreetly induced apoptosis in normal and cancer cells (Figure 1 *g*).

Flowcytometric validation apoptosis induction upon treatment with TMX

The effect of TMX treatment on apoptosis induction in colon cell lines was studied by annexin V/PI staining in flowcytometry. As in previous experiments it was observed that TMX treatment could induce apoptosis much more efficiently than 5FU in all colon cancer cell lines. TMX treatment showed no apoptosis in normal colon cell line at a high dosage of 60 μM in contrast to 5FU which showed induction of apoptosis in normal cell line (Figure 1 *h*).

Validation of cellular cytotoxicity induced by TMX in cancer cells as ROS generated

Cellular cytotoxicity induced by TMX in cancer cells was significantly downregulated upon co-treatment of ROS scavenger NAC at a dosage of 3 μM (Figure 2 *a*). The data were validated by cell-cycle analysis, which showed a dramatic decrease in SUBG0 followed by an increase in G0G1 upon co-treatment with NAC. This signifies that upon treatment with ROS scavenger and TMX apoptosis induction, TMX is abrogated, thereby leading to the conclusion that apoptosis induction in colon cancer cells upon TMX treatment is ROS-dependent (Figure 2 *b*). The fact that co-treatment of NAC and TMX scavenged intracellular ROS was proved by staining with di-chlorofluorescein-diacetate (DCFH-DA), which showed a significant decrease in intracellular ROS in cancer cell lines, whereas no change in intracellular ROS was observed in a normal cell line (Figure 2 *c*). Furthermore, upon co-treatment with NAC, there was an increase in red fluorescence, i.e. formation of J-aggregates, which indicates healthy cells. This proves that co-treatment with NAC-restricted TMX induces apoptosis in cancer cells (Figure 2 *d*).

Molecular mechanism of induction of apoptosis by TMX against colon cancer cell lines

The molecular mechanism of apoptosis by TMX was studied by analysing the RNA expression of proliferation and apoptosis-related genes using qRT-PCR. Upon treating the colon cancer cells with TMX, PCNA which is a cell proliferation marker was significantly downregulated. PCNA interacts with P21, thereby sequestering P21. Upon severe downregulation of PCNA, it cannot sequester P21 which in turn transactivate P53, thus leading to apoptosis[19]. Here P53–P21-mediated apoptosis was seen in all the colon cancer cell lines upon TMX treatment, followed by downregulation of PCNA. Apoptosis was validated by downregulation of BCL2 and followed by upregulation of BAX. Cyclin D1 is a proliferation marker that is highly expressed in cancer cells, as demonstrated by heightened G0G1 in all cancer cells, and is significantly downregulated by TMX treatment (Figure 2 *e*).

Chemosensitization effect of TMX on treatment with 5FU in cancer cells

CaCo2 cell line separately showed IC-50 against TMX and 5FU at 6 and 8 μM respectively, but when given together, their joint IC-50 against the CaCo2 cell line decreased to 0.5 μM for TMX and 1 μM for 5FU. Therefore CI for TMX and 5FU for CaCO2 cells reduced to 0.2, which signifies a synergistic effect. Individually IC-50 for TMX for HCT-116, HT-29, SW 480 and SW 837 are 4 μM, 4 μM, 5 μM and 3 μM and the IC-50 for 5FU for same cell lines are 6 μM, 5 μM, 4 μM and 4 μM. When these drugs were given together their combined IC-50 for the respective cell lines such as HCT 116, HT29, SW480 and SW837 was 0.7 μM TMX and 1.2 μM 5FU, 0.5 μM TMX and 1 μM 5FU, 0.5 μM TMX and 1 μM 5FU, 1 μM TMX and 2 μM 5FU respectively. Therefore CI values for these two drugs for the cell lines were calculated according to the previously described formula and were found to be 0.375, 0.325, 0.35 and 0.833 respectively. Thus, in all the colon cancer cell lines, co-treatment with TMX and 5FU showed a synergistic effect. Thus TMX treatment sensitizes the cancer cells against treatment with 5FU (Figure 3 *a*). As in individual therapy, combining 5FU and TMX did not elicit any toxicity against the normal cell lines (Figure 3 *b*). The synergistic effect of TMX and 5FU co-treatment significantly increased intracellular ROS in cancer cells, while it did not elicit any effects on cancer cells. The level of ROS generation in cancer cells upon co-treatment with 5FU and TMX was much higher than when separately applied (Figure 3 *c*). Caspase-3 activation assay is one of the confirmatory assays for apoptosis. The combined effect of TMX and 5FU causes significant increased caspase-3 cleavage, which signifies apoptosis. The combined IC-50 group induced apoptosis at a much

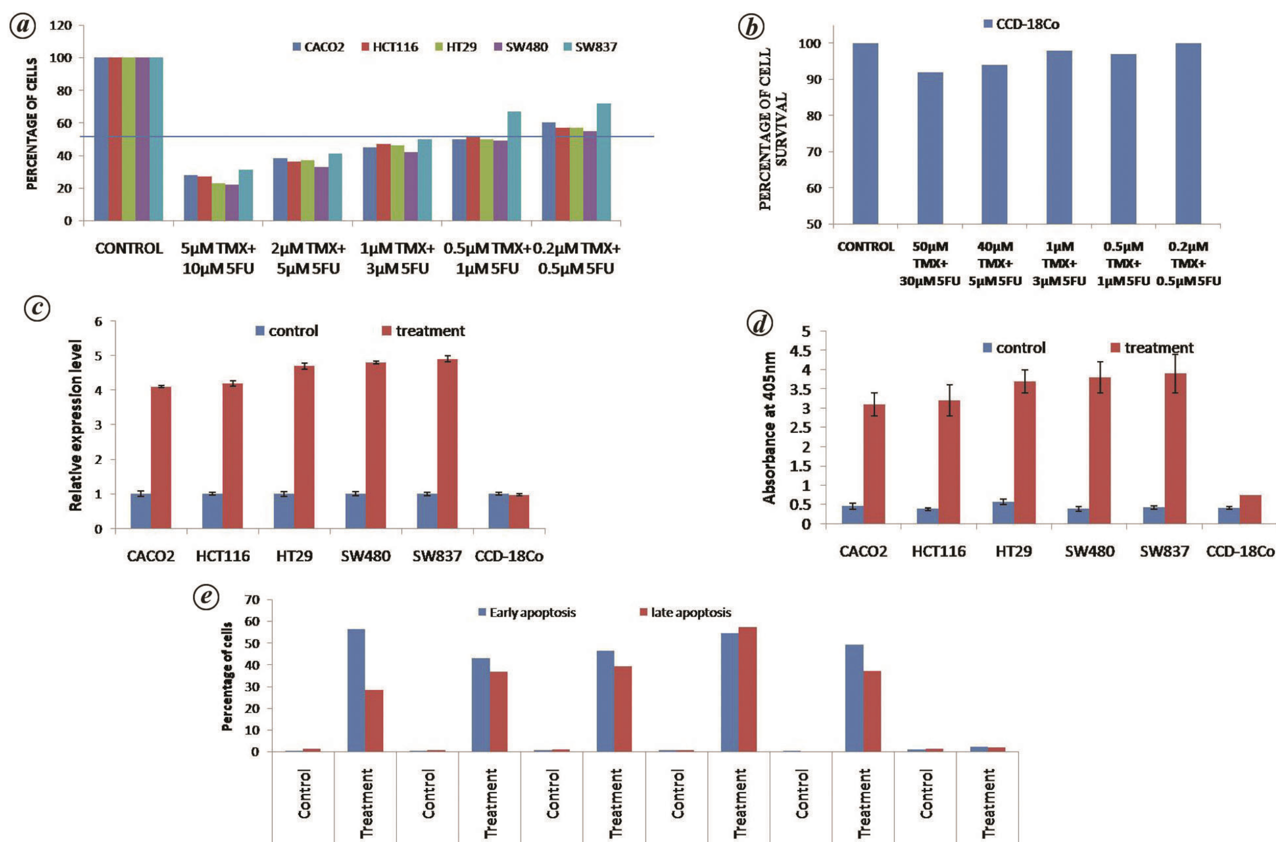

Figure 2. ***a***, Cellular viability assay by MTT analysis (cytotoxic effects) of TMX alone or co-treatment with ROS scavenger NAC measured after 48 h of treatment against CACO2, HCT116, HT29, SW480, SW837 and CCD-18Co cells. Data are presented as mean ± SD. Each experiment was done in triplicate and repeated at least three times. ***b***, Cell-cycle phase distribution upon treatment with IC-50 dosage of TMX and 3 µM NAC against both colon cancer and normal cells determined by propidium iodide staining in FACS. Data are represented as ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***c***, Intracellular ROS generation determination in the CACO2, HCT116, HT29, SW480, SW837 and CCD-18Co cells by DCFH-DA staining after treatment with TMX at IC-50 dosage and 3 µM NAC for cancer cell lines and highest dosage of 60 µM for the normal cell line. Data were taken 48 h after TMX treatment. Each value shows mean ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***d***, Mitochondrial membrane potential measured in the CACO2, HCT116, HT29, SW480, SW837 and CCD-18Co cells by JC1 staining after treatment with TMX at IC-50 and 3 µM NAC dosage for cancer cell lines and the highest dosage of 60 µM for the normal cell line. Data were taken 48 h after TMX treatment. Each value shows mean ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***e***, RT-PCR analysis to determine the effects of TMX on PCNA, cyclin D1, P21, P53, BAX and BCL-2 mRNA levels. Data are normalized for each respective cell line taking GAPDH as the internal control. Data are represented as mean fold changes ± standard deviation. *Significant difference to control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times.

higher rate than their counterparts (Figure 3 *d*). This observation inferred from the caspase-3 activation assay was validated by annexin V/PI staining, which showed synergism between 5FU and TMX treatment, since significant increase in apoptosis of cancer cells at a lower dosage of IC-50 was observed followed by decrease in toxic effects of 5FU in cancer cells (Figure 3 *e*).

Discussion

A Chinese herbal medicine combined with 5FU, compared with 5FU alone, reduced the risk of death in different stages of colon cancer patients: stage I by 95%, stage II by 64%, stage III by 29% and stage IV by 75% (ref. 20). Thus natural cytotoxic products with proven therapeutic potential increase the efficacy of conventional chemotherapeutic drugs, and this type of combinatorial treatment enables overcoming the drawbacks of traditional chemotherapeutics[21]. Chemotherapeutic drugs work as an antimetabolite to avert cell proliferation; they work by downregulating the enzyme thymidylate synthase blocking thymidine formation required for DNA synthesis. 5FU has a short half-life but very high diffusion potential, enabling it to impart systemic toxicity in the host body, which is one of the major drawbacks that overshadows its efficacy[22]. Natural products possess the dual role of cytoprotection to normal cells while being cytotoxic to cancer cells by increasing ROS and targeting several key molecules[23]. Most of the time, the molecules targeted by natural

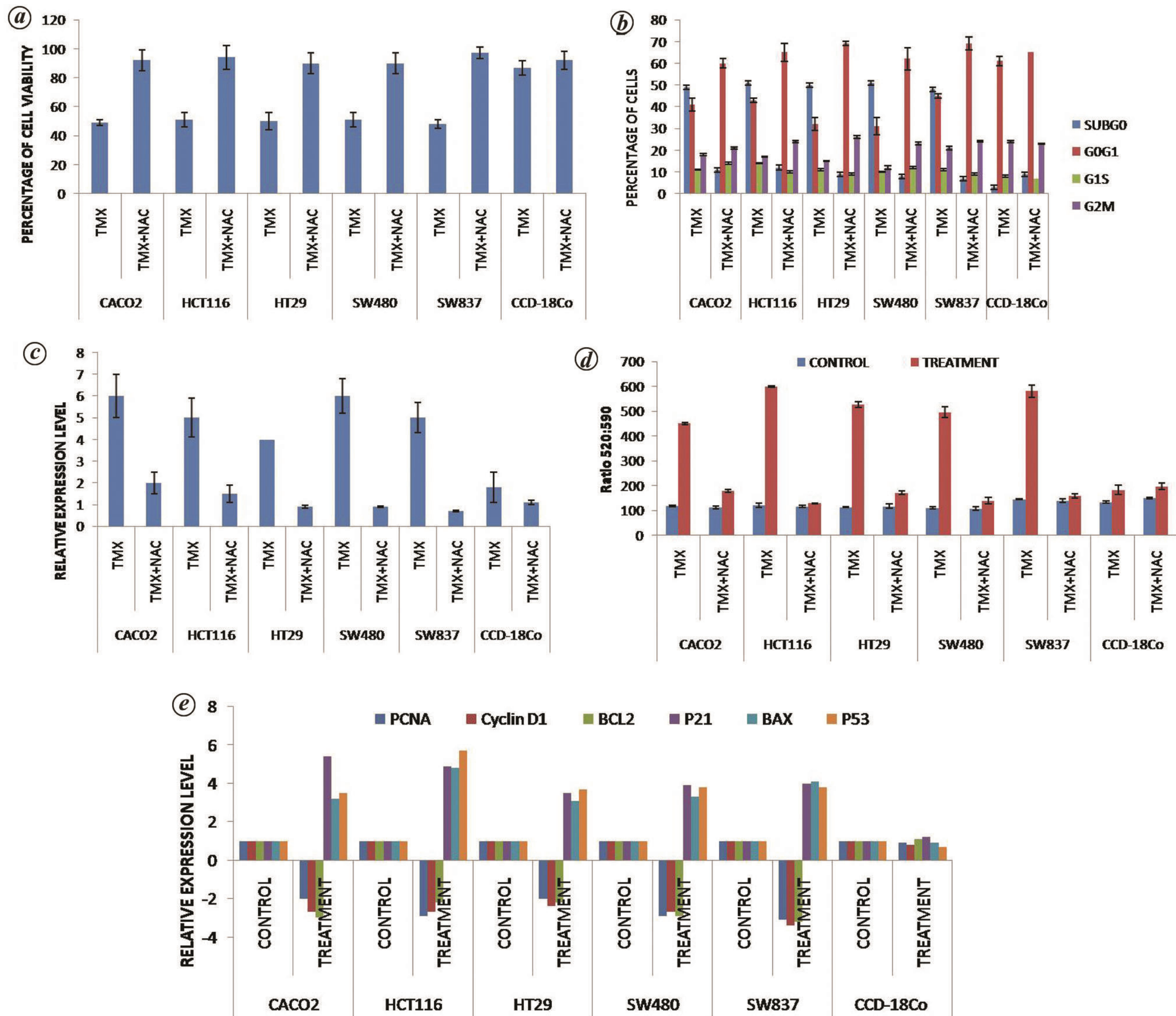

Figure 3. ***a***, Cellular viability assay by MTT analysis (cytotoxic effects) in combination with different doses of TMX and 5FU measured after 48 h of treatment against CACO2, HCT116, HT29, SW480 and SW837. Data are as mean ± SD. Each experiment was done in triplicate and repeated at least three times. ***b***, Cellular viability assay by MTT analysis (cytotoxic effects) in combination with different doses of TMX and 5FU measured after 48 h of treatment against CCD-18Co cells. Data are presented as mean ± SD. Each experiment was done in triplicate and repeated at least three times. ***c***, Intracellular ROS generation determination in the CACO2, HCT116, HT29, SW480, SW837 and CCD-18Co cells by DCFH-DA staining after treatment with a combination of TMX and 5FU at IC-50 dosage for cancer cell lines and the highest dosage of 50 μM TMX+ 30 μM 5FU for the normal cell line. Data were taken 48 h after TMX treatment. Each value shows mean ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***d***, Activity of caspase-3 measured in the CACO2, HCT116, HT29, SW480, SW837, and CCD-18Co cells by caspase-3 activation assay after treatment with a combination IC-50 dosage of TMX and 5FU for cancer cell lines and the highest dosage of 50 μM TMX+ 30 μM 5FU for the normal cell line. Data were taken 48 h after TMX/5FU treatment. Each value shows the mean ± S.D. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times. ***e***, Induction of apoptosis upon co-treatment of TMX and 5FU analysed in colon cell lines by annexin V/PI staining with a combination IC-50 dosage of TMX and 5FU for cancer cell lines and the highest dosage of 50 μM TMX+ 30 μM 5FU for the normal cell line. Data were taken 48 h after TMX/5FU treatment. Each value shows mean ± SD. *indicates a significant difference versus untreated control ($P < 0.005$). Each experiment was done in triplicate and repeated at least three times.

molecules impart chemoresistance against the conventional chemotherapeutic drugs. Therefore, several reports suggest that prophylactic use of natural products imparts protection against diseases and may increase usage of traditional chemotherapeutic drugs[24]. So a natural product with proven therapeutic efficacy that can combat the drawbacks and improve the effectiveness, i.e. exert synergistic action with the conventional chemotherapeutics, is the need of the hour. *S. chirata* is an ancient medicinal plant with proven multiferous uses. The main reason for its medicinal properties is the large reservoir of different groups of bioactive compounds. Among the group of bioactive compounds present in *S. chirata*, xanthones form an important group. Among the xanthones present, TMX

has been proven to be most bioactive with cytotoxic efficacy against breast and skin cancer cell lines while being non-toxic to normal cells[11]. In this study, TMX was able to exert its cytotoxic potential effectively on several cancer cell lines, while being non-toxic to normal colon cell lines. TMX showed comparable cytotoxic potential with the conventional chemotherapeutic drug 5FU. However, unlike 5FU, it was non-toxic to normal colon cells. This observation was validated by analysis related to the cell cycle, ROS generation and apoptosis. Here ten times (approximately) higher dosage of TMX than that which is cytotoxic to cancer cells was found to be non-toxic to normal colon cells. This observation was also validated by analysis related to the cell cycle, ROS and apoptosis. NAC is a free-radical scavenger that can be used to evaluate the effect of experimental drug-induced ROS generation on apoptosis. NAC pre-treatment is reported to effectively diminish cellular ROS and confirm the role of oxidative stress in drug treatment[25]. In the present study, NAC co-treatment abrogated TMX-induced apoptosis in all colon cancer cell lines; again, no effect on normal colon cell lines was seen with or without co-treatment of NAC and TMX. This observation was validated by analysis related to cell cycle, ROS and apoptosis. ROS upregulation can result in oxidative damage to mitochondrial and cellular proteins. In this study, ROS upregulation upon TMX treatment caused damage to the mitochondrial membrane proteins which in turn leads to the loss of mitochondrial membrane potential of cancer cells[26]. This observation was validated by JC-1 staining[27]. Molecular analysis showed that TMX restricts the colon cancer cell proliferation by downregulating proliferation marker PCNA which in turn upregulates P21, which transactivates tumour suppressor P53 which in turn drives the cell towards apoptosis. Upon establishing the efficacy of plant-derived small-molecule TMX, we examined whether it could increase the efficacy of conventional chemotherapeutic drug 5FU. For quantifying drug interactions between TMX and 5FU, and classifying the interactions of synergy, additivity or antagonism, CI was calculated[18]. Here it was found that for all the colon cancer cell lines, co-treatment of TMX and 5FU yielded a CI value that was less than that of 1, signifying synergism. Further validation of synergism was seen in a normal cell line which showed that upon co-treatment with TMX, cytotoxic effects of 5FU decreased significantly.

Conclusion

In conclusion, TMX from *S. chirata* successfully induced apoptosis in colon cancer cell lines while being non-toxic to the normal cell line. Further analysis revealed that this apoptosis induction was ROS-dependent, which was validated by co-treatment of ROS scavenger NAC with TMX. The chemotherapeutic potential of TMX was further validated by showing that it acts in synergism with 5FU by increasing its efficacy and decreasing its cytotoxic potential to normal cells.

Conflicts of interest: The authors declare that they have no conflict of interest.

1. Patil, P. S. *et al.*, Colorectal cancer in India: an audit from a tertiary center in a low prevalence area. *Indian J. Surg. Oncol.*, 2017, **8**, 484–490.
2. Brinkhaus, B. *et al.*, World Congress on Integrative Medicine & Health 2017: Part one. *BMC Complement. Altern. Med.*, 2017, **17**, 322.
3. Atanasov, A. G. *et al.*, Discovery and resupply of pharmacologically active plant-derived natural products: a review. *Biotechnol. Adv.*, 2015, **33**, 1582–1614.
4. Cossarizza, A. *et al.*, Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol.*, 2019, **49**, 1457–1973.
5. Atanasov, A. G., Zotchev, S. B., Dirsch, V. M. and Supuran, C. T., Natural products in drug discovery: advances and opportunities. *Nature Rev. Drug Discov.*, 2021, **20**, 200–216.
6. Barua, A., Choudhury, P., Mandal, S., Panda, C. K. and Saha, P., Therapeutic potential of xanthones from *Swertia chirata* in breast cancer cells. *Indian J. Med. Res.*, 2020, **152**, 285–295.
7. Barua, A., Choudhury, P., Mandal, S., Panda, C. K. and Saha, P., Anti-metastatic potential of a novel xanthone sourced by *Swertia chirata* against *in vivo* and *in vitro* breast adenocarcinoma frameworks. *Asian Pac. J. Cancer Prev.*, 2020, **21**, 2865–2875.
8. Barua, A., Choudhury, P., Panda, C. K. and Saha, P., Chemotherapeutic potential of novel xanthone sourced from *Swertia chirata* against skin carcinogenesis. *Asian J. Pharm. Clin. Res.*, 2020, **13**, 84–88.
9. Fang, C.-Y., Wu, C.-C., Fang, C.-L., Chen, W.-Y. and Chen, C.-L., Long-term growth comparison studies of FBS and FBS alternatives in six head and neck cell lines. *PLoS ONE*, 2017, **12**, e0178960.
10. Mohammadian, M., Feizollahzadeh, S., Mahmoudi, R., Toofani Milani, A., Rezapour-Firouzi, S. and Karimi Douna, B., Hsp90 inhibitor; NVP-AUY922 in combination with doxorubicin induces apoptosis and downregulates VEGF in MCF-7 breast cancer cell line. *Asian Pac. J. Cancer Prev.*, 2020, **21**, 1773–1778.
11. Barua, A., Choudhury, P., Maity, J. K., Mandal, S. B., Mandal, S. and Saha, P., Chemotherapeutic potential of novel non-toxic nucleoside analogues on EAC ascitic tumour cells. *Free Radic. Res.*, 2019, **53**, 57–67.
12. Park, J. W. *et al.*, 99mTc-MIBI uptake as a marker of mitochondrial membrane potential in cancer cells and effects of MDR1 and verapamil. *PLoS ONE*, 2020, **15**, e0228848.
13. Ernst, O. and Zor, T., Linearization of the Bradford protein assay. *J. Vis. Exp.*, 2010.
14. Ray, T., Kar, D., Pal, A., Mukherjee, S., Das, C. and Pal, A., Molecular targeting of breast and colon cancer cells by PAR1 mediated apoptosis through a novel pro-apoptotic peptide. *Apoptosis*, 2018, **23**, 679–694.
15. Zhang, Z., Guo, M., Zhao, S., Shao, J. and Zheng, S., ROS-JNK1/2-dependent activation of autophagy is required for the induction of anti-inflammatory effect of dihydroartemisinin in liver fibrosis. *Free Radic. Biol. Med.*, 2016, **101**, 272–283.
16. Yamamoto, T. *et al.*, Roles of catalase and hydrogen peroxide in green tea polyphenol-induced chemopreventive effects. *J. Pharmacol. Exp. Ther.*, 2004, **308**, 317–323.
17. Halasi, M., Wang, M., Chavan, T. S., Gaponenko, V., Hay, N. and Gartel, A. L., ROS inhibitor *N*-acetyl-l-cysteine antagonizes the activity of proteasome inhibitors. *Biochem. J.*, 2013, **454**, 201–208.

18. Huang, L., Jiang, Y. and Chen, Y., Predicting drug combination index and simulating the network-regulation dynamics by mathematical modeling of drug-targeted EGFR-ERK signaling pathway. *Sci. Rep.*, 2017, **7**, 40752.
19. Li, R., Hannon, G. J., Beach, D. and Stillman, B., Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. *Curr. Biol.*, 1996, **6**, 189–199.
20. McCulloch, M. *et al.*, Colon cancer survival with herbal medicine and vitamins combined with standard therapy in a whole-systems approach: ten-year follow-up data analyzed with marginal structural models and propensity score methods. *Integr. Cancer Ther.*, 2011, **10**, 240–259.
21. Senapati, S., Mahanta, A. K., Kumar, S. and Maiti, P., Controlled drug delivery vehicles for cancer treatment and their performance. *Signal Transduct. Target Ther.*, 2018, **3**, 7.
22. Zhang, N., Yin, Y., Xu, S.-J. and Chen, W.-S., 5-Fluorouracil: mechanisms of resistance and reversal strategies. *Molecules*, 2008, **13**, 1551–1569.
23. Zorov, D. B., Juhaszova, M. and Sollott, S. J., Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiol. Rev.*, 2014, **94**, 909–950.
24. Greenwell, M. and Rahman, P. K. S. M., Medicinal plants: their use in anticancer treatment. *Int. J. Pharm. Sci. Res.*, 2015, **6**, 4103–4112.
25. Chang, H.-W. *et al.*, Withaferin A induces oxidative stress-mediated apoptosis and DNA damage in oral cancer cells. *Front. Physiol.*, 2017, **8**, 634.
26. Guo, C., Sun, L., Chen, X. and Zhang, D., Oxidative stress, mitochondrial damage and neurodegenerative diseases. *Neural Regen. Res.*, 2013, **8**, 2003–2014.
27. Tan, H.-Y., Wang, N., Li, S., Hong, M., Wang, X. and Feng, Y., The reactive oxygen species in macrophage polarization: reflecting its dual role in progression and treatment of human diseases. *Oxid. Med. Cell. Longev.*, 2016, e2795090.

ACKNOWLEDGEMENTS. We thank Dr Koustubh Panda, Department of Biotechnology (University of Calcutta, Kolkata) for encouragement; Dr Sukta Das, Cancer Foundation of India for support; Dr Jayanta Chakrabarti (Director Chittaranjan National Cancer Institute, Kolkata) for providing infrastructural support. No funding was received for this study. This study was performed at Chittaranjan National Cancer Institute, NICED Kolkata and University of Calcutta.

Received 13 August 2021; revised accepted 12 November 2021

doi: 10.18520/cs/v122/i1/47-55