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Ameliorated in vitro anticancer efficacy of methotrexate D- α -Tocopheryl polyethylene glycol 1000 succinate ester against breast cancer cells

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Abstract

Background: Methotrexate (MTX), a folate anti-metabolite, has been used widely in the treatment of plenty of malignancies. However, the clinical use is limited because of its poor water solubility (BCS class II drug), nonspecific distribution, drug resistance, short circulation half-life, and toxicity. The objective of the present research was to synthesize the ester prodrug of MTX with D- α -Tocopheryl polyethylene glycol 1000 succinate (TPGS) and characterize for in vitro anticancer efficacy.

Results: The FTIR and NMR results revealed the successful synthesis of the prodrug. The assay and saturation solubility of the prodrug is found to be $23 \pm 2.5\%$ and 6.7 ± 1.3 mg/mL (MTX equivalent) respectively. The CMC of the prodrug in distilled water at room temperature is found to be 36.9 ± 2.6 μ g/mL. The prepared prodrug micelles showed a mean particle size of 166 ± 10 nm (PDI, 0.325 ± 0.09). Further, the TEM results confirmed the self-assembling character of the prodrug into micelles with a nearly spherical shape. The prodrug caused the significantly ($p < 0.01$) less hemolysis ($16.8 \pm 1.5\%$) when compared to plain MTX solution and significantly higher ($p < 0.01$) in vitro cytotoxicity, cell cycle arresting, and apoptosis against human breast cancer cells (MCF-7 and MDA-MB-231).

Conclusion: Our study results revealed the remarkable in vitro anticancer activity of MTX following its esterification with TPGS. However, further, in vivo studies are needed to prove its efficacy against different cancers.

Keywords: Methotrexate prodrug, TPGS, Cytotoxicity, Cell cycle analysis and apoptosis

Background

Cancer, uncontrollable growth of cells in the body, is one of the second major causes of death worldwide [1]. Among various conventional cancer treatment approaches, chemotherapy plays a vital role in the treatment of a variety of cancers [2]. Methotrexate (MTX), a folate anti-metabolite, has been primarily used in the treatment of rheumatoid arthritis. Besides, it is also used in the treatment of plenty of malignancies such as osteosarcoma, Hodgkin's disease, non-Hodgkin's lymphoma, and childhood acute lymphocytic leukemia. However, the clinical use of MTX is limited because of its poor water solubility (BCS class II drug), nonspecific distribution, drug

resistance, short circulation half-life, and toxicity. Besides, the multidrug resistance (MDR) is the prime cause of chemotherapy failure, including MTX [3–5].

Among the several novel nanoformulations approaches developed to overcome above conventional chemotherapeutic drug-related problems, the polymer-drug conjugate [6, 7] approach results in improved drug solubility, loading capacity, pharmacokinetic and dynamic characteristics, permeability, stability, targeting, and cancer cell sensitivity [1]. Also, these polymer-drug conjugates self-assemble into micelles in aqueous media and thus are used alone as drug delivery systems.

D- α -Tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS or simply TPGS) is an amphiphilic substance that self-assemble into micelles in aqueous media. Besides, it is an FDA-approved safe pharmaceutical

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adjuvant. The TPGS has been used in several formulations as a P-glycoprotein (P-gp) inhibitor to treat MDR cancers and to improve drugs oral bioavailability. The steric blocking of substrate binding [8], alteration of membrane fluidity, and inhibition of efflux pump ATPase are the few proposed potential mechanisms behind P-gp inhibition [9, 10]. For instance, the polymeric mixed micelles (PMMs) prepared with P-gp inhibitor (TPGS) are reported to reverse the MDR and increase the influx of drugs into tumor cells. It is also documented that the succinate esters of vitamin E are potent pro-apoptotic agents selective for cancer cells [9, 11]. Recently, Lorena and co-workers have proved the enhanced in vitro cytotoxicity of MTX-loaded deoxycholate-TPGS mixed nanomicelles against breast cancer cells [12]. Similarly, Sameer and co-workers have developed the lipid and TPGS-based novel core-shell type nanocapsular sustained release system of MTX for intravenous application [13]. Thus, the current study was aimed to synthesize MTX-TPGS conjugate and investigate its in vitro anticancer efficacy against MTX-sensitive (MCF-7) and MDR (MDA-MB-231) breast cancer cells.

Methods

Materials

Methotrexate was gifted by Emcure Pharmaceuticals Ltd., Pune. D- α -Tocopheryl polyethylene glycol 1000 succinate (TPGS), N,N'-dicyclohexylcarbodiimide (DCC) and 4-(Dimethylamino)pyridine (DMAP), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Annexin V-FITC, and propidium iodide (PI) were purchased from Sigma Aldrich, Mumbai. Dimethylformamide (DMF), HPLC grade acetonitrile, methanol, and water were purchased from Molychem, Mumbai. All other reagents used were of analytical reagent grade and were used without further purification.

Cell culture

The MTX-sensitive breast cancer cell line (MCF-7) and MDR epithelial human triple-negative breast cancer cell line (MDA-MB-231) were procured from ATCC, USA. Stock cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (100 μ g/mL) in a humidified atmosphere of 5% CO₂ at 37 °C until confluent.

Synthesis of MTX-TPGS conjugate (MTC)

The MTC was synthesized using the Steglich esterification reaction as per the previous report [14, 15]. The MTX and TPGS were reacted at 1:1 molar ratio in DMF containing DCC and DMAP at the same molar concentration. The prodrug synthesized was then confirmed using FTIR and NMR techniques.

Characterization of MTC

Assay

To determine the amount of MTX present in the conjugate, the 100 ppm and 50 ppm solutions of MTC in methanol were analyzed using a UV-visible spectrophotometer at 301 nm.

Aqueous saturation solubility

After shaking the saturated solutions of MTC for 24 h, the supernatants were analyzed using a UV-visible spectrophotometer at 301 nm.

Critical micelle concentration (CMC)

The CMC of MTC was determined using the iodine UV-visible spectrophotometric method as per the previous report [13]. Briefly, MTX-TPGS conjugate solutions of varying concentrations were prepared in double-distilled water and mixed with the same volume of standard KI/I₂ solution (25 μ L) and equilibrated for 12 h in the dark at room temperature. Finally, the absorbance of all samples was recorded at 366 nm using UV-visible spectrophotometer [15, 16].

Preparation of MTC micelles

MTC micelles were prepared using the film dispersion method. Briefly, 2.0 mg of MTC conjugate was dissolved separately in beakers containing 5 mL of methanol. The solvent was then allowed to evaporate at room temperature. The resultant film at the bottom of the beaker was then dispersed by adding double-distilled water (10 mL). The resultant solution was then bath sonicated for 5 min and centrifuged at 5000 rpm to obtain the clear supernatant micellar solution.

Mean particle size and zeta potential analysis

The mean particle size and zeta potential of prepared MTC micelles were determined using the nanoparticle analyzer, Horiba SZ-100 (Horiba Scientific, Japan). The measurements were performed in triplicate at 25 °C.

Confirmation of self-assembling nature by TEM

The self-assembling nature of MTC was assessed by TEM (FEI Tecnai T-20ST). Briefly, the air-dried micellar solution on a copper grid was mixed with a negative staining solution (phosphotungstic acid solution, 2% w/v), air-dried, and observed under TEM.

In vitro hemolysis study

The hemolytic ability of MTX and MTC solutions (5 and 50 μ g/mL in PBS, pH 7.4) was determined using human RBCs in comparison to negative control and positive control. The hemolytic effect was determined, after 1 h incubation at 37 °C, by measuring the supernatant absorbance at 420 nm [14, 15].

In vitro cytotoxicity

The cytotoxicity of MTX and MTC against human breast cancer cell lines (MCF-7 and MDA-MB-231) was determined using the MTT dye reduction assay. The 50,000 cells/well were seeded into each well, and after overnight incubation, cells were treated with different concentrations of MTX and MTC for 24 h and 48 h in 5% CO₂ atmosphere at 37 °C. The cytotoxic effect and then the IC₅₀ values were determined as per the previous protocol [14, 15].

Cell cycle analysis

1 × 10⁶ cells (MDA-MB-231) were seeded and cultured for 24 h in a 6-well plate containing 2 mL of medium. Cells were then incubated with the drug solution (10 μM, 2 mL) prepared in complete medium for 48 h. Cells were then harvested and centrifuged at 2000 rpm for 5 min at room temperature, and the supernatant was discarded carefully retaining the cell pellet. The cell pellet was washed twice by re-suspending in 2 mL of 1× PBS. Cells were then fixed by re-suspending in 300 μL of sheath fluid followed by the addition of 1 mL of chilled 70% EtOH drop by drop with continuous gentle shaking, and another 1 mL of chilled 70% EtOH was added at once. The cells were then stored at 4 °C overnight and centrifuged at 2000 rpm for 5 min, and the pellet was washed twice with cold 1× PBS (2 mL). The cell pellet was then re-suspended in 450 μL of sheath fluid containing 0.05 mg/mL propidium iodide (PI) and 0.05 mg/mL RNase A and incubated for 15 min in the dark. The

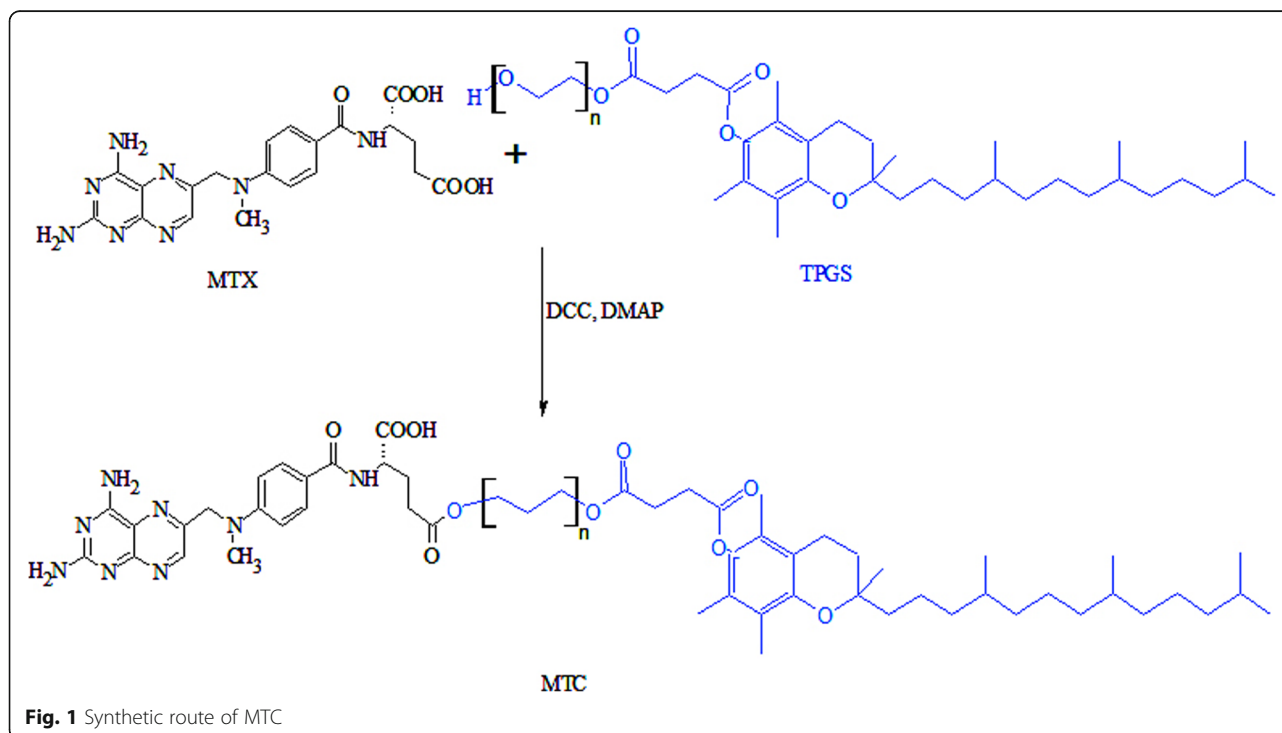
percentage of treated and untreated cell populations in various stages of the cell cycle was determined using FACS Caliber (BD Biosciences, San Jose, CA) [17].

Apoptosis study

1 × 10⁶ cells (MDA-MB-231) per well were seeded into a 6-well plate. After 24 h, the floating (dead) cells were removed by replacing the old culture medium with the new medium of the same volume containing drug solution (10 μM, 2 mL). After 48 h of incubation, the culture medium along with the cells was transferred into 15 mL tubes. The cell suspension was then centrifuged; cells were washed twice with cold PBS and then re-suspended in 1 mL of 1× Binding Buffer at a concentration of ~ 1 × 10⁶ cells/mL. Then, 500 μL of cell suspension was aliquoted and 10 μL of PI and 5 μL of Annexin V was added. The suspension was then incubated for 15 min at room temperature in the dark. Post incubation, the cells were analyzed by flow cytometer as soon as possible (within 1 h) [17].

In vitro release study

The in vitro release profile of MTX from MTC micelles was studied using the dialysis bag method and was compared with the plain MTX solution. Briefly, plain MTX solution prepared in phosphate buffer solution (PBS) of pH 7.4 and MTC micelles equivalent to 2 mg of MTX were placed into dialysis bags (MWCO = 12000 Da) and tightly sealed. The end-sealed dialysis bags were then submerged fully into 50 mL of release medium and PBS



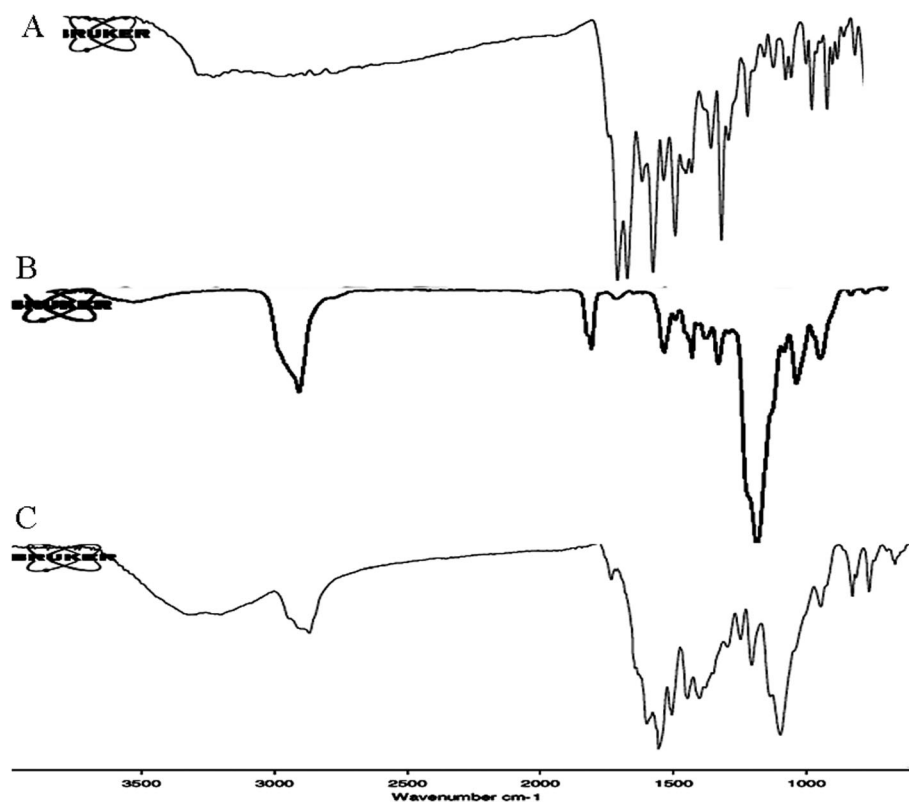


Fig. 2 FTIR spectrum of **a** MTX, **b** TPGS, and **c** MTC

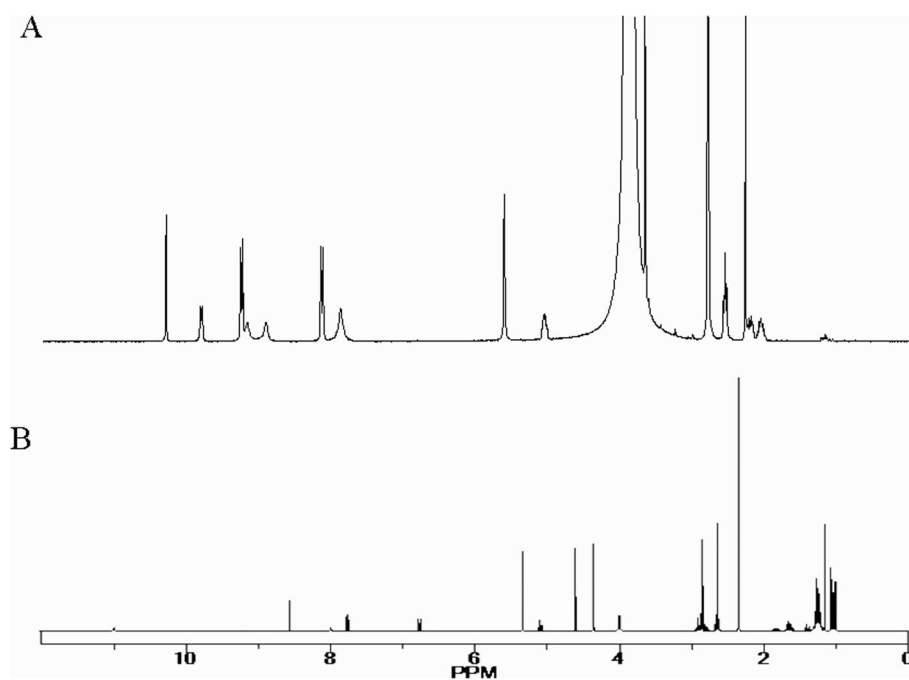


Fig. 3 NMR spectrum of **a** MTX and **b** MTC

of pH 7.4 containing 1% (w/v) tween-80 and maintained at $37 \pm 2^\circ\text{C}$. The release medium was kept under stirring at 150 rpm during the study. At pre-determined time intervals (1, 2, 4, 8, 16, and 24 h), 1 mL of sample was withdrawn from the release medium and the same volume was replaced with the fresh medium. The withdrawn samples were centrifuged at 2000 rpm for 5 min, and the supernatant was bath sonicated for 5 min and then analyzed using a UV-visible spectrophotometer at 301 nm. Experiments were performed in triplicate and release profiles were expressed in terms of percentage cumulative release and plotted vs. time [15].

Statistical analysis

Data are presented as the mean \pm standard deviation of three independent experiments. GraphPad Prism software version 8 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. The obtained results were analyzed using one-way ANOVA. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Synthesis and characterization of MTC

Figure 1 illustrates the synthetic route of MTC. The FTIR and ^1H NMR spectra of the MTX and MTC are shown in Figs. 2 and 3. In the FTIR spectrum of MTC (Fig. 2c), the peaks at 1205 cm^{-1} and 1732 cm^{-1} indicate the ester group formation with MTX. Moreover, the peak of C=O stretching vibration at 1732 cm^{-1} indicates the presence of a carbonyl group and confirmed the formation of MTC. Besides, we observed the peak at 1555 cm^{-1} corresponding to the C–O stretch of unreacted free carboxylic acid group of MTX. The peaks observed are in accordance with the previous reports [14, 15]. All characteristic peaks of MTX and TPGS are present in the spectrum of MTC indicating its successful synthesis.

The product was also confirmed with ^1H NMR analysis (Fig. 3b). The characteristic resonances for MTX have been observed in the ^1H NMR spectrum of MTC confirming the successful synthesis of the conjugate. In the ^1H NMR spectrum of MTC, the chemical shift value of 3.2 ppm indicates the formation of the ester linkage.

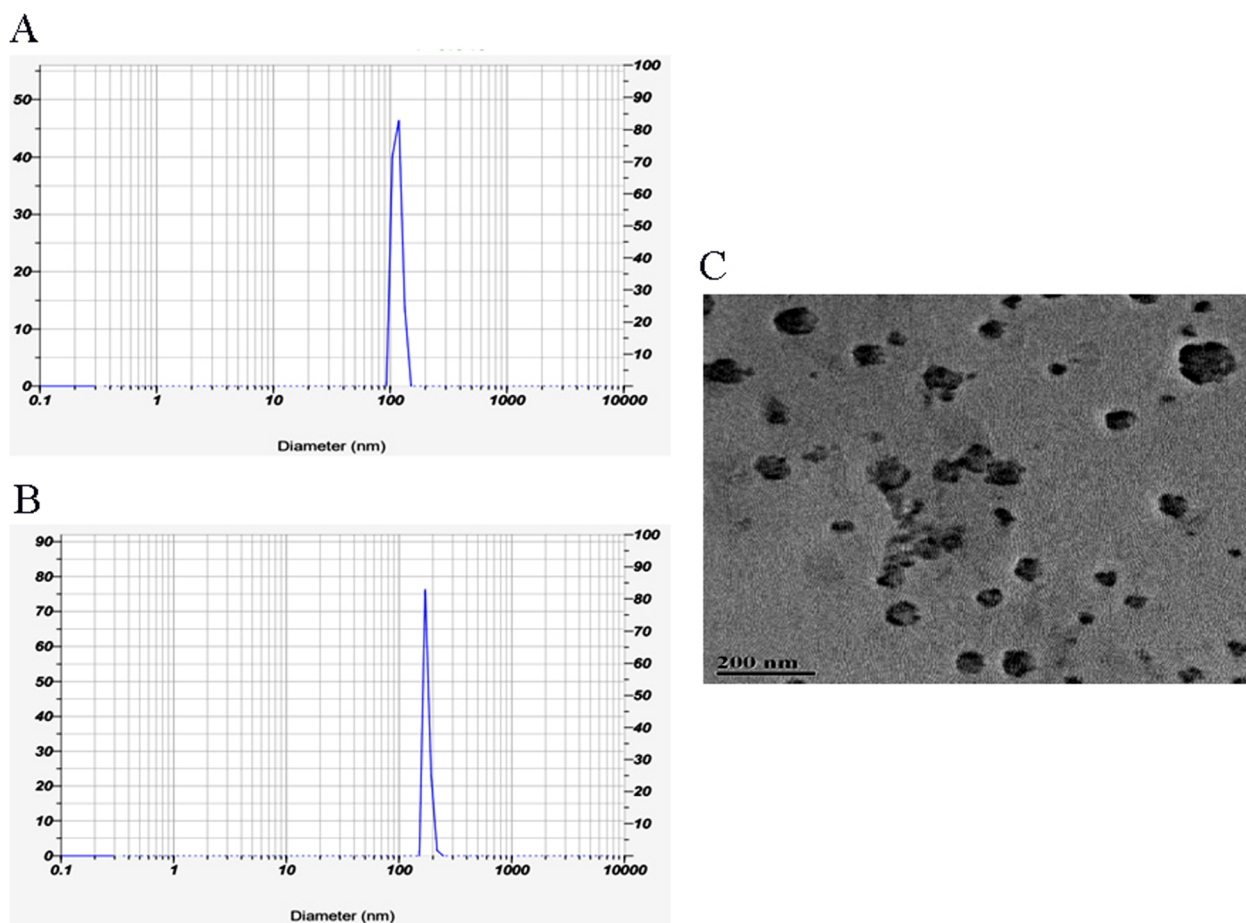


Fig. 4 The mean particle size **a** plain TPGS micelles and **b** MTC micelles and **c** TEM image showing self-assembling nature of MTC

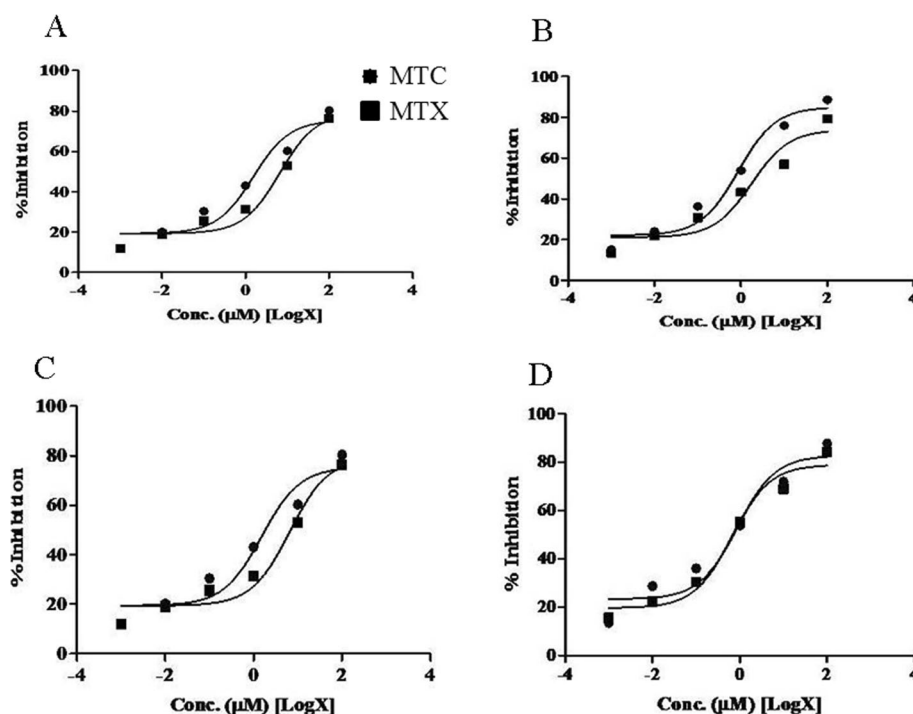


Fig. 5 Effect of MTX and MTC on percentage of MCF-7 cell growth after **a** 24 h and **b** 48 h and on MDA-MB-231 cell growth after **c** 24 h and **d** 48 h

The characteristic peaks of MTX with aromatic protons were observed at 7.0–8.0 ppm.

The assay of MTC is found to be $23 \pm 2.5\%$. The saturation solubility of MTC in distilled water is found to be 6.7 ± 1.3 mg/mL (MTX equivalent), and it is about 670 folds higher than the plain MTX (0.01 mg/mL) [15]. The CMC of plain TPGS and MTC are found to be 45.7 ± 2.7 and 36.9 ± 2.6 μ g/mL respectively.

Preparation and characterization of MTC micelles

The MTC micelles are prepared using film dispersion technique. The mean particle sizes of blank TPGS micelles (Fig. 4a) and MTC micelles (Fig. 4b) are found to be 108.5 ± 9 nm (PDI, 0.219 ± 0.088) and 166.4 ± 10 nm (PDI, 0.325 ± 0.09) respectively. The particle size of MTC micelles is found to be significantly ($p < 0.01$) larger than the blank TPGS micelles.

The self-assembling nature of MTC into micelles is confirmed by TEM analysis. The TEM image of MTC micelles (Fig. 4c) revealed that the self-assembled micelles are well dispersed as individual particles with nearly spherical in shape.

In vitro hemolysis

The MTX caused about $2.6 \pm 0.43\%$ and $19.6 \pm 3.5\%$ hemolysis at 5 and 50 μ g/mL respectively. The MTC caused significantly less hemolysis ($1.08 \pm 0.07\%$ and

$3.19 \pm 0.52\%$ at 5 and 50 μ g/mL respectively) than plain MTX at the same concentration.

In vitro cytotoxicity

The cytotoxicity of plain MTX is compared with its TPGS prodrug. The percentage cell growth inhibition caused by MTX and MTC treatments is presented in Fig. 5, and their IC_{50} values are tabulated in Table 1. Both MTX and MTC showed the concentration and time-dependent cell growth inhibition. The MDA-MB-231 cells are found more sensitive to plain MTX treatment as compared to the MCF-7 cells after both time points. The MTC caused significantly higher ($p < 0.01$) cytotoxicity (low IC_{50} values) as compared to the MTX against both cell lines after 24 h treatment. Further, these obtained results are in agreement with the previous report [15].

Table 1 Comparison of IC_{50} values (μ M) of tested substances against breast cancer cell lines

Cell lines	IC_{50} values (μ M)			
	24 h		48 h	
	Plain MTX	MTC	Plain MTX	MTC
MCF-7	13.27 ± 2.6	1.2 ± 0.21	1.76 ± 0.39	0.86 ± 0.06
MDA MB-231	6.57 ± 1.5	1.52 ± 0.34	0.66 ± 0.02	0.88 ± 0.05

Values are mean \pm SD ($n = 3$)

Cell cycle analysis and apoptosis

In the present study, the effect of MTX and MTC on the MDA-MB-231 cell replication phase is determined (Fig. 6a–c). The MTX treatment resulted in about 7% more cells gated in G0/G1 phase as compared to the MTC (Fig. 6b and Table 2), whereas the MTC treatment caused 5% and 2% more cells in the S phase and G2/M phase as compared to MTX treatment (Fig. 6c and Table 2). In conclusion, MTX caused more cell distribution at the G0/G1 phase, whereas MTC caused more cell distribution at the S and G2/M phase.

In the present study, the apoptotic activity of MTX and MTC against MDA-MB-231 breast cancer cell line is determined by Annexin V-FITC and propidium iodide (PI) staining method using FACS (Fig. 6d–f). The MTX treatment resulted in about 20% of cells in early apoptosis (lower right square) as compared to MTC treatment (Fig. 6e and Table 3). Surprisingly, the MTC treatment resulted in about 25% of cells in late apoptosis (upper right, square) and about 25% less viable cells

(lower left square) as compared to MTX treatment (Fig. 6f and Table 3). Besides, MTC treatment resulted in about 3% more necrotic cells (upper left square) as compared to MTX. The above results revealed the high efficacy and quick effect of MTX following chemical modification with amphiphilic TPGS.

In vitro release study

The in vitro release profiles of MTX from plain MTX solution and MTC micelles are determined using a dialysis bag technique (Fig. 7). It is observed that more than 85% of MTX is released from the plain MTX solution after 24 h. In the case of MTC micelles, we observed significantly less ($p < 0.01$) and sustained release of MTX (only 31 ± 2.6 %) after 24 h of the study.

Discussion

The resistance to MTX develops through several mechanisms including increased DHFR activity, altered binding to MTX due to DHFR mutations, and decreased MTX

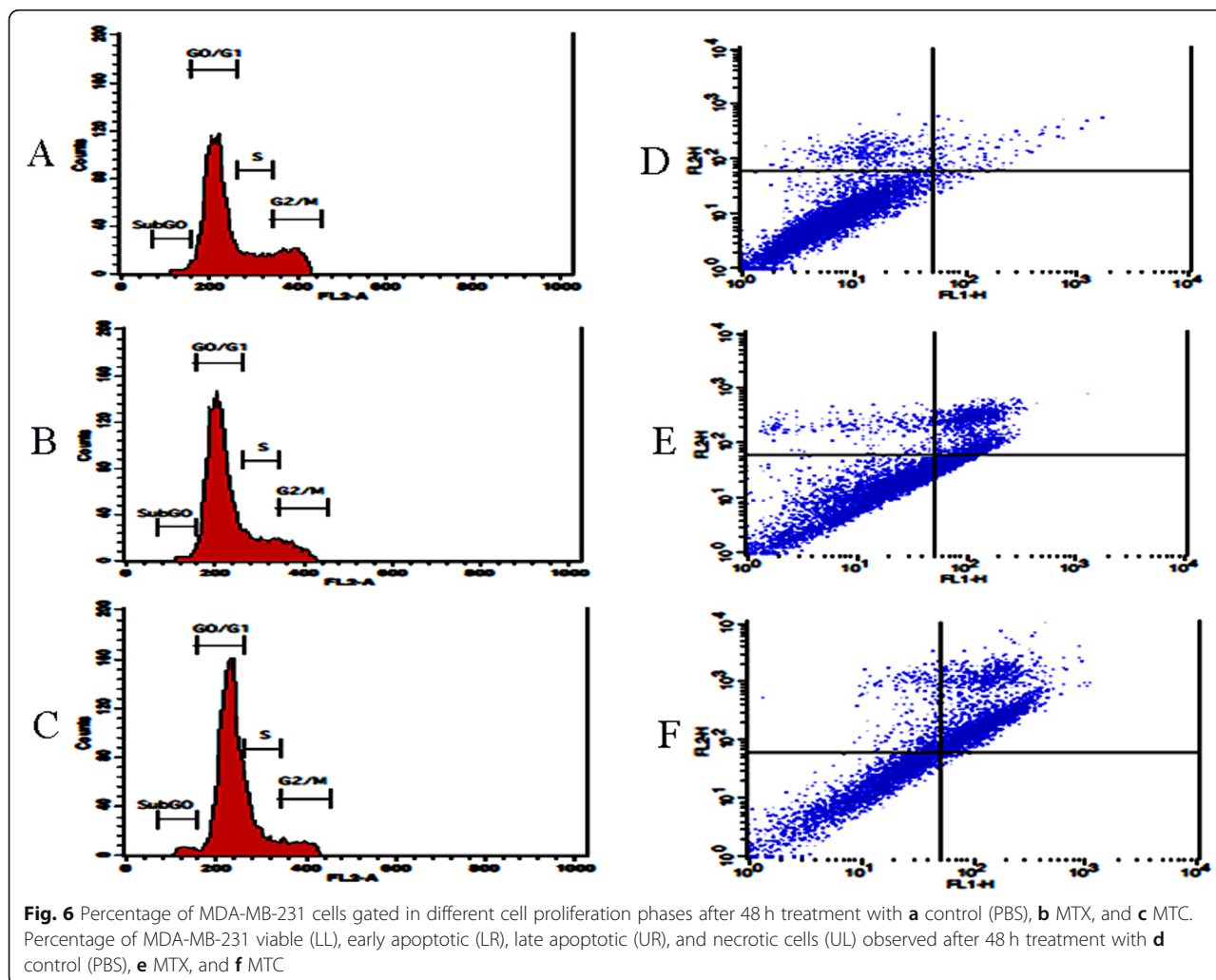


Table 2 Effect of tested substances on MDA-MB-231 cell replication phases after 48 h treatment

Cell line	Formulation (10 μ M)	SUBG0	G0/G1	S	G2M
MDAMB-231	Control	1.84 \pm 0.64	72.01 \pm 2.6	11.81 \pm 2.7	14.85 \pm 0.33
	MTX	1.54 \pm 0.30	78.37 \pm 3.11	13.68 \pm 0.92	6.99 \pm 2.33
	MTC	1.54 \pm 0.72	71.2 \pm 3.36	19.15 \pm 1.68	8.93 \pm 2.19

Values are mean \pm SD (n = 3)

uptake secondary to lack of expression of the MTX transporter protein (reduced folate carrier (RFC)). The reduction in MTX influx as a result of alterations in RFC is one of the main reasons decreasing the therapeutic efficacy of the drug. Further, many reports have been revealed that the MTX serves as a substrate for P-gp [3, 18].

TPGS, as an FDA-approved biocompatible drug excipient, acts not only as a simple drug carrier but also as an assistant molecule with various bio-functions to improve anticancer efficacy. It inhibits P-glycoprotein, enhances drug absorption, induces mitochondrial-associated apoptosis or other apoptotic pathways, promotes drug penetration and tumor accumulation, and even inhibits tumor metastasis. As a result, many formulations by using TPGS, TPGS-drug conjugates, or TPGS copolymers were prepared, and an enhanced therapeutic effect was achieved in different tumor models, especially in multidrug-resistant and metastatic tumors. Considering its effectiveness in tumor treatment, the TPGS-based drug delivery systems appear to be one of the best candidates for future clinical applications. Thus, in the current study, we developed and screened the anticancer effect of MTC against MTX-sensitive (MCF-7) and MTX-resistant (MDA-MB-231) breast cancer cells [19].

In the present study, the MTC is synthesized using the Steglich esterification reaction. Among the two aliphatic COOH groups (α and γ), the α -COOH (highly reactive than γ group) of MTX is expected to react with the hydroxyl group of TPGS. Post reaction, the reaction mixture was subjected for dialysis against water to remove solvent DMF and unreacted DMAP. The dialysis bag contents were then filtered to remove the precipitated dicyclohexylurea and unreacted MTX. The MTC (obtained after evaporation of the filtrate under reduced pressure) was recrystallized in diethyl ether thrice and used for further characterization.

The FTIR and ^1H NMR analysis confirmed the MTC formation. The MTC displayed the substantially enhanced water solubility as a result of its amphiphilic nature following conjugation. This amphiphilic MTC self-assemble (above its CMC) in an aqueous medium to form micellar systems, and this was confirmed by TEM analysis of aqueous MTC solution.

The CMC is an indicator of in vivo stability of micelles upon dilution in the systemic circulation. The moderately lower CMC value of MTC than plain TPGS indicates its better physical integrity and stability upon in vivo dilution.

In the current study, the MTC micelles showed a moderately increased mean particle size as compared to plain TPGS micelles. This increased mean particle size is might be due to enlarged micellar corona as a result of MTX presence in the corona. However, the size of MTC micelles is smaller (below 200 nm) for better accumulation in tumor site following intravenous administration through enhanced permeability and retention (EPR) effect.

The in vitro hemolytic test is performed to know the safety and suitability of MTC for intravenous administration. The MTC micelles, as compared to plain MTX, caused significantly less hemolysis revealing their safety, hemocompatibility, and suitability for intravenous administration.

The antitumor efficacy of plain MTX and MTC against MCF-7 and MDA-MB-231 cells is determined using the MTT dye reduction assay. The MTC demonstrated significantly higher cytotoxicity as compared to plain MTX. Analysis of a population of cells in each replication state can be achieved by fluorescence labeling of the nuclei with PI staining and then the estimation of DNA content by FACS technique. In the present study, MTX caused more cell arrest in the G0/G1 phase, whereas MTC caused more cell arrest in both S and G2/M phases. The apoptotic activity is always routed with the translocation of phosphatidylserine from the cytosol to the cell membrane. Phosphatidylserine specifically

Table 3 Apoptotic activity of tested formulations against MDA-MB-231 cells after 48 h treatment

Cell line	Formulation (10 μ M)	Viable cells	Early apoptotic	Late apoptotic	Necrotic cells
MDAMB-231	Control	95.57 \pm 0.98	0.51 \pm 0.18	0.72 \pm 0.46	3.2 \pm 0.33
	MTX	63.23 \pm 2.74	21.86 \pm 1.0	12.93 \pm 2.93	1.97 \pm 0.82
	MTC	37.90 \pm 0.38	2.0 \pm 0.13	37.90 \pm 0.38	5.37 \pm 0.03

Values are mean \pm SD (n = 3)

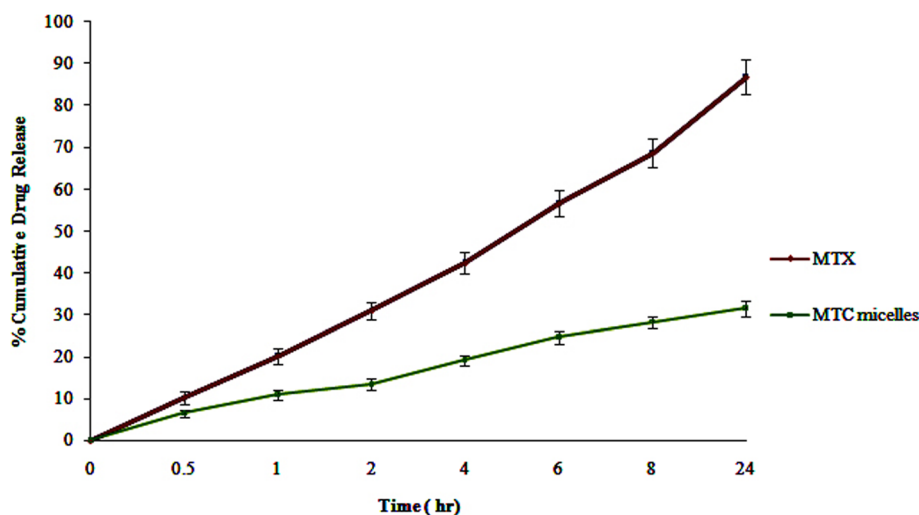


Fig. 7 Cumulative percentage of MTX released from the plain MTX solution and MTC micelles

binds with FITC-tagged Annexin V and can be accurately estimated quantitatively by the FACS technique [17]. We observed significantly increased cell apoptosis in the presence of MTC as compared to plain MTX. In the current study, the increased cytotoxicity, cell arresting, and apoptosis observed with MTC are attributed to the presence of TPGS and its inherent cytotoxic effect, efflux pump (P-gp) inhibition [20, 21], increased cell permeation, synergistic anticancer activity, and proapoptotic characteristics [15, 22].

In vitro release profile of MTX from MTC micelles and plain MTX solution is determined using the dialysis bag technique. The MTX release is found significantly higher from plain solution at all time points. This indicates the free diffusion of MTX through the dialysis membrane into the release medium. The MTC micelles showed the sustained release of MTX as a result of slow diffusion of MTC aggregates (micelles) or MTC unimers through the dialysis membrane. These results indicate the sustained release of MTX in circulation, which in turn causes less systemic toxicity, increased circulation half-life, and more passive accumulation of MTX into tumor tissues.

Conclusion

In the present research, the ester prodrug of methotrexate with TPGS was successfully synthesized and screened for its in vitro anticancer characteristics. Its remarkable aqueous solubility and lower hemolytic behavior indicate suitability for systemic administration. Further, its remarkable in vitro anticancer activity against MDR breast cancer cells indicates potential application in the treatment of various MTX-resistant cancers. However, further detailed in vivo biopharmaceutical studies are needed to validate its fate against various sensitive and MDR cancers.

Abbreviations

BCS: Biopharmaceutical classification system; CMC: Critical micelle concentration; DCC: *N,N'*-Dicyclohexylcarbodiimide; DHFR: Dihydrofolate reductase; DMAP: Dimethylaminopyridine; DMF: Dimethylformamide; FBS: Fetal bovine serum; MDR: Multidrug resistance; MTC: MTX-TPGS conjugate; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTX: Methotrexate; P-gp: P-glycoprotein; PMMs: Polymeric mixed micelles; RFC: Reduced folate carrier; TPGS: D- α -Tocopheryl polyethylene glycol 1000 succinate

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Authors' contributions

RK and SD performed all above research activities. AM designed, monitored, and coordinated the research activities. JD participated in the statistical analysis and data interpretation. PK was involved in the preparation and drafting of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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