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# Montmorillonite/poly-(ε-caprolactone) composites as versatile layered material: Reservoirs for anticancer drug and controlled release property

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ABSTRACT

This work evaluates intercalation of tamoxifen (Tmx) in interlayer gallery of $Na^+$-MMT (Montmorillonite, MMT) (Tmx–MMT), which is further compounded with poly-(ε-caprolactone) (PCL) (Tmx–MMT/PCL, MPs), for oral chemotherapy of breast cancer. The X-ray diffraction patterns, thermal and spectroscopic analyses indicated the intercalation of Tmx into the MMT interlayer that stabilized in the longitudinal monolayer mode by electrostatic interaction. No significant change in structural and functional properties of Tmx was found in the MMT layers. *In vitro* study of drug release profiles showed controlled release pattern. The genotoxic effect of drug was *in vitro* evaluated in human lymphocyte cell culture by comet assay, and results indicated moderate reduction in DNA damage when pristine Tmx was intercalated with MMT and formulated in composites. The Tmx–MMT hybrid efficacy was also confirmed on HeLa and A549 cancer cells by *in vitro* cell viability assay. *In vivo* pharmacokinetics (PK) of formulated Tmx in rats was examined and the results showed that plasma Tmx levels were within therapeutic window as compared to pristine Tmx. Therefore, Tmx–MMT hybrid and microcomposite particles (MPs) can be of considerable value in chemotherapy of malignant neoplastic disease with reduced side effects. This study clearly indicated that MMT not only plays a role as a delivery matrix for drug, but also facilitates significant increase in the delivery proficiency.



## 1. Introduction

Breast cancer is the most harmful cancer today in women and the mortality rate is extremely high in both primary and metastases neoplasia (Jemal et al., 2008; Sahana et al., 2010). The prognosis of cancer treated with routine treatments such as chemotherapy is still far from acceptable, because the high degree of drug eliminated is by P-glycoprotein (P-gp) or multidrug resistance protein (MRP) (Gottesman, 1993; Terwogt et al., 1998; Matheny et al., 2001; Troutman and Thakker, 2003; Foger et al., 2006; Devalapally et al., 2008).

The oral chemotherapy presents many challenges and its success could become a revolution in the history of chemotherapy. The preservation of an appropriate concentration in the circulation for a prolonged exposure of cancerous cells to the drugs has made oral chemotherapy in the first place for better therapeutic effects and less side effects as well as for convenience and life quality of the patients. For over two decades, tamoxifen (Tmx) is the most widely used orally administered drug for the treatment of advanced breast cancer. Tmx is a member of the class of non-steroidal triphenylethylene derivatives and is the earliest selective estrogen receptor modulator (SERM) (Jordan, 2003; Jain et al., 2011). Tmx acts as an anti-estrogen by binding to the estrogen receptor (ER) positive cancer cells. The Tmx–ER complex formation and its binding to DNA can alter or obstruct subsequent mRNA transcription leading to programmed cell-death (PCD, Apoptosis) (Cameron et al., 1997; Hu et al., 2006).

As Tmx therapy is long–term drug prescribed from three to five years, oral chemotherapy is the favored route of administration and the main drawback associated with oral therapy is serious complications to patients. For successful breast cancer therapy overcoming to side effects and increasing drug efficacy are enor-

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mously required, which might possibly be achieved by control drug delivery systems. Recently, various inorganic hybrid composites have emerged as an important class of drug delivery systems in pharmaceutical field. Among them, layered silicate material e.g. montmorillonite (MMT), have attracted a great deal of attention due to their ability to release drugs in a controlled manner, mucoadhesiveness and potent detoxification eventually leading to high efficacy of drugs (Dong and Feng, 2005; Feng et al., 2009; Kevadiya et al., 2010).

In this study, we employed Tmx intercalation in MMT interlayer gallery and the Tmx–MMT composites was further modified with poly-(ε-caprolactone) (PCL) to form microcomposite particles (MPs) and characterized. The Tmx–MMT hybrid and MPs were evaluated for *in vitro* release characteristics, computational and Langmuir model fitting for details Tmx–MMT interaction. *In vitro* genotoxicity assessments of Tmx were measured by degree of DNA (%) damage. *In vitro* cancer cell viability assay and *In vivo* Pharmacokinetics (PK) in rats were also evaluated after oral administration.

## 2. Materials and methods

### 2.1. Starting materials and reagents

For the present study, tamoxifen citrate (Tmx) was obtained as a gift sample from Khandelwal Laboratories Pvt. Ltd (Mumbai, India). Poly-(ε-caprolactone) (PCL) (mp: 60 °C; density 1.145), cellulose acetate dialysis tube (MW: 07014), trizma base, SYBR green I and lectin/PHA-P were purchased from Sigma–Aldrich, USA. Triton X-100, low melting point agarose (LMP), normal melting point agarose (NMP), RPMI-1640 (Roswell Park Memorial Institute 1640), Trypan blue, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide), 0.25% trypsin and 0.02% EDTA mixture, streptomycin-penicillin-amphotericin mixture and DMSO were procured from Himedia laboratory, Mumbai, India. FBS (GIBCO) (fetal bovine serum) were procured from Invitrogen, UK. Dichloromethane (HPLC grade), diethyl ether (HPLC grade), methanol (HPLC grade), EDTA and tween-80 were procured (S.D. Fine Chemicals, India) and polyvinyl alcohol (PVA, Mw. 20,000, National Chemicals, Vadodara, India) were used as received. All the other reagents were of HPLC grade and were used as received. Millipore water was prepared by a Milli-Q plus System (Millipore Corporation, USA).

### 2.2. Preparation of drug loaded Tmx–MMT hybrid

#### 2.2.1. Purification of MMT

The MMT rich bentonite was collected from Akli mines, Barmer district, Rajasthan, India and purified as reported earlier (Kevadiya et al., 2010). The purified MMT was obtained by dispersing 150 g of raw clay in 10 L deionized water and collecting the supernatant dispersion of particles <2 μm at the pre calculated time (10 h) and height (15 cm) at 30 °C. The dispersion was then reacted three times with 0.1 M NaCl solution, centrifuged, and washed with de-ionized water until free of chloride. The MMT dispersion was dried at 90–100 °C and ground to pass through the 200 mesh sieve (ASTM).

#### 2.2.2. Influence of pH and initial drug concentration on drug-clay intercalation

The effect of pH and the intercalation amount of Tmx in MMT was studied at fixed time (12 h) and fixed concentration of Tmx at room temperature.10 mL (25 mg) solution of Tmx in methanol was added to 25 mg of MMT powder, taken in a 25 mL glass vial, and stirred at 600 rpm. The pH was adjusted from 2 to 7 by HCl and NaOH solutions. The remaining concentrations of Tmx in the filtrates were measured absorbance at $\lambda_{max}$ = 274 nm using UV–visible spectrophotometer UV 2550 (Shimadzu, Japan), equipped with a quartz cell having a path length of 1 cm. To study the effect of initial drug concentration on the intercalation of Tmx into MMT, the intercalation was carried out at different initial concentration of Tmx at optimized pH = 5.5. Ten milliliter solutions of Tmx in methanol containing different quantity of Tmx were treated with 100 mg of MMT for 12 h at room temperature in a 25 mL glass vial with continuous stirring at 600 rpm. The reaction mixtures were filtered and absorption of Tmx in the filtrates was determined by UV–visible spectrophotometer. For preparing the bulk sample of drug-clay hybrid, 2 g of clay in 100 mL methanol under vigorously stirring for 1 h. 2 g Tmx solution (2 wt.% in methanol, pH 5.5) was added drop wise (2 mL/min) into the clay dispersion within 1 h at room temperature using peristaltic pump (Master flex L/S 7518-00, Cole–Parmer, USA). The mixed solution was further stirred (600 rpm) for 12 h, filtered, washed several times with methanol to remove the non-intercalated Tmx, dried at 60 °C and ground with mortar and pestle to obtain fine powder. This sample was designated as Tmx–MMT hybrid.The entire intercalation studies were performed in triplicates and the values were averaged for data analysis.

### 2.3. Preparation of Tmx–MMT/PCL microcomposites particles (MPs)

The Tmx–MMT/PCL microcomposites particles (MPs) were prepared with the oil in water (o/w) solvent evaporation method. 1 g of poly-(ε-caprolactone) (PCL) was dissolved in 100 mL dichloromethane and sonicated for 20 min, to which Tmx–MMT hybrid (PCL: Tmx–MMT = 1:0.5 w/w) was added and further sonicated for 10 min. The organic phase was added drop wise (0.5 mL/min) into the external aqueous phase containing 5% w/v of polyvinyl alcohol (500 mL) with stirring till dichloromethane evaporation, The microcomposite particles were collected using filter paper, washed five times with Milli-Q water and resuspended in 25 mL Milli-Q water, frozen in liquid nitrogen and lyophilized (Jagadeesh and Devi, 2010). The sample was designated as MPs.

### 2.4. Characterization

X-ray diffraction (XRD) analysis was carried out on Phillips powder diffractometer X' Pert MPD using PW3123/00 curved Ni-filtered Cu-Kα radiation with a scanning of 0.3°/min in $2\theta$ range of 2–10°. Fourier transform infrared spectra (FT-IR) were recorded on Perkin-Elmer, GX-FTIR as KBr pellet in 4000–400 $cm^{-1}$ range. Thermo gravimetric analysis (TGA) was carried out within 50–800 °C at the heating rate 10 °C/min under nitrogen flow (20 mL/min) using by TGA/SDTA 851e, Mettler-Toledo, Switzerland. The particles was analyzed basis on the dynamic light scattering technique (DLS) and Zeta potential was estimated on the basis of electrophoretic mobility under an electric field by zeta sizer (Nano-ZS90, Malvern instruments Ltd., Malvern, UK). The morphology of microcomposites particles (MPs) were observed by scanning electron microscope (SEM), LEO-1430VP, UK. The comet images were capture by using Carl zeiss Axio-Cam-MRc camera attached to Axio-Scope fluorescence microscope (Germany).

### 2.5. In vitro release behavior of Tmx

*In vitro* release behavior of Tmx was carried out with the help of USP eight stage dissolution rate test apparatus (Veego, Mumbai, India) using dialysis bag technique (Joshi et al., 2009; Kevadiya et al., 2010). The Tmx release experiments were carried out in phosphate buffer solution (PBS) of pH 7.4 containing 1% w/v Tween 80 at 37° ± 0.5 °C. Tween 80 was used to enhanced the solubility of Tmx in the buffer solution and evade the binding of Tmx to the container (bowl) wall. The dialysis bags were equilibrated with

the release medium for 2 h prior to release studies. The weighed quantities of hybrid and MPs (corresponding to 20 mg of entrapped Tmx) were suspended in dialysis bag containing 5 mL of the release medium. The dialysis bags were then placed in the stainless steel baskets and were immersed in container containing 500 mL of release medium. The rotation frequency of basket was kept at 100 rpm. 5 mL aliquots were withdrawn at regular time interval and the same volume was restored with fresh release medium. Samples were analyzed for Tmx content by UV–visible spectrophotometer. These studies were performed in triplicates for each sample and the average values were used in data analysis.

### 2.6. Genotoxicity assessments

#### 2.6.1. Lymphocyte culture and exposure to Tmx

The normal human peripheral blood lymphocyte culture was used for alkaline single cell gel electrophoresis assay for evaluation of DNA damage by action of pristine Tmx, Tmx–MMT hybrid and MPs. DNA lesions were expressed as the % DNA damage in comet tail. For lymphocyte culture, 5 mL of whole blood was collected in heparinised vial by venipuncture method from a non-smoker healthy volunteer. Lymphocyte culture was seeded in autoclaved micro-centrifuge tubes (2 mL) as described by Hungerford, (Hungerford, 1965) with slight modifications. In brief, 100 μL of whole blood was re-suspended in 1.88 mL RPMI-1640 and 20 μL PHA-P containing micro-centrifuge tubes and transferred in $CO_2$ incubator (Heracell 150i, Thermo Fisher Scientific) maintained at 37 °C and 5% $CO_2$ for incubation. Cultures were set in five groups with $G_1$ (control) as untreated group, $G_2$ as drug carrier control (MMT, 150 μg), $G_3$ as positive control (Tmx, 18.5 μg), $G_4$ as Tmx–MMT hybrid and $G_5$ with MPs. $G_4$ and $G_5$ were further ramified by eight cultures in each set. Each of these cultures were treated with Tmx–MMT (37 μg) and MPs (160 μg), respectively in series at 60th h after seeding. The culture was harvested after 1, 3, 5 and 24 h of treatment considering efficacy of Tmx release from hybrid and MPs in the medium.

#### 2.6.2. Single cell gel electrophoresis (comet assay)

The genotoxicity of Tmx was determined by calculating DNA damage using comet assay, based on the measurement of DNA migration under electrophoresis as per slightly modified technique given by Singh et al. (1988). In brief, 180 μL of 1% normal melting point agarose (NMP) was gelled on fully frosted slide (75 × 25 mm) and 100 μL 0.5% low melting point agarose-containing cell suspension (20 μL) was layered on the top of the NMP agarose. After formation of cell suspension containing layer, additional 100 μL of LMP agarose was added to fill the residual hole and to form an additional layer to increase the distance between the cells and the gel surface. After agarose gel solidification, the slides were placed in a lysis solution [2.5 M NaCl, 100 mM EDTA and 10 mM Tris HCl (pH 10), plus 1% Triton X-100 and 10% DMSO were added just before use] for 24 h at 4 °C, incubated in an alkaline electrophoresis buffer (300 mM NaOH/1 mM EDTA, pH >13) for 40 min and electrophoresed (Midi-Submarine electrophoresis Unit-7050, Tarsons, Kolkata, India) for 35 min. After the comets were formed, the alkaline gel was neutralized by rinsing the slides with a suitable buffer (0.4 M Tris, pH 7.5) three times. The slides were then stained with the fluorescent dye SYBR Green I (SG) and the comet images were captured under fluorescence microscope. The proportion of DNA damaged was scrutinized by scoring 100 comets for each group (Tri-Tek Comet-Score™ V1.5 software, Germany).

### 2.7. Cell cultures

HeLa (Human cervical cancer cell line) and A549 (Human lung adenocarcinoma epithelial cell line) were obtained from National Repository of Animal Cell Culture, National Centre for Cell Sciences (NCCS), Pune, India. Both cell lines were cultured in 25 $cm^2$ tissue culture flasks maintained at 37 °C in a humidified environment of 5% $CO_2$ and were grown in RPMI-1640 with 10% FBS, Streptomycin (1000 U/mL)-penicillin (100 μg/mL)-amphotericin (0.25 μg/mL) mixture replenished every three days.

#### 2.7.1. In vitro cytotoxicity of Tmx and Tmx–MMT hybrid

Cancer cell viability of the Tmx and Tmx–MMT hybrid was evaluated by the MTT assay. 150 μL of HeLa and A549 cells were seeded in 96-well plates (Becton Dickinson (BD), USA) at the density of $4 \times 10^4$ viable cells/well (HeLa) and $1.1 \times 10^4$ (A549) viable cells/well and incubated 24 h to allow cell attachment. Following attachment, the medium was replaced with complete medium (150 μL/well) containing the pristine Tmx and Tmx–MMT hybrid at equivalent drug concentrations ranging from 0.1–30 μg/mL for 72 h. Following treatment, the cells were washed with PBS and incubated with 100 μL/well fresh medium containing 0.5 mg/mL MTT. The MTT-containing medium was removed after 3 h incubation in dark condition. The MTT formazan was dissolved in 100 μL/well DMSO and optical density was determined at 570 nm using an ELISA plate reader (Bio-Tek, USA). Cell viability was calculated by the following equation:

$$\text{Cell viability}(\%) = (A_s/A_{\text{control}}) \times 100 \quad (1)$$

where, $A_s$ is the absorbance of the cells incubated with the Tmx and Tmx–MMT hybrid and $A_{\text{control}}$ is the absorbance of the cells incubated with the culture medium only. $IC_{50}$, the drug concentration at which inhibition of 50% cell growth was observed in comparison with that of the control sample, was calculated by the curve fitting of the cell viability data.

### 2.8. In vivo pharmacokinetics (PK)

#### 2.8.1. Animals and dosing

Ten to Twelve weeks old female wistar rats of 200–250 g were supplied by the Laboratory Animals Centre of Zydus Research Center (ZRC), Ahmedabad, India and were maintained at the Animal Holding Unit of Institute of Pharmacy, Nirma University, Ahmedabad (India). The animal caring, handling and the protocols were approved by the Institutional Animal Ethics Committee (IAEC), Nirma University, Ahmedabad (India). The animals were acclimatized at temperature of 25 ± 2 °C and relative humidity of 50–60% under natural 12 h/12 h light/dark conditions for one week before experiments. All animals were fasted for 24 h before the studies, and during the course of the studies; water was available *ad libitum*. The animals were arbitrarily distributed into three groups each containing six animals. First group of animal received oral pristine Tmx (suspension), while the second group of animals received Tmx–MMT hybrid (suspension) and third group received MPs (suspension). All the formulations were administered orally at a dose of 20 mg/kg body weight ($n = 6$). All animals were observed for their general condition, clinical signs, and mortality. The blood samples (approximately 0.3–0.4 mL) were collected from the retro orbital plexus under mild anesthesia into the micro centrifuge tubes containing EDTA (1.8 mg/mL blood). The blood collection time gaps were kept at 0 (predose), 1, 3, 6, 9, 12, 24, 48 and 72 h after administration of the drug. Plasma samples were harvested by centrifugation (Kubota-6500, Kubota Corporation, Japan) at 10,000 rpm for 15 min at 5 °C and stored at −20 °C for reverse-phase (RP) HPLC analysis.

#### 2.8.2. The sample preparation and drug quantification by HPLC

In order to effectively separate Tmx from plasma components, the method of extraction used was a slightly modified from as described by de Santana et al. (2008). 150 μL of ammonium hydroxide

(1 mol/L) and 250 μL of diethyl ether (step repeated for three times) were added to 100 μL of plasma in micro-centrifuge tube. Then, the mixture was vortex-mixed for 2 min. The phases were separated by centrifugation at 10,000 rpm for 15 min at 5 °C. The aqueous phase was frozen in bathing of dry ice and acetone for 5 min. The organic phase was then transferred to a new set of clean micro-centrifuge tubes and evaporated to dryness, under a stream of nitrogen. The residue was reconstituted in 100 μL of methanol when vortex-mixed for 10 min. The quantification of Tmx in plasma was determined using a validated RP-HPLC method reported in literature with slight modifications (Hu et al., 2006; De Santana et al., 2008; Jain et al., 2011). Briefly, subsequent to extracting of drug in methanol, analysis by high-performance liquid chromatography (HPLC) system consisting of Photodiode array detector (Waters Alliance model: 2695 separation module with Waters 2996 Photodiode Array Detector, Waters Corporation, Milford, MA, USA) and a reverse-phase C18 column (Phenomenex® make Luna C18 (2) HPLC column with Length = 25 cm, ID = 4.6 mm, Particle size = 5.0 μm, Phenomenex Inc, Torrance, CA, USA) was carried out. Tmx containing methanol samples were transferred to auto sampler vials, capped and placed in cassettes of the HPLC auto sampler. Mobile phase employed for analysis was the mixture of methanol and water containing 1.0% (v/v) triethylamine (89:11 v/v). The injection volume was 80 μL and retention time of Tmx was found to be 15 min. The detection wavelength ($\lambda_{max}$) for Tmx was 265 nm. Tmx concentration in plasma was determined using the standard curve obtained for known concentrations of Tmx in plasma processed under identical condition. The curve was found to be linear with a $R^2$ = 0.9978. The pharmacokinetics parameters like total area under the curve $(AUC)_{0-\infty}$,the mean residence time (MRT), peak plasma concentration ($C_{max}$) and time to reach the maximum plasma concentration ($T_{max}$) were determined on the basis of plasma drug concentration–time data analyzed by software (Origin 8.0, USA).

## 3. Results and discussions

### 3.1. The chemistry of drug-clay intercalation

A very elevated quantity of intercalation of Tmx in MMT layers was attained at a stable value of ~397 mg/g MMT within 12 h. The maximum adsorption was observed in the pH of 5.5, Fig. 1. There was a rapid decline in intercalation of Tmx in MMT, above pH 6 and below pH 5. The pKa of Tmx is ~8.9 (weak base) (Hu et al., 2006; Buchanan et al., 2007), which implies that at pH ⩽ 7.0, the Tmx exists as mono charged cations due to protonation of amines. Below pH 5, decrease in the intercalation of Tmx in the clay lattice due to the competition between the cationic drug and $H^+$ ions (Joshi et al., 2009). Fig. 2(a) shows the adsorption isotherm of Tmx in MMT. The adsorption data were fitted in Langmuir and Freundlich equations. The monolayer capacity of the adsorbent can be represented as the Langmuir equation,

$$Ce/Cs = 1/(C_{max}K_L) + Ce/C_{max} \quad (2)$$

where Cs was the equilibrium Tmx concentration on the adsorbent (mg $g^{-1}$), Ce: the equilibrium Tmx concentration in solution (mg $L^{-1}$), $C_{max}$: the monolayer capacity of the adsorbent (mg $g^{-1}$), and $K_L$: the Langmuir adsorption constant (L $mg^{-1}$). A plot of Ce/Cs vs. Ce gave a straight line and from slope and intercept of the straight line the values of $C_{max}$ (400 mg $g^{-1}$) and $K_L$ (383 × $10^{-5}$ L $g^{-1}$) were obtained with $r^2$ = 0.9992. The adsorption data fitted well with the Langmuir equation (Fig. 2(b)).

### 3.2. Characterization of drug–clay hybrid

#### 3.2.1. XRD

The powder XRD patterns of the pristine MMT and Tmx–MMT hybrid are shown in Fig. 3. Pristine MMT showed a typical XRD pattern with the basal spacing of 1.20 nm (2θ = 7.3°). The intercalation of Tmx leads to a significant increase in the interlayer basal spacing (2.13 nm, 2θ = 4.1°). As the thickness of MMT sheet is 0.96 nm (Kevadiya et al., 2010) the height of gallery of Tmx–MMT hybrid could be estimated to be 1.17 nm. This value was slightly lower than the longitudinal molecular length (1.30 nm) of Tmx, but little higher than their lateral length (1.10 nm) of Tmx molecule. It can be presumed from the gallery height that the intercalated Tmx molecules were arranged in longitudinal monolayer mode in MMT.

#### 3.2.2. Thermal analysis

Fig. 4(A) illustrates the differential thermal analysis (DTA) pattern and Fig. 4(B) TGA pattern of dried MMT and Tmx–MMT hybrid. The initial weight loss and endothermic peak at ~100 °C in MMT corresponded to the loss of adsorbed water. The weight loss at 400–500 °C was due to the dehydroxylation of the MMT (Lin et al., 2002; Bergaya et al., 2006; Joshi et al., 2009; Kevadiya et al., 2010). Tmx–MMT hybrid showed three steps of mass losses at 300 °C, 400–500 °C and 630 °C. One strong and two weak endothermic peaks were observed for Tmx–MMT hybrid. The weight loss of MMT (~16%) and Tmx–MMT hybrid (23.14%) was due to loss of water and structural hydroxyl group. (Supplementary data:

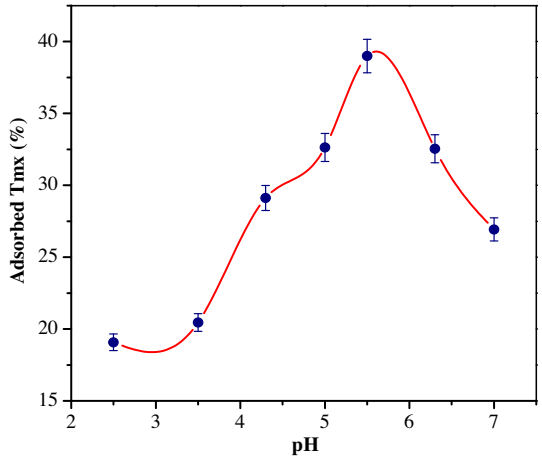


**Fig. 1.** Influence of pH on Tmx intercalation in gallery of MMT.

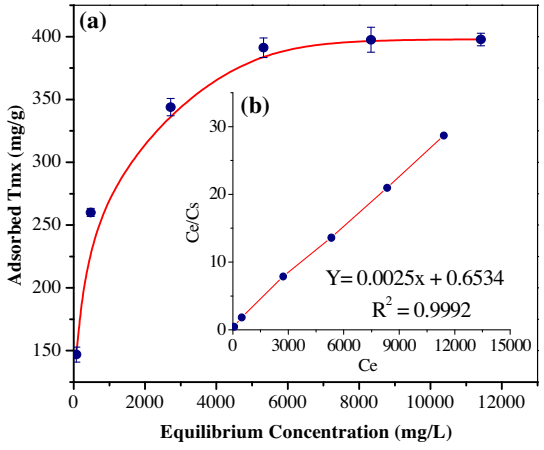


**Fig. 2.** Intercalation of Tmx in gallery of MMT. (a) Effect of concentration gradients with (b) Langmuir model fitting.

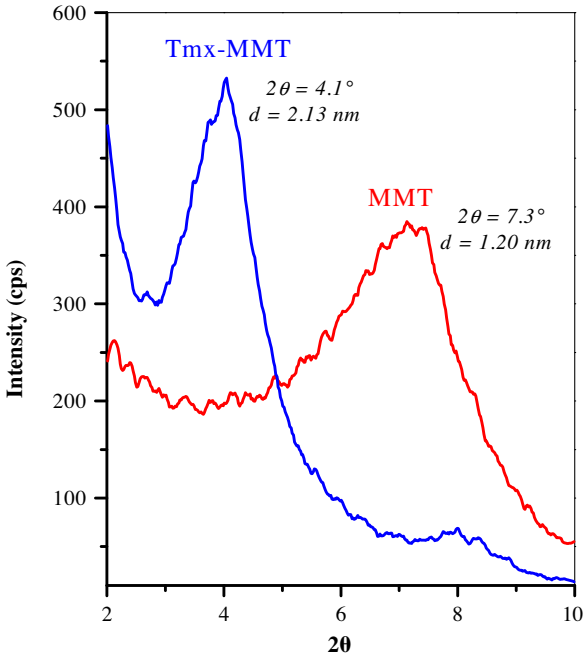


**Fig. 3.** XRD pattern of MMT and Tmx–MMT hybrid.

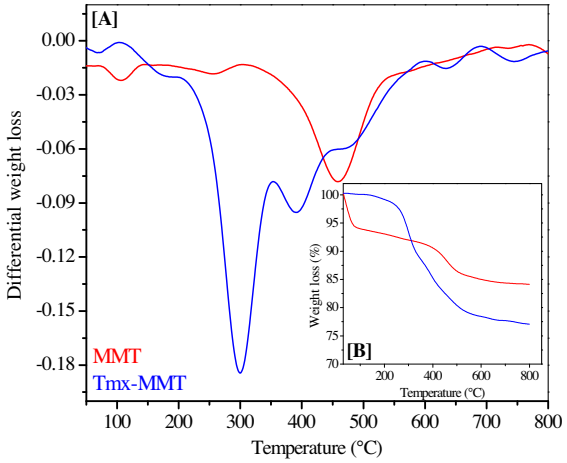


**Fig. 4.** (A) DTA and (B) TGA pattern of the MMT and Tmx–MMT hybrid.

Computation Model (Fig. S1), FT-IR (Fig. S2), Particle size distribution and Zeta potential analysis (Fig. S3) and SEM analysis (Fig. S4)).

### 3.3. In vitro Tmx release study

The release profile of the Tmx from Tmx–MMT hybrid and MPs are shown in Fig. 5. The formulation exhibited controlled release profile up to 72 h. The Tmx released from MPs showed the controlled release pattern with ~26% of drug released in 10 h followed by sustained release up to >72 h (32%). No initial burst release was observed from MPs. The release of the drug from Tmx–MMT hybrid was somewhat faster compared to that from MPs, where ~40% of the intercalated drug was released in 5 h and ~62% the drug was released in 72 h.

The sustained release of Tmx from MPs can be attributed to the wrapping of the Tmx–MMT hybrid plates by PCL matrix and, due to low permeability of the water in interior of MP (Jagadeesh and

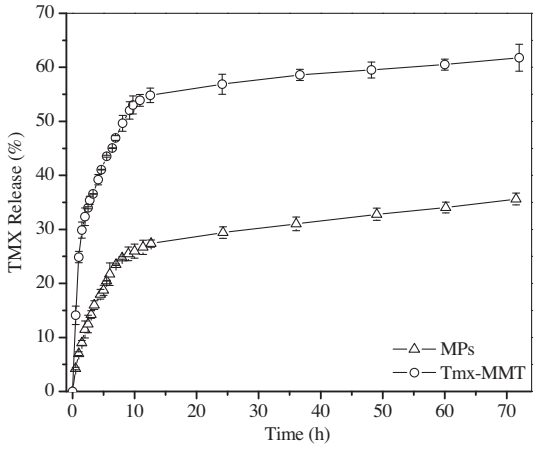


**Fig. 5.** *In vitro* release profiles of Tmx in simulated intestinal fluid (pH 7.4) at 37 ± 0.5 °C.

Devi, 2010). The burst effect must not be measured a negative circumstance in all cases. At the later stage, the Tmx release from Tmx–MMT hybrid was more controlled and sustained, whose rate may have been determined by the de-intercalation of drug from the clay plates.

### 3.4. The genotoxicity assessments

It has been reported that Tmx reacts with the estrogen receptor and is generally considered to be the mechanism by which its pharmacological property is mediated (Clemons et al., 2002; Shang and Brown, 2002). Unfortunately, Tmx has shown some severe side effects revealing strong genotoxic activity (Greaves et al., 1993; Martin et al., 2003; Poirier and Schild, 2003). Tmx appears to require metabolic activation to exert genotoxic action. It has been shown that Tmx is transformed to the genotoxic epoxide, as well as 4- and 2-hydroxy metabolites via enzymatic activation either by liver cytochrome P450 monooxidase or by different peroxidases or by hepatic α-oxidation of the Tmx ethyl group (Poon et al., 1993; Lim et al., 1994; Phillips, 2001). These metabolites could covalently attach to DNA, lipids and proteins inducing the irreversible damage to these biologically vital molecules and membrane structures. Tmx metabolites may also be oxidized by molecular oxygen giving rise to the ROS formation in the reduction/oxidation cycling process (Han and Liehr, 1992; Pagano et al., 2001).

The images of individual comets of different groups are presented in Fig. 6 and time dependent DNA (%) damage determined for different exposed time e.g. 1, 3, 5 and 24 h is shown in Fig. 7. Tmx treated group showed ~80% DNA damage within 1 h, while, DNA damage in Tmx–MMT and MPs treated cultured lymphocyte recorded ~65% and ~76% DNA damaged, respectively as compared to pristine Tmx (Fig. 7). The degree of DNA damage is lower in formulated Tmx, is due to slow liberation of Tmx from MMT/MPs. Earlier reports indicated that Tmx damages DNA inducing mainly DNA strand breaks. Tmx may induce chromosomal breaks by two possible mechanisms: (a) directly by inducing DNA double strand breaks (DSBs) by oxidative DNA damage or (b) defective translesion DNA synthesis (TLS) at sites of Tmx-induced damage, which may result in an increase in the number of gaps, some of which may then be converted to DSBs (Wozniak et al., 2007). The results indicated that the preservation or intercalating of Tmx within MMT interlayer gallery and further compounding with PCL leads to significantly reduced degree of genotoxic activity as compared to pristine Tmx treated group. However, both the Tmx containing

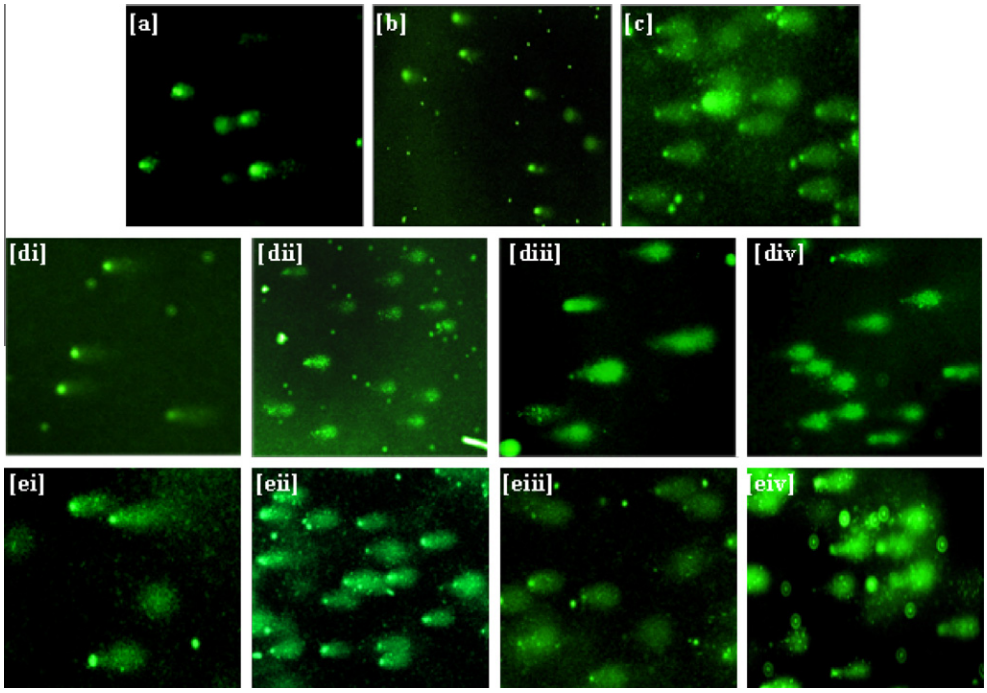


**Fig. 6.** The comet images of cultured human lymphocyte, (a) control group ($G_1$), (b) carrier control group ($G_2$) treated with MMT (c) positive control group ($G_3$) treated with Tmx with exposure time of 1 h, (d) treated by Tmx–MMT hybrid ($G_4$) with different exposure times e.g., (di) 1 h (dii) 3 h (Diii) 5 h (div) 24 h, (e) Treated by MPs ($G_5$) with different exposure times e.g., (ei) 1 h (eii) 3 h (eiii) 5 h (eiv) 24 h.

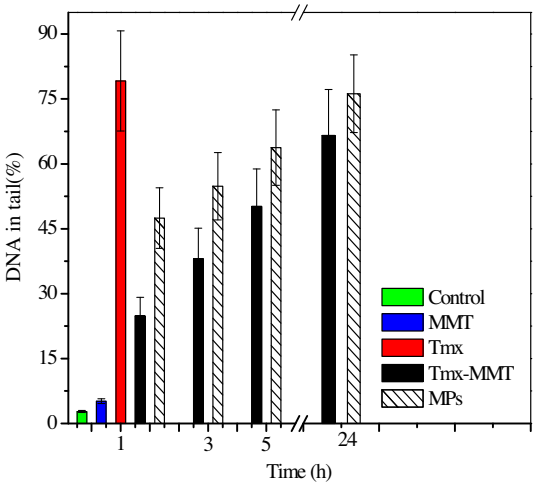


**Fig. 7.** Genotoxic action of Tmx as evaluated by the DNA damage (%) versus different exposure time to drug/formulation in human lymphocyte cell culture.

groups significantly damaged the DNA when compared to the normal and carrier control groups.

### 3.5. *In vitro* cell viability of drug-clay hybrid

Fig. 8 shows a comparison of *in vitro* viability of HeLa (Fig. 8A) and A549 cells (Fig. 8B) after exposure to Tmx and Tmx–MMT hybrids at the different concentrations after 72 h of incubation. The Tmx and Tmx–MMT hybrid showed dose-dependent reduction in cell viability in HeLa and A549 cells. The *in vitro* effect of Tmx and Tmx–MMT hybrids was quantitatively evaluated by $IC_{50}$ which is defined as the drug concentration at which 50% cells were killed at a given time period. The viability of HeLa was dose-dependent

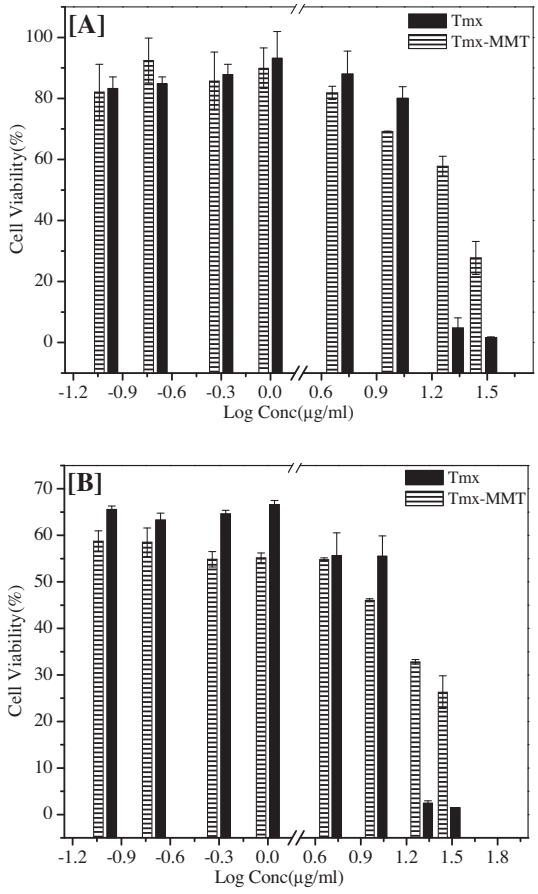


**Fig. 8.** *In vitro* cell Viability of (A) HeLa (Human cervical cancer cell line) and (B) A549 (Human lung adenocarcinoma epithelial cell line) cancer cells after 72 h of incubation with Tmx and Tmx intercalated in MMT at the same 0.1,0.2 0.5, 1, 5,10, 20, 30 µg/mL Tmx doses, respectively, ($n = 6$).

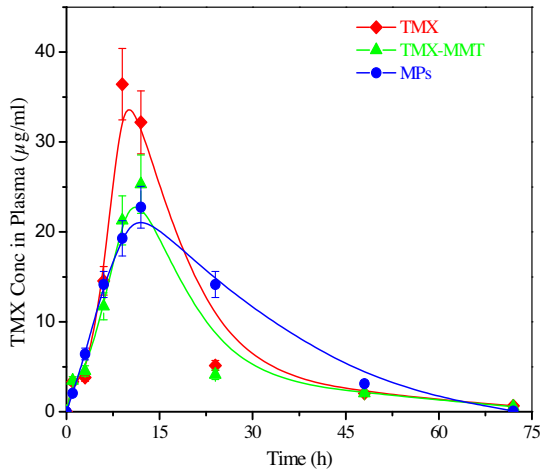


**Fig. 9.** Time profiles of relative plasma concentrations of Tmx after oral administration to female wistar rats when formulated in the MMT and MPs as compared to pristine Tmx, results are shown as means ± SD of six animals per group.

by Tmx and Tmx–MMT with an $IC_{50}$ = 2.30 μg/mL and 4.90 μg/mL, respectively. The HeLa cells are generally less susceptibility to Tmx–MMT as compared to A549 cells. However, A549 cell viability was inhibited by $IC_{50}$ = 1.18 μg/mL of Tmx–MMT is equivalent to $IC_{50}$ = 1.19 μg/mL of pristine Tmx. While, HeLa and A549 cells are more susceptible to Tmx and Tmx–MMT in case of elevated concentrations (20–30 μg/mL). Therefore, A549 cells were considered to be extremely sensitive to MMT. However, results advocated that Tmx could preserve its antitumor effectiveness subsequent to its intercalation in clay.

### 3.6. In vivo pharmacokinetics (PK) after oral administration

Fig. 9 shows the *in vivo* pharmacokinetics, i.e. the plasma drug concentration with respect to time after a single oral administration of pristine Tmx, Tmx intercalated in MMT and Tmx–MMT hybrid was compounded in MPs to female wistar rats at the same concentration of Tmx i.e., (20 mg/kg) body weight ($n$ = 6). The key PK parameters were analyzed and the results are listed in Table 1, which include $C_{max}$ (μg/mL) and $T_{max}$ (h) – the maximum drug concentration encountered after the drug administration and the time at which $C_{max}$ is reached, MRT (h) – the mean residence time of the drug in the plasma and $AUC_{0-\infty}$ (μg h/mL) – the total area under the curve which represents the *in vivo* therapeutic effects of drug. A few significant advantages of the MMT and MPs can be concluded from PK data. The $C_{max}$ and MRT of pristine Tmx were about ~36.5 μg/mL and ~14.5 h, respectively. The mean values obtained for $AUC_{0-\infty}$ and peak plasma time ($T_{max}$) were ~527 μg h/mL and 9 h, respectively. On the other hand, when Tmx was captured inside gallery of MMT, further compounded in MPs and orally administered to rats, higher drug concentrations were detected in plasma up to ~60 h with MPs (Fig. 9).

The plasma level curve obtained by the oral administration of Tmx–MMT and MPs could be described as divided into two parts; the first part involves the absorption phase until the achievement of $C_{max}$ (12 h) followed by maintenance of plasma concentration up to ~72 h, Upon comparing the MRT of pristine Tmx, it was observed that the Tmx–MMT and Tmx loaded MPs slightly increased the residence time of the Tmx in the plasma by ~15 h and ~18.5 h, respectively. Thus, an oral administration of Tmx by loading into the MMT and MPs there was only minor alteration in residence time of drug in plasma within the chemotherapeutic window.

**Table 1**
Pharmacokinetics of pristine Tmx, Tmx–MMT hybrid and MPs in Female wistar rats after single oral administration of same drug dose; data represent mean ± SD, ($n$ = 6).

| PK parameters | Tmx | Tmx–MMT | MPs |
|---|---|---|---|
| $C_{max}$ (μg/mL) | 36.43 ± 3.9 | 25.30 ± 3.2 | 22.75 ± 2.3 |
| $T_{max}$ (h) | 9 | 12 | 12 |
| $AUC_{0-\infty}$ (μg h/mL) | 526.91 ± 56.9 | 405.07 ± 36.05 | 582.54 ± 54.7 |
| MRT (h) | 14.50 ± 1.0 | 15.12 ± 1.3 | 18.53 ± 1.8 |

## 4. Conclusions

$Na^+$-MMT as drug carriers, Tmx–MMT hybrids and MPs containing the anti-estrogen modulator, tamoxifen (Tmx), were successfully prepared and characterized. *In vitro* release study showed controlled release pattern of drug for more than 72 h. The genotoxic effects of drug evaluated in human lymphocyte cell culture by *in vitro* comet assay indicated considerable reduction of drug genotoxicity by drug intercalation in clay. *In vitro* cell viability assay indicated that the drug efficacy in A549 cells remain unchanged after incorporation in clay. *In vivo* pharmacokinetics study in rat model showed plasma drug levels within therapeutic window as compared to pristine Tmx. These results indicated that clay has excellent prospective as a drug carrier for the treatment of breast cancer with reduced side effects.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejps.2012.04.009.

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