T1107 polymer expressed potential anti-tumor effects against HepG2 cell line

El-Refaie Kenawy, Mohamed L. Salem, Noura E. Sanoh and Mohamed M. Azaam
T1107 polymer expressed potential anti-tumor effects against HepG2 cell line

El-Refai Kenawy¹, Mohamed L. Salem²,³, Noura E. Sanoh¹,³ and Mohamed M. Azaam¹
¹Chemistry Department, Faculty of Science, Tanta University, Tanta, Egypt
²Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt.
³Center of Excellence in Cancer Research, Tanta University Educational Hospital, Tanta University, Tanta, Egypt

ABSTRACT

Background: Polymeric nanoparticles (NPs) are obtained naturally, semi-synthetically or synthetically by a polymerization reaction. Tetronics based ethylene oxide-propylene oxide copolymers have gained a great interest. It demonstrated interactions with cell membranes with potential for developing new biomaterials for application in nanomedicine. Aim: The primary aim of the current study is to test the direct anticancer activity of unmodified tetronic (T1107), P-HB+aminated tetronic and N, N DMAB+aminated tetronic polymers and the associated by apoptosis and cell cycle. Materials and Methods: Synthetic polymers were prepared and characterized to confirm the modification. Anti-tumor activity was examined in vitro using human hepatocellular carcinoma (HepG2) cell line. Cell viability (MTT), cell cycle and apoptosis were evaluated by flow cytometry. Results: Unmodified tetronic (T1107) decreased HepG2 viability (by 30%) than untreated HepG2 cells. Treatment with P-HB+aminated tetronic and N,N DMAB+aminated did not give a significant effect on Hepg-2 cells. Treatment of Hepg-2 with unmodified tetronic (T1107) induced cell cycle arrest at GO phase (39.4%), while P-HB+aminated tetronic and N,N DMAB+aminated tetronic induced cell cycle arrest at G1 phase (by 54.8% and 48.2%, respectively as compared to treatment with the doxorubicin (DOX) as a reference drug, which induced Hepg-2 cell cycle arrest at GO phase (by 39.1%). The unmodified tetronic (T1107) increased the numbers of late apoptotic cells (by 49.4%), while P-HB+aminated tetronic and N,N DMAB+aminated tetronic did not induced significant apoptosis. Conclusion: Unmodified Tetronic T1107 induces an anticancer effect more than modified Tetronic polymers.

Keywords: Apoptosis, Tumor, Cell Cycle, Tetronic, Polymer

INTRODUCTION

In recent years, poloxamers and poloxamides have received increasing attention in the pharmaceutical field, mainly for their advantages as potential nanosystems (Ref). Thereby, in order to create new alternative treatments for current pathologies, like cancer, alterations in the poloxamine could promote the ability to incorporate a drug or a nucleic acid molecule, this nanosystem has the potential to be used in cancer treatment (Ref). Block copolymers contain at least two incompatible blocks, so they form solid-state microdomains and self-assemble in selective solvents (Bahadur 2001, Riess 2003), these characteristics, combined with advances in polymerization technology, make polymeric surfactants very useful in the material (Nakashima and Bahadur, 2006). Tetronics® is a commercially available poly ethylene oxide (PEO) poly propylene oxide (PPO) block copolymer with unique temperature-dependent micellization, surface activity, and reversible thermal rheological behavior(Gonzalez-Lopez et al. 2008). Due to their low immunomodulatory activity and non-toxic effect, some of these copolymers have now been approved by the Food and Drug Administration (FDA) (Singh-Joy and McLain 2008). Their emerging applications in the manufacture of mesoporous materials (Sang...
and Coppens. 2011, Chen and Chang. 2014) synthesis of nanoparticles (Habas et al. 2004, Singh et al. 2014) and nanocarriers as drug delivery systems (Hedberg et al. 2004, Oh et al. 2004, Csaba et al. 2005, Sezgin et al. 2006, Fernandez et al. 2008) presented them as valuable biomaterials with potential medical applications (Hamley, 1998, Alexandridis and Lindman 2000). Tetronics® (also known as poloxamines) present an X-shaped structure made of an ethylenediamine central group bonded to four chains of PPO–PEO blocks. Tetronics® are synthesized by the sequential reaction of the acceptor ethylenediamine molecule first with propylene oxide (PO) and then with ethylene oxide (EO) precursors, resulting in a four-arm PEO-terminated molecular structure. The unique structure of Tetronics® provides them with multistimulus responsiveness.

Chemotherapy as Doxorubicin (DOX) is a chemotherapeutic drug that has been around since the late 1960s and is considered one of the most powerful broad-spectrum antitumor drugs. It’s often used to treat a variety of cancers, including leukemia, lymphomas, soft-tissue sarcomas, and solid tissue sarcomas (Lefrak et al. 1973). There are more than 100 diverse chemotherapy drugs that cause diverse common side impacts such as bone marrow concealment (Khouri et al. 2008), leucopenia shows up at the 10th day of the chemotherapeutic course whereas thrombocytopenia after 10-14 days(Hadland B.K., Longmore G.D. 2009), and spewing foot, weakness, queasiness, handerythrodysesthesia, cardiotoxicity. Moreover, thromboembolism, pericardial thickening, or cardiac arrhythmias (Chen and Di. 2016). The present study aimed to test the antitumor effect of Unmodified Tetronic (T1107), synthetic P-HB+Aminated Tetronic and, on HepG2 cells in vitro and to assess their antitumor effects in vitro determining whether these unmodified and modified polymers affect apoptosis and cell cycle of HepG2 cells compared to doxorubicin.

MATERIALS AND METHOD
Chemicals and reagents
Tetronic 1107 (T1107) (Average molecular weight: 15000 g/mol) was purchased from BASF Corporation (New Jersey, USA). Chloroacetyl chloride and Glacial acetic acid were obtained from El-Gomhouria Chemicals Company (Cairo, Egypt). Pyridine was obtained from El-Nasr Pharmaceutical Chemicals (Cairo, Egypt). p-Phenylene diamine was purchased from Acros Organics (Belgium). p-Hydroxybenzaldehyde, and p-dimethyl amino benzoaldehyde were purchased from Aldrich (USA). All aldehydes were used without further purification. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent were purchased from Millipore, Merck, Germany. Dulbecco’s modified Eagle’s medium (DMEM) and sterile phosphate buffered saline were purchased from Lonza, USA, fetal bovine serum were purchased from Life Science Production, UK.

Cancer cell line
Human Hepatocellular Carcinoma (HepG2) cell line was purchased from Medical Technology Center, Medical Research Institute, Alexandria University, Egypt

Synthesis of Schiff base of T1107
Chloroacetylation of T1107
In a round flask (100 ml), 19 ml of Pyridine (240 mmol) was added to a solution of T1107 (10g, 20 mmol) in dry ethanol and the mixture was cooled to 0 oC. Chloroacetyl chloride (19 ml, 240 mmol) was added to the cold mixture dropwise with vigorous stirring (DAIHAN MaXtir™ 500S Hi-performance Digital Magnetic Stirrers, SRICO, South Korea). The reaction mixture was stirred at 0°C for 3h and at room temperature for further 48h. The excess ethanol was removed by a rotary evaporator (IKA RV 10V Digital Rotary Evaporator, 115VAC, Germany). The precipitate was washed with diethyl ether and the product was dried under vacuum (Model VD 53, BINDER, Germany) for 10h (Figure.1).

Amination of chloroacetylated T1107
To a solution of p-phenylenediamine (13g, 120 mmol) in dry ethanol, chloroacetylated T1107 (5g, 6 mmol) were added portionwise. After the addition was completed, the system was fitted to reflux at 80°C for 4 days with continuous stirring. The product was washed with ethanol to remove the unreacted diamine. The product
was then dried under vacuum for 48h (El-Safty et al. 2021).

**Modification of the aminated tetronic with p-Dimethylamino benzaldehyde**

A mixture of p-(dimethylaminobenzaldehyde) (1.06g, 7.13 mmol), aminated tetronic (1g, 0.89 mmol), and 1mL glacial acetic acid in 15mL methanol was stirred at room temperature for 48h. The system was fitted to reflux for 10h at 80°C. The product was filtered off and washed with methanol to remove the unreacted species. The product was collected as a dark red powder and dried under vacuum at room temperature for 48h (El-Safty et al. 2021).

**Modification of aminated tetronic with p-hydroxy benzaldehyde**

A mixture of 4-hydroxybezaldehyde (0.87g, 7.13 mmol), aminated tetronic 1107 (1g, 0.89 mmol) and 1mL glacial acetic acid in 15mL methanol was stirred at room temperature for 48h. The system was fitted to reflux for 10h at 80°C. The product was filtered off and washed with methanol to remove the unreacted species. The product was collected as dark orange powder and dried under vacuum at room temperature for 48h (El-Safty et al. 2021).

**Measuring Cell Viability in vitro Using MTT**

Adherent cells were harvested with 2 ml trypsin-EDTA 0.25% then counted by hemocytometer. After that cells were seeded into 96-well plates in 100 µl (5000 cell/well) for each well, the cells were then incubated in 5% CO2 at 37°C incubator for 24h. Stock solutions of Unmodified Tetronic (T1107), synthetic P-HB Aminated Tetronic and N,N DMAB Aminated Tetronic were prepared based on optimal concentration of these polymers as used in drug delivery as a carrier(20mg/ml), by dissolving different concentration of the polymers in DMF (0.1%), while Doxorubicin concentration (ref. drug) was (1mg/ml). The plates were incubated for 24 hrs. at 37°C and in 5% CO2. After 24h of incubation, the medium was discarded from the plates and then the plates were washed with 100 µl PBS, then the cells were treated with 50µl of each polymers concentration diluted in 200µl of complete media, the plates were incubated for 24 h, MTT assay was performed for determining the cytotoxicity by adding 25 µl of MTT (5mg/ml) to each well and the plates were then incubated for 4. Then, cells were washed with PBS and 150µl DMSO was added to each well. The absorbance was measured at 545 nm using Plate Reader. The cell viability analysis was performed using Graph Pad Prism 6.0.

**Measuring Apoptosis of HepG2 cells by Flow Cytometry**

Based on the results of the cytotoxicity assay, HepG2 cells were collected from a 6-well plate previously treated with the stock solution of 5mg/ml (with final concentration 1.25mg/ml) of Unmodified Tetronic (T1107), synthetic P-HB Aminated Tetronic and N,N DMAB Aminated Tetronic. HepG2 cells were washed twice with ice-cold PBS, the cell density was calculated, and the cells were re-suspended in 1× annexin-binding buffer to obtain a final density of 1×10⁶ cells/ml. Then, 100µl of the cell suspension was placed into 1.5-ml Eppendorf tubes and 5µl annexin V-fluorescein isothiocyanate (FITC) and 1µl PI (100µg/ml) working solution was added. Stained HepG2 cells were then incubated at room temperature for 15 min followed by the addition of 400 µl of 1× annexin-binding buffer with gentle mixing; then, the samples were kept on ice. The cells were then analyzed by flow cytometry.

**Measuring Cell Cycle of HepG2 cells by Flow Cytometry**

Based on the results of cytotoxicity assay, HepG2 cells were harvested from a 6-well plate previously treated with the stock solution of 5mg/ml (with final concentration 1.25 mg/ml), synthetic P-HB Aminated Tetronic and N,N DMAB Aminated Tetronic. HepG2 cells were prepared at a concentration of 1×10⁶ cells/ml, washed twice with ice-cold PBS, and fixed with 70% ethanol at 4°C overnight. The fixed cells were re-suspended in 300– 500 µl PI/Triton x100 staining solution (1000µl of 0.1% Triton+ 40µl PI+ 20µl RNase), for 30 min at 37°C in the dark. The cells were then centrifuged at 1000xg, and the number of cells at the different phases of the cell cycle was analyzed using flow cytometry (BD FACSComp IIflow cytometry, BD Biosciences, USA) and the data were analyzed using FlowJo software.
RESULTS
Measuring modification of the aminated T1107 with p-hydroxy benzaldehyde by FT-IR spectra
The FT-IR spectrum of T1107 Schiff base (IV) showed the disappearance of the primary amino group, instead a strong peak appeared at 1603 cm⁻¹ which belongs to the azomethine group (C=N) due to the reaction of aminated T1107 with p-hydroxy benzaldehyde that confirms the formation of the Schiff base (Figure 2).

Modification of the aminated T1107 with dimethyl amino benzaldehyde
The FT-IR spectrum of T1107 Schiff base (V) showed the disappearance of the primary amino group, instead a strong peak appeared at 1599 cm⁻¹ which belongs to the azomethine group (C=N) due to the reaction of aminated T1107 with p-hydroxy benzaldehyde that confirms the formation of the Schiff base (Figure 2).

Sample size characterization by zetasizer analysis
Size characterization of samples was made by dynamic light-scattering (DLS) measurements using the Zetasizer Malvern, the result show distribution of and N,N DMAB+Aminated Tetronic, of P-HB+Aminated Tetronic in particles size 1339nm, 2112 nm, respectively in a volume equal to 100 ml of distl water, while the unmodified Tetronic particle size is 5.6 nm (Figure 3).

Effect of Unmodified Tetronic (T1107) and modified Aminted Tetronic polymers on HepG2 cells viability using MTT
Treatment of HepG2 cells with Unmodified Tetronic (T1107), P-HB+Aminated Tetronic and N,N DMAB+Aminated Tetronic compared to DOX treatment however there was some aggregations in media (Figure 4), the different concentrations of P-HB+Aminated Tetronic and N,N DMAB+Aminated Tetronic did not show any effect on the cells viability, while the low concentration (stock conc=5mg/ml which diluted into 1.25mg/ml) of Unmodified Tetronic (T1107) showed that reduced HepG2 cells viability to (67%) compared to negative control Hepg-2 cells (100%) and Dox (45%) (Figure 5).

Effect of unmodified and modified Tetronic (T1107) polymer on apoptosis of HepG2 cells in vitro
Healthy and apoptotic cell percentage of HepG2 cells was analysed using flow cytometry after treatment with Unmodified Tetronic (T1107), P-HB+Aminated Tetronic and N,N DMAB+Aminated Tetronic and phenotyping distribution of healthy and late apoptotic cells (Figure 6) and % of healthy, early apoptotic, late apoptotic cells and necrotic cells (Figure 7). The results showed that treatment with Unmodified Tetronic (T1107) increased the late apoptotic cells % to 49.2% compared to N,N DMAB+Aminated Tetronic and P-HB+Aminated Tetronic and DOX, 1.77%, 0.65 % and 29.2% respectively.

Effect of unmodified and modified Tetronic (T1107) polymer on cell cycle of HepG2 cells in vitro
Nuclear DNA content of Hepg-2 cells was analyzed using flow cytometry after treatment with P-HB+Aminated Tetronic and N,N DMAB+Aminated Tetronic and unmodified Tetronic at (5 mg/ml) respectively, compared to free DOX as reference drug, results showed that DNA content of Hepg-2 cells undergoes different phases (sub G0, G1, S, and G2M) of the cell cycle before mitotic division and graphs of fractional DNA content (PI fluorescence, X-axis) versus cell counts (Y-axis) are displayed in Figure 8. Cell cycle distribution of Hepg2cells from Hepg-2 cells treated with Unmodified Tetronic (T1107)induced cell cycle arrest at G0 phase (39.4%) while, P-HB+Aminated Tetronic and N,N DMAB+Aminated Tetronic arrested the cell cycle at G1 phase 54.8% and 48.2% respectively compared to free DOX, which induced HepG2 cell cycle arrest at G0 phase 39.1% (Figure 8).

Statistical analysis
Numerical data obtained from each experiment were expressed as mean ± SE and statistical differences between experimental and control groups were assessed using One-way Analysis of Variance (ANOVA). Graph Pad Prism (Graph Pad Software, Inc., San Diego, CA) was used to analyze P values. P values <0.05 were considered statistically significant.
T1107 polymer expressed anticancer potential against HepG2 cell line

Figure 1. Scheme of Synthesis of Aminated T1107

Figure 2. FT-IR spectrum of Schiff base aminated tetronic+ dimethyl amino benzaldehyde (DMAB+Aminated Tetronic) and aminated tetronic+ para hydroxyl benzaldehyde (PHB+Aminated Tetronic).
Figure 3. Particle size distribution of P-HB+Aminated Tetronic, \( \text{N,N DMAB+Aminated Tetronic} \) and Unmodified Tetronic measured on a Zetasizer Marvlen, (A) P-HB+Aminated Tetronic, (B) \( \text{N,N DMAB+Aminated Tetronic} \), (C) Unmodified Tetronic(T1107).

**DISCUSSION**

Since, DOX is widely used as a chemotherapeutic drug for treating a variety of cancers such as liver cancer. However, it associates with significant side effects. The present study was undertaken to evaluate the capability of Unmodified Tetronic (T1107), \( \text{N,N DMAB+Aminated Tetronic} \) and P-HB+Aminated Tetronic as anticancer materials, if any, and to compare the anti-tumor effects of these materials to DOX, using Human Hepatocyte Carcinoma (HepG2) cell line in vitro.

According to (El-Safty et al. 2021) \( \text{N,N DMAB+Aminated Tetronic} \) and P-HB+Aminated Tetronic were synthesized by Schiff Base of Tetronic 1107. The FT-IR spectrum of T1107 Schiff base showed the disappearance of the primary amino group azomethine group (C=N) due to the reaction of aminated T1107 with p-hydroxy benzaldehyde forming P-HB+Aminated Tetronic, and reaction with p-Dimethylamino benzaldehyde forming N,N DMAB+Aminated Tetronic, this chemical modification could led to greater stability of the modified copolymer compared to the unmodified Tetronic (Jonathan et al., 1998). The free amino groups caused an increase in the phagocytosis in experiments carried out with HepG2 cells.
T1107 polymer expressed anticancer potential against HepG2 cell line

**Figure 4.** Effect of modified Tetronics on HepG2 cells (A) HepG2 cancer cells, (B) Doxorubicin, (C) Unmodified Tetronic, (D) N,N DMAB+ Aminated Tetronic, (E) P-HB+ Aminated Tetronic in vitro on cancer cells viability. HepG2 cells were cultured in vitro for 24 hr and then assessed under inverted microscope.

**Figure 5.** The effect of modified Tetronic on HepG2 viability. HepG2 cells were seeded in complete DMEM medium at (37°C, 5%CO2) and treated with N,N DMAB+ Aminated Tetronic and (F) P-HB+ Aminated Tetronic and HepG2 cell viability was measured using MTT assay. Data were represented as mean ± SE (n=3). ***p=0.0006, **** P < 0.0001, Ns (Not significant) statistically significant comparison of control group and other treated group.

When the amine groups were capped (p-hydroxy benzaldehyde and p-Dimethylamino benzaldehyde, the coating was 1-2 µm, the surface recovered the anionic character, and the uptake by the cells was reduced, although it was still higher than for Unmodified Tetronic nanospheres. Minor differences were observed regarding biodistribution: tetramine poloxamine enhances splenic uptake, while the capped poloxamine derivative reverts this effect and slightly enhances the fraction of nanoparticles at blood 3 hours after injection (Jonathan et al. 1998).

The zetasizer showed the presence of the unmodified Tetronic (T1107) in nano size and it did not exceed 10 nm, It is well known that particle size is crucial for biomedical applications during circulation and biodistribution inside the living system. For nanomedicine, particles smaller than 10 nm can be easily removed from the kidney or by extravasation, while larger particles have an adverse effect on diagnostic sensitivity and therapeutic effects, and are easier to remove by the reticuloendothelial system. Furthermore, compared to unmodified Tetronic (T1107), the particle size range of DMAB+ Aminated Tetronic and PHB+ Aminated Tetronic is 12 µm, and the effect of these modified polymers as antitumor materials is not significant. Cytotoxicity analysis showed that, compared to DMAB+ Aminated Tetronic and PHB+ Aminated Tetronic, unmodified tetronic T1107 showed higher cytotoxicity to HepG2 cancer cells at a stock concentration of 5mg/ml (with final concentration 1.25 mg/ml). This effect is due to the existence of free functional groups with unmodified tetronic, which may be due to the induction of cytotoxicity due to the reaction with specific cell receptors. In DMAB+ Aminated Tetronic and PHB+ aminated Tetronic, functional groups limit main stability Prevent reaction with cell receptors. Several anticancer compounds have been shown to have growth inhibitory effects by blocking the cell cycle at specific checkpoints of the cell cycle or by inducing apoptosis or the collective effect of cycle arrest and apoptosis (Gerard El, Karen VH. 2001). Therefore, this study analyzed the effects of unmodified Tetronic T1107, DMAB+ aminated Tetronic, and PHB+ aminated Tetronic on these parameters. They exhibit different characteristics in the cancer cell cycle.
Figure 6. The effect of modified Tetronic on HepG2 cell apoptosis. HepG2 cells were treated with modified pluronic for 48h then phenotyping distribution of HepG2 cells and % of healthy, early apoptotic, late apoptotic cells and necrotic cells were assessed.

Figure 7. The effect of modified Tetronic on HepG2 cell apoptosis. HepG2 cells were treated with modified Tetronic for 48h then phenotyping distribution of Hepg-2 cells and % of healthy, early apoptotic, late apoptotic cells and necrotic cells were assessed. Data were represented as mean ± SE (n = 3), * p ≤ 0.05, ** p ≤0.01, *** p≤ 0.001, **** P ≤ 0.0001 statistically significant comparison of control group and other treated.
T1107 polymer expressed anticancer potential against HepG2 cell line

Figure 8. Nuclear DNA contents of Hepg-2 cells after treatment with modified and unmodified Tetronic. HepG2 cells were treated with modified polymers for 48 hr and then HepG2 cell cycle was analyzed. (A) Hepg-2 cancer cells, (B) Doxorubic, (C) Unmodified Tetronic, (D) N,N DMAB+Aminated Tetronic, (E) P-HB+Aminated Tetronic.

Unmodified Tetronic T1107 stops about 39.4% of cancer cell cycle in G0 phase. This means that the cells remain metabolically active in this phase but do not proliferate; the cells are not actively dividing or preparing to divide. On the other hand, when the cells enter the cell cycle they show the different phases of cell cycle as shown after treatment with combination of N,NDMAB and aminated Tetronic or after combination of PHB and Aminated Tetronic.

With regard to apoptosis of HepG2 cells, the current study revealed that treatment with the unmodified Tetronic T1107 showed higher numbers of Hepg-2 in late apoptosis. By contrast, treatment with DMA/Aminated Tetronic and P-HB+Aminated Tetronic did not show a significant effect on Hepg-2 cells as compared with free DOX. These data suggest that the anti-cancer effects of the unmodified Tetronic T1107 are mediated by both arresting the cell cycle of HepG-2 as well as inducing apoptosis.

CONCLUSION

Unmodified Tetronic T1107 induces an anticancer effect more than modified Tetronic polymers. Further studies are needed to optimize the beneficial effects of the obtained data and to understand the underlying mechanisms.

ABBREVIATIONS

- DOX: Doxorubicin
- N,N DMAB+Aminated Tetronic: N,N Dimethyl Amino Benzaldehyde Aminated Tetronic
- P-HB+Aminated Tetronic: Para Hydroxy Benzaldehyde Aminated Tetronic

AUTHORS’ CONTRIBUTION

All authors participated in the design of the study and performed the statistical analysis, all authors contributed to the manuscript revision, all authors approved the final manuscript.

CONFLICT OF INTEREST

All authors declare no conflicts of interest.
FUNDING

No fund was received for this work.

REFERENCES


Bahadur P. 2001, Block copolymers-their microdomain formation (in solid state) and surfactant behaviour (in solution). Curr. Sci. 80, 1002–1007.


Hedberg EL, Shih CK, Solchaga LA, Caplan AI, Mikos AG. 2004, Controlled release of hyaluronan oligomers from biodegradable polymeric microparticle carriers. J. Control. 100, 257–266.


