Effect of several PEO-PPO amphiphiles on *bax*, *bcl-2* and *hTERT* mRNAs: an insight into apoptosis and cell immortalization induced in hepatoma cells by these polymeric excipients

Cynthia Celeste Castronuovo, [1,2] María Luján Cuestas, [1,2] José Raúl Oubiña, [1,2] Verónica Lidia Mathet[*,1,2]

[1]Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPAM), UBA-CONICET, Facultad de Medicina, Universidad de Buenos Aires, Ciudad Autónoma de Buenos Aires, Argentina. [2]CONICET, Ciudad Autónoma de Buenos Aires, Argentina.

***Corresponding author:**

Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPAM), UBA-CONICET, Facultad de Medicina, Universidad de Buenos Aires. Paraguay 2155 piso 11. Ciudad Autónoma de Buenos Aires (C1121ABG), Argentina.

Phone: + 54 11 5950 9500 ext. 2175.

Fax: 54 11 4964 2554.

E-mail: vmathet@yahoo.com

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ABSTRACT

Recent data have shown that synthetic polymers and nanomaterials display phenotypic effects in cells and signal transduction mechanisms involved in inflammation, differentiation, proliferation and apoptosis. Aims: to investigate the effect of poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) block copolymers with a wide range of biomedical and pharmaceutical applications on apoptosis and/or cell immortalization, by flow cytometry and multiplex RT-PCR for *bax*, *bcl*-2 and *hTERT*. Results: PEO-PPO amphiphiles up-regulated *bax* and *hTERT* and induced apoptosis of two human hepatoma cell lines. Conclusions: PEO-PPO block copolymers -considered safe for human use- can drastically alter gene expression profiles of genes related to apoptosis / cell proliferation.

Keywords: *bax, bcl-2, hTERT*, multiplex RT-PCR, poloxamines, poloxamers

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AV-FITC, annexin V- fluorescein isothiocyannate; GRAS, generally recognized as safe; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; hTERT, human telomerase reverse transcriptase; MTS/PMS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate; PEO-PPO, poly(ethylene oxide)-poly(propylene oxide); PI, propidium iodide; RT-PCR, reverse transcription polymerase chain reaction.

INTRODUCTION

Hepatocarcinogenesis is a slow and complex process associated with the accumulation of genetic and epigenetic changes that occur during initiation, promotion and progression of the disease that progressively alter the hepatocellular phenotype producing cellular intermediates that evolve into hepatocellular carcinoma (HCC) (1). During the long-lasting preneoplastic stage, hepatocyte cycling is accelerated by upregulation of mitogenic pathways that leads to the production of monoclonal populations of aberrant and dysplastic hepatocytes showing telomere erosion and telomerase re-expression, sometimes microsatellite instability, and occasionally irreversible structural aberrations in chromosomes and genes that function in different regulatory pathways, including p73, p53, Rb, APC, DLC-1, p16, PTEN, IGF-2, β-catenin, c-myc and cyclin D1 (2,3).

Apoptosis -one form of programmed cell death- is a physiological process that plays a crucial role in controlling the number of cells in development, although it is also involved in a wide range of pathological conditions such as cancer.

At the molecular level, apoptosis is tightly regulated by the activation of the aspartate-specific cysteine protease (caspase) cascade. One of the pathways leading to the activation of these proteins depends upon the participation of mitochondria (4), which is in turn regulated by the Bcl-2 family of proteins, either inhibiting (Bcl-2, Bcl-X_L, Bcl-w, Bfl-1 and Mcl-1) or promoting apoptosis (Bax, Bak, Bad, Bcl-X_S, Bid, Bik and Hrk) (5,6). Heterodimerization between pro- and anti-apoptotic members of this family and relative levels of both types of proteins (e.g., Bcl-2/Bax) may determine the susceptibility to a given apoptotic stimulus and the cell fate (7).

Cell immortalization involves activity of the telomerase, a ribonucleoprotein enzyme required for the addition of telomeric repeats to the ends of chromosomes and thus, for chromosome stability (8). Two major subunits contribute to enzymatic activity: a structural RNA component that contains the template region and the catalytic subunit with reverse transcriptase activity (hTERT). The RNA component of telomerase is expressed constitutively in both normal and neoplastic tissues. Conversely, the expression of the *hTERT* (mRNA) is exclusively expressed in germ line and certain stem cells, and in almost all tumor-derived immortalized cells, preventing progressive telomere shortening during each cell division (9).

Substantial experimental data demonstrated that the transcriptional regulation of *hTERT* expression represents the primary and rate-limiting step in the activation of telomerase activity in most cells (9). This activity disappears during development differentiation in most somatic cells but remains active in some tissues, such as male germ cells, activated lymphocytes, and –among other cell types- hematopoietic proliferating stem-like cells (10).

In brief, analyzing the mRNA expression of both apoptotic cell death regulators as well as an immortalization associated telomerase control could be useful in antitumoral studies. Therefore, a multiplex RT-PCR was developed for the simultaneous detection and semi-quantitative analysis of *bax, bcl-2* and *hTERT* mRNAs in cellular samples. This assay also includes an internal control to determine the efficiency of both RNA extraction and amplification steps.

In the last few years, polymer nanomaterials have attracted a considerable interest as vehicles used for diagnostic and therapeutic agents. One promising example of a polymer nanomaterial used for safe and efficient delivery of drugs,

genes and imaging molecules is represented by linear and non-ionic Pluronic® block copolymers and by branched and ionic Tetronic® block copolymers (also known as poloxamers and poloxamines, respectively) (11). Due to their amphiphilic character, these block copolymers of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) can interact with hydrophobic surface and biological membranes, and in aqueous solutions they can self-assemble into micelles (12). Incorporation of low molecular mass drugs into such polymeric micelles may increase drug solubility and stability and may improve drug pharmacokinetics and biodistribution (13). However, polymer-based drug delivery systems consider such molecules as biologically inert excipients that protect biological agents from degradation, prolong their exposure to tissues and enhance transport of biological agents into cells (14). However, such consideration is under revision since it was demonstrated that at least when these amphiphilic molecules are combined with drugs, DNA or antigens, may alter genetically cellular responses to these compounds (15, 16).

Our research group is devoting efforts to improve the pharmacotherapy of HCC by using several PEO-PPO block copolymers -approved for their biomedical use by the FDA- as drug delivering systems. However, the term GRAS (generally recognized as safe) applied for these amphiphilic molecules could be a matter of concern since –as we mentioned above- some accumulative data about their toxicity has emerged during the past few years (16).

In the current study, by using a multiplex RT-PCR reaction technique, we investigated whether *bcl-2/bax* mRNA expression ratio or *hTERT* mRNA expression are respectively correlated with tumor-cell apoptosis or tumor-cell

proliferative activity in two HCC cell lines exposed to PEO-PPO block copolymers, as a way –in this particular case- to improve pharmacotherapy in cancer. Noteworthy, these studies also intend to support the need for assessing pharmacogenomic effects of polymeric materials used as pharmaceutical excipients or as biomedical devices.

2. MATERIALS AND METHODS

2.1. Polymeric amphiphiles

Poloxamines Tetronic® T904 (average molecular weight *Mw* = 6.7 kDa, % PEO = 40, HLB 12-18), T304 (*Mw* = 1.65 kDa, % PEO = 40, HLB 12-18) and T1107 (*Mw* = 15 kDa, % PEO = 70, HLB 18-23) and the poloxamer Pluronic® F127 (*Mw* = 12.6 kDa, % PEO = 70, HLB 18-23; BASF Corporation, New Milford, CT, USA) were used. To eliminate synthesis residues that could be cytotoxic, PEO-PPO samples were solubilized in distilled water, dialyzed against pure water (cellulose membrane, MWCO = 1000-3500, Spectra/Por Dialysis Membrane, Spectrum Laboratories, Inc., USA) over five days and freeze-dried. Aqueous solutions of these PEO-PPO surfactants were then prepared in distilled water and sterilized by filtration (0.22 μm, Millipore Ireland B.V., Carringtwohill, Co. Cork, Ireland).

2.2. Cells and culture conditions. The human hepatoma cell lines SKHep1 and PLC/PRF/5 were purchased from ATCC (SKHep1 ATCC N°: HTB-52; PLC/PRF/5 ATCC N°: CRL-8024). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies Corp.) and maintained at 37°C in a humidified atmosphere of 5% CO_2.

2.3. Cytotoxicity assays. Cells were resuspended in 96-well plates ($2.0x10^4$ cells/well), grown overnight and treated with T904, T304, T1107 and F127 at dissimilar concentrations (0.01, 0.1 and 1%). The cytotoxic activity was determined after 24 and 72 h by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS) assay (Promega, Madison, WI). The absorbance at λ=495 nm was determined by using a FlexStation 3 (Molecular Devices, LLC, Sunnyvale, CA, USA).

Quantification of hepatic transaminases (aspartate aminotransferase [AST] and alanine aminotransferase [ALT] in all supernatants of both cell lines was carried out by means of the IFCC kinetic method to complete cytotoxicity studies.

2.4. RNA extraction and cDNA synthesis*.* SKHep1 and PLC/PRF/5 cell lines were cultured in a 24-well plate ($2.0x10^5$ cells/well) and incubated in DMEM supplemented with 10% FBS for 24 h, at 37°C. Then, culture medium was removed from every well and replaced with fresh medium containing the corresponding PEO-PPO block copolymer solutions at those concentrations that turned out to be non-cytotoxic by any of the methods described above. Cells containing the surfactant solutions were incubated for 24 and 72 h, at 37°C. After treatment, cells were removed from the plates and lysed with a lysis buffer containing Tris HCl (pH 8.4), NaCl 130 mM, EDTA 5mM. Total RNA was extracted by using TRIzol® (Life Technologies, Carlsbad CA, USA) according to the manufacturer's specificactions. One microgram (as determined by spectrophotometry) of total denatured RNA was reverse transcribed by using 100 ng/µl random primers (Promega) and 1 µL of MMLV reverse transcriptase

(Life Technologies Corp.) in a total volume of 20 µl. The reaction was performed for 10 min at 25˚ C, 60 min at 37˚ C, and 45 min at 95˚ C.

2.5. Multiplex PCR.

Specific primers for *β-actin*, *bax*, *bcl-2* and telomerase mRNA were chosen expanding across adjacent exons (Table 1).

The multiplex PCR was performed in a 20 µl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 10 mM dNTPs, 2 mM $MgCl_2$, 10 µM of each primer and 0.05 U of *Taq* DNA Polymerase (Life Technologies Corp.) and 2 µl of cDNA. The reaction was initiated by heat denaturation at 94°C for 3 min and the amplification was carried out in two steps: 6 cycles with primers for *bax*, *bcl-2* and *hTERT* at 94°C for 30 s, 63°C for 30 s and 72°C for 30 s; followed by 30 cycles with the addition of β-actin primers, as follows: 94°C for 3 min, 9 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s and extension at 72°C for 30 s; and further 21 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 30 seconds. The final extension step was carried out at 72°C for 7 min. *β-actin* primers were added at a later stage in order to obtain a comparative amount of PCR products to be subsequently used for normalization. PCR products were resolved in 1.5% agarose gels, stained with ethidium bromide, visualized under UV light transillumination and photographed. Relative intensity of bands was quantified by densitometry scanning by using ImageJ 1.45 software and normalized to *β-actin* values.

The expected sizes of the amplified products were 523 bp for *β-actin*, 365 bp for *bax*, 293 bp for *bcl-2* and 209 bp for *hTERT*.

2.6. Apoptotic assays. Apoptosis was thoroughly evaluated in both liver-derived cancer cells by using annexin V - fluorescein isothiocyanate (FITC) in

conjunction with the vital dye Propidium Iodide (PI) as previously reported (17). Briefly, SKHep1 and PLC/PRF/5 cells lines were seeded into 6-well plates ($6.0x10^5$ and $8.0x10^5$ cells/well, respectively) and incubated in DMEM supplemented with 10% FBS for 24 h, at 37°C. Then, culture medium was removed from every well and replaced by fresh medium containing the corresponding PEO-PPO surfactant solutions at non-cytotoxic concentrations. Cells were incubated for 24 and 72 h, at 37°C and analysis of phosphatidylserine exposure during apoptosis was performed by flow cytometric analysis of annexin V-FITC/PI stained cells at both times (FITC Annexin V Apoptosis Detection Kit, BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ, USA) by using a FACScan cytometer (Becton Dickinson). A minimum of 10,000 events was analyzed.

2.7. Statistical analysis. All experiments were carried out in triplicate and data were analyzed by two-way ANOVA followed by Bonferroni's post-test to compare replicate means, by using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA). Results are presented as means ± SD and statistically significant differences regarding control values ($p < 0.05$) were indicated with the symbol (*).

3. RESULTS

3.1. Cytotoxicity assays. When the cytotoxic activity was determined in both cell lines, PLC/PRF/5 and SKHep1, using the MTS/PMS assay after 24 and 72 h post-exposure either to T904, T304, T1107 or F127 at dissimilar concentrations (0.01, 0.1 and 1%), we observed that: (i) 0.1-1% T904 was cytotoxic for both cell lines at both 24 and 72 h post-exposure; (ii) 1% T304

was cytotoxic for both cell lines only at 72 h post-exposure; and (iii) 1% F127 was cytotoxic only for SKHep1 at 72 h post-exposure. T1107 at all concentrations studied and the remaining concentrations of the copolymers mentioned above turned out to be cytocompatible.

Consistent results with those mentioned above were achieved by analyzing the supernatants´ levels of AST and ALT **(Fig. 1)**.

3.2. Multiplex cDNA amplification. Multiplex RT-PCR was assayed in two human hepatoma cell lines, SKHep1 and PLC/PRF/5. *β-actin*, *bax*, *bcl-2* and *hTERT* mRNAs were successfully detected in all of them, with the exception of *bcl-2* in PLC cells, which turned out to be negative for its expression (**Fig. 2**).

bax values ranged from 1 for controls (untreated cells) to 1.3 ± 0.07 and 1.3 ± 0.04 for SKHep1 cells at 24 h-exposure to 1 and 0.1% T1107, respectively. This poloxamine was the only PEO-PPO block copolymer that significantly increased the mRNA levels of *bax* at 24 h. In contrast, at 72 h post-exposure, mRNA values of *bax* significantly increased in all samples from 1 for untreated controls to 1.6 ± 0.07 for 0.1% F127. In addition, 0.01% T304 was the only assayed sample that did not significantly affect the mRNA levels of *bax*. No significant variations were observed in the mRNA levels of *bcl-2* in SKHep1 cells exposed to any copolymeric amphiphiles studied herein at both times, except for 0.1% F127, which at 72 h post- exposure, significantly increased the mRNA levels of *bcl-2* (1.4 ± 0.1). Different mRNA levels for the *bcl-2*/*bax* ratio ranged from 1 for the untreated controls to 0.7 ± 0.1 for cells exposed to 0.1% T304 at 72 h post-exposure. At this time point, all the copolymers studied herein displayed a significant decrease in the *bcl-2*/*bax* ratio compared to the untreated controls, except 0.01% T304 which did not display a significant variation in such ratio. At

24 h post-exposure, only SKHep1 cells treated with 1% and 0.1% T1107 showed a significant decrease in the *bcl-2*/*bax* ratio (0.7 ± 0.04 and 0.8 ± 0.006, respectively). Regarding the mRNA values of *hTERT*, at 24 h post-exposure, only 0.1% T304 significantly decreased their mRNA values (0.6 ± 0.03). At 72 h post-exposure, *hTERT* values were significantly increased in 0.01% T904 (1.3 ± 0.06), 0.1% T304 (1.3 ± 0.1), 1% T1107 (1.3 ± 0.1), 0.01% F127 (1.2 ± 0.04) and 0.1% F127 (1.5 ± 0.01) (**Fig. 3**).

In PLC/PRF/5 cells, *bax* values varied from 1.1 ± 0.06 for the untreated controls to 1.9 ± 0.02 for 0.01% F127 at 72 h post-exposure. Except for 0.01% T904, all copolymers assayed at 24 h post-exposure, displayed a significant decrease in the mRNA values of *bax*. At 24 h post-exposure, *hTERT* mRNA values were significantly increased in PLC/PRF/5 samples treated with 0.01% T904 (1.5 ± 0.1) and significantly decreased in those treated with 1% F127 (0.7 ± 0.05). At 72 h post-exposure, *hTERT* values were significantly increased in 0.01% T904 (2.0 ± 0.2), 0.01% T1107 (1.8 ± 0.3), 0.1% T1107 (1.4 ± 0.3), 1% T1107 (1.4 ± 0.2), 0.01% F127 (4.7 ± 0.4), 0.1% F127 (3.5 ± 0.2) and 1% F127 (2.2 ± 0.1) (**Fig. 4**).

3.3. Apoptotic assay with annexin V (AV)-FITC/PI. Apoptosis was studied in both liver derived cancer cells by using AV-FITC in addition to PI. Quantification of fluorescence levels is presented in **Table 2**. Exposure of PLC/PRF/5 cells to non-cytotoxic concentrations of T904, T304, T1107 and F127 for 24 h did not significantly induce any detectable sign of early or late apoptosis when compared to controls. However, at 72 h post-exposure, a significant increase in the number of early apoptotic cells (AV+/PI-) was observed in those cells treated with 0.1% T304 (12.5 ± 1.3), 0.01-1% T1107 (16.2 ± 1.7, 15.0 ± 0.1 and

15.9 ± 0.1, respectively) and 0.01-1% F127 (16.2 ± 0.3, 10.4 ± 0.3 and 19.0 ± 2.1, respectively). Late apoptosis (AV+/PI+) was only observed with 0.1% T304 (15.5 ± 3.4), 1% T1107 (18.1 ± 0.2) and 0.01-1% F127 (17.5 ± 0.2, 16.4 ± 1.2 and 17.7 ± 1.7, respectively).

In the case of SKHep1 cells, 1% T1107 was the only PEO-PPO copolymer that at 24 h post-exposure induced apoptosis with a significant variation in the number of cells binding only AV-FITC (early apoptosis, 3.6 ± 0.1). However, at 72 h post-exposure, a significant increase in the number of early and late apoptotic cells treated with 0.01% T904 (3.8 ± 0.2 and 10.3 ± 0.6, respectively), 0.1% T304 (7.6 ± 1.8 and 9.6 ± 0.7, respectively), 0.01% T1107 (7.9 ± 0.2 and 8.2 ± 0.9, respectively), 0.1% T1107 (6.0 ± 0.5 and 5.9 ± 0.3, respectively), 1% T1107 (5.8 ± 0.8 and 9.0 ± 1.3, respectively), 0.01% F127 (3.2 ± 0.3 and 7.0 ± 0.4, respectively) and 0.1%F127 (8.4 ± 1.2 and 9.2 ± 1.2, respectively) was observed.

4. DISCUSSION

HCC is a primary malignancy that affects hepatocytes with worldwide increasing incidence (18). It is considered the most frequent primary liver cancer and the third leading cause of cancer-related death (19). Its etiology is well-known, with a diversity of factors that include infections with hepatitis B virus (HBV) and/or hepatitis C virus (HCV) and alcohol intake as the most predominant, leading to liver cirrhosis, which culminates generally in HCC (20-23). In recent years, there were major advances in the understanding of this complex disorder which have focused on the development of new therapy strategies to overcome the ability of cancer cells to avoid apoptosis (24). Our

research group is investigating strategies to improve the pharmacotherapy of HCC. In this context, the present study investigated for the first time the effect on *bax*, *bcl-2* and *hTERT* mRNA expression of PEO-PPO block copolymers that, at present, are considered as intrinsically inert amphiphiles used in the preparation of micellar drug formulations, among which, micellar formulations with antitumoral agents are regarded as promising examples.

Apoptosis is a controlled and organized active process regulated by multiple factors, and its evasion is often due to a deregulated balance between anti-apoptotic (Bcl-2) and pro-apoptotic factors (Bax) (25, 26). Bax and Bcl-2 are members of the Bcl-2 family of proteins that controls cellular fate. When Bcl-2 is overexpressed, the ratio between them is altered, which means that cell death can be avoided by tumor cells (27-29). This feature is crucially responsible for treatment failure.

Another important factor is the telomerase activity, present in approximately 80% of tumor malignancies (30-34), including most cases of HCC (35-36). Tumor cells have the ability to become immortal through an unlimited replicative potential due to the activation of telomerase, which is the limiting step to cell immortalization (37,38).

In the present work, a multiplex RT-PCR was developed for the simultaneous detection and semi-quantitative analysis of *bax*, *bcl-2* and *hTERT* mRNAs in cellular samples exposed to PEO-PPO block copolymers at dissimilar concentrations. This multiplex RT-PCR combines both rapidity and specificity for detecting mRNAs involved in both apoptotic and immortalization events, providing a convenient tool for the study of the early steps of cell cycle regulation in various clinical or experimental situations. All copolymers studied

herein were able of up-regulate the mRNA levels of the pro-apoptotic protein, Bax and that of the cell immortalization enzyme, h-TERT; moreover, at least in SKHep1 cells, where Bcl-2 is expressed, the *bcl-2/bax* mRNA expression ratio was also evaluated and turned out to be significantly decreased, especially at 72 h post-exposure, suggesting a pro-apoptotic effect. At present, we have no firm explanation for an apparently contradictory result, e.g. the apoptotic event and the mRNA *hTERT* increase. However, it could reflect a compensatory process, where the initial synthesis of mRNA *hTERT* (at 72 h) might attempt to counteract the ongoing apoptotic event observed at such experimental time. A thorough study of the *hTERT* promoter activity might represent a useful tool to shed some light on one of the potential mechanisms of action of the PEO-PPO copolymers.

According to the results obtained with AV/PI, the PEO-PPO block copolymers studied herein displayed pro-apoptotic effects. It was reported that amphiphilic polymer surfactants such as Pluronics, bind to cellular membranes, leading to the incorporation of the hydrophobic PPO blocks into the lipidic interior, while the hydrophilic PEO blocks remain exposed to the external solution (39). This effect may result in cell membranes fluidization. Besides, it was also demonstrated that some Pluronic copolymers are able to accelerate the interleaflet exchange of the lipids (lipid flip-flop) (40).

Apoptosis analysis using AV in conjunction with PI revealed flip-flop of phosphatidylserine to the outer leaflet of the cell membrane. This event constitutes an early stage of apoptosis. Thus, all PEO-PPO surfactants studied in the present work significantly induced apoptosis of both human hepatoma cell lines, at least at 72 h post-exposure. These results suggests the ability of these

amphiphiles to be incorporated in pharmaceutical formulations as a way to improve the activity of the antitumoral agents used in the treatment of many solid tumors such as HCC.

In addition, the study described in this paper presents a new evidence of the significant effects that synthetic polymers can promote on signal transduction pathways, gene expression, cell apoptosis and cell immortalization. Thus, this work contributes to better characterize the clinical significance of the polymer genomics as a new field aimed to maximize clinical outcomes and to improve human health. Although in this particular case, where copolymers were assayed on human hepatoma cells, the effect of these surfactants on cell apoptosis constitutes a way to improve the pharmacotherapy of cancer, it must be emphasized that these copolymers should be used with caution due to their effect on genes related to apoptosis and/or cell immortalization.

5. CONCLUSIONS

PEO-PPO block copolymers displayed selective phenotypic effects in two human hepatoma cell lines (SKHep1 and PLC/PRF/5), affecting expression levels of genes related to apoptosis and cell immortalization. However, collectively considered, these copolymers displayed a pro-apoptotic effect, thus constituting a potential tool for improving the pharmacotherapy of antineoplastic agents. These studies also support the need for assessing pharmacogenomics effects of polymeric materials used in pharmacy and medicine to maximize clinical outcomes and have an insight into the pharmacological and toxicological effects of such polymeric formulations.

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Conflict of Interest

None to declare.

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Figure Legends

Figure 1. Effect of poloxamines T904, T304, T1107 and poloxamer F127 (0.01%, 0.1% and 1%) at (A) 24 h and (B) 72 h in PLC/PRF/5 cells, and at C) 24 h and D) 72 h in SKHep1 on transaminases levels. * $p < 0,05$.

Figure 2. Multiplex RT-PCR for *β-actin*, *bax*, *bcl-2* and *hTERT* mRNAs. Lane 1: molecular weight marker, lane 2: SKHep1 cells and lane 3: PLC/PRF/5 cells.

Figure 3. Effect of poloxamines T904, T304, T1107 and poloxamer F127 (0.01%, 0.1% and 1%) on relative expression levels of *bax*, *bcl-2* and *hTERT* in SKHep1 cells. (A) *bax*, (B) *bcl-2* and (C) *hTERT* mRNA levels and (D) *bcl-2*/*bax* ratio at 24 and 72 h post-exposure to copolymers. *Statistically significant respect to control ($p < 0.05$).

Figure 4. Effect of poloxamines T904, T304, T1107 and poloxamer F127 (0.01%, 0.1% and 1%) on relative expression levels of *bax*, and *hTERT* in PLC/PRF/5 cells. (A) *bax* and (B) *hTERT* mRNA levels at 24 and 72 h post-exposure with copolymers. *Statistically significant respect to control ($p < 0.05$).

Table 1. Pairs of primers used for Multiplex PCR

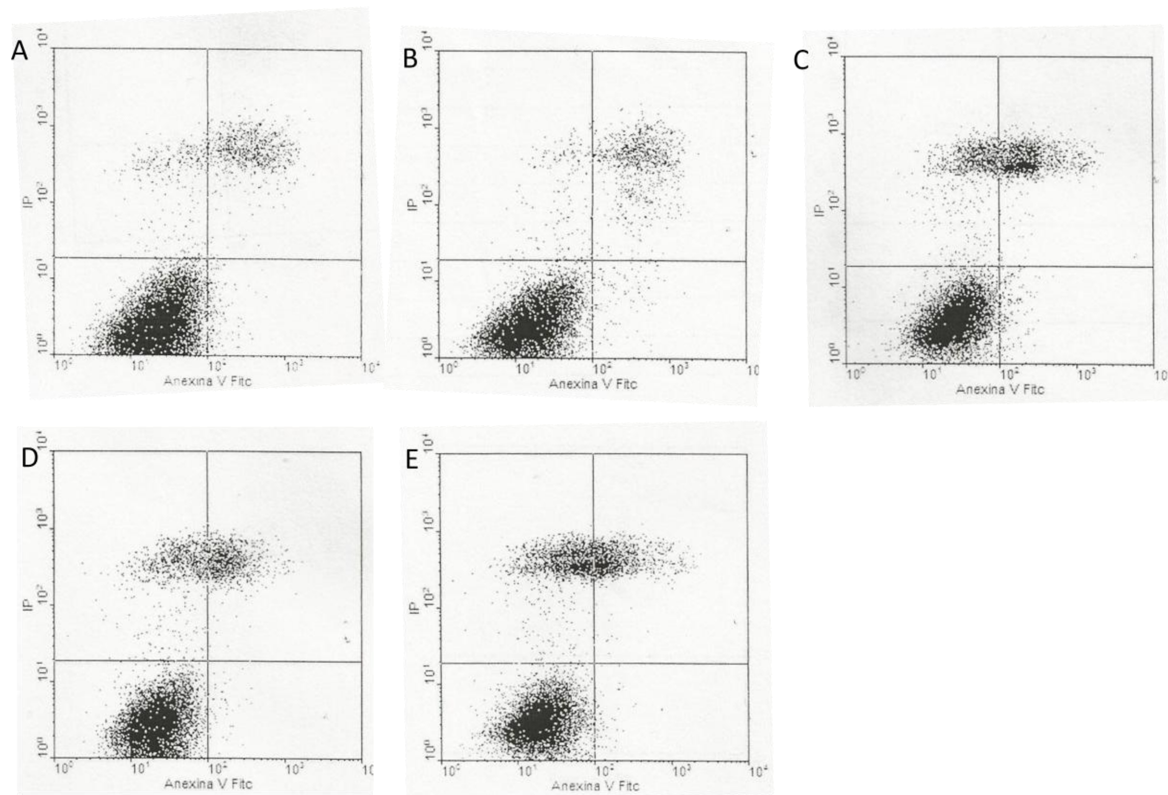
Gene	Forward primer (5’→3’)	Reverse primer (5’→3’)
bax	ACCAAGAAGCTGAGCGAGTGTC	ACAAAGATGGTCACGGTCTGCC
bcl-2	TGCACCTGACGCCCTTCAC	AGACAGCCAGGAGAAATCAAACAG
h-TERT	TCCTGCGTTTGGATGATTTCTTG	GCCGCACCAGGGGAATAGGC
β-actin	GGACCTGACTGACTACCTCATGAA	GATCCACATCTGCTGGAAGGTGG

Table 2. Early and late apoptosis determined using AV/IP at 24 and 72 h post-exposure to different PEO-PPO block copolymers in (A) PLC/PRF/5 and (B) SKHep1 cells. * $p < 0.05$.

		PLC/PRF/5			
		24 h		72 h	
		Early apoptosis	Late apoptosis	Early apoptosis	Late apoptosis
	Control	4.5 ± 0.50	6.5 ± 0.50	8.6 ± 0.04	13.9 ± 0.80
T904	**0.01%**	4.05 ± 2.20	6.7 ± 0.20	**11.0 ± 1.40***	14.6 ± 2.20
T304	**0.01%**	3.0 ± 1.50	7.2 ± 0.80	9.4 ± 1.20	11.4 ± 0.30
	0.1%	2.8 ± 0.70	6.3 ± 0.70	**12.5 ± 1.20***	**15.5 ± 3.40***
T1107	**0.01%**	4.8 ± 0.20	5.6 ± 1.50	**16.2 ± 1.70***	13.1 ± 0.40
	0.1%	4.8 ± 0.20	7.9 ± 0.10	**15.0 ± 0.10***	12.9 ± 0.20
	1%	5.2 ± 0.30	8.4 ± 0.50	**18.9 ± 0.10***	**18.1 ± 0.20***
F127	**0.01%**	7.0 ± 0.08	8.9 ± 0.07	**16.2 ± 0.30***	**17.5 ± 0.20***
	0.1%	7.2 ± 0.30	9.2 ± 0.30	**10.4 ± 0.30***	**16.4 ± 1.20***
	1%	7.6 ± 0.06	9.5 ± 0.07	**19.1 ± 2.10***	**17.7 ± 1.70***

		SKHep1			
		24 h		72 h	
		Early apoptosis	Late apoptosis	Early apoptosis	Late Apoptosis
	Control	2.8 ± 0.10	4.7 ± 0.20	3.0 ± 1.30	2.8 ± 0.20
T904	**0.01%**	2.8 ± 0.30	6.0 ± 0.50	**3.8 ± 0.20***	**10.3 ± 0.80***
T304	**0.01%**	3.3 ± 0.80	5.8 ± 0.40	4.7 ± 1.60	6.6 ± 1.60
	0.1%	2.5 ± 0.10	5.5 ± 0.20	**7.6 ± 1.80***	**9.6 ± 0.70***
T1107	**0.01%**	2.0 ± 0.70	5.2 ± 0.70	**7.9 ± 0.20***	**8.2 ± 0.90***
	0.1%	2.5 ± 0.60	4.8 ± 0.50	**6.0 ± 0.60***	**5.9 ± 0.30***
	1%	**3.6 ± 0.10***	4.7 ± 0.10	**5.8 ± 0.80***	**9.0 ± 1.30***
F127	**0.01%**	1.6 ± 0.30	4.4 ± 0.60	3.2 ± 0.30	**7.0 ± 0.40***
	0.1%	1.8 ± 0.40	3.2 ± 0.30	**9.4 ± 1.20***	**9.4 ± 1.20***

Supplementary Figure 1.



Flow cytometric analysis of apoptosis by using AV binding and PI uptake in PLC/PRF/5 (A, B and C) and SKHep1cells (D and E): (A) Control; (B) cells treated with 0.01% T904; (C) cells treated with 0.1% T304; (D) control; (E) cells treated with 0.01% F127.