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# Poloxamines Display a Multiple Inhibitory Activity of ATP-Binding Cassette (ABC) Transporters in Cancer Cell Lines

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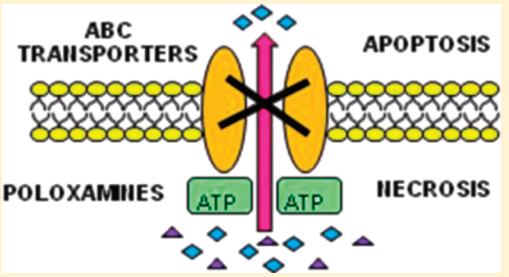
Supporting Information

**ABSTRACT:** Primary hepatocellular carcinoma is the third most common fatal cancer worldwide with more than 500,000 annual deaths. Approximately 40% of the patients with HCC showed tumoral overexpression of transmembrane proteins belonging to the ATP-binding cassette protein superfamily (ABC) which pump drugs out of cells. The overexpression of these efflux transporters confers on the cells a multiple drug resistance phenotype, which is considered a crucial cause of treatment refractoriness in patients with cancer. The aim of this study was to investigate the inhibitory effect of different concentrations of pH- and temperature-responsive X-shaped poly(ethylene oxide)-poly(propylene oxide) block copolymers (poloxamines, Tetronic, PEO-PPO) showing a wide range of molecular weights and EO/PO ratios on the functional activity of three different ABC proteins, namely P-glycoprotein (P-gp or MDR1), breast cancer resistance protein (BCRP), and multidrug resistance-associated protein MRP1, in two human hepatocarcinoma cell lines, HepG2 and Huh7. First, the cytotoxicity of the different copolymers (at different concentrations) on both liver carcinoma cell lines was thoroughly evaluated by means of apoptosis analysis using annexin V and propidium iodide (PI). Thus, viable cells (AV−/PI−), early apoptotic cells (AV+/PI−) and late apoptotic cells (V-FITC+/PI+) were identified. Results pointed out copolymers of intermediate to high hydrophobicity and intermediate molecular weight (e.g., T904) as the most cytotoxic. Then, DiOC2, rhodamine 123 and vinblastine were used as differential substrates of these pumps. HeLa, an epithelial cell line of human cervical cancer that does not express P-gp, was used exclusively as a control and enabled the discerning between P-gp and MRP1 inhibition. Moderate to highly hydrophobic poloxamines T304, T904 and T1301 showed inhibitory activity against P-gp and BCRP but not against MRP1 in both hepatic cell lines. A remarkable dependence of this effect on the copolymer concentration and hydrophobicity was found. No inhibitory effect against these ABC pumps was observed with the hydrophilic T1107. These findings further evidence the potential usefulness of these Trojan horses as both drug nanocarriers and ABC inhibitors in hepatic MDR tumors and infections that involve the activity of these efflux transporters.




**KEYWORDS:** ATP-binding cassette protein superfamily inhibition, P-gp, BCRP, MRP1, multidrug resistance, poloxamines

## 1. INTRODUCTION

Multidrug resistance (MDR) is generally accepted as a key cause of treatment failure in patients with infectious diseases [e.g., chronic hepatitis B (CHB), infection by the human immunodeficiency virus (HIV)] and different kinds of cancer such as primary hepatocellular carcinoma (HCC).[1]

Epidemiological studies have shown that HCC is the fifth most common cancer worldwide and the third cause of cancer mortality, with one million annual deaths.[2] Almost 80% of the HCC cases are etiologically associated with chronic infection with the hepatitis B virus (HBV) which affects approximately 400 million people around the globe.[3]

To eliminate persistent viral replication and prevent the progression to CHB, cirrhosis and HCC, several therapeutic strategies have been evaluated.[4] However, the emergence of drug resistant mutants that intrinsically withstand the current antiviral therapy constitutes a great limitation in the treatment of the HBV infection. The presence of cellular factors affecting the antiviral efficacy, such as the increased efflux of nucleos(t)ide analogues (NAs, e.g. lamivudine, adefovir, entecavir, telbivudine and tenofovir) via pumps of the ATP-binding cassette (ABC) superfamily, is another well-established mechanism of cellular resistance and therapeutic debacle.[5] For example, Han et al. have found that the X protein of HBV (HBx) increases the transcriptional activity and the expressed levels of P-glycoprotein (P-gp or MDR1) in the H4IIE rat hepatoma cell line;[6] P-gp was the first human ABC transporter shown to confer multidrug resistance

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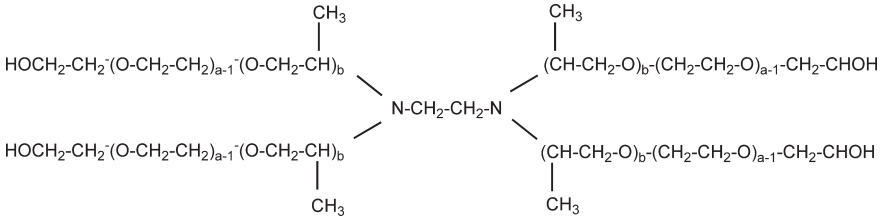
**Scheme 1. General Molecular Structure of Poloxamines**




and the most extensively investigated one.[7] On the other hand, the treatment of HCC is also hampered by the development of resistance to the diverse agents employed in antitumoral pharmacotherapy, especially doxorubicin (DOX).[8] These facts lead to extremely high mortality tolls.[9]

The molecular characterization of various hematological and solid tumors displaying the MDR phenotype resulted in the identification of several genes that code for different cellular efflux transporters.[10−12] To date, there are 48 identified human ABC genes.[13]

These transmembrane pumps are available in liver, heart, spleen, kidneys, lungs, pancreas, placenta, prostate, stomach,[13] the intestinal epithelium,[14] the central nervous system (e.g., microglia),[15] and many anatomical barriers such as the blood−brain barrier (BBB).[14] Some of them are ubiquitous, and others are tissue-specific.

The activity of the ABC proteins involves the binding of ATP and the use of the generated energy to pump molecules of different chemical nature across extra- and intracellular membranes against a concentration gradient.[13] Thus, drug removal from target cells results in subtherapeutic concentrations and eventually in therapeutic failure.[16]

Overexpression of P-gp, multidrug resistance-associated proteins (MRP1 and MRP2) and the breast cancer resistance protein (BCRP) were reported to mediate resistance and cross-resistance to multiple drugs in HCC.[8,17,18] In addition to drug resistance, it was reported that MDR-positive cells block the release of mitochondrial cytochrome *c* into the cytoplasm, preventing apoptosis in tumor cells.[19,20]

Many pharmacologically active compounds have shown the ability to inhibit the activity of ABC transporters. For example, elacridar (GF120918) is a combined BCRP/P-gp inhibitor currently under clinical trials as MDR reversal agent.[21] The coadministration of low doses of VER, another P-gp substrate, with DOX increased the bioavailability of the anticancer drug in rodents.[22] Some of these inhibitors are highly toxic (e.g., fumitremorgin) and have been investigated only *in vitro*. However, their clinical implementation could be from controversial to unfeasible.

The effective inhibition of ABC pumps by means of different self-assembly amphiphilic copolymers (e.g., poly(ethylene oxide)-poly(propylene oxide); PEO-PPO) has been well documented.[23,24] Kabanov et al. investigated the activity of linear PEO-PPO-PEO triblocks (poloxamers, Pluronic) on the inhibition of efflux transporters overexpressed in MDR cells.[25−27] It is worth stressing that the inhibitory activity has been ascribed mainly to the unimers. Recently, the fast uptake and the colocalization of these copolymers with the mitochondria were shown to hinder different stages of the respiratory chain and to constrain oxygen consumption, thus depleting ATP levels and inhibiting the activity of the pump.[27] In the first study assessing the inhibitory activity of branched PEO-PPOs (poloxamines, Tetronic), we recently reported on the structure-related inhibitory activity of pristine and *N*-methylated poloxamines on P-gp pumps using a human colon carcinoma cell line (Caco2) model;[28] poloxamines display a higher functionality than poloxamers and a dual temperature and pH-responsive behavior that results in different and more complex aggregation patterns.[29,30] Moreover, the polymeric micelles formed by these self-assembly copolymers above the critical micellar concentration constitute versatile nanocarriers to encapsulate poorly water-soluble drugs[31] and to passively target them to tumors by means of the enhanced permeation retention (EPR) effect.[32]

**Table 1. Physical Properties of Poloxamines T304, T904, T1107 and T1301**

| Tetronic | MW(kDa) | no. of EO units (a) | no. of PO (b) | HLB |
|---|---|---|---|---|
| 304 | 1650 | 16 | 16 | 12−18 |
| 904 | 6700 | 60 | 68 | 12−18 |
| 1301 | 6800 | 16 | 104 | 1−7 |
| 1107 | 15,000 | 238.6 | 80 | 18−23 |

**Table 2. Relative Ability of P-gp (MDR1), MRP1 and BCRP To Efflux Rho 123 and DiOC2 and Their Susceptibility To Be Inhibited by VIN**

| | Rho 123 | DiOC2 | inhibition by VIN |
|---|---|---|---|
| MDR1 | +++ | +++ | +++ |
| MRP1 | ++ | − | +++ |
| BCRP | − | + | − |

Our research group investigates nanotechnology strategies to improve the pharmacotherapy of CHB, chronic hepatitis C (CHC) and HCC.[4] In this context, the present study investigated for the first time the multiple inhibitory effects of intrinsically inert amphiphiles on the functional activity of P-gp, BCRP and MRP1 in two human liver carcinoma cell lines. Overall results would support the potential of these copolymers in the therapy of human liver diseases that are refractive to the pharmacotherapy due to the activity of these pumps.

## 2. MATERIALS AND METHODS

**2.1. Poloxamines.** Poloxamines Tetronic 304 (T304, MW 1.65 kDa, 40 wt % PEO), 904 (T904, MW 6.7 kDa, 40 wt % PEO), 1107 (T1107, MW 15 kDa, 70 wt % PEO) and 1301 (T1301, MW 6.8 kDa, 10 wt % PEO) were a gift of BASF Corporation (New Milford, CT, USA). The general molecular

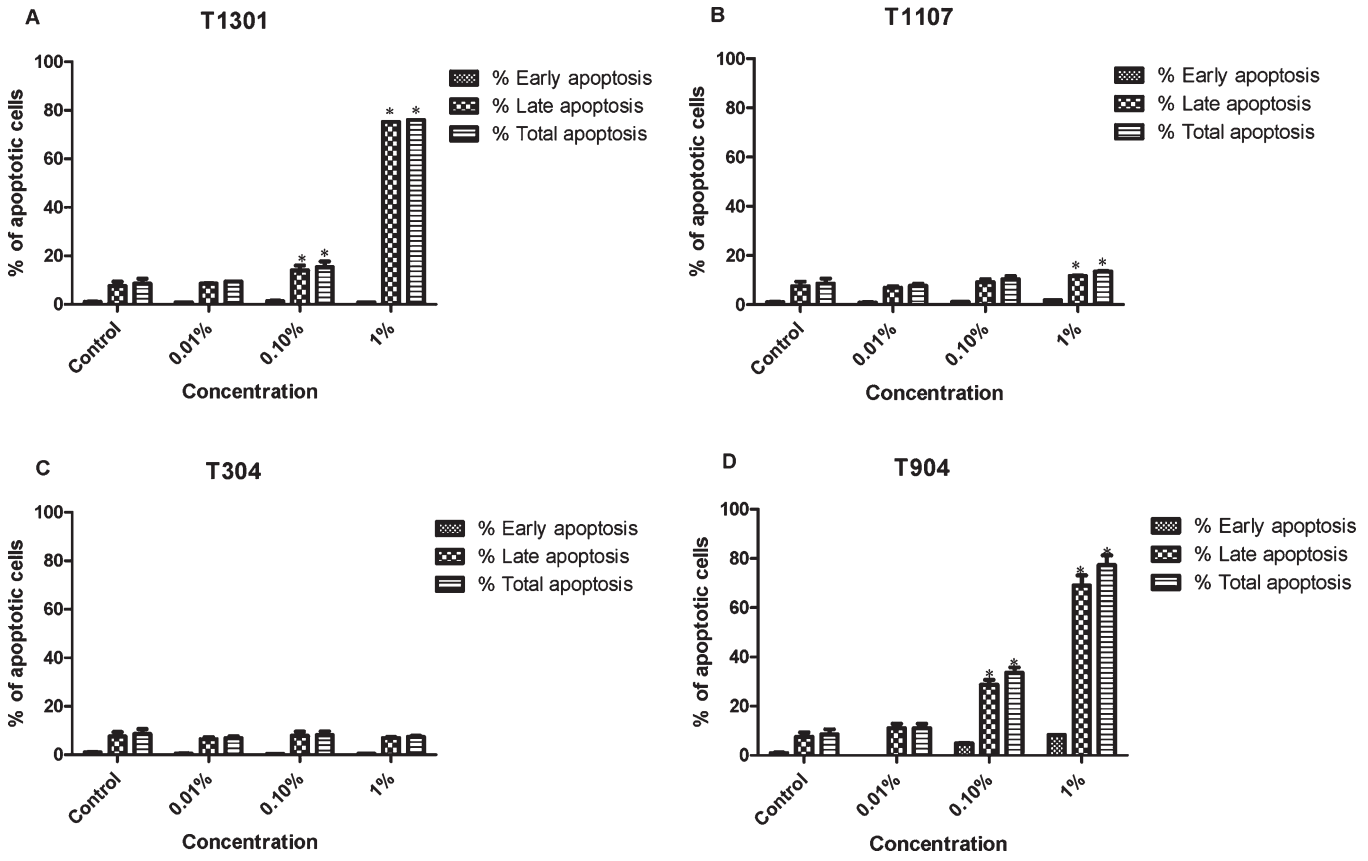



**Figure 1.** Effect of poloxamines (A) T1301, (B) T1107, (C) T304 and (D) T904 on apoptosis in Huh7 cells. *Statistical significance was calculated with ANOVA, with respect to untreated controls ($p < 0.05$).

structure of these copolymers is depicted in Scheme 1, and their physical properties are summarized in Table 1. To eliminate synthesis residues that could be cytotoxic, poloxamine samples were solubilized in distilled water, dialyzed against pure water (cellulose membrane, MWCO = 1000−3500, Spectra/Por Dyalisis Membrane, Spectrum Laboratories, Inc., USA) over five days and freeze-dried.

**2.2. Preparation of Poloxamine Solutions.** Poloxamine stock solutions (10% w/v) were prepared by dissolving the required amount of copolymer in distilled water, at 4 °C. Then, they were sterilized by filtration (0.22 $\mu$m, Millipore Ireland B.V., Carrigtwohill, Co. Cork, Ireland). Solutions were equilibrated at 37 °C at least 1 h before use.

**2.3. Cell Cultures.** Human cell lines derived from cervical cancer (HeLa) and from hepatocellular carcinoma (HepG2 and Huh7) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS, Life Technologies Corp). HeLa and HepG2 cells were from ATCC, whereas Huh7 cells were kindly provided by Prof. Dr. M. Mizokami (Nagoya City University Graduate School of Medical Sciences, Japan). All cells were maintained at 37 °C in a humidified 5% $CO_2$ atmosphere. Cells were harvested by trypsinization, and the number of live cells was determined with trypan blue (Life Technologies). Protein expression of P-gp, MRP1 and BCRP in Huh7, HepG2 and HeLa cell lines was corroborated by Western blot using specific monoclonal antibodies (Figure S1 in the Supporting Information).

**2.4. Poloxamine Cytotoxicity.** Prior to the study of the inhibitory activity, the cytotoxicity of the different copolymers (at different concentrations) on both liver carcinoma cell lines was comprehensively evaluated by means of apoptosis analysis using annexin V in conjunction with propidium iodide (PI). Annexin V (AV) is a $Ca^{2+}$-dependent, phospholipid-binding protein with high affinity for phosphatidylserine (PS). Thus, this protein can be used as a sensitive marker for PS exposure on the outer leaflet of the cell membrane.[33] The staining with annexin V-fluorescein isothiocyanate (FITC) in conjunction with vital dyes such as PI allows one to distinguish viable cells (AV−/PI−), early apoptotic cells (AV+/PI−) and late apoptotic cells (AV+/PI+). Since necrotic cells also stain with both annexin V-FITC and PI (AV+/PI+), they cannot be distinguished from cells undergoing late apoptosis.

Briefly, HepG2 and Huh7 cells were seeded in 6-well plates ($1.3 \times 10^6$ and $0.5 \times 10^6$ cells/well, respectively) and incubated in DMEM supplemented with 10% FCS for 24 h, at 37 °C. The culture medium was removed, copolymer solutions in culture medium (2 mL, copolymer final concentration 0.01%, 0.1% and 1%) were added, and cells were incubated again for 24 h, at 37 °C. The concentration of hepatic transaminases (aspartate aminotransferase, AST and alanine aminotransferase, ALT) in the supernatants of both hepatocyte cell cultures was determined by means of the IFCC kinetic method. Flow cytofluorometric analyses were performed by using rhodamine 123 (1 $\mu$g/mL, Rho 123, Sigma) for mitochondrial transmembrane potential ($\Delta\Psi_m$) quantification and PI (1 $\mu$g/mL, Sigma) for determination of cell hypodiploidy. Analysis of PS exposure during apoptosis was also performed by flow cytometric analysis of AV-FITC/PI-stained cells (FITC Annexin V Apoptosis Detection

Kit I, BD Pharmingen, Becton Dickinson, Franklin Lakes, NJ, USA) using a FACScan cytometer (Becton Dickinson). A minimum of 10,000 events was acquired gating the forward and side scatters to exclude cell debris and analyzed in FL-1 and FL-2. All data are expressed as means ± standard deviation (SD) of at least three independent experiments. Comparison of treatments against controls was made with ANOVA using Bonferroni's post-test to compare replicate means. The significance level chosen for statistical analysis was $p < 0.05$.

**2.5. Functional Activity Assay.** The drug efflux assay was performed by using the Multidrug Resistance Direct Dye Efflux Assay (Chemicon International Inc., Temecula, CA, USA) based on the differential affinity of the efflux pumps P-gp, MRP1 and BCRP to vinblastine (VIN), DiOC2 (3,3′-diethyloxacarbocyanine iodide) and Rho 123. DiOC2 is a highly specific substrate of P-gp, and it is a weak substrate of BCRP, while Rho 123 is effluxed by P-gp and to a lesser extent by MRP1. Both P-gp and MRP1 are inhibited by VIN (Table 2). To discern between the inhibitory effect of poloxamines on MRP1 and P-gp, a cell line lacking the expression of P-gp (HeLa cells) was used for comparison. It is worth stressing that the goal of the present work was to investigate the effect of the copolymers on liver cell lines and the use of HeLa cells was limited only to this assay. The assay was performed following the manufacturer's instructions. In brief, HeLa, HepG2 and Huh7 cells ($7.5 \times 10^4$ cells) were suspended in cold buffer (RPMI-1640 medium supplemented with 10% FCS chilled at 4 °C) and incubated in the presence of Rho 123 or DiOC2 on ice for 60 and 15 min, respectively. After this incubation time, Rho 123- and DiOC2-loaded cells were resuspended in cold buffer and incubated (i) in the presence of poloxamines and 0.1% dimethylsulfoxide (DMSO) or VIN, at 37 °C (optimal conditions for pump activity) and (ii) in the presence of poloxamines and cold buffer at 4 °C (metabolic inhibition of pump activity) for 1 h. The final copolymer concentrations considered for this study were those that did not show significant cytotoxicity (see above). After washing, cells were resuspended in cold buffer containing 1 μg/mL of PI and analyzed by flow cytometry. Single-cell fluorescence was quantified by counting a minimum of 10,000 events by using a FACScan flow cytometer and analyzed in FL-1 and FL-2. Results are expressed as mean fluorescence values. Statistical analysis was performed by using the Student *t* test [two-sided test]. The *p* values of less than 0.005 were considered significant.

## 3. RESULTS

**3.1. Poloxamine Cytotoxicity.** Exposure of Huh7 cells to 0.01% T904, 0.01% T1301, 0.01−0.1% T1107 and 0.01−1% T304 for 24 h did not significantly induce any detectable sign of early or late apoptosis when compared to the controls (Figure 1). In contrast, 0.1% and 1% T904 and T1301 as well as 1% T1107 showed a significant cell viability loss (Figure 1). However, no significant increase in the number of early apoptosis cells (AV+/PI−) was observed. It is worth mentioning that, regardless of the statistically significant cytotoxicity, 1% T1107 led to a relatively low cell death of approximately 12%. Flow cytometer dot plots corresponding to Huh7 cells untreated and treated with 1% T304 and T904 are presented in Figure 2. In the case of HepG2 cells, T304 was not cytotoxic, values being comparable to those of the controls. In contrast, T1301 turned out to be highly cytotoxic at all the tested concentrations (Figure 3). T1107 and

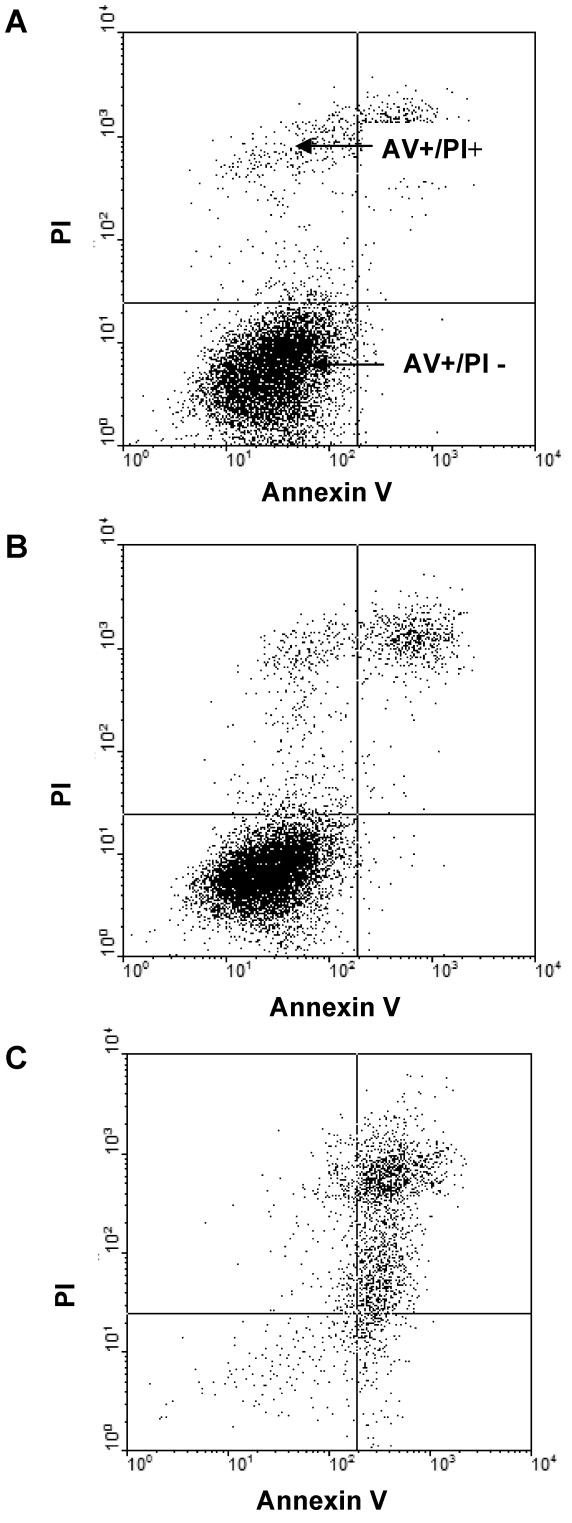



**Figure 2.** Flow cytometric analysis of apoptosis using AV binding and PI uptake in Huh7 cells: (A) control, (B) cells treated with T304 1% and (C) cells treated with T904 1%.

T904 showed a moderate to high cytotoxicity, cell death percentages ranging between 20% and 25% and between 15% and 32%, respectively. When HepG2 cells were exposed to 0.1% T904, a small fraction of the cell population was clearly in early apoptosis (AV+/PI−), as evidenced by a significant increase in the number of

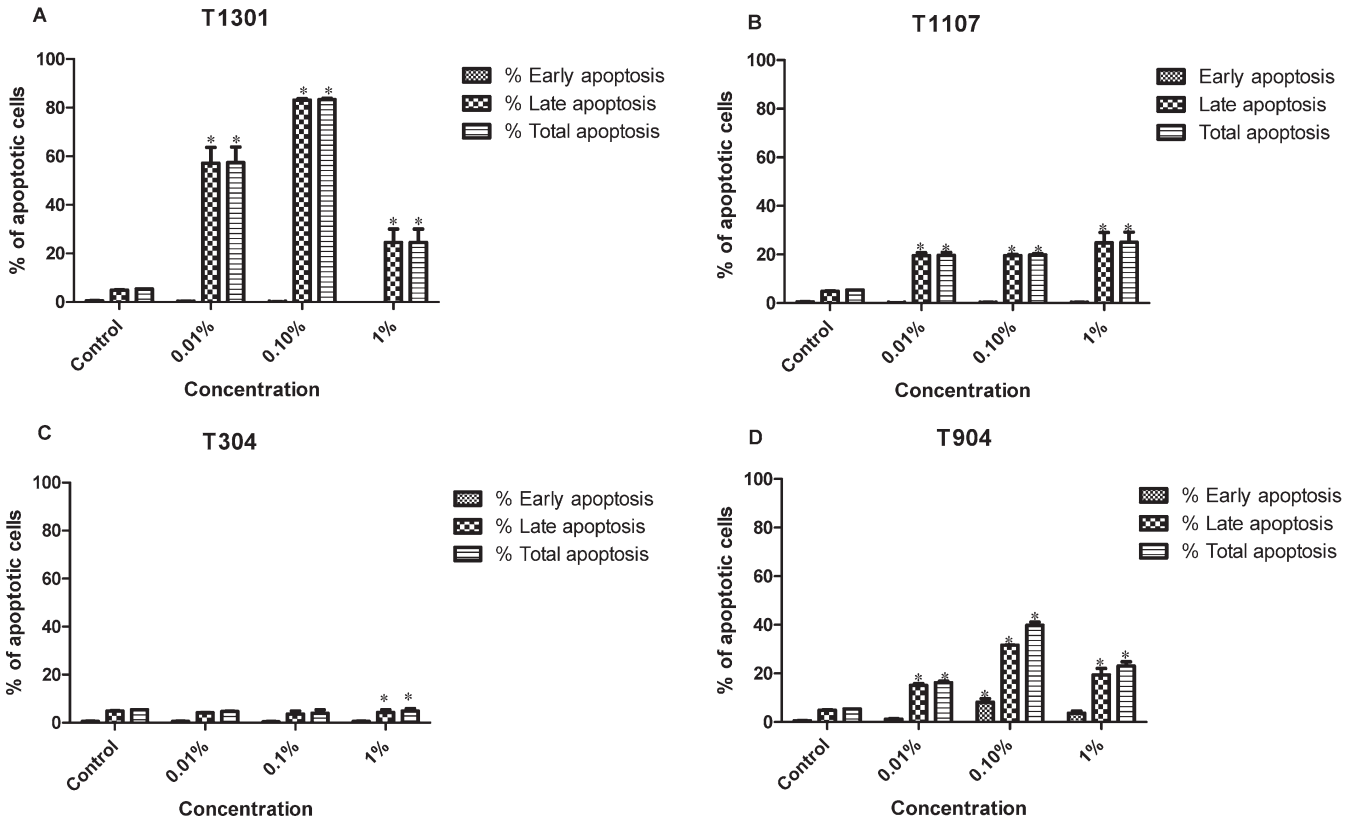



**Figure 3.** Effect of poloxamines (A) T1301, (B) T1107, (C) T304 and (D) T904 on apoptosis in HepG2 cells. *Statistical significance was calculated with ANOVA, with respect to untreated controls ($p < 0.05$).

cells binding only annexin V-FITC (8.2% ± 2.1). No significant variation was observed in the number of early apoptotic cells treated with the remaining copolymers at the three concentrations studied. The fact that, with HepG2 exposed to 1% T904, the number of early apoptotic cells (3.7% ± 1.2) was not significantly greater than that with 0.1% T904 (8.2% ± 2.1) suggested the strong cytotoxicity of this copolymer that leads to a high percentage of cell debris. The number of AV+/PI+ dead cells in cultures exposed to 1% T904 and 1% T1301 (19.4% ± 3.7 and 24.5% ± 7.8, respectively, data not shown) was lower than that observed with the same copolymers at 0.1% (31.6% ± 0.1 and 83.1% ± 0.8, respectively, data not shown). Again, the results observed with HepG2 cells supported the strong cytotoxicity of T904 and T1301 that results in a high percentage of cell debris; the higher the copolymer concentration, the more pronounced the cell death.

As a way to gain further insight into the potential cytotoxicity of the copolymers, the concentration of hepatic transaminases, AST and ALT, was measured in the supernatants of both liver carcinoma cells. An AST/ALT ratio (de Ritis ratio) greater than 1 strongly suggests hepatocyte necrosis.[34] On the other hand, the AST/ALT value was compared to that of the controls. Thus, AST/ALT ratios greater than 1.0 and 3.0 were considered an indicator of necrosis in Huh7 and HepG2 cells, respectively; HepG2 cell controls (without poloxamine) showed AST/ALT values of approximately 3.0. Data suggested that the mechanism of cell death provoked by T904, T1301 and T1107 at different concentrations was different (Table 3). According to AST/ALT data, Huh7 cells treated with 1% T1107 and 0.1% and 1% T904 (AST/ALT ratio = 1.5, 7 and 18, respectively) underwent necrosis, while those exposed to 0.1% and 1% T1301 (AST/ALT ratio = 1) underwent apoptosis. HepG2 cells followed a similar trend; cells exposed to 1% T1107 and 0.1% and 1% T904 showed AST/ALT values of 5.0, 21 and 20, respectively, indicating a necrotic pathway.

According to the results obtained with AV/PI, we found that Huh7 cells had a similar $\Delta\Psi_m$ to that of control cells (as quantified with Rho 123), with the exception of those exposed to 0.1% and 1% T904 (Figure 4A); the latter showed a significantly high accumulation of Rho 123, indicating much higher mitochondrial damage, and therefore early apoptosis. The same was observed in HepG2 cells exposed to 0.01−1% T904, T1107 and T1301 (Figure 4B).

Hypodiploid DNA content analyses strictly mirrored those obtained with AV-FITC/PI (Figure 5). Thus, the number of apoptotic cells, measured by the appearance of a hypodiploid population (sub-$G_0$-$G_1$) was significantly increased in Huh7 cells in contact with 1% T904 (17.5%), 0.1% T904 (8.9%), 1% T1301 (12.1%), 0.1% T1301 (9.1%) and 1% T1107 (4.4%). This phenomenon was more pronounced in HepG2 cells. For example, 0.01%, 0.1% and 1% T904 led to hypodiploidy percentages of 69.8%, 87.9% and 49.5%, respectively. Figure 6 exemplifies different DNA histograms showing the cell cycle arrest of Huh7 cells exposed to 1% T904 and T304 in comparison with control cells.

**3.2. Functional Activity Assay.** No significant change in the ability of P-gp, MRP1 and/or BCRP to efflux DiOC2 and Rho 123 was observed in Huh7, HepG2 and HeLa cells exposed to 0.01% T304 and 0.01−1% T1107. In both liver carcinoma cell lines, 0.01% T904 and 0.1% T304 inhibited the functional

**Table 3. Effect of Poloxamines T304, T904, T1301 and T1107 (0.01%, 0.1% and 1%) in Both Hepatocarcinoma Cell Lines Huh7 and HepG2 on the AST/ALT Ratio**

| poloxamine | concentration (%) | AST ± SD (IU/L) | ALT ± SD (IU/L) | AST/ALT[a] |
|---|---|---|---|---|
| | | Huh7 | | |
| control | | 2.0 ± 0.1 | 2.0 ± 0.1 | 1 |
| T304 | 0.01 | 2.0 ± 0.1 | 2.0 ± 0.2 | 1 |
| | 0.1 | 2.0 ± 0.2 | 2.0 ± 0.1 | 1 |
| | 1 | 2.0 ± 0.1 | 2.0 ± 0.1 | 1 |
| T904 | 0.01 | 2.0 ± 0.1 | 2.0 ± 0.1 | 1 |
| | 0.1 | 14.0 ± 0.7 | 2.0 ± 0.3 | 7 |
| | 1 | 36.0 ± 0.8 | 2.0 ± 0.1 | 18 |
| T1301 | 0.01 | 2.0 ± 0.1 | 2.0 ± 0.1 | 1 |
| | 0.1 | 2.0 ± 0.1 | 2.0 ± 0.1 | 1 |
| | 1 | 2.0 ± 0.2 | 2.0 ± 0.2 | 1 |
| T1107 | 0.01 | 2.0 ± 0.3 | 2.0 ± 0.1 | 1 |
| | 0.1 | 2.0 ± 0.1 | 2.0 ± 0.2 | 1 |
| | 1 | 3.0 ± 0.1 | 2.0 ± 0.1 | 1.5 |
| | | HepG2 | | |
| control | | 9.0 ± 0.3 | 3.0 ± 0.2 | 3 |
| T304 | 0.01 | 9.0 ± 0.3 | 3.0 ± 0.3 | 3 |
| | 0.1 | 9.0 ± 0.1 | 3.0 ± 0.2 | 3 |
| | 1 | 9.0 ± 0.1 | 3.0 ± 0.2 | 3 |
| T904 | 0.01 | 9.0 ± 0.1 | 3.0 ± 0.1 | 3 |
| | 0.1 | 84.0 ± 0.7 | 4.0 ± 0.3 | 21 |
| | 1 | 334.0 ± 0.6 | 17.0 ± 0.5 | 20 |
| T1301 | 0.01 | 9.0 ± 0.3 | 3.0 ± 0.3 | 3 |
| | 0.1 | 9.0 ± 0.1 | 3.0 ± 0.2 | 3 |
| | 1 | 43.0 ± 0.4 | 33.0 ± 0.5 | 1.3 |
| T1107 | 0.01 | 9.0 ± 0.1 | 3.0 ± 0.2 | 3 |
| | 0.1 | 9.0 ± 0.1 | 3.0 ± 0.1 | 3 |
| | 1 | 10.0 ± 0.2 | 2.0 ± 0.1 | 5 |

[a] Necrosis defined as AST/ALT ratio > 1 in Huh7 cells and as AST/ALT ratio > 3 in HepG2 cells.

activity of BCRP and, at a higher concentration, T304 also inhibited the activity of P-gp. The same result was displayed by 0.01% T1301 in Huh7 which only inhibited the functional activity of BCRP, and at higher concentration also the activity of P-gp. The inhibitory effect of poloxamines on the functional activity of P-gp, MRP1 and BCRP in both Huh7 and HepG2 cells was confirmed using HeLa cells that lack P-gp. This is the conventional approach to differentiate if poloxamines inhibit the functional activity of P-gp and/or MRP1 in the two liver carcinoma cells. At last, assays performed in HeLa cells indicated that none of the specimens included in this study and that inhibited the activity of BCRP affected the performance of MRP1 (Figures 7, 8 and 9). Table 4 summarizes the inhibitory activity of the different specimens on P-gp, BCRP and MRP1 in the different cancer cell lines; more detailed data is included as Supporting Information (Tables S1, S2 and S3).

## 4. DISCUSSION

According to the hydrophilic−lipophilic balance (HLB), poloxamines are classified into three groups: (i) hydrophilic (HLB > 18; e.g., T1107); (ii) medium hydrophobic (HLB 12−18; e.g., T304 and T904); and (iii) highly hydrophobic (HLB 1−7; e.g., T1301).[30] Regardless of the higher physicochemical versatility of these derivatives with respect to the linear ones, their study as drug nanocarriers and ABC-pump inhibitors has been much more limited. Aiming to explore their potential application in the pharmacotherapy of infectious and tumoral liver diseases, the present study comprised two stages. First, the cytotoxicity of different poloxamines (T304, T904, T1107 and T1301) on two human liver carcinoma cells (HepG2 and Huh7) was thoroughly assessed by means of the cell death mechanism. To evaluate the effect of compositional parameters, copolymers displaying a broad range of molecular features were employed. Then, the inhibition of the functional activity of several ATP-dependent transporters belonging to the ABC family of proteins (P-gp, BCRP and MRP1) was investigated.

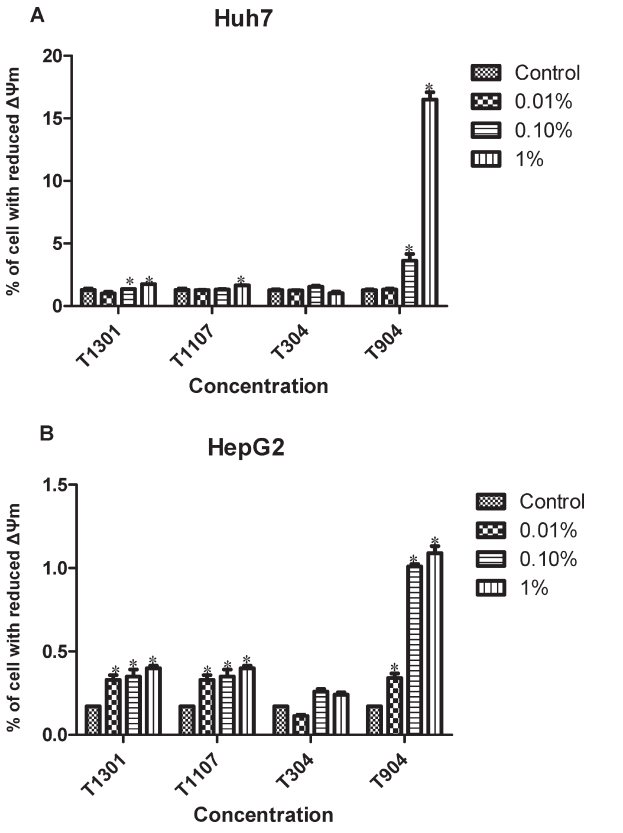



**Figure 4.** Effect of poloxamines T1301, T1107, T304 and T904 0.01−1% on Rho 123 accumulation as a marker of mitochondrial function and early apoptosis in (A) Huh7 and (B) HepG2 cells. *Statistically significant with respect to the control ($p < 0.05$).

**4.1. Poloxamine Cytotoxicity.** The concept of apoptosis was first introduced in 1972[35] to describe a form of programmed cell death different from necrosis. The latter pathway results in the systematic and efficient removal of damaged or unnecessary cells,[36] while in the former, a cell actively participates in its own destruction. In the early stages of apoptosis, the most remarkable events are (i) the translocation of PS from the inner to the outer side of the plasma membrane, thus exposing PS on the external surface of the cell, without increasing the cell permeability, and (ii) changes in $\Delta\Psi_m$. In the late stages of apoptosis, DNA fragmentation and loss of cell membrane permeability take place.

Huh7 and HepG2 cells showed a differential susceptibility to the same copolymers. Poloxamines display identical general

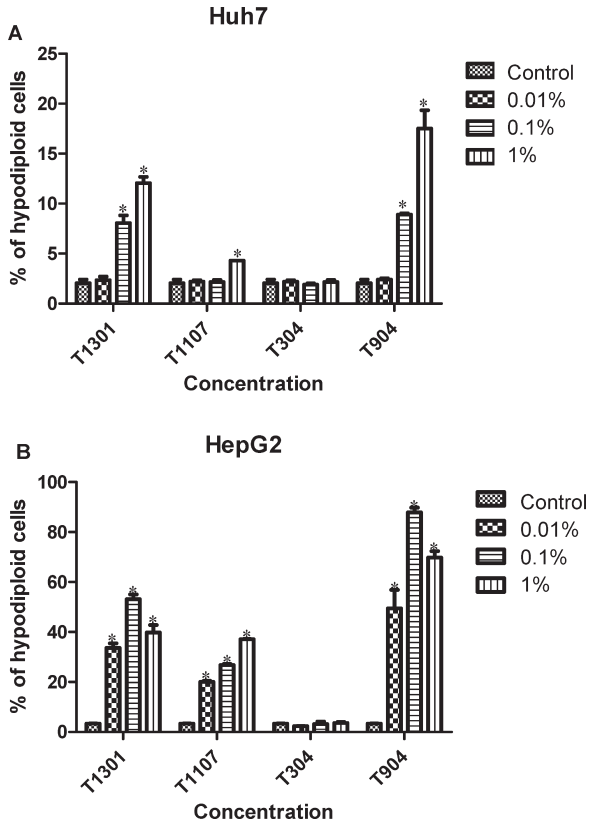



**Figure 5.** Effect of poloxamines T1301, T1107, T304 and T904 0.01−1% on apoptosis calculated as the hypodiploid population (sub-$G_0$-$G_1$) region of the DNA content in (A) Huh7 and (B) HepG2 cells. Hypodiploidy was analyzed by PI incorporation in permeabilized cells and flow cytometry. *Statistically significant with respect to the control ($p < 0.05$).

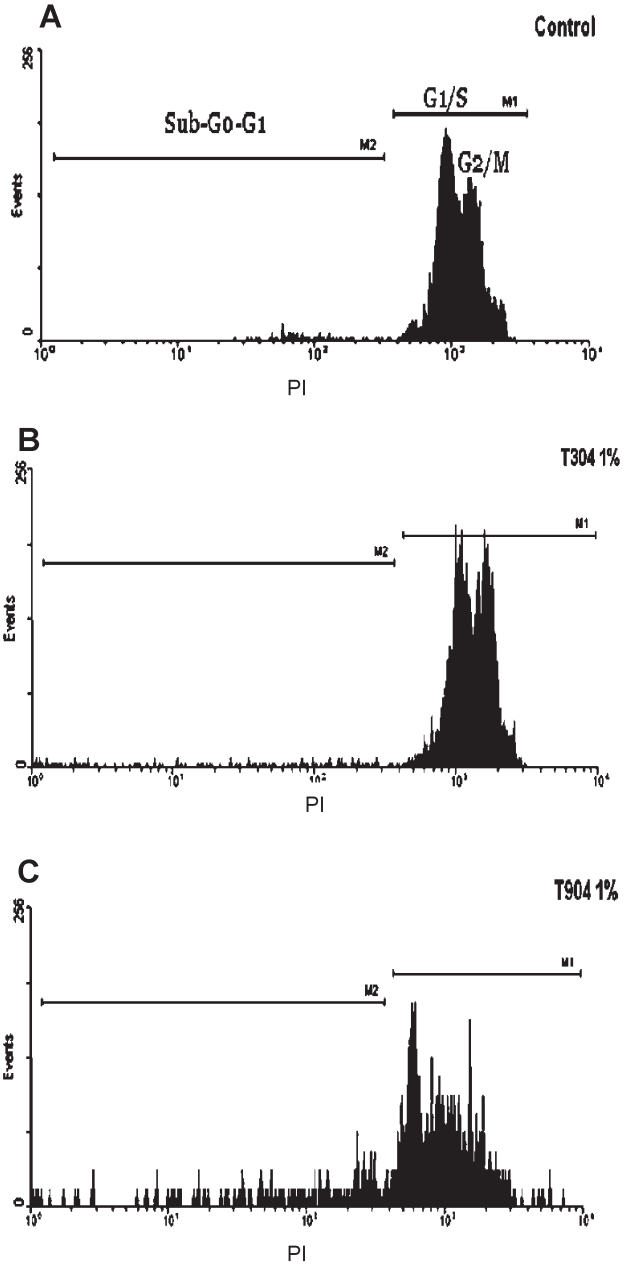



**Figure 6.** Effect of 1% poloxamines (B) T304 and (C) T904 on the cell cycle of Huh7 cells in comparison with a control (A). Cells with DNA content less than $G_0$-$G_1$ indicates apoptotic cells (hypodiploidy).

structure. However, the molecular weight, the HLB value and the length of the hydrophobic blocks govern the copolymer−cell interaction;[40] copolymer molecules can intercalate in the cellular membrane with both PEO external blocks exposed at the surface or by the opposite, chains can span the bilayer. In this context, the cytotoxicity needs to be evaluated for each specific derivative. In general, the higher the copolymer concentration, the more pronounced the cell death. Highly hydrophilic T1107 displayed good cytocompatibility as previously reported in studies exploring the encapsulation of HepG2 cells within T1107 cross-linked matrices.[37,38] Conversely, moderate to highly hydrophobic poloxamines with an intermediate molecular weight (T904 and T1301) displayed a more pronounced cytotoxicity as recently shown with Caco2[28] and Balb/3T3.[39]

Due to an identical staining pattern, late apoptosis cannot be distinguished from necrosis by means of annexin V-FITC and PI. To gain further insight into the potential cytotoxicity of the copolymers, the concentration of two characteristic hepatic transaminases, AST and ALT, was measured in the supernatants of both liver carcinoma cells. Data suggested that the mechanism of cell death caused by T904, T1301 and T1107 at different concentrations was different. On the other hand, many reports speculated that apoptosis and necrosis might act together or sequentially. In fact, according to Majno and Joris, necrosis is not a form of cell death but the final stage of any cell death process, including apoptosis.[41] Thus, although necrosis and apoptosis were for many years considered as two opposite forms of cell death, nowadays the difference between them is more controversial.[42] As stated by Farber, "*there is no field of basic cell biology and cell pathology that is more confusing and more unintelligible than is the area of apoptosis versus necrosis.*"[43] Irrespective of these theoretical considerations and the mechanism of cell death, it appears to be clear that 0.1% and 1% T904 and T1301 were highly cytotoxic for both liver carcinoma cell lines. The same is valid for 0.01% T904 and T1301 in HepG2 cells. Even though values of cell death for T1107 were significantly greater than those of the controls, the detrimental effect was substantially milder. It is also worth mentioning that 50% has been previously stated as a reference value to indicate cytotoxicity.[44] Noteworthy, HepG2 cells exposed to 1% T1301 showed an AST/ALT ratio of

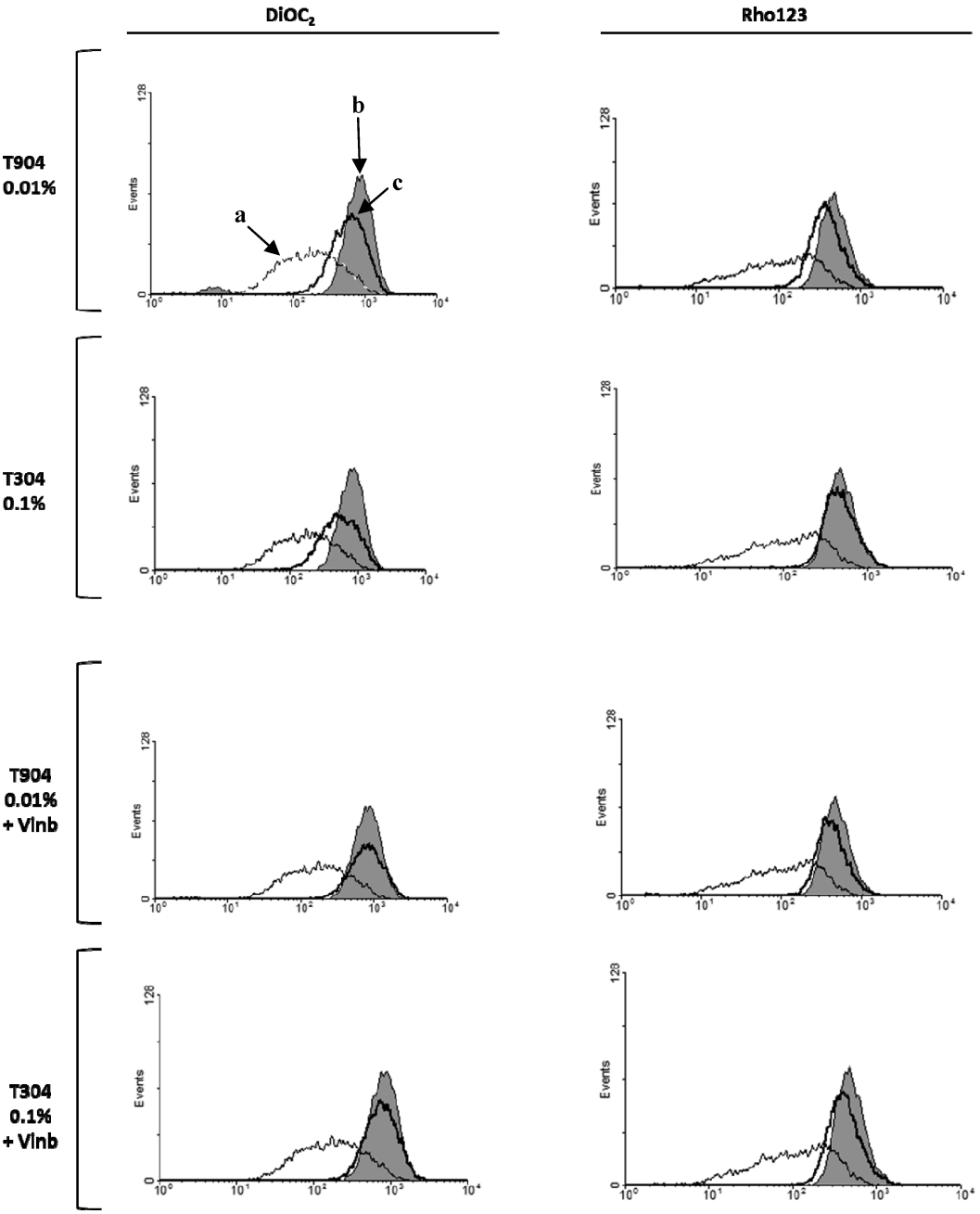



**Figure 7.** Inhibitory effect of poloxamines 0.01% T904 and 0.1% T304 on the functional activity of P-gp, MRP1 and BCRP analyzed by flow cytometry by using the fluorescent dyes DiOC2 and Rho123, in Huh7 cells. a: Control cells at 37 °C (optimal conditions for pump activity = lesser intracellular fluorescence). b: Control cells at 4 °C (metabolic inhibition of pump activity = maximum intracellular fluorescence). c: Cells in the presence of poloxamines with or without vinblastine (Vinb).

1.3. This value would suggest no hepatotoxicity. However, transaminase levels increased to 43 and 33 IU/L for AST and ALT, respectively (data not shown), suggesting that this copolymer would be highly hepatotoxic.

The two major and best characterized pathways that lead to apoptosis are the intrinsic and the extrinsic one.[41] In both pathways, mitochondria are critical for the execution of the cell death stage. Mitochondrial membrane permeabilization (MMP) and/or massive caspase activation are considered the "point-of-no-return" in many models of programmed cell death.[45] MMP affects the inner and outer mitochondrial membranes and dissipates the $\Delta\Psi_m$.[46] A very recent study elegantly showed that poloxamers colocalize with mitochondria, inhibiting different stages of the inhibitory chain, decreasing oxygen consumption[27] and possibly triggering the loss of $\Delta\Psi_m$. Moreover, these effects are selective and pronounced for MDR cells compared to non-MDR counterparts and were demonstrated for both drug-selected and Pgp-transfected cell models.

According to the results obtained with AV/PI, we found that Huh7 cells had a similar $\Delta\Psi_m$ to that of control cells (as quantified with Rho 123), with the exception of those exposed to 0.1% and 1% T904. These later results suggested mitochondrial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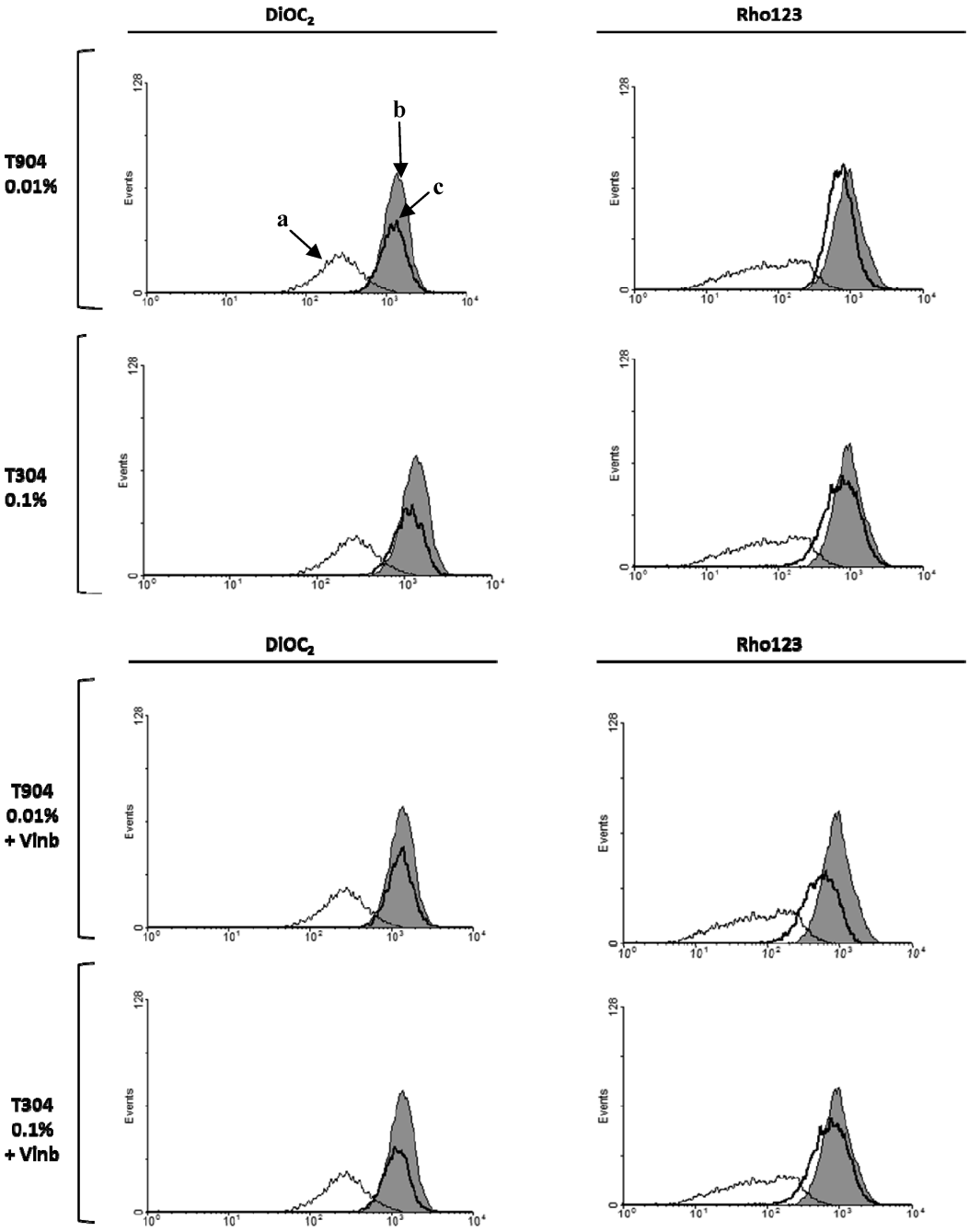


**Figure 8.** Inhibitory effect of poloxamines that displayed slightly or no cytotoxicity (0.01% T904 and 0.1% T304) on the functional activity of P-gp, MRP1 and BCRP, analyzed by flow cytometry by using the fluorescent dyes DiOC2 and Rho123, in HepG2 cells. a: Control cells at 37 °C (optimal conditions for pump activity = lesser intracellular fluorescence). b: Control cells at 4 °C (metabolic inhibition of pump activity = maximum intracellular fluorescence). c: Cells in the presence of poloxamines with or without vinblastine (Vinb).

damage, and therefore early apoptosis. The same phenomenon was observed in HepG2 cells exposed to 0.01−1% T904, T1107 and T1301. Thus, although in HepG2 cells exposed to 1% T1107 and to 0.1% and 1% T904 we detected signs of early apoptosis, AST/ALT values greater than 3.0 indicated that necrosis also took place. In contrast, these poloxamines, at the remaining concentrations, only showed signs of apoptosis and not necrosis. Interestingly, and taking into account the results obtained with AV/PI, $\Delta\Psi_m$ and de Ritis ratio, we might hypothesize that the mechanism of cell death in Huh7 cells exposed to 1% T1107 was mainly necrotic. These studies showed some differences with the AV-FITC/PI method in some cases because (i) early apoptosis is an event that temporarily might not match changes in the $\Delta\Psi_m$ and (ii) the sensitivity of both techniques is different. Further experiments should be conducted to determine whether the mechanism by which poloxamines trigger loss of $\Delta\Psi_m$ is direct by colocalizing with mitochondria or indirect by interacting with some intracellular factor or molecule.

Cell death was also measured by the detection of hypodiploidy, where necrotic and mid/late stage apoptotic cells are labeled


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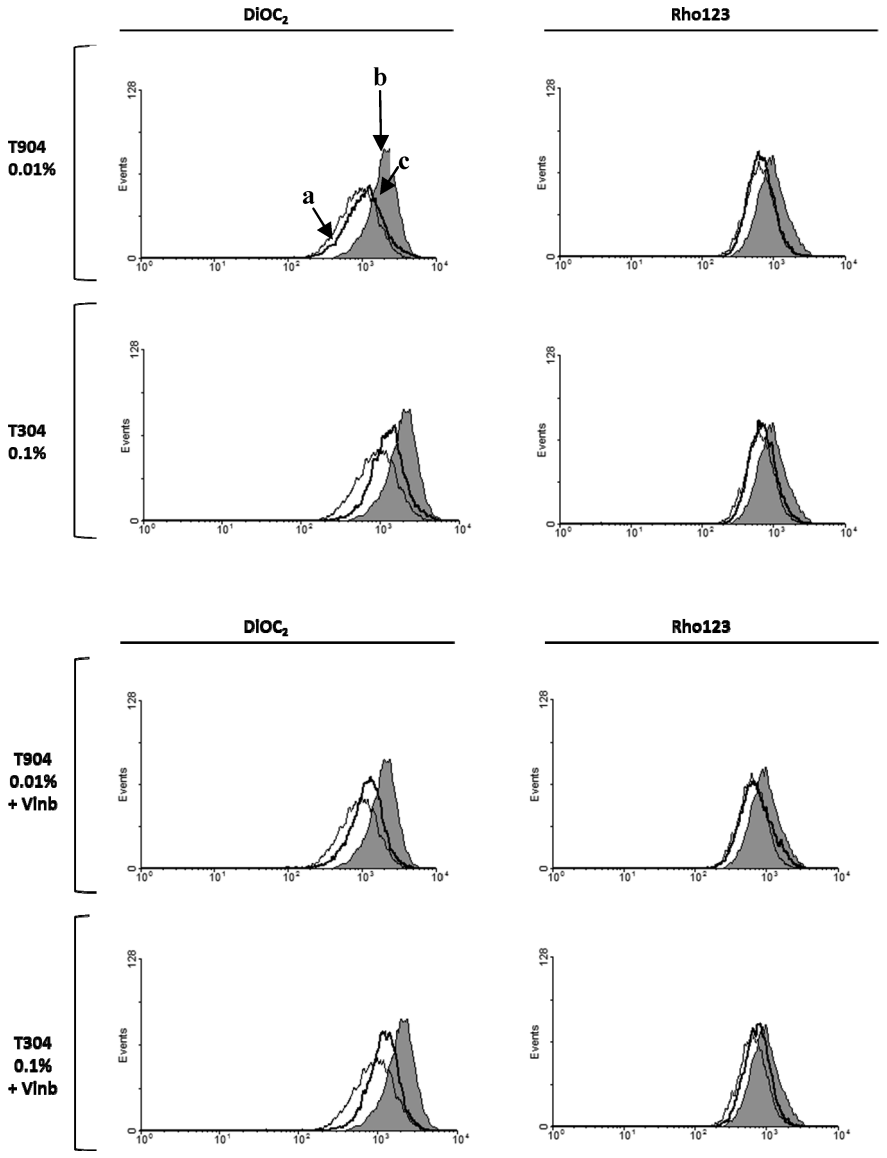


**Figure 9.** Inhibitory effect of 0.01% T904 and 0.1% T304 on the functional activity of MRP1 and BCRP analyzed by flow cytometry by using the fluorescent dyes DiOC2 and Rho123, in HeLa cells. a: Control cells at 37 °C (optimal conditions for pump activity = lesser intracellular fluorescence). b: Control cells at 4 °C (metabolic inhibition of pump activity = maximum intracellular fluorescence). c: Cells in the presence of poloxamines with or without vinblastine (Vinb).

with the fluorescent dye PI.[47] This method facilitates simultaneous quantitative analysis of cytotoxicity, perturbations in the cell cycle and aneuploidization.[46] Apoptotic cells containing degraded DNA appear as cells with hypodiploid DNA content, and they are represented in the so-called sub-$G_0$-$G_1$ region on DNA histograms.[48] Quantification of apoptotic cells was thus calculated as the percentage of cells in the sub-$G_0$-$G_1$ region (hypodiploidy) in the cell cycle analysis. Hypodiploid DNA content analyses strictly mirrored those obtained with AV-FITC/PI. One of the key observations of this study was that T304 at all the concentrations assayed did not produce any arrest of the cell cycle. Thus, the antiproliferative activity of T904, T1107 and T1301 toward Huh7 and HepG2 cells was probably due to their ability to promote an arrest in the G2/M phase. Furthermore, the proapoptotic activity of poloxamines may be due to a similar mechanism to that described by Alakhova et al. for Pluronic which consisted of an impaired mitochondrial function in MDR cells that leads to the release of cytochrome *c*, thus contributing to enhanced cell death by apoptosis.[27] Cell cycle arrest in G2 phase is a common response to a variety of DNA-damaging agents.[49] Activation of checkpoint kinase 2 (Chk2) in response to DNA damage and a link between Chk2 activation and cell cycle arrest was well documented.[50] Further studies should be made to elucidate the mechanism by which the arrest in G2 phase is produced by poloxamines T904, T1301 and T1107. It is well-known that an impaired activation of cdc2—cyclin B complexes might lead to cell cycle arrest at G2 phase.[51] For example, Tsao et al. had elegantly shown that camptothecin induced G2 arrest by triggering an altered regulation of p34cd2/cyclin B.[49] Visanji et al. had shown that induction of G2/M phase cell cycle arrest by carnosol and carnosic acid was associated with alteration of cyclin A and cyclin B1 levels.[52] It was also well documented that the G2 arrest was independent of the tumor suppressor protein p53, and involved a block in the cdc25-dependent

**Table 4. Inhibitory Effect of Poloxamines T904, T304, T1301 and T1107 on the Functional Activity of P-gp, MRP1 and BCRP, Using the Fluorescent Dyes DiOC2 and Rho123**

| poloxamine | P-gp | MRP1 | BCRP |
|---|---|---|---|
| | Huh7 | | |
| control | − | − | − |
| T904 0.01% | + | + | + |
| T304 1% | + | + | + |
| T304 0.1% | − | − | + |
| T304 0.01% | − | − | − |
| T1301 0.1% | + | + | + |
| T1301 0.01% | − | − | + |
| T1107 1% | − | − | − |
| T1107 0.1% | − | − | − |
| T1107 0.01% | − | − | − |
| | HeLa | | |
| control | ne[a] | − | − |
| T904 0.01% | ne | − | + |
| T304 1% | ne | − | + |
| T304 0.1% | ne | − | + |
| T304 0.01% | ne | − | − |
| T1301 0.1% | ne | − | + |
| T1301 0.01% | ne | − | + |
| T1107 1% | ne | − | − |
| T1107 0.1% | ne | − | − |
| T1107 0.01% | ne | − | − |
| | HepG2 | | |
| control | − | − | − |
| T904 0.01% | + | + | + |
| T304 1% | + | + | + |
| T304 0.1% | − | − | + |
| T304 0.01% | − | − | − |

[a] Not expressed.

activation of the mitotic cyclin dependent kinase (CDK) complexes.[53] Having expressed this, further studies are demanded to shed light into the mechanisms that are affected by poloxamines.

**4.2. Functional Activity Assay.** The study of the inhibition of the functional activity of P-gp, BCRP and MRP1 in Huh7 and HepG2 cells was conducted employing Rho 123, DiOC2 and VIN, at copolymer concentrations that displayed slight or no cytotoxicity. Since the staining protocol used does not enable the discerning of P-gp and MRP1, a cell line lacking the expression of P-gp (HeLa cells) was used as control. Taking together the findings of this assay, we can conclude that T904 was the most effective inhibitor of the ABC transporters; 0.01% T904 inhibited both P-gp and BCRP. T304 and T1301 showed a less effective performance, inhibiting both pumps at 0.1%. T1301 at the lowest concentration inhibited only BCRP. Findings indicated that, considering the copolymer structure−activity relationship, poloxamines of both intermediate to low molecular weight and medium to high hydrophobicity were efficient inhibitors of Pgp and BCRP; the greater the copolymer concentration, the stronger the inhibition.

Different mechanisms for overcoming drug resistance by means of Pluronic block copolymers were described elsewhere. Among them, (i) decrease in membrane microviscosity, (ii) ATP depletion, (iii) inhibition of drug efflux transport systems, (iv) reduction in GSH/GST detoxification system and (v) drug release from acidic vesicles in the MDR cell were the best studied.[24] It is highly probable that poloxamines act similarly. However, it was surprising that none of the poloxamines studied herein showed inhibition of the functional activity of MRP1. Since the activity of this drug efflux transporter is closely tied to the GSH/GST detoxification system in MDR cells,[54] we propose that poloxamines, in contrast to poloxamers, have no effect in the intracellular levels of both GSH and GST activity in at least Huh7, HepG2 and HeLa cells. Further studies should be performed to confirm this hypothesis.

Finally, the fact that 0.1−1% T304 and 0.01% T904 displayed no cytotoxicity in both liver carcinoma cell lines, and displayed an inhibitory effect on the activity of P-gp and/or BCRP, makes these poloxamines appealing candidates for modulating the activity of ABC transporters to overcome cellular drug resistance in HCC, in CHB and in CHC and for improving the bioavailability at clinically relevant viral reservoirs of HBV and HCV.

## 5. CONCLUSIONS

The potentially beneficial effect of amphiphilic copolymers to improve the pharmacotherapy in hepatic diseases by means of the inhibition of efflux transporters has been assessed for the first time in two hepatocarcinoma cell lines. First, several complementary techniques such as PS exposure on the outer leaflet of the cell membrane, changes in the $\Delta\Psi_m$, detection of hypodiploidy and determination of the AST/ALT ratio were employed. Results clearly indicated that the cytotoxicity is intimately associated with the structure of the derivatives. The fact that none of the poloxamines studied had an inhibitory effect on the activity of MRP1 suggested that the inhibition mechanism does not rely on the intracellular levels of GST and GSH. T304 was the only derivative that showed no cytotoxicty and no arrest of the cell cycle at all concentrations tested and, at the same time, inhibited the functional activity of P-gp and/or BCRP in a concentration-dependent manner. These data point out T304 as a good and safe candidate to overcome drug resistance associated with efflux transport in HCC, CHB and CHC. Aiming to improve our understanding of the mechanisms behind the inhibitory activity, ongoing investigations are addressing the effect that these copolymers have on the expression of ABC-related genes. These results will be published separately.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Figure S1 and Tables S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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