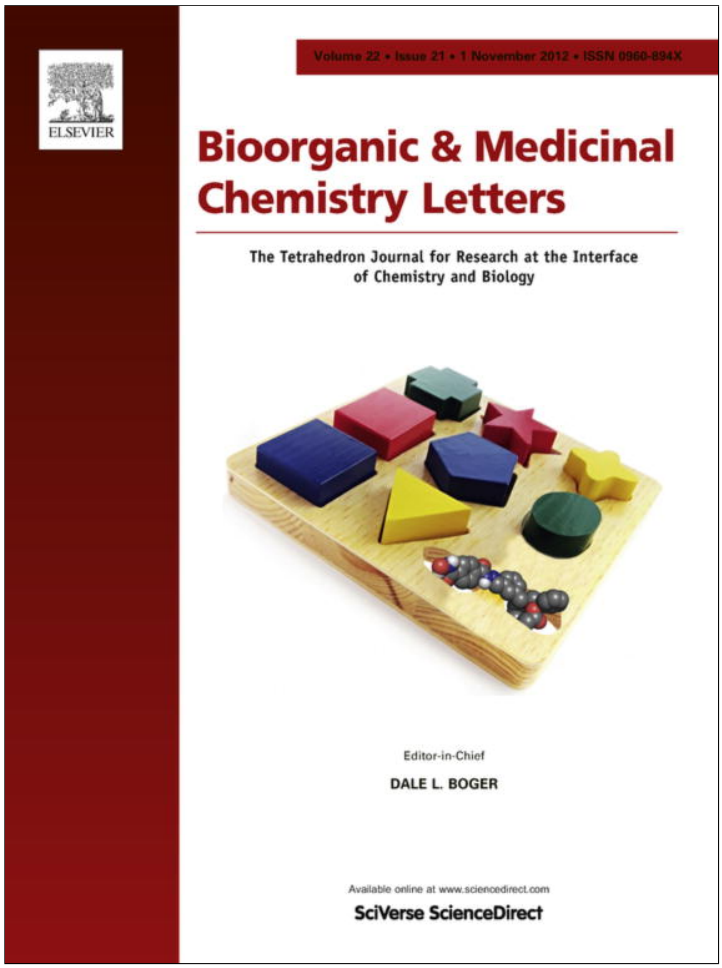

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Downregulation of *mdr1* and *abcg2* genes is a mechanism of inhibition of efflux pumps mediated by polymeric amphiphiles

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ABSTRACT

The ability of cells to acquire resistance to multiple pharmaceuticals, namely multidrug resistance (MDR), is often mediated by the over-expression of efflux transporters of the ATP-binding cassette (ABC) superfamily; for example P-glycoprotein (P-gp or MDR1), breast cancer resistance protein (BCRP or ABCG2), and multidrug resistance-associated protein MRP1. ABCs pump drug molecules out of cells against a concentration gradient, reducing their intracellular concentration. The ability of polymeric amphiphiles to inhibit ABCs as well as the cellular pathways involved in the inhibition has been extensively investigated. This work investigated for the first time the effect of branched poly(ethylene oxide)-poly(propylene oxide) block copolymers (poloxamines) on the levels of mRNA encoding for MDR1, BCRP and MRP1, in a human hepatoma cell line (Huh7). Copolymers with a broad range of molecular weights and hydrophilic-lipophilic balances were assayed. Results confirmed the down-regulation of *mdr1* and *abcg2* genes. Conversely, the *mrp1* gene was not affected. These findings further support the versatility of these temperature- and pH-responsive copolymers to overcome drug resistance in cancer and infectious diseases.

Over the last decades, great efforts have been devoted to understand the role of efflux transporters of the ATP-binding cassette superfamily (ABC) in the regulation of (i) drugs pharmacokinetics and (ii) drug access to the intracellular space.[1] The ability of cells to acquire resistance to multiple drugs is called multidrug resistance (MDR) and it is often mediated by the over-expression of these transmembrane transporters.[2] Of the 48 human ABC transporters identified, three are widely associated with MDR: (i) P-glycoprotein (P-gp) encoded by the gene *abcb1* (or *mdr1*), (ii) the multidrug resistance-associated protein-1 (MRP-1) encoded by *abcc1* (or *mrp1*) and (iii) the breast cancer resistance protein (BCRP or ABCG2) encoded by *abcg2*.[3] Since their identification, these ABCs have been the subject of extensive investigations in oncology and more recently in infectious diseases. However, attempts to exploit these proteins as therapeutic targets are still under clinical scrutiny.[4] In addition, the activity of some inhibitors such as fumitremorgin C has been investigated only in vitro and their clinical implementation is from controversial to unfeasible owing to their high toxicity. The effective inhibition of ABC pumps by means of inherently inert amphiphilic poly(ethylene oxide)-poly(propylene oxide) (PEO-PPO) block copolymers opened a new therapeutic alternative and their activity has been ascribed to the hindrance of different cellular pathways.[5–8] Remarkably, none of the previous studies investigated the effect of these block copolymers on the regulation of genes encoding for ABCs.

Herein we report for the first time the effect of branched PEO-PPO block copolymers (poloxamines) on the expression of mRNA encoding for the main ABCs in a human hepatoma cell line.

Poloxamine stock solutions (10%) were prepared by dissolving the required amount of copolymer in distilled water, at 4 °C. Then, they were sterilized by filtration (0.22 μm, Millipore Ireland B.V.) and equilibrated at 37 °C at least 1 h before use. Tetronic® T304 (MW 1.65 kDa, HLB 12–18), T904 (MW 6.7 kDa, HLB 12-18), T1107 (MW 15 kDa, HLB 18–23) and T1301 (MW 6.8 kDa, HLB 1–7) were a donation of BASF Corporation (New Milford CT, USA).

Human hepatoma cell line, Huh7, was seeded in 6-well plates (0.5×10^6 cells/well) and incubated in Dulbecco's modified Eaglés medium (DMEM, Life Technologies) supplemented with 10% fetal calf serum (FCS, Life Technologies) for 24 h at 37 °C. The culture medium was then removed and solutions of poloxamine samples at a final copolymer concentration in the culture medium of 0.01%, 0.1% and 1% were added, and cells were incubated for 72 h at 37 °C to further quantify the relative amounts of *mdr1*, *mrp1* and *abcg2* transcripts, by quantitative real time PCR (qPCR, see below). Prior to this study, poloxamine cytotoxicity was performed

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by flow cytometric analysis of annexin-V (AV)-fluorescein isothiocyanate (FITC)/propidium iodide (PI)-stained cells (FITC Annexin V Apoptosis Detection kit I, BD Pharmingem, Becton Dickinson, USA) using a FAC Scan cytometer (Becton Dickinson) as previously reported.[8]

The multiple inhibitory effect of poloxamines on the functional activity of MDR1, MRP1 and ABCG2 in the human liver carcinoma cell line Huh7, was performed as previously described[8] by using 3,3′-diethyloxacarbocyanine iodide ($DiOC_2$) and rhodamine 123 (Rho 123) as differential substrates for P-gp, MRP-1 and ABCG2. $DiOC_2$ and Rho 123 were purchased from Sigma Company (USA).

Total RNA from control (without poloxamine) and samples (with poloxamine) was extracted using TRIzol® (Life Technologies, USA) and cDNAs were synthesized by reverse transcription using MMLV reverse transcriptase (Life Technologies) with random primers (Promega, USA). The reaction was performed for 10 min at 25 °C (annealing), 60 min at 37 °C (cDNA synthesis), and 45 min at 95 °C (enzyme denaturation).

To detect the relative amounts of *mdr1*, *mrp1* and *abcg2* transcripts, comparative quantitative real time PCR (qPCR) was performed with specific primers (Table 1) using an iQ5 Real Time PCR Detection System (Bio-Rad). Primers were designed using the Beacon Designer 4.0 software (PREMIER Biosoft International, USA). Standard curves using a 'calibrator' cDNA (chosen among the cDNA samples) were prepared for each target and reference gene. Fluorescent signals were generated during each of the 40 cycles and were proportional to quantities of double-stranded DNA (denaturation 20 s at 95 °C; annealing and extension 60 s at 60 °C). The relative quantification was analysed by Rotor-Gene Q 1.7.94 software (Qiagen, Germany); only values between 90–110% were taken as valid. Results were normalised to 18S. The initial amount of each sample template was determined as a relative expression compared to one of the samples chosen as reference (in this case, the control sample) which is considered the '1 × sample'. Relative quantification was done using the ΔC_T method. Results are expressed as mean ± SD of at least 4 independent determinations for each gene. To determine statistical significance means were compared by the Student *t* test [two-sided test] with 95% confidence interval; *p* values of less than 0.05 were considered significant. All data were independently analyzed in duplicate by using both the statistical package R Development Core Team (http://www.R-project.org) and the Tadpole program.

Prior to the study of the effect of poloxamines T304, T904, T1107 and T1301 on the expression of mRNA encoding for P-gp, MRP-1 and ABCG2 in a human hepatoma cell line, the 72 h cytotoxicity of these amphiphilic copolymers at different concentrations (0.01–1%) was assayed by flow cytometric analyses using AV-FITC/IP. According to this, only exposure of Huh7 cells to 0.01% and 0.1% T304, 0.01% T904, 0.01% T1107 and 0.01% T1301 for 72 h did not induce any detectable signs of cytotoxicity (data not shown). When the functional activity assay was performed with these poloxamines at the non-72 h cytotoxic concentration at 4 °C and 37 °C using $DiOC_2$, Rho 123 and VIN, similar results to those obtained in our previous work were obtained.[8] Thus, of all poloxamines assayed, only 0.01% T304, 0.01% T904 and 0.01% T1301 inhibited the activity of ABCG2 although 0.01% T904 was also able to inhibit the activity of P-gp. T304 could also inhibit the activity of P-gp but at a higher concentration (1–0.1%) (Table 2). The comparative qPCR studies were performed to quantity the relative amounts of *mdr1*, *mrp1* and *abcg2* transcripts after a 72 h exposure with poloxamines at a non-cytotoxic concentration. As it is observed in Figure 1, mRNA levels of *mrp1* did not significantly differ from those detected in control cells, except for cells exposed to 0.01% T1301 (0.24 ± 0.06). Conversely, mRNA levels of *abcg2* appeared to be down-regulated during treatment with 0.01% T904 (0.54 ± 0.01), 0.01%T1107 (0.21 ± 0.03), 0.01% T1301 (0.21 ± 0.02), 0.1% T304 (0.41 ± 0.04) and 1% T304 (0.59 ± 0.01), whereas *mdr1* transcripts were decreased with 0.01% T904 (0.63 ± 0.14), 0.01% T1301 (0.27 ± 0.11) and 1% T304 (0.57 ± 0.19); and increased in the presence of 0.1% T304 (1.96 ± 0.24). No significant variation was documented in the levels of those transcripts in Huh7 cells treated with 0.01% T1107 (1.33 ± 0.22). Regarding T304, the effect on *mdr1* depends clearly on the concentration. At high concentration, T304 decreased mRNA levels, while at a low concentration it increased them. Noteworthy, of all poloxamines assayed, only the highly hydrophobic 0.01% T1301 induced a statistically significant decrease in the mRNA levels of the three ABC transporters studied herein.

Many studies using Pluronic® suggested different mechanisms for overcoming drug resistance in MDR cells. Among them: (i) membrane fluidization, (ii) ATP depletion, (iii) direct interaction with the ABC, (iv) reduction in GSH/GST detoxification system, (v) drug release from acidic vesicles, and (vi) incorporation into the mitochondrial membrane, inhibiting respiration.[6,9] A recent work demonstrated that Pluronic® P85 prevented the development of doxorubicin-induced MDR in leukemia cells as determined by RT-PCR analysis, though no significant changes in *mdr1* mRNA levels were observed.[10] In contrast, with cells selected with doxorubicin alone, mRNA levels of *mdr1* were significantly increased.

In a previous study we investigated the multiple inhibitory effects of the intrinsically inert amphiphiles PEO-PPO block copolymers on the functional activity of P-gp, BCRP and MRP1 in two human liver carcinoma cell lines.[8] Aiming to improve our understanding of the mechanisms behind the inhibitory activity, more investigations have been addressed to elucidate the effect that these copolymers have on the expression of ABC-related genes.

In this context, in the present study we investigated the effect of different concentrations of Tetronic® block copolymers showing a wide range of molecular weights and EO/PO ratios on the mRNA levels of *mdr1*, *mrp1* and *abcg2* of a human hepatoma cell line. These three genes are linked to treatment refractoriness due to MDR. A significant decrease in the mRNA levels of *mdr1* and *abcg2* in Huh7 cells treated with several poloxamines after 72 h was observed. However, no significant changes in *mrp1* mRNA transcripts levels were detected except for 0.01% T1301. These results were in very good agreement with those obtained by measuring the

Table 1
Quantitative Real time PCR primers employed in this study

Gene	Primer	Primer sequence
mdr1/*abcb1*	Forward	5′TGCTCAGACAGGATGTGAGTTG 3′
mdr1/*abcb1*	Reverse	5′AATTACAGCAAGCCTGGAACC 3′
mrp1/*abcc1*	Forward	5′GCCAAGAAGGAGGAGACC 3′
mrp1/*abcc1*	Reverse	5′AGGAAGATGCTGAGGAAGG 3′
BCRP/*abcg2*	Forward	5′TATAGCTCAGATCATTGTCACAGTC 3′
BCRP/*abcg2*	Reverse	5′GTTGGTCGTCAGGAAGAAGAG 3′
18s	Forward	5′TAACCCGTTGAACCCCATT 3′
18s	Reverse	5′CCATCCAATCGGTAGTAGCG 3′

Table 2
Inhibitory effect of T304, T904, T1107 and T1301 on the functional activity of P-gp, MRP-1 and ABCG2 at 72 h non-cytotxic concentrations

Poloxamine	72 h non-cytotoxic concentration (%)	ABC pump		
		P-gp	MRP-1	ABCG2
T304	0.01	–	–	+
	0.1	+	–	+
T904	0.01	+	–	+
T1107	0.01	–	–	–
T1301	0.01	–	–	+

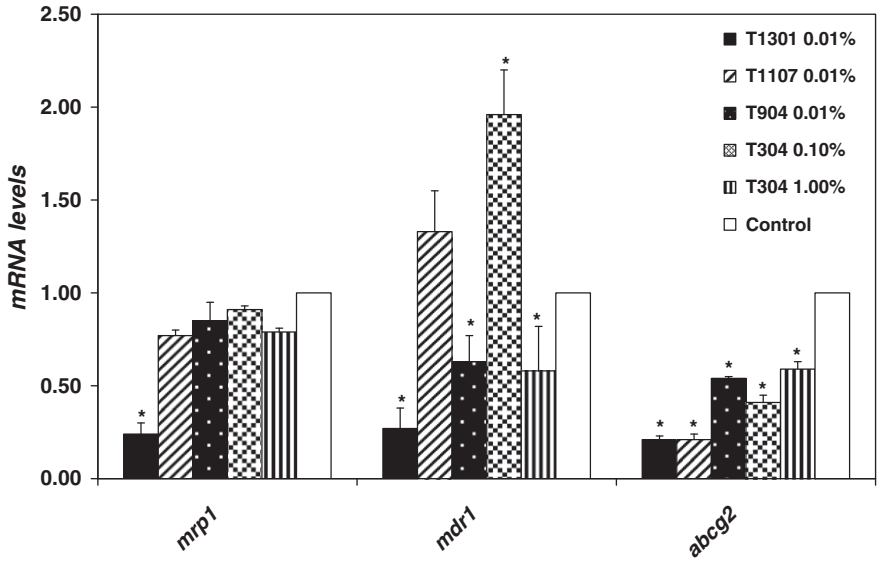

Figure 1. Levels of mRNA encoding for MRP1, Pgp and BCRP in Huh7 cells in contact with different poloxamines over 72 h. *Differences are statistically significant (p <0.05).

functional activity of these ABCs with $DiOC_2$ and Rho 123 at 4 °C and 37 °C, except for some discrepancies observed for 0.01% T1301 in *mrp1*, 0.01% T1107 in *abcg2* and 0.1% T304 in *mdr1*. These discrepancies between both studies might be explained taking into account that mRNA levels were determined at 72 h whereas the functional activity assays were determined after incubating cells with poloxamines at a 72 h non-cytotoxic concentration for 1 h at 4 °C and 37 °C.[8]

Interestingly, 0.01% T1301 down-regulated *mrp1*, while a functional inhibition of this ABC was not observed. Moreover, our findings demonstrate that the inhibitory activity of poloxamines can be traced down to the cell genome and open new and more basic instances that could be targeted to overcome resistance. Because there are still likely several complex molecular pathways associated with MDR mediated by ABC transporters, it will be of great relevance to further understand such mechanisms to maximize the options to interrupt these pathways. This reinforces the use of Tetronic® block copolymers for improving the pharmacotherapy with antitumorals and antivirals for liver diseases such as hepatocellular carcinoma or chronic hepatitis B and C. However, in full agreement with Kabanov et al.[9], the term GRAS ('generally regarded as safe') cannot be used for copolymer excipients such as poloxamines that are commonly considered as inert, owing to their effect on the expression of at least *abc* genes. Further studies on the pharmacogenomic effect of poloxamines may help to predict their therapeutic potential for overcoming MDR as well as their side effects associated with the inhibition of the physiological functions of ABCs in hepatic detoxification processes.

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