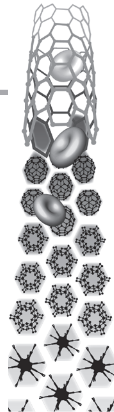

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Inhibition of P-glycoprotein pumps by PEO–PPO amphiphiles: branched versus linear derivatives

Inhibition of the activity of efflux transporters may relevantly improve the chemotherapy of cancer and infectious diseases. **Aim:** To explore the ability of poloxamines (Tetronic®, X-shaped structure with a central ethylendiamine group and four branches of poly[ethylene oxide]–poly[propylene oxide] [PEO–PPO]) to inhibit the activity of P-glycoprotein (P-gp) on Caco-2 cell monolayers and to elucidate the incidence of the molecular architecture of PEO–PPO block copolymers on the intracellular accumulation of a relevant substrate, doxorubicin, by comparison with poloxamers (Pluronic®, linear triblock copolymers), well-known inhibitors of this efflux transporter. **Methods:** Both pristine and *N*-methylated poloxamines displaying a wide range of molecular weights and EO/PO ratios were tested regarding cytocompatibility and accumulation of doxorubicin in Caco-2 monolayers. Verapamil was used as a control. **Results:** The most active anti-P-gp poloxamines (which enhanced two- to three-fold doxorubicin accumulation compared with verapamil) resulted to be pristine medium-to-high hydrophobic T304, T904, T1301, T901 and T150R1. A notable dependence of the anti-P-gp activity on the copolymer concentration was found. A joint diagram of the inhibitory activity of poloxamers and poloxamines as a function of the effective length of the PPO block is proposed. **Conclusion:** The anti-P-gp activity is maxima for block copolymers possessing a low-to-medium hydrophilic–lipophilic balance and an 'effective number' of PO units ranging from 30 to 50.

KEYWORDS: cancer ▪ doxorubicin ▪ drug efflux ▪ HIV/AIDS ▪ P-glycoprotein inhibition ▪ poloxamer ▪ poloxamine ▪ polymeric micelle ▪ transport

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Effective chemotherapy in cancer and in infectious diseases, such as HIV/AIDS, relies on the attainment of sufficiently high intracellular drug concentrations. However, diverse integral membrane proteins that belong to the ATP-binding cassette superfamily are involved in the ATP-powered efflux of drug molecules in the basolateral-to-apical direction [1]. Drug removal from the target organ results in subtherapeutic concentrations and, very often, in therapeutic failure [2]. Efflux transporters are found in the intestinal epithelium [3,4], the CNS (e.g., microglia) [5] and in many anatomical barriers, such as the blood–brain barrier [4], the blood–cerebrospinal fluid barrier [4], the blood–retinal barrier [6] and the blood–testis barrier [7]. In cancer, the upregulated production of efflux pumps is a well-known mechanism of multidrug resistance (MDR) [8]. In HIV infection, these transporters distort drug absorption in the GI tract after oral administration of protease inhibitors and decrease their oral bioavailability [9]. They also delimit cellular (e.g., macrophages) and anatomical viral reservoirs (e.g., brain and testes) and represent a crucial hurdle towards the eradication of the virus from the host [10].

Aiming to effectively inhibit the activity of the efflux pumps and to enhance the bioavailability of their substrates in different tissues and organs, various intrinsically inert copolymers have been explored over the years [11,12]. Poly(ethylene oxide)–poly(propylene oxide) (PEO–PPO) amphiphiles are the most broadly investigated self-assembly temperature-sensitive polymers [13]. According to the structure of the main chain, PEO–PPO block copolymers can be classified into two main groups: the linear and bifunctional poloxamers (Pluronic®); and the branched poloxamines (Tetronic®) (Table 1). Due to their good biocompatibility and approval by the US FDA and the European Medicines Agency (EMEA) for pharmaceuticals and medical devices [14–16], they have been applied in drug delivery [17,18] and tissue engineering [19,20]. Kabanov's group has investigated profusely the inhibition of different efflux transporters over-expressed in MDR cells by a broad spectrum of poloxamers and have outlined the structural features governing the inhibition process [21–24]. Two main mechanisms would be involved: the pronounced depletion of ATP intracellular levels, due to the cellular uptake of copolymer molecules and the perturbation of different metabolic pathways; and the fluidization and the decrease of the membrane microviscosity that alters the pump conformation and ATP binding [21]. Another study revealed that poloxamers act as mobile

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Table 1. Structure and compositional features of the pristine poly(ethylene oxide)–poly(propylene oxide) derivatives used in the present study.

Copolymer	Name	MW (kDa)†	Number of PO repeating units (N_{PO})‡	HLB†
Poloxamines	T304	1650	16	12–18
	T901	4700	72	1–7
	T904	6700	68	12–18
	T908	25,000	84	> 24
	T1107	15,000	80	18–23
	T1301	6800	104	1–7
	T1307	18,000	92	> 24
	T150R1	7900	116	1–7
Poloxamers	L43	1850	22	7–12
	L92	3650	50	1–7
	P103	4950	60	9
	P123	5750	69	7–12
	P85	4600	40	12–18
	F68	8400	29	> 24
	F87	7700	40	> 24
	F127	12,600	65	18–23

Poloxamines: $[HOCH_2-CH_2-(O-CH_2-CH_2)_{a-1}-(O-CH_2-CH(CH_3))_b]_2N-CH_2-CH_2-N[(CH(CH_3)-CH_2-O)_b-(CH_2-CH_2-O)_{a-1}-CH_2-CHOH]_2$

Poloxamers: $HOCH_2-CH_2-(O-CH_2-CH_2)_{a-1}-(O-CH_2-CH)-(CH(CH_3)-CH_2-O)_b-(CH_2-CH_2-O)_{a-1}-CH_2-CHOH$

†*Information provided by BASF, CT, USA. MW values are expressed as average molecular weight.*
‡*Data taken from* [13].
HLB: Hydrophilic–lipophilic balance.

carriers and form ion channels, facilitating both neutral drug and ion transport across the lipid bilayer; the former would rely on the presence of unimers and dimers, and the latter on the formation of aggregates [25]. A recent study confirmed that poloxamers rapidly cross the cellular membrane of MDR cells and colocalize with the mitochondria, inhibiting different stages of the respiratory chain and decreasing oxygen consumption [26]. In the clinical arena, SP1049C (Supratek Pharma Inc., Montreal, Canada), a doxorubicin (DOX)-loaded mixed micellar system (22–27 nm) composed of the hydrophobic Pluronic L61 and hydrophilic F127, has successfully undergone Phase I and II clinical trials and it recently entered Phase III studies [27,28]. It has been also granted orphan drug designation by the FDA in 2005 for the treatment of esophageal cancer [29]. In recent years, poloxamers were also explored as boosting agents in the anti-HIV pharmacotherapy [30–32]. For example, Pluronic P85 enhanced the *in vitro* accumulation of nelfinavir, a P-glycoprotein (P-gp) substrate, in different cell lines and the absorption and the bioavailability of the drug in the brain of a CNS HIV animal model, after intravenous administration [31]. Other amphiphiles, such as PEG-co-poly(ε-caprolactone) diblocks, were also shown to display anti-P-gp activity *in vitro* [33].

Poloxamines present a central ethylenediamine group that confers pH dependency and thermodynamic stability on the copolymer upon aggregation [34]. This dual responsiveness has been exploited to fine-tune the aggregation pattern and the solubilization ability of poloxamine polymeric micelles under different pH conditions [35–37]. In addition, *N*-alkylation of tertiary amine groups renders the chains positively charged, independent of the pH of the medium [38]. Quaternized molecules show lower aggregation number and the size of the aggregates is smaller than that of unmodified derivatives [39,40]. This chemical modification also alters the drug/micelle interaction and the physical stability of the drug-loaded micelles under dilution [39,40]. Moreover, quaternization has been shown to improve cell attachment to poloxamine-based hydrogels due to the stronger electrostatic interaction with the negatively charged cell membrane [38]. Based on these structural differences, changes in the anti-P-gp activity of the branched copolymers with respect to the linear counterparts can be hypothesized *a priori*.

The present preliminary study reports, for the first time, on the P-gp inhibitory performance of pristine and *N*-methylated poloxamines. The intracellular accumulation of DOX (an exclusive substrate of P-gp) in Caco-2 cell monolayers (human colon adenocarcinoma cell line), an *in vitro* model of intestinal epithelium with a relatively high expression of P-gp, was investigated. Their performance was compared with that of poloxamers displaying a broad variety of molecular weights and

hydrophilic–lipophilic balance (HLB) values. The structural implications of the copolymers were studied and discussed.

Materials & methods

Materials

Tetronic 304 (T304), 901 (T901), 904 (T904), 908 (T908), 1107 (T1107), 1301 (T1301) and 1307 (T1307) and reverse sequential 150R1 (T150R1) and Pluronic L43, L92, P103, P123, P85, F68, F87 and F127 were a gift from BASF Corporation (New Milford, CT, USA) and were used as received. Their structural properties are summarized in Table 1. *N*-methylated derivatives of T904 (met-T904), T908 (met-T908), T1107 (met-T1107), T1301 (met-T1301), T1307 (met-T1307) were synthesized following a technique described elsewhere [38]. The degree of methylation was between 44 and 45% (i.e., approximately one out of two N atoms was methylated), as determined by argentometry [38]. DOX, verapamil (VER) and Triton X-100 were provided by Sigma (St. Louis,MO, USA). Bodipy® phalloidin was from Molecular Probes Inc. (Eugene, OR, USA). Other reagents were of analytical grade.

Caco-2 human colon carcinoma cell line (ATCC, LGC Standards SLU, Barcelona, Spain) was maintained in high-glucose content DMEM medium (Gibco, Invitrogen, Carlsbad CA, USA) supplemented with glucose (0.45%), penicillin (100 U/ml), streptomycin (0.1%, Gibco), non-essential aminoacids, L-glutamine (1%) and fetal bovine serum (FBS; 10%, HyClone, South Logan, UT, USA) at 37°C and 5% CO_2.

Intrinsic inhibitory activity of the different copolymers

The cytotoxicity of the different copolymers was evaluated by the crystal violet method [41]. This method has shown good correlation with other methods, such as MTT [42]. Furthermore, it has been previously reported (and we have also observed this phenomenon in our experiments) that DOX could interfere in the formation of formazan crystals and make the MTT assay unreliable [43]. Caco-2 cells were seeded in 96-well plates (1×10^5 cells/ml, 100 µl/well) and incubated in supplemented medium for 24 h. Copolymer solutions in phosphate-buffered saline (PBS; 1×) were added (0.1, 1 and 10% concentration, 20 µl; final copolymer concentrations in culture medium were 0.01, 0.1 and 1% w/v after 1:10 dilution in culture medium) and cells incubated at 37°C for 48 h. The culture medium was removed and cells fixed with glutaraldehyde (10 µl, 11%) for 15 min and washed (3 × 200 µl) to eliminate glutaraldehyde residues. Cells were stained with crystal violet solution (100 µl, 0.1%) in pH 6 buffer (orthophosphoric acid 200 mM, formic acid 200 mM, and 2-*N*-morpholine-ethanesulfonic acid, 200 mM) at room temperature for 15 min. The staining solution was soaked and cells washed with distilled water (3 × 200 µl) and dried. Finally, cells were treated with 100 µl of 10% acetic acid at room temperature for 15 min under gentle stirring and the absorbance was measured in a plate reader (λ = 595 nm; Tecan Ultra Evolution, Männedorf, Switzerland). A copolymer-free PBS (1×) was used as control of sample vehicle and showed 2–4% inhibition with respect to cells incubated in serum-containing medium. Experiments were carried out in triplicate. Percentage of viability was calculated as follows:

$$\%\ viability = (A_S / A_0) \times 100$$

A_s and A_c being the absorbance of the sample and the PBS control, respectively.

To determine the intrinsic DOX cytotoxicity, a similar assay was performed with DOX solely solutions of increasing concentration. A curve of concentration versus percentage of inhibition was plotted and a nonlinear adjustment carried out with software GraphPad Prism 2.01 1996 (GraphPad Software Inc., La Jolla, CA, USA). The inhibitory potency and the inhibitory efficacy were estimated from the half maximal inhibitory concentration 50 (the concentration that inhibits the growth of 50% of the cells, IC_{50}) and the maximum effect (E_{max}), respectively. IC_{50} was 11 µM and E_{max} 77%.

In vitro quantitative analysis of DOX accumulation

The effect of the different poloxamer and poloxamine (including *N*-methylated ones) solutions on the intracellular accumulation of DOX was evaluated according to a procedure described elsewhere [44]. Briefly, cells were seeded in 24-well plates (2×10^5 cells/ml, 500 µl/well) and incubated in supplemented medium for 48 h. The medium was replaced by serum-free DMEM containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (25 mM, pH = 7.4). Copolymer solutions were added (0.1, 1 and 10% concentration, 20 µl; final copolymer concentrations in culture medium were 0.01, 0.1 and 1% w/v) and cells incubated at 37°C (30 min). Copolymer-free medium and VER solution (100 µM) were used as blank and positive control, respectively. Then, 50 µl of DOX solution (100 µM in water) was

added and samples incubated for an additional 60 min. The medium was soaked and the cells washed (PBS, 3 × 500 µl) to remove DOX and copolymer residues. Cells were lysed (1% Triton X-100, 300 µl, 20 min), supernatant aliquots (200 µl) were transferred to opaque 96-well plates and the fluorescence was measured in a plate reader (λ_{exc} = 485 nm; λ_{em} = 580 nm). The remaining 100 µl was diluted (1:10, PBS) and the protein contents measured as depicted previously. DOX-free medium was used as blank. DOX concentrations were calculated using a calibration curve in the 0.2 pmol to 0.2 nmol range (R^2 = 0.997). Determinations were carried out three separate times, each in triplicate. Data of DOX concentration were normalized to the protein content in each well. DOX accumulation factors (f_{DOX}) were calculated as follows:

$$f_{DOX} = AD_s / AD_0$$

AD_s and AD_0 being the accumulated amounts of DOX (DOX nmoles/mg protein) for the sample and for DOX solution without copolymer or VER, respectively. f_{DOXmax} represents the highest f_{DOX} determined for a certain copolymer at any of the concentrations investigated, namely 0.01, 0.1 or 1%. Statistical differences ($p < 0.05$) of DOX accumulation with respect to DOX/VER controls were analyzed using the Dunnett's multiple comparison test. Statistical significance ($p < 0.05$) was evaluated by ANOVA (*post hoc* Dunnet´s T3) with SPSS 15.1 software.

Determination of protein concentration

The protein concentration per well after exposure to the DOX/copolymer samples was used to normalize the accumulated amount of DOX and was determined by the method of Bradford [45]. Cells exposed to culture medium and VER were used as blank and control, respectively. Data were also used to estimate the percentage of viable cells (% viability) upon exposure to DOX/copolymer as follows:

$$\%\,viability = (P_s / P_0) \times 100$$

P_s and P_0 being the protein contents obtained with the DOX/copolymer sample and the blank, respectively.

Confocal microscopy

Doxorubicin intrinsic fluorescence can be capitalized to qualitatively assess the integrity of the cell monolayer by means of confocal microscopy. Cells exposed to 0.1% polymer/DOX solutions (as for the DOX accumulation studies) were washed with cold PBS (pH 5, 3 × 10 min) to remove DOX residues, fixed with paraformaldehyde solution (4%, PFA, 10 min) and rewashed with PBS (pH 7.4, 10 min) to remove residual PFA. Then, specimens were incubated in darkness with a solution of the high-affinity probe for f-actin Bodipy phalloidin (30 µl/ml, 30 min) in 0.2% Triton X-100 (permeabilizer). Samples were washed again with PBS (pH 7.4, 3 × 10 min), mounted on glass slides using an antifading solution (Bio-Rad Laboratories, CA, USA) and cells visualized at 20× and 63× with a Confocal Spectral Microscope Leica TCS-SP2 (LEICA Microsystems Heidelberg GmbH, Mannheim, Germany); DOX is visualized as red (λ_{exc} = 561nm) and Bodipy phalloidin as blue (λ_{exc} = 633 nm).

Results & discussion

DOX accumulation

P-glycoprotein pumps expressed in the apical surface of the intestinal epithelium [46] represent a crucial barrier for the effective oral absorption and bioavailability of a broad variety of drugs, and a main reason for therapeutic debacle. Poorly water-soluble drugs classified into class II and IV of the Biopharmaceutics Classification System (BCS) are main substrates of this pump [47]. P-gp is also overexpressed in different diseased tissues and organs, leading to subtherapeutic drug concentrations. In this framework, the investigation of nonionic amphiphiles that generate polymeric micelles in aqueous medium and could perform as both drug carriers and P-gp inhibitors is an area of great interest [18,40]. The primary goal of the present work was to compare the implications of the molecular structure of poloxamer and poloxamine families of PEO–PPO block copolymers in the inhibitory activity of P-gp. In addition, the influence of *N*-alkylation on the inhibition was also investigated. VER, a well-characterized selective P-gp inhibitor, is currently in clinical trials as an MDR reversal agent [48]. Coadministration of low doses of VER with DOX has been shown to increase the bioavailability of the anticancer drug in rodents [49]. In this context, the inhibitory performance of the copolymers was assessed and compared with that of VER (control). VER increased DOX accumulation 1.45-fold with respect to a DOX solely solution (blank).

For comparative purposes, we first evaluated the inhibitory performance of low (F68, F87, F127), medium (L43, P123) and high (P85,

L92, P103) anti-P-gp activity poloxamers [21]. The 0.01% hydrophilic poloxamer solutions (F68, F87 and F127) were less effective than VER and did not improve DOX accumulation; these copolymer concentrations were lower or close above the critical micellar concentration (CMC). f_{DOX} values were between 0.64 and 0.75 (FIGURE 1). The f_{DOX} values below 1 would indicate that DOX accumulated intracellularly to a lower extent than in the basal level (only DOX). However, differences were not statistically significant due to relatively large standard deviations found in the determination of the basal levels.

An increase in the copolymer concentration to 0.1% led to an increase of f_{DOX} to values similar to VER (i.e., 1.53, 1.13 and 1.85 for F68, F87 and F127, respectively), indicating some P-gp inhibitory activity of the copolymers. A further increase in the copolymer concentration to 1% resulted in a decreased intracellular accumulation, this phenomenon being related to the drug sequestration by the formed polymeric micelles (see below). In spite of the slight-to-moderate improvement in DOX accumulation, it is remarkable that these amphiphiles were as active as VER at reasonably low concentrations (0.1%). The 0.01% systems of hydrophobic low-molecular-weight poloxamers of relatively long (P123) or short (L43) PPO blocks showed a similar trend with f_{DOX} values slightly below 1. When higher concentrations were tested, a sharp increase in the activity was observed, f_{DOXmax} of L43 (1%) being 2.24. Interestingly, our findings indicate that this copolymer is slightly more effective than P123 in a Caco-2 model. Shaik *et al.* [31] have described a differential P-gp ATPase inhibitory activity of F127 that depends on the substrate investigated; that is, F127 was not effective to block the binding of nelfinavir to the pump, while it effectively inhibited the interaction with VER. These results indicated that different substrates may interact with different epitopes in the transmembrane protein; the presence of more than one binding domain was also described elsewhere [50]. Moreover, a recent work reported on the inhibition of the P-gp-mediated basolateral-apical transport of ciloprolol by Pluronic F68 in Caco-2 monolayers [51]. It is worth stressing that both FDA-approved F127 and F68 have been previously described as relatively inefficient P-gp inhibitors [21]. Overall, our data highlight that the prediction of the inhibitory activity of a certain copolymer with respect to a specific substrate is very complex and needs to be assessed experimentally. Having expressed this, there exists solid evidence supporting that more hydrophilic PEO–PPO derivatives usually show a less effective inhibitory performance. Hydrophobic poloxamers of intermediate PPO length showed the greatest inhibitory activity (e.g., L92 [0.1%], P85 [1%] and P103 [1%] provided f_{DOXmax} values of 3.83, 5.18 and 2.57, respectively). P85 was the only 0.01% system displaying a significant increase of f_{DOX} when compared with VER, the value being 2.07. The f_{DOX} values for 0.1 and 1% P85 systems were also significantly higher than the control. Despite the sharp increase showed by P85 with respect to L92 and P103, differences between f_{DOXmax} values were not statistically significant. These findings were in full agreement with previous reports [21].

In general, when the poloxamer concentration increased from 0.1 to 1%, a decrease in DOX intracellular accumulation was apparent. This phenomenon probably stems from the sequestration of DOX molecules by the increasing number of polymeric micelles in the medium. Since the inhibitory activity has been ascribed to the unimers [21], any additional concentration increase over this value does not result in a beneficial effect. In this context, the higher the molecular weight of the copolymer, the sharper the f_{DOX} decrease observed. However, this decrease was statistically significant only for F127 and P123, both polymers sharing two features: especially low CMC values (0.0025% and 0.0035%, respectively, at 37°C) [52]; and long PEO segments (N_{PO} > 65). In the case of P103

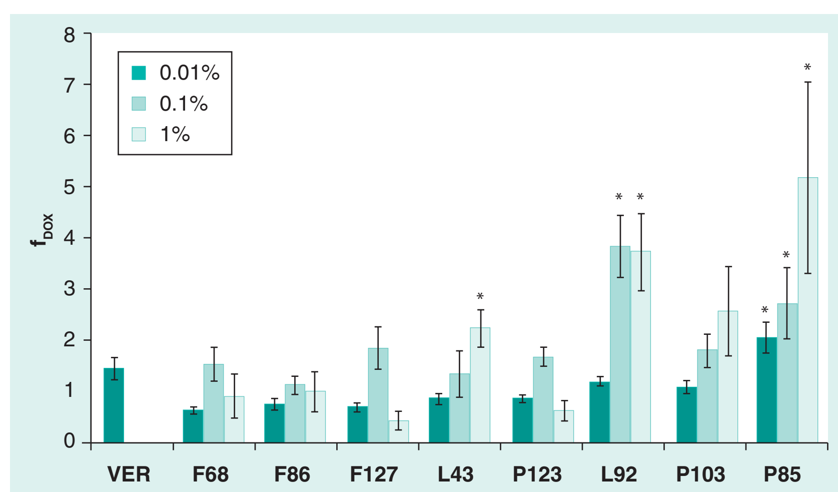

Figure 1. Doxorubicin accumulation, expressed as f_{DOX}, in the presence of different poloxamer concentrations. A measurement of 100 µM VER was used as a control. All the samples contained 100 µM DOX (n = 3).
*Statistically significant increase (p < 0.05) of DOX accumulated amount when compared with DOX/VER.
DOX: Doxorubicin; VER: Verapamil.

and P85, a concentration change from 0.1 to 1% showed a slight, but not statistically significant, increase in f_{DOX}. These findings indicated that, regardless of the generation of micelles (CMC values of P103 and P85 are 0.003 and 0.03%, respectively [51]), they do not encapsulate DOX efficiently; the sequestration capability is probably shown only by larger micelles. The low DOX encapsulation capacity of P85 has been previously reported [53]. L43 presents a CMC value of 0.40% [52]. Thus, an increase in the copolymer concentration from 0.1 to 1% resulted in a fourfold increase of the number of unimers and a stronger inhibitory activity, the f_{DOXmax} value being 2.24 for a 1% system.

Another interesting observation is that 1% P103/DOX and P85/DOX systems led to a pronounced decrease in the relative protein content to approximately 45 and 60% of the VER/DOX control, respectively. This phenomenon results in the concomitant increase of the normalized values of accumulated DOX (see below).

A main structural characteristic of poloxamines is that the total %PPO is the sum of four shorter blocks linked to a central pH-sensitive ethylenediamine group, as opposed to poloxamers that present a single central PPO segment. According to the HLB value, the poloxamines (and their *N*-methylated derivatives) were classified as: hydrophilic (HLB > 18; T1107, T1307 and T908); medium hydrophobic (HLB 12–18; T304 and T904); and highly hydrophobic (HLB 1–7; T901, T1301 and T150R1).

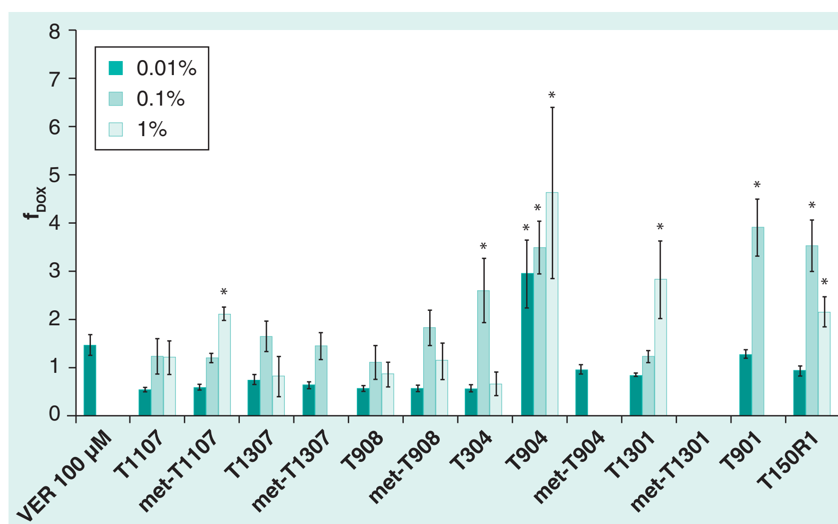

Figure 2. Doxorubicin accumulation, expressed as f_{DOX}, in the presence of different pristine and methylated poloxamines. A measurement of 100 µM VER was used as a control. All the samples contained 100 µM DOX (n = 3).
*Statistically significant increase ($p < 0.05$) of DOX accumulated amount when compared with DOX/VER.
DOX: Doxorubicin; VER: Verapamil.

Due to the molecular architecture and the monoprotonated state under physiological conditions, poloxamines display slightly higher CMC values than poloxamers of similar molecular features [39,40]; protonation increases the repulsion between the polymer chains and hinders the aggregation process. Diluted (0.01%) T1107, T1307 and T908 solutions did not enhance DOX accumulation; f_{DOX} values were comparable to those of hydrophilic poloxamers: 0.53, 0.73 and 0.55, respectively (FIGURE 2). Even though these values were lower than the basal level, the differences were not statistically significant.

An increase in the copolymer concentration to 0.1% resulted in a pronounced f_{DOX} increase to 1.22, 1.63 and 1.09, these extents being similar to VER. In coincidence with hydrophilic poloxamers, 1% T1107, T1307 and T908 solutions showed a decrease in f_{DOX}, probably due to the drug sequestration by the micelles. On the other hand, 0.01% solutions of the most hydrophobic poloxamines (HLB 1–7) presented f_{DOX} values similar to highly hydrophobic poloxamers (e.g., L92, L43, P103 and P123), the values being 0.84, 0.92 and 1.26 for T1301, T150R1 and T901, respectively. Similarly to poloxamers, the lower the molecular weight and the shorter the PEO segments (e.g., T901), the more effective the inhibition found. It is remarkable that a reverse-sequential poloxamine (T150R1) combining internal PEO with external PPO blocks was also active. An increase of the copolymer concentration to 0.1% led to a sharp increase in the amount of accumulated DOX, especially for T901 that showed a remarkable f_{DOXmax} of 3.88. This value was comparable to that of L92, a polymer displaying a similar HLB, although a much lower N_{PO}. The 1% T901/DOX sample had a strong cytotoxic effect (total cell death) and no accumulation could be measured. The 1% T1301 was less cytotoxic and a clear increase of f_{DOX} to 2.81 was apparent. This profile was almost super-imposable to those of L43 and P103. T304, a polymer that combines very low molecular weight and intermediate HLB also showed a maximal inhibition at 0.1% (f_{DOXmax} = 2.58). T904 showed the most remarkable performance among the most diluted poloxamers and poloxamines, f_{DOX} being 2.93 and representing a twofold increase with respect to VER. This extent was greater than the one shown by 0.01% P85, the most effective poloxamer tested. More concentrated systems led to a steady increase of the accumulation to f_{DOXmax} of approximately 4.6, a behavior comparable to that of P85. On the other hand, it is

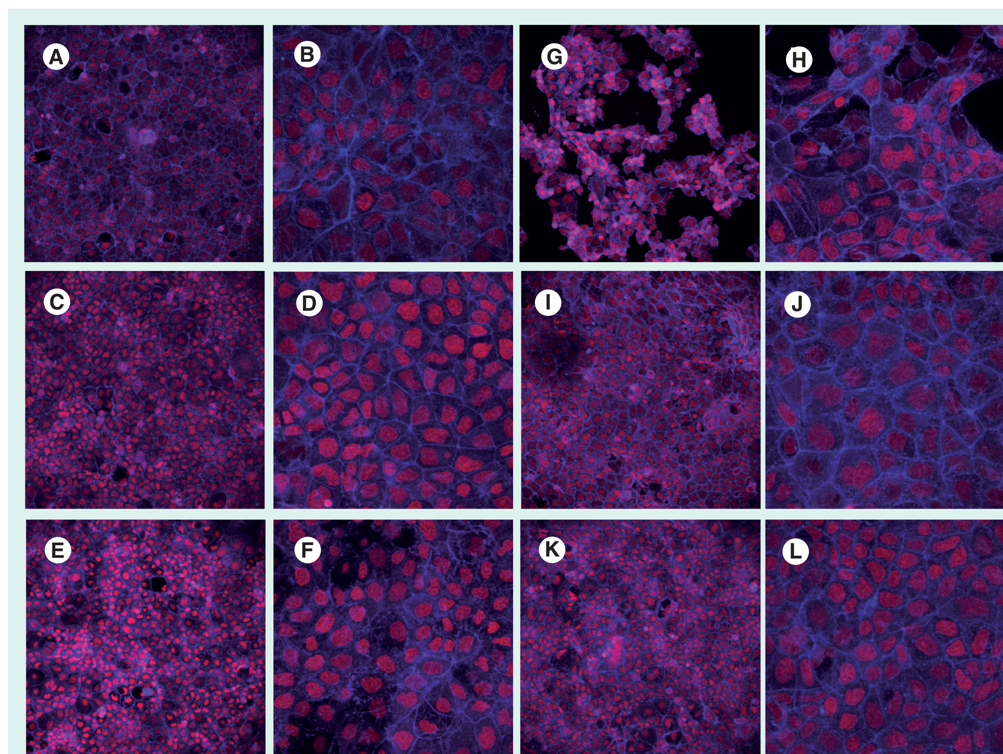

Figure 3. Confocal microscopy of Caco-2 cells exposed to 0.1% copolymer solutions. Fluorescent micrographs of Caco-2 monolayers exposed to doxorubicin (DOX; blank, **A & B**), verapamil (VER)/DOX (control, **C & D**), 0.1% P85/ DOX **(E & F)**, 0.1% T904/DOX **(G & H)**, 0.1% T1307/DOX **(I & J)** and 0.1% met-T1307/DOX **(K & L)**. Nuclei are stained with DOX (red) and *f*-actin with Bodipy® phalloidin (blue). Magnification 20× **(A, C, E, G, I & K)** and 63× **(B, D, F, H, J & L)**.

important to remark that 1% T904 was cytotoxic *per se*. The 0.01% *N*-methylated derivatives of the most hydrophilic poloxamines showed a very similar behavior to that of their pristine counterparts, and the accumulated DOX amounts were slightly lower, although not statistically different, than the basal. At a higher concentration (1%), met-T1107 led to a significantly higher DOX accumulation than the unmodified polymer and the VER control. Furthermore, met-T908 showed a better performance than T908, the increase being especially relevant for 0.1% solutions; f_{DOXmax} values were 1.81 and 1.09 for met-T908 and T908, respectively. By contrast, 0.1% met-T1307 showed a similar behavior to that of unmodified T1307. On the other hand, 1% met-1307/DOX systems showed high toxicity. Highly hydrophobic *N*-methylated poloxamines (e.g., met-T1301) combined with DOX killed all the cells even at concentrations as low as 0.01%, thus the measurement of DOX accumulation was not possible. A less hydrophobic derivative (met-T904) was better tolerated by the cells but only at very low concentrations (0.01%) and the performance was worse than the pristine derivative. These findings would suggest that *N*-methylation leads to only a slightly better copolymer–cell electrostatic interaction and greater anti-P-gp activity only for highly hydrophilic polymers that, *per se,* show a low-to-moderate inhibitory capacity. More hydrophobic derivatives were substantially more cytototoxic. Thus, this modification does not encompass any advantage over the pristine derivatives.

To evaluate the integrity of Caco-2 monolayers (and qualitatively assess the localization and intensity of DOX), cells exposed to 0.1% polymer/DOX solutions (as for the DOX accumulation studies) were fixed and observed under the confocal microscope (Figure 3). In general, confluent monolayers remained well attached, although T904/DOX samples resulted in some level of detachment, probably due to some cytotoxicity. F-actin staining confirmed the characteristic Caco-2 morphology of all the monolayers. All the specimens showed the nuclear localization of DOX. This behavior is characteristic of Caco-2 cells that are sensitive to DOX [54]; in resistant strains DOX is usually colocalized in the cytoplasm and the nucleus. Having expressed this, samples exposed to DOX/VER and DOX/copolymer showed a more intense staining than the DOX solely solution, indicating the inhibition of the efflux mechanism to some extent. In

addition, P85 and T904 showed a higher intensity than VER (control), supporting the quantitative analysis described above that showed the pronounced increase in the accumulated DOX.

From these data it is still unclear if the viability loss found for several copolymer/DOX systems was intrinsic of the copolymer or if it was the result of the increasingly high intracellular DOX concentrations that led to a more pronounced cell death. Thus, the viability of cells exposed to DOX-free and DOX-containing copolymer solutions were also evaluated.

Intrinsic cytotoxicity of pristine copolymers

The good cell compatibility of linear [20] and some branched [19,55,56] hydrophilic PEO–PPO copolymers has been previously reported. Moreover, the cytotoxicity of pristine and *N*-alkylated poloxamines was recently investigated by means of mitochondrial dehydrogenase (MTT) and lactate dehydrogenase (LDH) assays [40]. Regardless of some discrepancies in the results among the methods, in general both pristine and *N*-alkylated hydrophilic derivatives displayed good cytocompatibility, even at concentrations as high as 5%. By contrast, a sustained viability loss was found for increasing concentrations of copolymers of intermediate to high hydrophobicity [40]. This phenomenon was more pronounced for the *N*-alkylated counterparts that showed a sharp viability loss, even at concentrations as low as 0.1%; the greater cytotoxicity of crosslinked *N*-methylated poloxamine hydrogels was depicted elsewhere [38]. In the present study, f_{DOX} values are calculated by rationing the accumulated DOX amounts by the protein content of the sample measured immediately after the exposure to the copolymer/DOX solution. To discern whether the cell death stems from the intrinsic cytotoxicity of the copolymers or, conversely, it relies on a combined or synergistic cytotoxicity due to the copolymer and the DOX, the viability of Caco-2 monolayers exposed for 48 h to DOX-free pristine and *N*-methylated poloxamines in a broad range of concentrations (0.01–1%) was measured by the crystal violet method [43]. The intrinsic cytotoxicity of poloxamers was not assayed, although Pluronic L92 was evaluated for comparison. Findings indicated the excellent cytocompatibility of all the pristine hydrophilic poloxamines T1107, T1307 and T908; percentage viability ranging between 91–97 and 81–86% for 0.01 and 1% solutions, respectively (Figure 4). In addition, a lower cytocompatibility was evidenced for *N*-methylated counterparts only in 1% solutions, the viability values being 26 and 57% for met-T1307 and met-T908, respectively. The 0.01% T904 and T901 showed relatively high viability (>80%) although higher copolymer concentrations led to a sharp decrease to 20%. These results are in full agreement with our previous work and stress the intimate association between the HLB and the molecular weight and the cytotoxicity of the copolymer [40]. Met-T904 was the most cytotoxic of all the tested copolymers, the viability being less than 60% for the 0.01% solution. Finally, L92 also showed some cytotoxicity that gradually increased at higher copolymer concentrations (e.g., viability of 0.01 and 1% solutions was approximately 60 and 40%, respectively). This result was especially interesting, as several works report on the high inhibitory activity of the copolymers based on the measurement of accumulated amounts of substrate. However, since this quantitative analysis is carried out on a protein content basis, copolymers displaying moderate cytotoxicity could lead to some overestimation of the inhibitory activity due to the reduced protein content upon cell death.

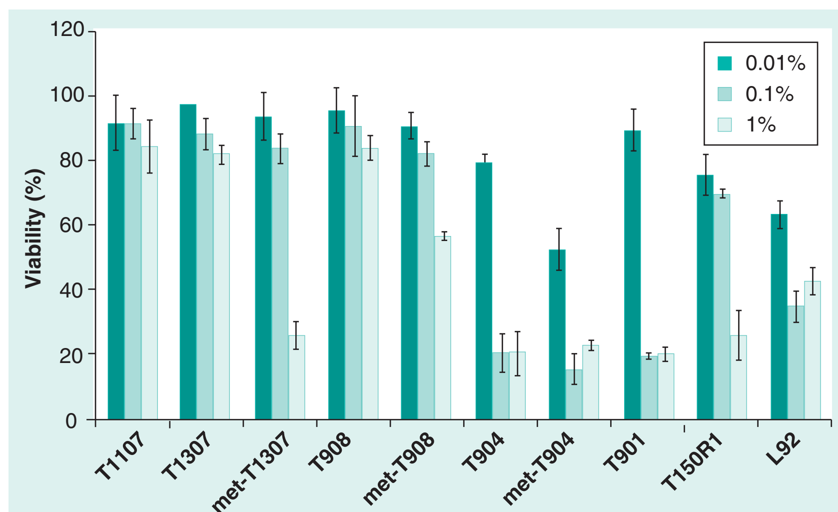

Figure 4. Cell viability after the exposure of Caco-2 monolayers for 48 h to different poloxamers, poloxamines and *N*-methylated poloxamines. Determined by the crystal violet method (n = 3).

Cytotoxicity of DOX/copolymer systems

Doxorubicin is an effective agent against colorectal and other cancers of the GI tract [57]. Since Caco-2 is a colonic tumoral cell line, it is expected that higher DOX intracellular concentrations due to P-gp inhibition would lead to increased cell death and decreased protein amounts after the assay. This phenomenon, in turn, will increase the calculated f_{DOX} value. On one hand, it would

be more reliable to test the P-gp inhibitory activity of the copolymers only in the case of samples displaying high intrinsic cytocompatibility. On the other hand, since different viability tests might lead to divergent results, establishing the criteria for sample inclusion in the study is not straightforward. Thus, we decided to report on the full data and compare the DOX/copolymer cytotoxicity to that of DOX-free (only copolymer) samples. The viability of cells exposed to DOX/polymer samples for just 1 h was estimated by measuring protein amounts. Met-T1301/DOX samples killed all the cells and the protein content could not be measured. For the rest of the systems, when 0.01% copolymer concentrations were tested, viability levels remained greater than 87% (Figure 5A & 5B). In general, an increase in the concentration to 0.1% led to a slight to moderate viability loss (e.g., L92/DOX showed a more pronounced decrease to 52%) (Figure 5A). This viability was comparable to that of the copolymer alone (Figure 4), although only after 1 h exposure. Standard deviations were relatively high and, in most of the cases, viability changes were not statistically significant. Cytotoxicity was more noticeable for 1% polymer/DOX of T901 and met-T1307 that showed no viable cells even after only 1 h exposure (Figure 5B). It is worth stressing that DOX-free T901 and met-T1307 samples showed viability levels of approximately 20% (Figure 4). Intermediate cytotoxicity was found for DOX-containing 1% met-T1107, P103, P85 and T904 samples; with percentage viability between 42 and 56%. The most hydrophilic derivatives of both families (HLB > 18) showed similar viability to that of VER/DOX, values usually being greater than 90% (Figure 5A & 5B). These findings also indicated that the more hydrophobic and the lower the molecular weight of the copolymer, the higher the cytotoxicity of DOX/ copolymer combinations. In addition, *N*-methylated derivatives of hydrophobic poloxamines led to a more detrimental effect than their precursors. Overall, cytotoxicity values were slightly higher than those observed with the DOX-free systems of identical copolymer concentration. However, it should be stressed that in this assay, the cells were in contact with the DOX/copolymer mixture only for 1 h, as opposed to the 48 h of the previous experiment with copolymer solely solutions. These findings indicate that DOX-containing systems lead to much faster viability losses and strongly support that the increased intracellular accumulation of DOX results in more pronounced cell death. Moreover, the intracellular DOX concentrations found in the presence of the different copolymers were in the range of 0.23 to 3.65 μM, values in the same order of the measured IC_{50} (11 μM). These additional data constitutes further evidence that the viability loss stems from both some copolymer toxicity and the copolymer-enhanced DOX accumulation.

Copolymer structure–activity relationship

A preliminary (and direct) analysis of the structure–activity relationship suggested that poloxamines do not directly comply with the structural requirements previously stated by Batrakova *et al.* to attain maximal inhibition with poloxamers [21]: intermediate-length PPO blocks (30–60 PO units); and relatively low HLB values. The structure–activity relationship of poloxamines is presented in Figure 6. For example, T904

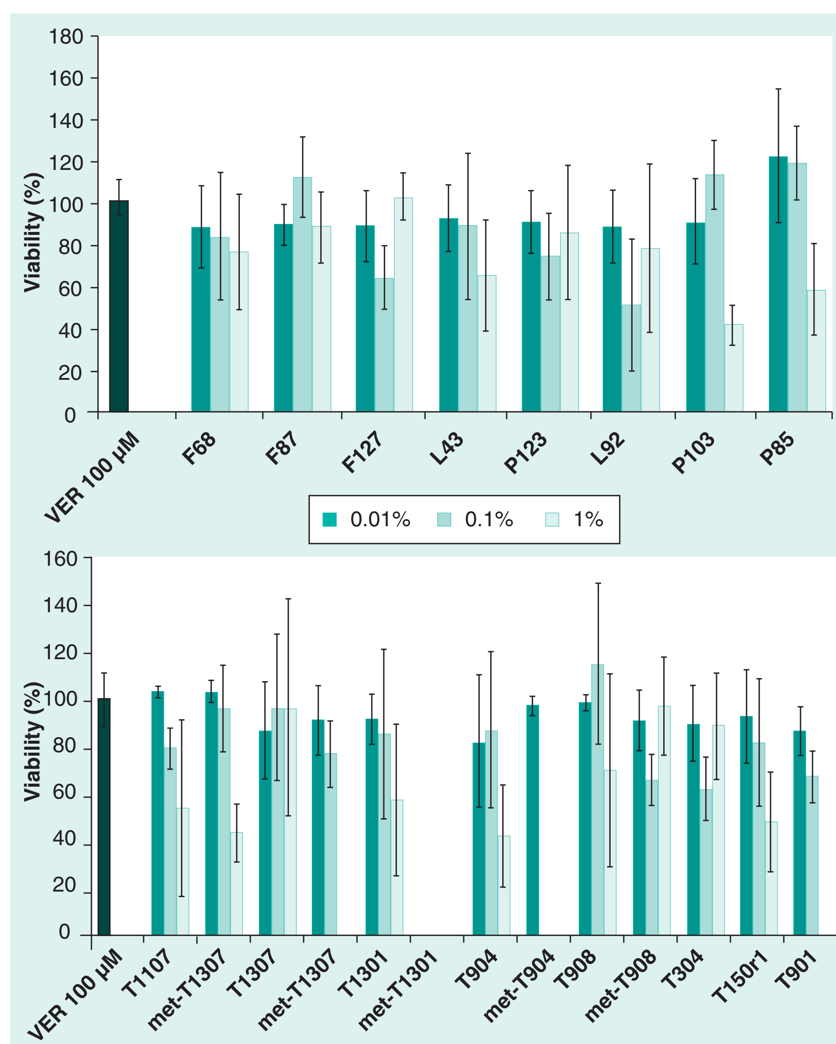

Figure 5. Cell viability after the exposure of Caco-2 monolayers for 1 h to doxorubicin in the presence of different poloxamers (A), poloxamines and *N*-methylated poloxamines (B). 100 μM VER was used as a control (black column). All the samples contained 100 μM doxorubicin (n = 3).
VER: Verapamil.

(N_{PO} = 69) and P85 (N_{PO} = 40) displayed similar inhibition profiles regardless of the substantially different structural features. T904 was the most effective inhibitor even at the lowest concentration tested (0.01%). A similar behavior was found for L92 (N_{PO} = 50) and T901 (N_{PO} = 73). In addition, more hydrophobic poloxamines (HLB < 19), such as T304 and T1301 with extremely low or high N_{PO} values of 16 and 104, respectively, showed effective inhibition as expressed by f_{DOXmax} values of 2.58 and 2.81. Poloxamers with similar relatively short or long PPO segments (L43 and P123) showed lower inhibitory efficiency.

Previous studies indicated N_{PO} as the critical parameter that rules the association of poloxamers to phospholipid bilayers [58,59]. PEO–PPO–PEO with very short PPO segments (e.g., F38, 15 PO units) and radius of gyration (Rg) smaller (7 Å) than hydrophobic tails of the lipid bilayer (~20 Å) probably intercalates in the cellular membrane, with both PEO external blocks exposed at the surface [55]. When polymers bearing longer PPO were investigated (e.g., F88, 39 PO units, Rg ~15 Å), results suggested that the copolymer chains span the bilayer.

Fragmentation of the PPO component of poloxamines into four short arms appears to be a key structural feature affecting the structure–inhibitory activity relationship. However, the molecule of poloxamine could also be considered as two PEO–PPO–PEO triblocks covalently linked by the central ethylenediamine group. Assuming this, the number of 'effective' PO units in the central PPO block of each of the 'triblocks' in poloxamine would be $N_{PO}/2$. In this situation, two PO units in poloxamines would have a similar impact on the dimensions of the hydrophobic domain, such as one PO unit in a poloxamer molecule. In this framework, a new diagram of the inhibitory activity of different poloxamers and poloxamines according to the 'effective' number of PO units in the PPO blocks is proposed (Figure 7). According to this, three main areas of low (f_{DOXmax} > 2), medium (2 > f_{DOXmax} > 3) and high (f_{DOXmax} > 3) inhibitory activity could be defined for all the PEO–PPO block copolymers; these ranges being in partial agreement with those previously defined exclusively for poloxamers [21]. A main difference resides in the fact that copolymers of both intermediate-to-low HLB are now included in the high-inhibition group. For example, the most active poloxamines (f_{DOXmax} > 3) display an effective PO number ($N_{PO}/2$) between 34 (T904) and 52 (T1301). In addition, VER should probably be considered as relatively weak, although highly specific, P-gp inhibitor with effectiveness comparable to that of all the hydrophilic poloxamers and poloxamines in concentrations of approximately 0.1%. Nevertheless, it is clear from Figure 7 that the 'effective' PO number itself does not totally explain the differences in inhibition activity and that the HLB also has to be taken into account; those copolymers with high HLB (>18) resulting in lower DOX accumulation. Remarkably, due to *N*-methylation, T1107 underwent from a low to an intermediate activity. This result indicates that minor molecular modifications can notably affect the interaction of these copolymers with cells and alter the inhibitory performance. In this context, additional mechanistic studies are demanded to elucidate the potential pathways involved in the inhibitory activity of poloxamines and their chemically modified derivatives.

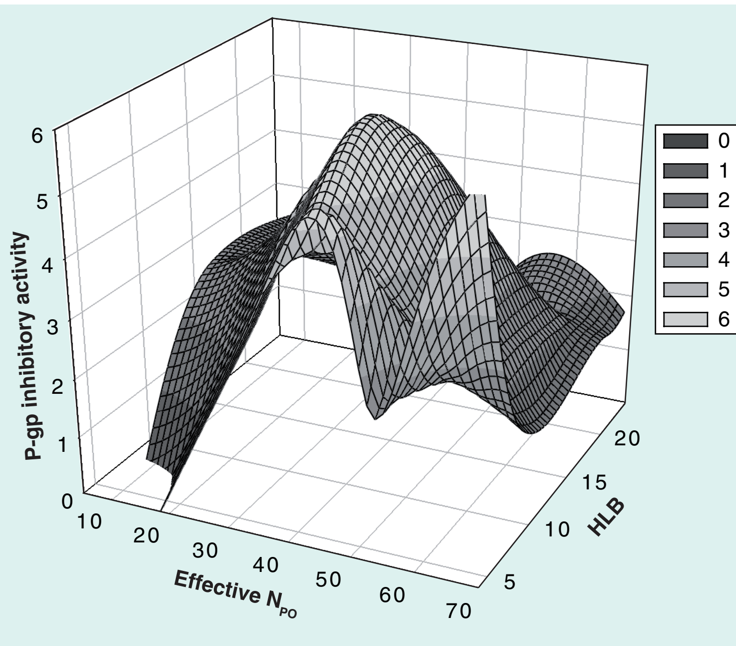

Figure 6. Structure–activity relationship of poloxamines and poloxamers. The 'effective' number of PO units in poloxamines was calculated as PO/2.
HLB: Hydrophilic–lipophilic balance.

Conclusion

A comprehensive study of the inhibition of P-gp-mediated DOX efflux by pristine and *N*-methylated branched PEO–PPO block copolymers was conducted and compared with that of the linear counterparts. Findings indicated that all the poloxamines were active to some extent, this activity being equal or greater than that of VER. In accordance to previous studies with poloxamers, poloxamines of intermediate hydrophobicity (low-to-medium HLB) and with effective N_{PO} ($N_{PO}/2$) ranging from 30 to 50 displayed the highest inhibitory activity. The

positively charged character of poloxamines due to protonation under physiological conditions and *N*-alkylation, both leading to a stronger affinity for the cellular membrane, did not appear to play a crucial role in ruling the interaction. Conversely, *N*-alkylation increased the cytotoxicity of the copolymers, this phenomenon being especially remarkable in the case of hydrophobic derivatives. Overall results support the versatility of poloxamines as both dually responsive drug carriers and inhibitors of efflux transporters.

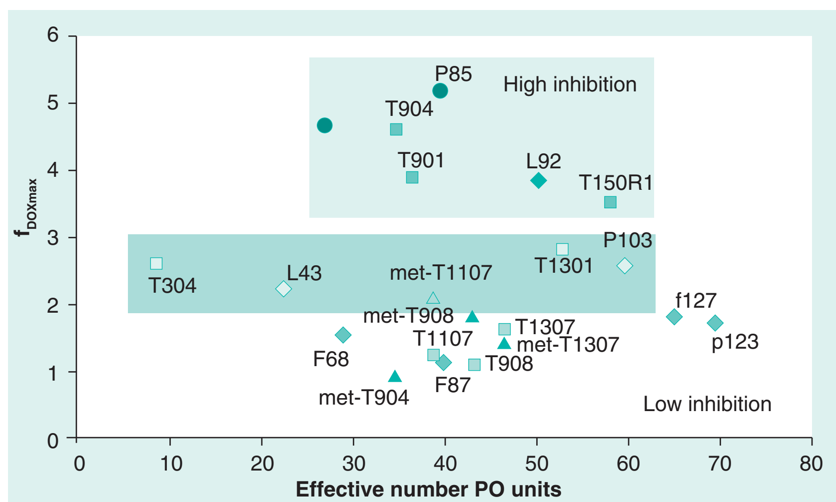

Figure 7. Inhibitory efficacy of different poloxamers, pristine and *N*-methylated poloxamines versus the 'effective' number of PO units (N_{PO}/2 in the case of poloxamines).

Future perspective

As recently stated, the role of block copolymers is evolving from plain 'inert drug nanocarriers' to relevant 'biological response modifiers' [24]. Up to now, linear PEO–PPO–PEO block copolymers of the Pluronic family have been the aim of most pharmaceutical and biomedical studies. The results obtained with poloxamines (Tetronic family) highlight the role of the spatial arrangement of the blocks and the central linker (the ethylenediamine group) on their ability to inhibit the activity of P-glycoprotein. A better knowledge of the way the copolymer interacts with the cells and localizes on or inside them is crucial for understanding the effects of minor structural changes on the biological performance of the block copolymers. In addition, the implementation of procedures for designing tailor-made block copolymers with specific and improved ability to regulate certain biological functions may offer novel ways to overcome certain body malfunctions/diseases. One may envision some block copolymers as very active coadjuvant substances that may remarkably enhance the therapeutic efficiency of nanomedicines, or even some day as the nanomedicine themselves.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- The fragmented nature of the poly(propylene oxide) domain of poloxamines is a key feature affecting the cell/copolymer interaction.
- Hydrophilic poloxamines (hydrophilic–lipophilic balance [HLB] > 18; T1107, T1307 and T908) showed maximum anti-P-gp activity at 0.1% (compared to 0.01 or 1%) with doxorubicin accumulation values similar to those achieved with verapamil control. *N*-alkylation improved the performance.
- Medium hydrophobic poloxamines (HLB 12-18; T304 and T904) showed greater anti-P-gp activity, being T904 the most effective copolymer at the lowest concentration tested 0.01%.
- Highly hydrophobic poloxamines (HLB 1-7) 0.1% T901, 1% T1301 and 0.1 or 1% T150R1 were more effective than verapamil control. *N*-alkylation led to a deleterious effect on cell compatibility.
- Poloxamines T901, T904 and T150R1 are as efficient inhibitors of efflux pumps as Pluronics® P85 and L92.
- The effective number of PO units in the poloxamine structure (i.e., the total number divided by 2) enables comparison of the performance of the X-shaped copolymers with that of the linear counterparts.

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