A combination drug delivery system employing thermosensitive liposomes for enhanced cell penetration and improved *in vitro* efficacy

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**ABSTRACT**

Drug-loaded thermosensitive liposomes are investigated as drug delivery systems in combination with local mild hyperthermia therapy due to their capacity to release their cargo at a specific temperature range (40–42 °C). Additional benefit can be achieved by the development of such systems that combine two different anticancer drugs, have cell penetration properties and, when heated, release their drug payload in a controlled fashion. To this end, liposomes were developed incorporating at low concentration (5 mol%) a number of monoalkylether phosphatidylcholine lipids, encompassing the platelet activating factor, PAF, and its analogues that induce thermoresponsiveness and have anticancer biological activity. These thermoresponsive liposomes were efficiently (> 90%) loaded with doxorubicin (DOX), and their thermal properties, stability and drug release were investigated both at 37 °C and at elevated temperatures. In vitro studies of the most advantageous liposomal formulation containing the methylated PAF derivative (methyl-PAF, edelfosine), an established antitumor agent, were performed on human prostate cancer cell lines. This system exhibits controlled release of DOX at 40–42 °C, enhanced cell uptake due to the presence of methyl-PAF, and improved cell viability inhibition due to the combined action of both medications.

**1. Introduction**

The application of elevated temperatures topically for curing certain diseases including cancer was known for thousands of years as evidenced from historical scripts dating back to ancient Egypt (3000 BCE), China and India, as well as to the Greek physician Hippocrates of Kos (400 BCE) (Pang 2016; Seegenschmiedt and Vernon, 1995). In modern times local hyperthermia is widely studied and employed in medicine, especially in oncology to augment the therapeutic outcome of various cancer treatments such as chemotherapy or radiotherapy (Cihoric et al., 2015; Kok et al., 2015; Pang 2016). The therapeutic benefits of local hyperthermia are attributed, among others, to increased tissue oxygenation, blood flow and microvascular permeability of drugs, as well as to the modulation of the immune system by activating natural killer cells and phagocytes (Datta et al., 2015; Frey et al., 2012; Mallory et al., 2016 Schildkopf et al., 2010; Song, 1984).

With the advent of drug delivery systems that can, either passively (due to the enhanced permeation and retention effect), or actively (when specific targeting groups are introduced) (Torchilin, 2010; Torchilin, 2011), target tumors, coupled with the ability to heat selectively deeply located solid tumors employing up-to-date radiofrequency, ultrasound- or microwave-based methods (Koning, 2010), the research is further expanding to combine drug delivery with hyperthermia through the development of temperature-triggered (or thermoresponsive) nano drug delivery systems (Shao et al., 2011). The aim is to combine drug delivery systems with local mild hyperthermia therapy (regional hyperthermia) in which a tissue is heated up to 40–42 °C (Urano, 1999). Topically increased temperature treatment results in improved tumor blood flow, vascular permeability, and up-regulated drug influx in the tumor. It is, therefore, advantageous to combine mild hyperthermia with suitably designed thermally triggered long-circulating drug delivery systems, as in this case the drug would be released almost exclusively in the heated area. The most widely studied and promising thermoresponsive nanosystems that are designed to release their payload abruptly above a certain temperature are either polymeric systems of various architectures (Kojima, 2010; Tenhu and...
Winnik, 2011; Ward and Georgiou, 2011) or liposomes.

Thermosensitive liposomes are investigated as drug delivery systems, especially in cancer therapy, due to the property of phosphatidylcholine liposomal bilayer membrane to become, up to a certain extent, permeable to water-soluble molecules at temperatures that are close to the main gel to liquid crystal phase transition temperature ($T_m$), being practically impermeable at lower temperatures (Gaber et al., 1997; Papahadjopoulos et al., 1973; Yatvin et al., 1978). Liposomes based on dipalmitoylglycercophosphocholine (DPPC) that have a $T_m$ of about 41 °C were intensively exploited by many groups for attaining temperature-triggered release of liposomal encapsulated water soluble drugs. A major advance on the subject was the work of Needham et al. who proved that the introduction in the bilayer of low quantities (10%) of a lysophospholipid, i.e. monoalkyl chain phosphatidylcholine (lyso-PC), resulted in fast release of encapsulated molecules (Anyarambahatla and Needham, 1999; Needham et al., 2000; Needham and Dewhirst, 2001). Ever since, various liposomal formulations, summarized in several review articles (Ta and Porter, 2013; van Elk, 2016; Viard and Puri, 2015), exhibiting fast release at biologically relevant temperatures have been investigated. As an alternative to lyso-PC, a variety of molecules have been proposed for further improving the thermoresponsive properties of the bilayers, for instance, monoalkyl chain lipids of various chemical structures (Lindner et al., 2008), simple surfactants (Tagami et al., 2011a) or thermoresponsive polymers such as poly(N-isopropylacrylamide) (Kono, 2001; Kono and Takagishi, 2004). However, the lyso-PC containing liposomal formulation encapsulating the anticancer drug doxorubicin is still considered the most advanced, being currently in Phase 3 clinical trial under the trade name ThermoDox (Celsion.com/thermodox, 2018; Dou et al., 2017).

Doxorubicin (DOX), a well-known and widely used anticancer drug in chemotherapy for various tumors including prostate cancer (Gewirtz, 1999; Torti et al., 1983), acts as a potent DNA intercalator (Fornari et al., 1994). However, its use is associated with severe side effects including normal tissue cytotoxicity and cardiotoxicity (Barr et al., 2007). In addition, although it initially results in a positive response against cancer cells, it often induces acquired drug resistance (Guo et al., 2016; Mahon et al., 2011). Thermosensitive liposomal formulations lead to increased tumor accumulation of DOX (Kono, 2001; Sadeghi et al., 2018), thereby reducing unwanted side effects, but it has been shown that clinical trials failed to unequivocally improve progression-free survival (Kono, 2001). It has also been reported that these liposomes do not effectively facilitate transport of the drug in the interior of the cells and that primarily extracellular DOX release is observed (Horovitz et al., 1992). DOX administered in thermoresponsive liposomes was shown to stop blood flow in tumors thus acting as an anti-vascular agent (Chen et al., 2004; Ponce et al., 2006). This was attributed to the very fast release of the drug upon heating (typically within less than 1 min) that mostly results in the release of the free drug in the tumor vasculature or in the interstitial space but not inside cancerous cells (Manzoor et al., 2012). This points out the need of a thermoresponsive system that allows a slower drug release, with the additional ability to effectively transport the drug inside cells.

Among the strategies employed to overcome DOX-induced multidrug resistant is its combination with suitable drugs that can act synergistically with DOX. In this context, a class of monoalkyl ether phospholipids analogues of the platelet-activating factor (PAF) is known to exhibit antineoplastic activity (Houlihan et al., 1995). These compounds are considered of particular interest because they represent a strategy for killing neoplastic cells that is fundamentally different from that of doxorubicin as there is no evidence that they interact directly with DNA. Among the monoalkyl ether phospholipids family, one derivative of PAF, 1-O-octadecyl-2-O-methyl-rac-glycerol-3-phasphocholine (methyl-PAF, also known as edelfosine or ET-18-OCH₃), is the most active of this class and the most studied as an antitumor phospholipid agent (Ausili et al., 2018; Bonilla et al., 2015; Chen et al., 2004; Gajate and Mollinedo, 2014; Teixeira et al., 2018). This compound accumulates in plasma membrane lipid rafts, endoplasmic reticulum and mitochondria inducing apoptosis in cancer cells while, more importantly, it is known to be selectively taken up by malignant but not by normal cells (Gajate et al., 2000; Manzoor et al., 2012; Mollinedo et al., 1997; Quesada et al., 2004). Its activity is attributed mainly to its binding affinity to lipid rafts of the bilayer and the disorganization it exerts in liquid-ordered membrane structures, which can affect the transmembrane proteins and the overall membrane permeability as well (Andresen et al., 2004; Ausili et al., 2018; González-Fernández et al., 2018; Mahadeo et al., 2015). The in vivo toxicity is high towards a large number of cancer cell lines (IC₅₀ in the order of μM), and the preclinical studies presented low systemic toxicity in animals (Houlihan et al., 1995). Consequently, many clinical studies were undertaken showing, however, limited success attributed mainly to its metabolic instability, lack of sensitivity, high hemolytic potential, and gastrointestinal toxicity (Briglia et al., 2015; Pachioni et al., 2013; Ríos-Marco et al., 2017; van Blitterswijk et al., 2013). However, it was found effective and beneficial for leukemic patients requiring autologous bone marrow transplantation so that currently it is widely used for this purpose (Zarémba et al., 2019).

To overcome the limitations of this otherwise potent medication, a number of lipid or liposomal nanoparticle formulations of methyl-PAF were developed and studied (Busto et al., 2008; González-Fernández et al., 2015; González-Fernández et al., 2018; Mayhew et al., 1997). It was found that methyl-PAF containing liposomes were > 20 times less hemolytic but about two times less effective in in vitro cell studies than free methyl-PAF (Peters et al., 1997). In vivo experiments in normal mice showed that these liposomal formulations showed no toxicity but only partially inhibited tumor growth (Ahmad et al., 1997). It has been suggested that methyl-PAF containing liposomes promote drug diffusion into cells due to its membrane permeability enhancing properties (Andresen et al., 2004). It was also suggested that when methyl-PAF is located in stable liposomes the nonspecific cell membrane-binding affinity, which is associated with lytic effects, is reduced (Peters et al., 1997). Recent work provided evidence that edelfosine-loaded lipid nanoparticles are effective against primary osteosarcoma tumors in vivo and inhibit lung metastasis using osteosarcoma animal models (González-Fernández et al., 2018).

In several studies methyl-PAF, either free or encapsulated in lipid nanoparticles, has been used in combination with DOX to yield a positive synergistic effect against cancer cells because it attacks cancer cells in a fundamentally different way than DOX, i.e. without direct interaction with DNA. In addition, due to its membrane localization property, it enhances drug uptake in cancer cells without affecting DOX efficiency, suggesting that it can be a route for reducing DOX cardiotoxicity (Evig et al., 2004; González-Fernández et al., 2017; Noseda et al., 1988; Principe et al., 1992). Moreover, it has been suggested that the modulation of plasma membrane composition by methyl-PAF localization in lipid rafts of the membrane could increase the uptake of DNA intercalators such as DOX by neoplastic cells (Chen et al., 2004).

A series of novel PEGylated long-circulating liposomes containing monoalkyl ether phospholipids that induce thermoresponsive properties at low concentrations (5%) have been recently reported by our group in a comparative experimental and computational study (Eleftheriou et al., 2016). It has been shown that they are stable at normal body temperature, but exhibit superior release properties of their load at 40–42 °C at low (5%) ether phospholipid incorporation. In this study, PAF and a number of its analogues including methyl-PAF (Scheme 1) were employed and tested as thermoresponsive liposomal drug delivery systems aiming at slow and controlled drug release upon heating near $T_m$ and concurrently at improved therapeutic efficiency due to the combination of two complementary anticancer drugs, one of which has also the property to target malignant, but not normal, cells. These liposomes were efficiently (> 90%) loaded with doxorubicin and their physicochemical properties, stability, and drug release were
investigated. Following formulation optimization, in vitro cell studies of the most advantageous methyl-PAF containing formulations were performed following various heating schemes to confirm increased uptake of drugs at elevated temperatures, and to evaluate the effectiveness of doxorubicin-loaded thermosensitive liposomes employing the DOX-resistant DU145 and PC3 human prostate adenocarcinoma cancer cell lines.

2. Materials and methods

2.1. Materials

The phospholipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG) were obtained from Lipoid GmbH (Ludwigshafen, Germany) and Avanti Polar Lipids (Alabaster, Alabama, USA), respectively. The 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PC) was obtained from NOF Co. (Tokyo, Japan), while 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphocholine (PAF), 1-O-octadecyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PAF), and 1-O-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine (methyl-PAF, eldefosine) were obtained from Sigma Chemicals Co. (St. Louis, MA, USA). Sephadex G-50 (medium) and thiazolyl blue tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MA, USA). Doxorubicin hydrochloride (DOX) was kindly donated by Regulon SA (Athens, Greece). Nucleopore filters of 100 nm pore size (Whatman) were employed for liposome extrusion. Phosphate-buffered saline (PBS) was prepared according to the manufacturer’s instruction employing Gibco® PBS Tablets (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). DMEM, fetal bovine serum (FBS), penicillin/streptomycin, and L-glutamine were purchased from Invitrogen Ltd. (Paisley, UK).

2.2. Preparation of thermosensitive liposomes

For the preparation of thermosensitive liposomes the protocol employed by Mills and Needham has been followed (Mills and Needham, 2004). Small unilamellar liposomes were prepared by the extrusion method (Olson et al., 1979) employing a laboratory extruder (LiposFast-Pneumatic, Avestin Inc.) (MacDonald et al., 1991). In a typical experiment for preparing a 1 mL dispersion of liposomes, 20.0 mg of DPPC, 3.4 mg of DSPE-PEG (molar ratio of DPPC:DSPE-PEG = 91:4), and the corresponding amounts of monoalkyl phospholipids for the preparation of thermoresponsive liposomes at a molar ratio of 91:4:5, were dissolved in a chloroform/methanol solution (2:1 (v/v)). The solvents were evaporated in a rotavapor (30–32 °C) for the formation of lipid films. Each film was further extensively dried under vacuum overnight and subsequently hydrated with 1 mL of a solution composed of PBS containing 1 μM of the appropriate monoalkyl phospholipid at 50 °C. The obtained suspension was extruded through two stacked polycarbonate filters of 100 nm pore size. Twenty-five cycles were applied at 50 °C. DOX encapsulation was realized by the pH gradient active-loading method (Eleftheriou et al., 2016; Mayer et al., 1986; Madden et al., 1990). In short, the above mentioned DPPC:DSPE-PEG:monoalkyl phospholipids dry lipid films prepared as above, were hydrated at 50 °C with 1 mL of isotonic (157 mM) citric acid buffer, pH 4.0, and extruded through 100 nm polycarbonate filters as above. The concentration of the citric acid in the buffer was chosen as to have the same total osmolality, as determined using the OSMOMAT® 030 osmometer (Gnotec, Berlin, Germany), with that of PBS (300 mOsm/L). Following extrusion and cooling at room temperature, the external aqueous solution of the liposomal dispersions was exchanged by passing the liposomes through a Sephadex G-50 size exclusion minicolumn preconditioned with PBS, employing the same PBS buffer as eluent. Following this step, drug loading was realized by the addition of 1.2 mg DOX per 1 mL of each of the above liposomal dispersions and kept at 37 °C for 30 min under gentle stirring. Finally, the liposomal dispersions are cooled to room temperature and then passed through a Sephadex G-50 medium minicolumn (conditioned in PBS, pH 7.4) to remove non-encapsulated DOX. Non-loaded liposomes were also prepared and used as controls for the characterization and in vitro experiments. For their preparation the same as above procedure was followed, albeit without the DOX-loading step.

The encapsulated DOX concentration in all liposomal formulations was monitored by employing a Cary Eclipse fluorescence
spectroscopy (Mulgrave, Victoria, Australia) and registering the fluorescence intensity at 592 nm (excitation wavelength = 492 nm) of a known amount of liposomal dispersion (typically 5 μL) in carefully deaerated 2.7 mL of PBS after the addition of 100 μL of triton X-100 (2% w/w) solution for the complete release of encapsulated DOX. Subsequently, DOX concentration was determined employing a separately constructed calibration curve of DOX (0.1–5 μM) in PBS. In all liposomal formulations of the various monoalkyl phosphatidylcholines, active loading resulted in DOX concentrations of 1.95 ± 0.15 mM (1.13 ± 0.09 mg/mL) corresponding to >90% DOX encapsulation efficiency. The total lipid weight in the liposomal dispersions, after all the purification steps, was 22.5 ± 0.5 mg/mL and the drug/total lipid ratio ca. 5% (wt/wt). All liposomal samples were sterile filtered (0.22 μm, Millipore, Danvers, MA), and either used immediately or stored at 4 °C and used within the next day. Within this short time period no significant leakage of doxorubicin from the liposomes was detected either by fluorescence spectroscopy (see below) or by gel filtration.

Especially in the case of methyl-PAF containing liposomes that were used for the in vitro cell experiments, the final concentration percentage (%) of methyl-PAF relative to the total lipids in the final liposomal dispersions was determined using NMR spectroscopy by registering the 1H NMR spectrum of the lyophilized DPPC:methyl-PAF (5%) liposomal dispersion (Supplementary Fig. S1). Specifically, by comparing the ratio of the peak located at 3.45 ppm, attributed to the methoxy group of methyl-PAF, to the peak at 5.20 ppm, attributed to the methine group of DPPC, or to the peak at 2.30 ppm, attributed to the methylene groups relative to the ester bonds of DDPC, it was found that 3.6 ± 0.3% methyl-PAF was incorporated into the final liposomal formulations. The calculation was verified by performing the same peak analysis in the NMR spectra of a series of DPPC:methyl-PAF mixtures with 3, 4 and 5% methyl-PAF content. The result denotes that ca. 70% of the initially incorporated monoalkyl ether lipid is present in the final liposomal bilayer (i.e. after the size exclusion purification step), which is taken into account especially for the in vitro experiments.

2.3 Characterization techniques

The obtained liposomes were investigated by dynamic light scattering, Z-potential experiments, and differential scanning calorimetry, while the permeability of the bilayer at various temperatures was assessed by monitoring the release of DOX (see section 2.4.). The size and polydispersity of liposomal dispersions were determined at 22 °C by dynamic light scattering employing an AXIOM-150/EX (Triton, Hellas) apparatus with a 30 mW laser source and an Avalanche photodiode detector at an angle of 90°. For these experiments 100 μL of liposomes were diluted with 0.6 mL of PBS buffer. Ten scattering measurements were acquired for each dispersion and the results were averaged. Autocorrelation functions were collected for 20 s and analyzed using the CONTIN algorithm to obtain the apparent hydrodynamic radii distribution.

The Z-potential values were obtained at 22 °C using ZetaPlus of Brookhaven Instruments Corp. (Long Island, USA) equipped with a 35 mW solid-state laser emitting at 660 nm. From the obtained electrophoretic mobility, the Z-potential of the liposomal dispersions was calculated using the Smoluchowski’s equation. In a typical experiment, 50 μL of a liposomal dispersion was diluted with 1.5 mL of PBS and introduced into the instrument cell. Ten measurements were collected for each dispersion and the results were averaged. The standard deviation of the measurements was less than 1%.

The main lipid phase transition has been assessed by DSC for both the PEGylated DPPC:DSPE-PEG liposomes incorporating the different monoalkyl chain lipids as well as for the DOX loaded corresponding liposomal formulations. DSC measurements were performed by employing a MDSC 2920 calorimeter (TA Instruments, USA) under nitrogen flow (20 mL/min), using a heating rate of 2 °C/min, a temperature modulation amplitude of 0.31 °C every 60 s, and an empty pan as a reference. Two heating/cooling scans were carried out from 15 °C to 60 °C and the transition temperature, Tm, as well as the temperature width at half maximum of the DSC peak, ΔTm, were determined using the Origin® software (Microcal Inc., Northampton, MA). In all cases the error of at least 3 different batches was ±0.2 °C. Heat and temperature calibrations were performed by using indium as a standard. For each experiment 100 μL of liposomal dispersions were ultracentrifuged (90,000 rpm, 45 min, 10 °C) in an Optima™ Max Ultracentrifuge Beckman Coulter, Inc. (Fullerton, CA, USA) coupled with the MLA-130 rotor. The resulting wet pellet was diluted with 10 μL of the same external buffer and subsequently transferred and sealed into aluminum pans (Gaber et al., 1995). For comparison purposes, and in order to be in line with the in vitro cell experiments, differential scanning calorimetry runs have been also carried out with liposomes employing DMEM + 10% FBS as an external buffer.

2.4 Temperature and time-dependent release of encapsulated DOX

The property of DOX as a self-quenching compound at high concentrations as those obtained in the liposomal interior was utilized to detect and determine DOX concentration in the aqueous phase following its release from the liposomes. Thus, DOX release was monitored by continuously registering the fluorescence intensity of dispersions using a Cary Eclipse fluorescence spectrophotometer coupled with a Single Cell Peltier accessory (type SPVF 1x0) able to continuously stir and stabilize the temperature in the cell with 0.1 °C precision, both from Varian Inc. (Mulgrave, Victoria, Australia). It was possible to measure a single sample over the whole time period as the implemented xenon flash lamp is only active when a data point is acquired. Hence, constant excitation of DOX was avoided, and samples showed no photobleaching for a period of at least 1 h. Typically, 5 μL of DOX-loaded liposomes were diluted in 2700 μL of pre-heated and thoroughly deaerated PBS, or in other experiments with DMEM + 10% FBS, which was already placed and thermally equilibrated in the fluorescence cell at the pre-determined temperature. Upon addition of the liposomal dispersions in the preheated buffer under continuous stirring, the registering of the fluorescence intensity was immediately initiated. It was thus possible for the liposomes to reach the desired temperature within seconds and also to monitor continuously the content release over time from the first 10 s after the addition by following the fluorescence intensity over time, Io of released DOX (excitation wavelength = 492 nm, emission wavelength = 592 nm). Release measurements at 37, 38, 39, 40, 41, 42 and 43 °C were performed for 30 min. DOX release was calculated as release (%) = (Io − L)/Io) × 100. After 30 min, 150 μL of 10% Triton X-100 was added to the samples in order to drive all DOX in the aqueous media and obtain Io, at each specific temperature. The quantity of DOX-loaded liposomes (5 μL) added in the cuvette was selected so that the Io values are within the linear regime of the DOX fluorescence intensity vs. concentration curve. This was ensured by performing a separate experiment where DOX fluorescence intensity was registered for various DOX concentrations and determining the concentration range were the intensity vs concentration was linear (Mills and Needham, 2004). Io values of each sample were determined at 25 °C in separate experiments conducted as above. In addition, liposomal stability at room temperature was studied by incubating the liposomal dispersions at 25 °C for 24 h, registering their fluorescence intensity and calculating the DOX release as described above.

2.5 Cell culture

Cells used in this study were the human prostate carcinoma cell lines DU145 and PC3 (ATCC, Manassas, VA, USA). The cells were grown in DMEM with phenol red supplemented with 10% FBS, 1% penicillin/streptomycin and 1% L-Glutamine at 37 °C in a 5% CO₂...
2.6. Fluorescence and confocal laser scanning microscopy studies

DU145 and PC3 cells (10 × 10³) were inoculated on 22 mm glass cover slips housed in 35 mm petri dishes, for the fluorescence microscopy studies, or in Nunc™ glass base dishes (ThermoFisher Scientific, Rochester, NY, USA) for confocal laser scanning microscopy (CLSM) studies, and left to grow overnight in 2 mL of DMEM containing 10% FBS under the same conditions as elaborated above in the cell culture section. Cells were subsequently incubated with DOX, or DOX-loaded methyl-PAF liposomes for 2 h in DMEM.

Epifluorescence microscopy studies in cells were performed by washing the cover slips with PBS, inverting them onto microscope slides and placing them under the Olympus UPLFLN40X objective (NA 0.75) of a Nikon Eclipse microscope that is coupled with an Olympus DP71 digital color camera, utilized to obtain both brightfield and epifluorescence microscopy images. Fluorescence excitation was realized with a mercury USH 102D lamp (Ushio Inc., Tokyo, Japan) while fluorescence emission of DOX was imaged using the rhodamine isothiocyanate (RITC) filter (Chroma Technology Corp).

CLSM studies were performed on a multiphoton confocal microscope Leica TCS SP8 MP (Wetzlar, Germany) equipped with an Argon laser (excitation lines at 458, 476, 488, 496, and 514 nm), and an IR MaiTai DeepSee Ti:Sapphire laser (Spectra-Physics, Santa Clara, CA, USA) for multiphoton applications. Images were acquired with the spectral detector of the microscope using appropriate emission wavelength ranges: DOX was excited at 496 nm with the Argon laser and emission was recorded between 505 and 600 nm. Images were acquired with the LAS X software (Leica Microsystems CMS GmbH) and are presented without any post-processing.

2.7. Flow cytometry

The in vitro cellular uptake of free DOX and liposomal encapsulated DOX was determined by fluorescence-activated cell analysis. DU145 or PC3 human prostate cancer cells were grown overnight at a density of 25 × 10⁴ cells per well in 6-well plates. Subsequently, cells were exposed to free DOX, or DOX-loaded methyl-PAF liposomes at either 37 °C or 40 °C. The cells were then washed twice with phosphate buffered saline, trypsinized, serum deactivated, and the resulting cell suspensions were centrifuged for 5 min at 1000 rpm. Finally, the collected cells were resuspended in PBS and the uptake of DOX was analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

2.8. MTT cell viability assay

DU145 or PC3 human prostate cancer cells were inoculated (10 × 10³) into 96-well plates and left to incubate in 100 μL DMEM containing 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine for 24 h at 37 °C in a 5% CO₂ humidified atmosphere.

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2.9. Statistical analysis

All experiments were repeated independently at least three times. MTT data are shown as means of six independent values with error bars representing one standard deviation. Student’s two-tailed t-test was performed on the MTT cytotoxicity data obtained to determine the statistical significance of a difference between means. The statistical significance follows the assignment: * p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001.

3. Results and discussion

The commonly used lysophosphatidylcholine (lyso-PC) taken as the standard thermoresponsive liposomal formulation, and a number of monoalkyl chain ether-linked PAF analogues (Scheme I), were used for the preparation of PEGylated DPPC-based thermoresponsive liposomes of 100 nm diameter at a 5% monoalkyl lipid:DPPC molar ratio. Empty and DOX loaded liposomes were physicochemically characterized with respect to their size distribution, ζ-potential, and phase transition characteristics. This series of liposomal formulations were compared with regard to stability (37 °C) and release properties at elevated temperatures (39–43 °C) both in phosphate buffer, as well as in cell medium in the presence of FBS. The biological activity of methyl-PAF containing liposomes was evaluated at the cellular level employing the DOX resistant human prostate adenocarcinoma DU145 and PC3 cell lines (David-Beabes et al., 2000; Mahon et al. 2011), either at 37 °C or 40 °C. The cellular uptake of DOX was followed by fluorescence-activated cell sorting flow cytometry (FACS) and visualized by fluorescence and confocal laser scanning microscopy (CLSM), while inhibition of DU145 and PC3 cell proliferation was assessed by the MTT assay.

3.1. Physicochemical characteristics of liposomes

The presence of monoalkyl ether phosphatidylcholine lipids in the liposomal bilayer at the employed concentration (5 mol%) has a small but distinct effect on the size of their liposomal systems compared to parent DPPC:DSPE-PEG non-thermoresponsive liposomes or to the standard DPPC:DPPE-PEG:lyso-PC thermoresponsive liposomes. Their mean hydrodynamic radii were found to be 55 ± 5 nm, clearly larger compared to 45 ± 5 nm of the standard liposomes, Fig. 1A. This difference is consistent with enhanced plasticity of the bilayer in the presence of alkyl ether phospholipids, compared to simple or lyso-PC containing liposomes, given that all other variables during their preparation steps were kept the same. DOX-loaded liposomal formulations have a similar trend in their mean size, although not so noticeable as in the case of empty formulations (Fig. 1B). The size of these formulations lies well within 60 ± 5 nm, while the size distributions are comparable. This size increase is clearly due to the presence of DOX and can be the result of either the formation of DOX/citrate crystals in the form of gel inside the liposomal internal aqueous phase as is well-established in the literature (Li et al., 1998), or could be the result of the presence of DOX molecules near or at the bilayer as also reported in the literature (Gaber et al., 1998).

The electric potential of all formulations, as derived from ζ-potential measurements, is, within experimental error, equal in all cases. This is anticipated since all different lipids employed have the same polar
moieties. The negative values obtained (-3.5 ± 1.5 mV) are in line with values reported in the literature (Silvander et al., 2000), and are due to the presence of the negatively charged DSPE-PEG at 5 mol% concentration. The relatively low value is reflecting the reduced electrostatic mobility of the nanoparticles due to the presence of the long PEG chains that increase the hydrodynamic drag and move the location of the slipping plane further away from the particle surface (Mcdaniel et al., 1984; Silvander et al. 2000; Woodle et al., 1992). Given that the electrostatic membrane potential is of importance for the stability of the liposomal systems and also affects the interaction with charged species in the biological milieu, it is advantageous to have the same \( \zeta \)-potential values in studies that compare the release and drug delivery properties of different formulations.

The thermal properties of the liposomal formulations can provide information on the quality of these systems and, essentially, on the homogeneity of the bilayer (Heimb erg, 2007; Taylor and Morri, 1995), which is of importance given the incorporation of a variety of monoalkyl lipids in the membrane. Differential scanning calorimetry measurements were performed at the temperature range from 15 to 55 °C for the basic DPPC:DSPE-PEG liposomes, taken as control, and for liposomes incorporating monoalkyl lipids at 5 mol%. The obtained thermodynamic parameters are summarized in Table 1, while the respective DSC profiles are shown in the Supporting Information (Supplementary Fig. S2 and S3). The transition temperature, \( T_m \) of the main gel to liquid crystalline transition (\( P_{II} \rightarrow L_0 \)) of the basic DPPC:DSPE-PEG formulation is registered at 41.9 ± 0.2 °C in full agreement with literature (Koynova and Caffrey, 1998; Mouritsen, 1991). In all formulations only one single peak was registered, an indication that at the concentrations employed the systems are uniform and that the presence of the added lipids does not result in phase separation or inhomogeneities in the bilayer (Mouritsen and Jørgensen, 1994). From the data presented in Table 1, it is apparent that incorporation of lyso-PC, the standard lysolipid employed in the most well-known thermoresponsive liposomes, bears no significant change in \( T_m \) compared to parent DPPC:DSPE-PEG liposomes. In addition, the width of the DSC trace, expressed as the temperature width at half-maximum of the main peak (\( \Delta T_{1/2} \)) is also the same within experimental error, denoting that the cooperativity values of the transitions (\( \alpha \) 1/ \( \Delta T_{1/2} \)) are practically unaltered for both systems. On the other hand, the presence of ether-type lysolipids results in a small but clearly noticeable lowering of \( T_m \) values but no significant change in the molecular cooperativity of the transition, i.e. of the DSC peak width. Both parameters taken into account point to a small disturbance of the bilayer order without, however, any apparent effect on the areas that local order extends. Based on the small effect of these ether-type lysolipids on the thermodynamic parameters of the bilayers, the fact that these parameters are practically unaltered during the second heating run, and given their closely related chemical structure with DPPC, it is reasonable to conclude that these lipids fully dissolve in the bilayer forming homogeneous and thermally stable solutions with DPPC (Heimburg, 2007).

On the other hand, DOX-loaded liposomes containing monoalkyl lipids during the first heating run exhibit higher \( T_m \) values (by 0.4–0.5 °C) compared to the non-drug-loaded counterparts (Table 2), while the \( \Delta T_{1/2} \) values remain the same within experimental error. After the first heating/cooling run, during the second heating run, their \( T_m \) values are again registered at lower temperatures that are practically equal to those of the non-loaded formulations. Evidently, after the first heating run the encapsulated DOX is completely released from the liposomal interior to the external medium as will be shown in the next section and the bilayer resumes the same physical state as in the non-

### Table 1

DSC parameters (\( T_m \) and \( \Delta T_{1/2} \)) of the main lipid phase transition of DPPC:DSPE-PEG based thermoresponsive unilamellar liposomes in PBS or in culture medium (DMEM + 10% FBS), obtained during the first and second heating run.

<table>
<thead>
<tr>
<th>Formulation (% molar ratio of monoalkyl lipid)</th>
<th>( T_m ) (°C) First/heating run</th>
<th>( \Delta T_{1/2} ) (°C) First/heating run</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC:DSPE-PEG</td>
<td>41.9/41.9</td>
<td>2.4/2.4</td>
</tr>
<tr>
<td>in culture medium</td>
<td>41.6/41.5</td>
<td>2.5/2.5</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:lyso-PC (5%)</td>
<td>41.5/41.7</td>
<td>2.5/2.6</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:PAF (5%)</td>
<td>41.2/41.4</td>
<td>2.5/2.9</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:lyso-PAF (5%)</td>
<td>41.4/41.6</td>
<td>2.4/2.3</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:methyl-PAF (5%)</td>
<td>41.2/41.3</td>
<td>2.4/2.9</td>
</tr>
<tr>
<td>in culture medium</td>
<td>41.2/41.2</td>
<td>2.6/2.7</td>
</tr>
</tbody>
</table>

## Table 2

DSC parameters (\( T_m \) and \( \Delta T_{1/2} \)) of the main lipid phase transition of DOX-loaded DPPC:DSPE-PEG based thermoresponsive unilamellar liposomes in PBS or in culture medium (DMEM + 10% FBS), obtained during the first and second heating run.

<table>
<thead>
<tr>
<th>DOX-loaded liposomes Formulation (% molar ratio of monoalkyl lipid)</th>
<th>( T_m ) (°C) First/heating run</th>
<th>( \Delta T_{1/2} ) (°C) First/heating run</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC:DSPE-PEG</td>
<td>41.8/41.9</td>
<td>2.5/2.5</td>
</tr>
<tr>
<td>in culture medium</td>
<td>41.8/41.8</td>
<td>2.7/2.5</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:lyso-PC (5%)</td>
<td>41.7/41.4</td>
<td>2.3/2.4</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:PAF (5%)</td>
<td>41.7/41.5</td>
<td>2.4/2.4</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:lyso-PAF (5%)</td>
<td>41.7/41.4</td>
<td>2.4/2.4</td>
</tr>
<tr>
<td>DPPC:DSPE-PEG:methyl-PAF (5%)</td>
<td>41.6/41.4</td>
<td>2.4/2.4</td>
</tr>
<tr>
<td>in culture medium</td>
<td>41.3/41.1</td>
<td>2.6/2.6</td>
</tr>
</tbody>
</table>
DOX loaded formulations. In contrast, both parameters \((T_m, \Delta T_m)\) in DOX-loaded DPPC:DSPE-PEG liposomes are not affected in both 1st and 2nd heating/cooling runs being the same with the non-loaded system. Therefore, the differences observed for DOX-loaded thermoresponsive liposomes during the first run can be safely attributed to the presence of encapsulated DOX. Given that DOX is a weak base, a small part of DOX molecules are expected to be in the neutral (nonprotonated) state. It can be presumed that these neutral moieties are most probably interdigitated in the bilayer but only when the latter is fluid due to the presence of monoalkyl lipids. This is explaining the differences observed in \(T_m\) for the drug loaded systems and is consistent with the fact that the cooperativity values of drug-loaded thermoresponsive liposomes are equal with those on the non-loaded ones as well as with the parent non-thermoresponsive liposomes.

Finally, DSC experiments were also performed in cell culture medium \((\text{DMEM} + 10\% \text{ FBS})\) for the systems that were chosen to be employed in \textit{in vitro} studies. As shown in Tables 1 and 2, the presence of culture medium in the exterior of empty or DOX-loaded liposomes results to a decrease of ca. 0.3 °C for \(T_m\) and of ca. 0.2 °C for \(\Delta T_m\). Although these values lie within the experimental error, the observed deviation is always towards lower values for \(T_m\) and higher values for \(\Delta T_m\) demonstrating that the known interaction of culture media \((\text{DMEM} + \text{FBS})\) with liposomes (see Section 3.2), has also a small but detectable effect on liposomal main phase transition. Moreover, the DSC traces prove that this interaction does not lead to depletion of lysolipids from the bilayer, since this would result in a system that would be similar to the non-thermoresponsive parent liposomes and, therefore, would have increased \(T_m\) and loss of the temperature-triggered release property, which is not observed in this study (see the following section).

3.2. Time- and temperature-dependent drug release

The release of encapsulated DOX from all liposomal formulations was studied both at room temperature as well as at elevated temperatures. At room temperature DOX release after a 24-h period is lower than 1% for all formulations (Supplementary, Table S1). At elevated temperatures the time-dependent release of encapsulated DOX was continuously monitored for a period of 30 min. This period of time was chosen given that within this timeframe DOX release almost levels off for all systems studied, and also because this is relevant to the duration of heat as applied in medical practice (Dou et al., 2017). All tested thermoresponsive systems are satisfactory stable at 37 °C, releasing less than 10% DOX after the first 30 min while their release profiles also reach plateau values within this period (Supplementary Fig. S4). As expected, however, the parent non-thermoresponsive liposomes are definitely less leaky, due to the absence of lipids that could induce membrane fluidity.

Upon temperature increase, DOX release progressively escalates in all thermoresponsive preparations. Comparison of the time-dependent DOX release profiles of liposomal systems containing monoalkyl ether lipids to that containing the monoalkyl ester counterpart lyso-PC (Fig. 2), all at 5 mol% incorporation, clearly denotes that all ether lipid formulations behave similarly to each other, but exhibit slower release rates that the standard thermosensitive formulation with lyso-PC. The maximum attained release at 40 °C is relatively low in all cases (typically < 30%, Supplementary Fig. S5) while, as shown in Fig. 2, at 41 °C the release has not yet achieved its highest value, which is observed at 42 °C. This is in line with the DSC results, which indicate that the \(T_m\) for all DOX-loaded thermoresponsive formulations is 41.7–41.8 °C. It is also clear that the well-studied standard thermoresponsive DPPC:DSPE-PEG:lyso-PC formulation exhibits a fast release which, especially at 42 °C, leads to almost complete release within 1–2 min, while all monoalkyl ether containing formulations exhibit a much slower release rate.

Their release profiles provide evidence for a two-phase process, an initial burst release followed by a secondary slower one. Similar release profiles of DOX can be also found in the literature (Needham and Dewhirst 2001; Tagami et al., 2011b), being noticeable only in cases where the systems do not release their entire payload within a very short timeframe. It should be noted that the observed two-step behavior is exclusively registered in the case of active DOX-loaded liposomes. For comparison, the release of carboxyfluorescein (CF) from the same liposomal formulations is distinctly different. As evident from the data presented in Supplementary Fig. S6, the release profiles of CF at 41 and 42 °C do not show this complex behavior, exhibiting an abrupt fast release within the first ~1 min and first order kinetics thereafter. The initial burst phase which results in the release of ~20–30% of total DOX can be tentatively attributed to those DOX molecules that are residing within or at the vicinity of the membrane. As discussed in the previous section, the thermal analysis of the systems indicated that a number of DOX molecules are located in the bilayer (unlike CF, which for this reason is not following the same release pattern). This rationalization is corroborated by the fact that the non-thermoresponsive liposomes also release within the same period approx. the same amount of DOX (~20%) while thereafter no additional release is registered. The second phase is controlled by the enhanced permeabilization of the bilayer due to the presence of the monoalkyl lipids and is responsible for the gradual release of the drug in the outer phase. It is evident that almost complete release of DOX from the formulations with the PAF derivatives can be achieved after c.a. 15 min if the temperature is close to the \(T_m\) (42 °C), while at 1 °C lower the rate is decreased and approximately 1 h is needed for complete release.

The temperature-dependent DOX release diagrams (Fig. 3) indicate that up to 39 °C all formulations are sufficiently stable releasing less than 15% after 30 min. At 40 °C release is somewhat increased, up to approx. 20–30%. At 41 °C lyso-PC imparts higher release than the ether lipids, but at 42 °C this difference is abolished, suggesting that this is mainly due to the difference in the release rates and not to the values at equilibrium. After 30 min at 41 °C, the formulations containing the PAF derivatives release 70–80% of encapsulated DOX compared to 95% for the lyso-PC formulation. In addition, as more clearly shown in Fig. 3, they attain the same DOX release levels as the lyso-PC formulation after ca. 15 min at 42 °C. The various PAF derivatives do show a small but substantial variation in their release profiles, although, the final DOX released at temperature near \(T_m\) is almost 100% for all formulations. Methyl-PAF exhibits, at all temperatures tested, the lowest rate suggesting a different packing of this derivative that bears a methyl group at the 2-position. Overall, formulations containing methyl-PAF do show that are the most stable at low temperatures, while at higher temperatures release DOX in a more controlled quasi-linear fashion.

Based on the above results and on the additional fact that methyl-PAF is exploited as an anti-cancer drug per se or in combination with DOX, DOX loaded thermoresponsive liposomal formulations containing this ether lipid were chosen to be further studied employing \textit{in vitro} cell experiments. However, it is well established that the presence of serum or other components significantly affect the release profiles of drug-loaded liposomes (Chen et al., 2014; Gaber et al., 1995; Mittag et al., 2017). This is attributed to the presence of serum proteins such as albumin that is known to bind strongly to lysophospholipids (Kotting et al., 1992; Lindner et al. 2008; Mittag et al., 2017). In this respect it is important to note that this binding is highest at temperatures close to \(T_m\) (Hosokawa et al., 2003). The permeation enhancement in the presence of serum has been also attributed to the fact that at these temperatures the penetration of proteins into the membrane bilayer is facilitated (Hossann et al., 2012; Mittag et al., 2017). Alternatively, this observation is attributed to the interaction of rigid (i.e. below \(T_m\)) lipid bilayers with components of the complement system (Gaber et al., 1995) that are also present in serum. The latter is part of the immune system and, among others, has the ability to attack the cell membrane of pathogens.

Given that the culture medium used (DMEM + 10\% FBS) is known to alter significantly the release properties of liposome-encapsulated
drugs, further time- and temperature-dependent drug release experiments were initiated for monitoring DOX release in the culture medium instead of PBS. The obtained release profiles of DOX-loaded methyl-PAF containing liposomes proved quite different than above in many respects. The release profiles do not show an initial small burst release and a second distinct release rate as in PBS, but instead show a rather fast release reaching a maximum within the first 10–15 min as evidenced in the time-dependent release profiles (Fig. 4). The maximum release (close to 90%) is observed at 41 °C, in agreement with the DSC results that revealed the Tm of liposomes in culture media to be located at 41.2–41.3 °C. More importantly, however, is the considerable release of DOX at 37 °C (~40%) and the presence of an inflection point at the temperature-dependent release profiles at 39 °C (Fig. 4).

To further elaborate these findings, the same experiment was repeated employing drug-loaded non-thermoresponsive DPPC:DSPE-PEG and the well-studied thermoresponsive DPPC:DSPE-PEG:lyso-PC system. As shown in Fig. 5 and in Supporting Information (Supplementary Fig. S7), the non-thermoresponsive liposomal formulation DPPC:DSPE-PEG, even at physiological temperatures, does show increased DOX release, higher than that of the thermoresponsive analogues. The maximum release is observed at 39 °C which coincides with the onset temperature of the main lipid phase transition. Further temperature increase reduces the observed DOX release. Such behavior where maximum release in human or mouse plasma was registered at temperatures below Tm and close to the onset temperature was also reported in the literature (Chen et al., 2014). The lyso-PC based thermoresponsive formulation behaves as the methyl-PAF formulation, also exhibiting an inflection point at this specific temperature. At higher temperatures, above 39 °C, the release from both thermoresponsive formulations is further increased as evidenced in the temperature-dependent DOX release profiles (Fig. 5), reaching peak levels at the temperature corresponding to the maximum of their DSC trace. Most of the release is taking place within the first 15 min, since release after 30 min is only slightly increased (Supplementary Fig. S7). Formulations containing methyl-PAF seem more efficient in DOX release although the difference is small. It thus becomes apparent that in vitro experiments even the simple DPPC-PEG liposomes are thermoresponsive releasing almost 70% of the encapsulated DOX at 39 °C in the culture medium. However, at 41 °C their release is definitely lower, ca. 40%, and this must be taken into account in the rationalization of the following in vitro experiments. It should be noted that this behavior is only observed for systems that active DOX loading was performed, and is coupled to the presence of FBS, since DOX release in PBS + 10% FBS shows a similar behavior. Even DMEM without FBS has an intermediate, but not so significant, effect on DOX permeability (data not shown) as is also reported in the literature (de Smet et al., 2010; Tejera-Garcia et al., 2014).

Our results denote the emergence of membrane heterogeneity during the gel to liquid crystalline main phase transition that is particularly obvious in the presence of FBS. Indeed, enhanced permeability...
is nowadays explained by the widely accepted “bilayer lipid heterogeneity” model (Mills and Needham, 2005) that suggests the existence of solid “crystalline” phases and leaky interfacial regions in the grain boundaries (boundaries of lipid rafts). At lower temperatures the observed release is an inherent property of the DPPC lipid bilayers (after their interaction with components of FBS as noted above) since the release follows different kinetics and equilibrium values. At higher temperatures, the release is primarily due to the presence of monoalkyl lipids that lead to enhanced bilayer permeability. In our case, both release patterns are operating at high temperatures (40–41 °C) and this results in increased release rates compared to release rates in the absence of FBS. However, in addition to the above, we cannot exclude the effect of citrates in the overall behavior of the system. Upon heating near T_m, the enhancement of bilayer permeability will allow citrate ions to also cross the membrane and interact with components of the external medium such as inorganic ions. In this connection it has been shown that chelated Mg^{2+} enhances membrane permeability leading to substantial leakage of even small RNA molecules at room temperatures, and that the most effective chelator of Mg^{2+} was citrate (Adamala and Szostak, 2013; O’Flaherty et al., 2018). In this respect it is of interest to point out the work of Papahadjopoulos (Gaber et al., 1995) who first reported that in the presence of EDTA (which binds Mg^{2+} ions) the release of DOX was inhibited. Similar complexes can be formed not only with the Mg^{2+} ions that are present in the cell culture medium but with other similar ionic moieties leading to the more complex behavior observed. This observation can also explain the increased permeability registered when DMEM without FBS is present in the medium, and also can contribute to the overall permeability enhancement in the presence of DMEM + FBS.

3.3. In vitro Studies: Intracellular uptake

Liposomal DOX uptake in PC3 and DU145 cells was followed employing epifluorescence microscopy at either 37 or 40 °C. Cells were incubated with DOX-loaded DPPC:DSPE-PEG-methyl-PAF liposomes at 37 °C for 1, 2 and 4 h, and at 40 °C always for 1 h since this is the usually employed duration of regional hyperthermia in patients (Lindner et al., 2008). After the addition of liposomal DOX incubation at 40 °C was taking place either immediately, or cells were first allowed at 37 °C for 1 or 2 h and then placed at 40 °C. In this way different heating schemes were tested, simulating different treatment protocols employed in clinical practice, i.e. administering the drug prior to or after heating the tumor site.

Cells incubated with DOX-loaded methyl-PAF liposomes at 37 °C show some DOX internalization (Supplementary Fig. S8) exhibiting maximum fluorescence intensity after 4 h. On the contrary, incubation...
at 40 °C for 1 h greatly enhances fluorescence intensity and clearly shows that DOX is located in cell nuclei membrane and its interior (Fig. 6 and Supplementary Fig. S9 and S10). It should be noted that incubation with free DOX at the same concentration is hardly detectable even after incubation for a 2 h period at 37 °C (Supplementary Fig. S11, upper row). Furthermore, the different treatment schemes employed show noteworthy variations in the intensity of DOX fluorescence signals (Fig. 6 and Supplementary Fig. S9). Specifically, cells monitored directly after incubation at 40 °C show maximum DOX intensity, while cells allowed at 37 °C for various time intervals (up to 3 h) after 1 h incubation at 40 °C show reduced fluorescence signals in the nuclei as well as DOX signals in the cytosol that could suggest that either DOX efflux mechanism is active and readily observed in live cells after 2 or 3 h at 37 °C or that this is the outcome of cell stress induced by the drug.

Based upon the epifluorescence experiments, confocal microscopy experiments were performed after incubating both cell lines with free DOX or DOX-loaded in DPPC:DSPE-PEG:methyl-PAF liposomes at 37 and 40 °C. It is evident that at 37 °C DOX fluorescence intensity is low for DOX and minimal when DOX-loaded methyl-PAF liposomes were administered (Supplementary Fig. S11 and Fig. S12). Incubation at 40 °C (Fig. 7 and Supplementary Fig. S13) revealed a clear and distinct fluorescence signal predominantly in cells’ nuclei. At this temperature, cells administered with free DOX exhibit increased fluorescence signal compared to liposomal DOX, as is also the case when incubation is performed at 37 °C. Further analysis indicates that DOX is located close to the nuclear envelope and in nucleoli for both cell lines, possibly in the chromatin structures (Ou et al., 2017) (Fig. 7, lower row). Comparatively, incubation at 37 °C also indicates that DOX is located mostly in the nucleus and in the cytoplasm as well, but definitely at a considerably smaller amount.

To quantify the uptake of DOX upon incubation of PC3 and DU145 cell lines with 5 μM DOX, either free or encapsulated in DPPC:DSPE-PEG:methyl-PAF liposomes, at 37 or 40 °C for 1 h, fluorescence activated cell analysis was performed based on the intrinsic fluorescence of DOX (Fig. 8). At 37 °C the fluorescence intensity of DOX internalized in cells is relatively low as also observed in CLSM experiments. It is, however, clear that liposomal DOX is minimally internalized as the registered fluorescence intensity is close to that of control, while free DOX shows a statistically significant internalization. Incubation at 40 °C greatly increases internalization for both free and liposomal DOX.
although free DOX is again more effectively internalized corroborating the findings with optical and confocal microscopy.

3.4. Cell viability studies

Dose-dependent cytotoxicity studies of free drugs, i.e. DOX and methyl-PAF, as well as of their liposomal formulations were performed on the human prostate adenocarcinoma DU145 and PC3 cell lines which are known to be doxorubicin resistant (David-Beabes et al., 2000; Fale et al., 2015; Mahon et al. 2011). The toxicity of DOX, either free or encapsulated in the thermoresponsive liposomes, was tested in the concentration range of 2.5–10 μM, along with the toxicity of the non-drug-loaded carrier (methyl-PAF containing DPPC:DSPE-PEG liposomes) at the same concentrations as those employed in liposomal DOX. The toxicities of free methyl-PAF and of free methyl-PAF co-administered with DOX were also evaluated. In this case methyl-PAF concentrations in the 2–8 μM concentration range were employed as these methyl-PAF concentrations correspond to its amounts that are present in the DOX-loaded DPPC:DSPE-PEG:methyl-PAF liposomes (c.f. experimental Section 2.3). After the addition of the formulations cells were always incubated for a total of 4 h either at 37 °C, or at 40 °C for 1 h, followed by incubation for another 3 h at 37 °C. In another approach, cells were incubated, at 37 °C for 1 h, then at 40 °C for 1 h and finally for 2 h at 37 °C, in order to explore whether incubation at 37 °C is initially needed for the efficient liposomal internalization prior to heating at temperatures that trigger drug release. Subsequently drug containing media were replaced with complete media, further incubated at 37 °C for 24 h, and finally cell viability was assessed by the MTT assay.

As shown in Fig. 9, free DOX at the experimentally relevant concentrations of up to 10 μM, has at 37 °C a medium effect on cell viability (~80%) while methyl-PAF at the corresponding concentrations has no cytotoxic effect at all. The observed low toxicity of DOX is anticipated due to the chemoresistant character of the selected cell lines. The insignificant effect of methyl-PAF on cell viability is also justified given the low concentration used (up to 8 μM) and after taking into account that the reported IC50 towards DU145 cells is reported to be 14 μM after 72 h incubation (Busto et al., 2008). However, the combination of both drugs enhances effectiveness, leading to an increased toxicity (70% cell viability, p < 0.05, Fig. 9) in line with the reported synergy of both medications (Ahmad et al., 1997; Andesen et al., 2004; Evig et al., 2004; Peters et al., 1997). Incubation at elevated temperature (40 °C) further increases toxicity both for DOX and DOX/methyl-PAF (~60% cell viability in both cases).

Liposomal formulations on the other hand indicate a real synergy when the two compounds are both present in the same liposomal system. As expected empty DPPC:DSPE-PEG:methyl-PAF liposomes have a non-statistically significant effect on PC3 (Fig. 9) or DU145 cells (Supplementary Fig. S14) even after incubation at 40 °C. In this respect this system has the same behaviour with empty DPPC:DSPE-PEG liposomes. DOX-loaded DPPC:DSPE-PEG liposomes induce minor cytotoxicity at 37 °C, which increases after incubation at 40 °C (cell viability 80%). It should be noted that this system (liposomal DOX) is less effective that free DOX at both temperatures. The result is in line with the known fact that liposomal DOX is substantially less cytotoxic than free DOX. This is due to limited bioavailability that is mainly attributed to the existence of DOX entrapped, i.e. non-bioavailable, so that the benefit of simple liposomal DOX formulations lies in the passive targeting that can be attained due to the EPR effect (Horowitz et al., 1992; Laginha et al., 2005; Tagami et al., 2011b). Furthermore it was shown that inducing the release of DOX from such stable formulations by the addition of an ionofore resulted in an increase of cytotoxicity equivalent to that of free DOX (Horowitz et al., 1992), indicating the significance of triggering the release for increasing the therapeutic outcome.

DOX-loaded DPPC:DSPE-PEG:methyl-PAF liposomes on the other hand are more effective that DOX-loaded liposomes both at 37 °C (cell viability 65%) and at 40 °C (cell viability 60%) (p < 0.01, Fig. 9). More importantly they are equally potent with the non-liposomal combination of DOX/methyl-PAF, although as mentioned above, it would be expected for liposomal formulas to be less effective in in vitro cell studies. In fact, as shown above in the intracellular uptake
section, less DOX is internalized in cells when liposomal formulations are employed. This is, therefore, a clear indication that the presence of methyl-PAF in the liposomal bilayer, which by itself alone does not infer any toxicity, increases liposomal DOX cytotoxicity at 40 but also at 37 °C. The latter can be attributed to the fact that, even at 37 °C and under the experimental conditions employed these liposomes are also releasing a significant amount of their payload (Supplementary Fig. S7).

It is also of interest to note that incubation initially at 37 °C for 1 h and then at 40 °C for another one hour (Fig. 9, lower row, for PC3 cells, Michael Zachariadis: Funding acquisition. curation, Formal analysis, Methodology, Validation, Supervision, Investigation, Visualization. Methodology, Validation. CRediT authorship contribution statement

4. Conclusions

Incorporation of monoalkyl ether phosphatidylcholine lipids, including PAF and its analogues, in the bilayer induces thermosensitivity on DPPC-based doxorubicin-loaded liposomes displaying a controlled release profile at temperatures near 40–42 °C. Among them, methyl-PAF (edelfosine) containing liposomes are found to be effectively internalized in prostate cancer cell lines and enhance the therapeutic efficiency of DOX due to the combination of both medications. Given its passive targeting and cell internalization properties this system can be proved promising as a drug carrier combined with mild local hyperthermia.

CRediT authorship contribution statement

Kleopatra Eleftheriou: Data curation, Formal analysis, Methodology, Validation. Archontia Kaminari: Data curation, Formal analysis, Methodology, Validation, Visualization, Funding acquisition. Katerina N. Panagiotaki: Data curation, Formal analysis, Methodology, Validation, Funding acquisition. Zili Sideratou: Data curation, Formal analysis, Methodology, Validation, Supervision, Funding acquisition. Michael Zachariadis: Formal analysis, Investigation, Visualization. Jane Anastassopoulos: Writing - review & editing. Dimitris Tsiorvas: Conceptualization, Formal analysis, Project administration, Supervision, Investigation, Validation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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References


