

Ariana

Abawi

+33 (0)605265690

Villeurbanne, France

ariana.abawi@gmail.com 🖂

WORK HISTORY

Oct. 2021 – Oct. 2024	PhD STUDENT (French Ministry of Higher Education, Research and Innovation							
Lyon, France	financial supports) French national center of scientific research (ICBMS) Thesis supervisors : Pr Agnès Girard-Egrot and Dr Ofelia Maniti Research Project : Vector tumor cell membrane interactions for optimized delivery of medi							
	COMPLEMENTARY TEACHING ACTIVITY Claude Bernard Lyon 1 University Practical work in laboratory and tutorial for 1 St , 2 nd and 3 rd year Bachelor of Science Students Teaching unit of personal and professional project for 2 nd year Bachelor of Science Students							
	INTERNSHIP CO-SUPERVISION <i>French national center of scientific re</i> 6 Students Setting up an internship topic	l esearch (ICBI Superv Correct	MS) ision of their handling in the labo tion of their oral presentation and	oratory d internship report				
Jan. 2021 – Jun. 2021 _{Lyon, France}	RESEARCH INTERNSHIP French national center of scientific re Supervisors : Dr Ofelia Maniti Research Project: Targeting of tumor	esearch (ICBN	<i>IS, GEMBAS Team)</i> nade liposomes	Sector Se				
Apr. 2019 – May. 2019 Lyon, France	RESEARCH INTERNSHIP French national center of scientific r Supervisors : Dr Julien Bompard and Research Project: Preparation and c	esearch (ICBI d Dr Ofelia Ma characterizatic	MS, GEMBAS Team) aniti on of liposomes for tumor cell tar	geting				
EDUCATION)							
2021 - 2024	PhD OF BIOCHEMISTRY, Gradu Claude Bernard University Lyon 1, Fr	ated with hor ance	nours					
2019 - 2021	MASTER OF BIOCHEMISTRY-M Claude Bernard University Lyon 1, Fra	OLECULAR ance	BIOLOGY, Rank: 4/30					
2016 - 2019	DIPLOMA OF HIGHER EDUCATI Claude Bernard University Lyon 1, Fr	ON IN LIFE	SCIENCES BIOCHEMISTRY	PATHWAY				
2014 - 2019	A LEVEL, Life Sciences Major, Gr Private High School Institution Robin -	aduated with - Saint-Vincer	n honours nt de Paul, Vienne, France					
SKILLS								
Laboratory Technique	es Software - Micro	osoft office	Languages - French (mother tongue)	1				

- Cell culture
- Cell viability assays

- Microsoft offi
 ImageJ
- English (C1)

- Liposome preparation and characterization
- DLS, Zeta potential, SEC
- Fluorescence microscopy
- Confocal microscopy
- 3D cell printing
- Bioink development

PUBLICATIONS

- <u>Abawi A</u>, Wang X, Bompard J, Bérot Anna, Andretto V, Gudimard L, Devillard C, Petiot E, Joseph B, Lollo G, Granjon T, Girard-Egrot A, Maniti O. Monomethyl Auristatin E Grafted-Liposomes to Target Prostate Tumor Cell Lines. Int J Mol Sci. 2021 Apr 15;22(8):4103. doi: 10.3390/ijms22084103. PMID: 33921088; PMCID: PMC8071391.
- <u>Abawi A</u>, Thomann C, Lollo G, Granjon T, Petiot E, Bérot A, Oger C, Bultel-Poncé V, Guy A, Galano J-M, Durand T, Girard-Egrot A, Maniti O. Carrier-Tumor Cell Membrane Interactions for Optimized Delivery of a Promising Drug, 4(RS)-4-F4t-Neuroprostane. Pharmaceutics 2023 Dec 15(12):2739. doi: 10.3390/pharmaceutics15122739; PMID: 38140081; PMCID: PMC10748318.
- <u>Abawi A</u>, Trunfio Sfarghiu AM, Thomann C, Petiot E, Lollo G, Granjon T, Girard-Egrot A, Maniti O. Tailor-made vincristine-liposomes for tumor targeting. Biochimie. 2024 Jul 31:S0300-9084(24)00176-7. doi: 10.1016/j.biochi.2024.07.017. Epub ahead of print. PMID: 39094823.



Ariana **Abawi** +33 (0)605265690

Villeurbanne, France 💡

ariana.abawi@gmail.com

CONFERENCES

Jun. 2022	8th European Workshop on Lipid Mediators, Karolinska Institute – Stockholm (Poster)
Jul. 2022	22 nd Scientific day of Institution Multidisciplinaire de Biochimie des Lipides (IMBL), Avignon, France (Oral communication)
Nov. 2022	18th International lipidomics meeting (GERLI), Saint-Jean-Cap-Ferrat, France (Oral communication)
Apr. 2023	Cancer Research Forum (CLARA), Lyon, France (Poster)
Jul. 2023	15 th International Society for the Study of Fatty Acids and Lipids, Nantes, France (Poster) 3D Fab days, Lyon, France (Oral communication) ICBMS days, Lyon, France (Oral communication)
Oct. 2023	19 th International lipidomics meeting (GERLI), Paris, France (Best Poster Price) 8 th Scientific day of Ecole Doctorale Interdisciplinaire Sciences-Santé (EDISS), Lyon, France (Best Poster Price)
Oct. 2024	48 th Annual Meeting of the Brazilian Biophysical Society, Sao Paulo, Brazil Collaborative work with Prof. Pietro Ciancaglini, Riberao Preto, Brazil





Article Monomethyl Auristatin E Grafted-Liposomes to Target Prostate Tumor Cell Lines

Ariana Abawi ^{1,†}, Xiaoyi Wang ^{1,†}, Julien Bompard ¹, Anna Bérot ¹, Valentina Andretto ², Leslie Gudimard ¹, Chloé Devillard ¹, Emma Petiot ¹, Benoit Joseph ¹, Giovanna Lollo ², Thierry Granjon ¹, Agnès Girard-Egrot ¹, and Ofelia Maniti ^{1,*}

- ¹ Institut de Chimie et Biochimie Moléculaires et Supramoléculaires, ICBMS UMR 5246, Univ Lyon, Université Lyon 1, CNRS, F-69622 Lyon, France; ariane.abawi@etu.univ-lyon1.fr (A.A.); xiaoyiiiiii.wang@gmail.com (X.W.); bio.julien.bompard@gmail.com (J.B.); Anna.Brt@hotmail.fr (A.B.); leslie.gudimard-garampon@univ-lyon1.fr (L.G.); chloe.devillard@etu.univ-lyon1.fr (C.D.); emma.petiot@univ-lyon1.fr (E.P.); benoit.joseph@univ-lyon1.fr (B.J.); thierry.granjon@univ-lyon1.fr (T.G.); agnes.girard-egrot@univ-lyon1.fr (A.G.-E.)
- ² Laboratoire d'Automatique, de Génie des Procédés et de Génie Pharmaceutique, LAGEPP UMR 5007, Univ Lyon, Université Lyon 1, CNRS, F-69622 Lyon, France; valentina.andretto@univ-lyon1.fr (V.A.); giovanna.lollo@univ-lyon1.fr (G.L.)
- * Correspondence: ofelia.maniti@univ-lyon1.fr; Tel.: +33-(0)4-72-44-82-14
- † These authors have equally contributed to the manuscript.

Abstract: Novel nanomedicines have been engineered to deliver molecules with therapeutic potentials, overcoming drawbacks such as poor solubility, toxicity or short half-life. Lipid-based carriers such as liposomes represent one of the most advanced classes of drug delivery systems. A Monomethyl Auristatin E (MMAE) warhead was grafted on a lipid derivative and integrated in fusogenic liposomes, following the model of antibody drug conjugates. By modulating the liposome composition, we designed a set of particles characterized by different membrane fluidities as a key parameter to obtain selective uptake from fibroblast or prostate tumor cells. Only the fluid liposomes made of palmitoyl-oleoyl-phosphatidylcholine and dioleoyl-phosphatidylethanolamine, integrating the MMAE-lipid derivative, showed an effect on prostate tumor PC-3 and LNCaP cell viability. On the other hand, they exhibited negligible effects on the fibroblast NIH-3T3 cells, which only interacted with rigid liposomes. Therefore, fluid liposomes grafted with MMAE represent an interesting example of drug carriers, as they can be easily engineered to promote liposome fusion with the target membrane and ensure drug selectivity.

Keywords: liposomes; drug delivery; membrane fluidity; Monomethyl Auristatin E

1. Introduction

To increase targeting ability towards specific cells and tissues, active agents need an appropriate delivery system [1,2]. Over the past few decades, efforts were made not only in search of new therapies, but also in developing novel nanomedicines to deliver molecules with therapeutic potentials, overcoming drawbacks such as poor solubility, toxicity or short life time in body fluids [3,4]. Among the various types of nanomedicines, liposomes have been largely described as drug carriers [5,6], and several formulations are currently marketed [7–11] or in clinical trials [6,12].

The clinical interest of liposomes relies on their composition: they are nanosized vesicles made of lipid bilayers surrounding a hydrophilic aqueous core. Their structure resembles the ones of the cell's membranes, which makes them nontoxic, biocompatible and biodegradable, and prone to interact with cells. Like most sub-micrometer-sized drug carriers, liposomes attain the tumor site through a passive targeting mechanism [13–17], but active targeting strategies using modifications in membrane protein composition have also been described. In fact, liposomes grafted with ligands specific to overexpressed membrane



Citation: Abawi, A.; Wang, X.; Bompard, J.; Bérot, A.; Andretto, V.; Gudimard, L.; Devillard, C.; Petiot, E.; Joseph, B.; Lollo, G.; et al. Monomethyl Auristatin E Grafted-Liposomes to Target Prostate Tumor Cell Lines. *Int. J. Mol. Sci.* 2021, *22*, 4103. https://doi.org/10.3390/ ijms22084103

Academic Editor: Olga Abian

Received: 16 March 2021 Accepted: 12 April 2021 Published: 15 April 2021

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Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). receptors [18–21] or with lectins to target a change in the carbohydrate composition of the membrane [22] have already been reported in the literature as examples of active targeting.

A hallmark of proliferating cells, and more specifically of cancer cells, is the ability to increase de novo lipid production and to alter their lipid repertoire in favor of the monounsaturated and ceramide-based skeleton [23]. In cancer-related phenomena, membrane fluidity changes have been equally described. For instance, in 1987, Hattori et al. investigated membrane fluidity in the membrane phospholipids of 15 brain tumors and compared them to those of the white and grey matter by electro spin resonance (ESR) using a stearate spin probe [24]. Membrane fluidity was quantified by calculating the order parameter from the ESR spectra based on the spectral anisotropy motional averaging of the spin-labeled fatty acid. The order parameter increased from metastatic brain tumor, meningioma, grey and white matter, which indicates that membrane fluidity increased in metastatic tumors when compared with other pathologies and normal brain tissues. Membranes of murine B16 melanoma and L5178 lymphoma variants with high metastatic potential have lower cholesterol/phospholipid ratio and greater unsaturated phospholipid content [25]. Such modifications in lipid composition are also expected to increase membrane fluidity. Electron paramagnetic resonance analysis of the membrane fluidity of the non-cultured lung cancer tissues obtained from the resected tumor samples of 51 patients showed that the membranes of the tumor tissues were more fluid than those of normal lungs; the most fluid domains were enlarged and their order parameter decreased in comparison to normal tissue [26].

In a very complete study, Kaur et al. analyzed membrane fluidity alterations during the early stages of the carcinogenic transformations of colonic epithelial cells (induced in rats by 1,2-dimethylhydrazine dihydrochloride—DMH administration) using fluorescent probes and showed an increase in membrane fluidity and in membrane dynamics during the first stages of the carcinogenic transformations [27]. The fluidity of the plasma membranes of the normal murine thymocytes and leukemic GRSL cells was investigated by molecular dynamics. The translational and rotational degrees of freedom pointed out that the lateral self-diffusion coefficient of the lipids in leukemic cell membranes was almost double than that of the normal cell membranes. Furthermore, these data demonstrated quantitatively that leukemic cell membranes were more fluid than normal cell membranes in the case of thymocytes, which was in good agreement with the qualitative results obtained from fluorescence depolarization measurements [28].

In a previous study on PC-3 and WPMY-1 cells, we have shown that the membranes of the highly aggressive and metastatic PC-3 cells were less viscous and more prone to deformation than those of the control WPMY-1 cells [29].

Developing nanoparticles designed to maximize their biophysical interactions with membrane lipids to enhance drug delivery and overcome drug resistance are promising strategies in therapeutics and research applications [30]. For instance, differences in membrane fluidity were used for the selective delivery of hybrid liposomes (constituted of 90% DMPC and 10% polyoxyethylene dodecyl ethers) and obtained growth inhibitory effects in correlation with the membrane fluidity of cancer cells [31]. Hybrid liposomes were capable to discriminate between human hepatocellular carcinoma cells with more fluid membranes and normal hepatocytes [32].

Based on the abovementioned literature, we previously exploited liposome membrane fluidity to promote selective targeting to cancer cells on three prostatic tumor cell lines of increasing aggressiveness [33]. Differences in liposome uptake were recorded compared to nontumor cells and between the metastatic lines. These differences were related to the liposome membrane fluidity, as measured using an in-house produced fluorophore (European Patent EPO19306175.1) [34]. The mechanism of this interaction was also investigated following the internalization pathways of two fluorophores differently loaded in the system: calcein was encapsulated in the liposome hydrophilic compartment, while a fluorescent lipid, N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-

3-Phosphoethanolamine (NBD-PE), was embedded in the liposome membrane, revealing pronounced liposome fusion with the target membranes.

In the present study, we aim at answering a consequent question raised from the knowledge gained from our previous research: can fluidity-based selectivity ensure efficient drug delivery?

The amphiphilic properties of phospholipids allow liposomes to encapsulate both hydrophilic drugs, in the inner aqueous core, and hydrophobic drugs, in the hydrophobic space provided by phospholipid acyl chains in the bilayer. However, the encapsulation efficiency makes the liposome manufacturing processes difficult and limit their use at industrial scale. Passive encapsulation processes yield low drug entrapment efficiency (under 30%), which can be increased by active encapsulation processes such as pH or salt concentration gradients [35].

In this report, we focus on the targeted delivery of Monomethyl Auristatin E (MMAE), a synthetic derivative of dolastatin 10, a linear pentapeptide originally isolated from the extracts of the sea hare *Dolabella auriculari*, first described in the 1990s [36,37]. This molecule inhibits tubulin polymerization, thus blocking mitosis, exploiting a mechanism similar to the one of taxanes. The half maximal inhibitory concentration (IC₅₀) of MMAE and of MMAE-phosphate was determined to be approximately 2 and 48 nM, respectively, in PC-3 and C4-2B cell lines [38]. Being highly cytotoxic, MMAE is too effective to be used directly in chemotherapy, but it is widely used as a cytotoxic component of antibody–drug conjugates (ADCs). MMAE and its analog, Monomethyl Auristatin F (MMAF), gained large interest as ADC warheads thanks to their high potency, water solubility, stability in biological fluids and grafting possibilities. Starting with Brentuximab vedontin, marketed since 2011 against anaplastic large cell and Hodgkin lymphoma, several auristatin-based ADC have successfully reach clinical use or are in clinical trials [39,40].

The limitations of ADCs are overall related to hydrophobicity [41], the inhomogeneity [42,43] of the conjugates, and low drug/antibody ratio (the optimal range is 2–4 drug molecules per antibody). Therefore, hydrophilic drug-linker architectures have paved the way for highly drug-loaded ADCs, aiming at masking or minimizing the apparent hydrophobicity of the payloads and at overcoming the low drug to antibody ratio [44]. In a previous report, the synthesis of monodisperse polysarcosine-MMAE compounds and their use as hydrophobicity masking entities for the construction of highly loaded homogeneous β -glucuronidase-responsive ADCs was described [45]. In the present report, such a construct has been adapted to allow conjugation to liposomes of various fluidities. The selectivity of the MMAE-based liposomes towards prostate cancer cells, based on their membrane fluidity, was tested. We showed that fluid liposomes containing unsaturated lipids are best suited for a selective MMAE delivery to tumors.

2. Results and Discussion

2.1. DPPT-MMAE Compound Preparation

In a previous study, the use of monodisperse polysarcosine as hydrophobicity masking entity for the formulation of high drug-load ADCs having improved physicochemical properties was reported. Here, an analogue product was grafted on the lipidlike compound 1,2-DiPalmitoyl-sn-glycero-3-PhosphoThioethanol (DPPT). We used a previously described drug-linker platform [45] that includes the MMAE cytotoxin, a glucuronide trigger [46], a self-immolative linker [47,48], an autohydrolysable maleimide-based bioconjugation head [49] and a polysarcosine unit. The compound is represented in Figure 1A. As described in the Materials and Methods section, the maleimide-based linker was grafted on the thiol head of DPPT (Figure 1B). After 30 min incubation, the specific retention peak of the MMAE drug-linker platform (1.5 min retention time) disappeared in favor of the DPPT-MMAE component (8.8 min) (Figure 1C). The DPPT-MMAE- derivative was obtained with a yield of 60%.



Figure 1. Synthesis of the DPPT-MMAE derivative. (**A**) Structure of the DPPT-MMAE derivative: monomethyl auristatin E (MMAE) group (purple) covalently linked to a glucuronide trigger (orange) through a self-immolative linker (red) together with a polysarcosine unit (yellow). The maleimide part is covalently attached to the sulfur headgroup of DPPT (green). (**B**) Maleimide-SH coupling reaction scheme (**C**) HPLC chromatogram after 30 min of reaction.

2.2. Liposome Characterization

The obtained derivative was dissolved in chloroform and added to the lipid mixture at 5 μ M final concentration (2500:1 lipid to drug molar ratio) which represented 0.04 molar %. The liposomes were prepared as described in the Materials and Methods section with a classical freeze–thaw protocol followed by extrusion. The different lipid compositions prepared are listed in Table 1. A constant molar percentage (20%) of fusogenic lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was used to promote the fusogenicity

of the prepared liposomes. Due to its conical shape, this lipid promotes inverted hexagonal phase intermediates that favor membrane fusion [50–52]. The MMAE-DPPT derivative was added at 0.04%. For each liposome preparation, the remaining 79.96% of the lipid composition was made of a different phosphatidylcholine molecular specie (PC): 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), respectively, as detailed in Table 1.

Table 1. Lipid used for liposome preparations, with the lipid name, fatty acid composition, structure and phase transition temperatures (T_m) .

Molar	Liposome Preparation	Acyl Chain Composition	Lipid Name and Structure				
			POPC 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine				
	РО	16:0–18:1 PC	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	-4 °C			
			DMPC 1,2-dimyristoyl-sn-glycero-3-phosphocholine				
Main linid	DM	14:0 PC		24 °C			
(79.96%)			DPPC 1,2-dipalmitoyl-glycero-3-phosphocholine				
	DP	16:0 PC					
	DS	18:0 PC	DSPC 1,2-distearoyl-sn-glycero-3-phosphocholine	55 °C			
			DOPE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine				
Fusogenic lipid (20 %)	All preparations	18:1 (Δ9-Cis) PE	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	-16 °C			
	A 11		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~				
(0.04 %)	All 16:0 preparations		The state of the s	r+50			

It is expected that due to the presence of DPPT acyl chains, the MMAE-DPPT derivative inserts into the lipid bilayer, without preferential location in the inner or outer leaflet (Figure 2A). To check the quality of the preparation in view of in vivo administration, the hydrodynamic size and polydispersity of the MMAE-containing liposomes were measured (Figure 2B,C). A typical size distribution histogram showing a single peak centered around 160 nm was obtained for the DM, DP and PO-MMAE liposomes (Figure 2B). The PDI values ranged between 0.1 and 0.2, which is typical for liposomes obtained with extrusion processes. The MMAE-containing liposomes were larger than the liposomes prepared without the MMAE-derivative (Figure 2C). A more heterogeneous preparation was obtained for DS-MMAE liposomes, with an average diameter of 250 nm and a PDI of 0.4. It is of note that the DS liposomes were more dispersed in size in the absence of MMAE, with a tendency to aggregate. All liposome preparations had a negative zeta potential (Figure 2D) ranging between -15 and -25 mV. No significant variation was recorded between the liposomes containing MMAE and the liposomes devoid of MMAE, except for the DP ones. In this case, a lower zeta-potential value was obtained for DP-MMAE liposomes (-45 mV). The DPPC used in this preparation has the same acyl chains as the DPPT-MMAE derivative and we can tentatively suggest that in this case, a slightly different polar head orientation may induce a change in the surface charge.



Figure 2. Liposome size characterization: (**A**) Scheme of liposomes composed of 79.96% PC, 20% DOPE and 0.04% DPPT-MMAE. (**B**) Typical size distribution histograms of MMAE-liposomes. (**C**) Liposome average size and polydispersity index (PDI). (**D**) Zeta-potential. Gray bars, size of liposomes devoid of DPPT-MMAE derivatives, white bars, liposomes containing DPPT-derivative, full line PDI of liposomes devoid of DPPT-MMAE derivatives, dashed line, PDI of liposomes containing DPPT-derivative. Plot of representative means (±SD) of three independent experiments per liposome preparation.

2.3. Liposome Membrane Fluidity

To ensure that the membrane lipid composition translates into a range of membrane fluidity at 37 °C, the degree of membrane order was quantified using a homemade Laurdanderivative sensitive to the membrane polarity, named Dioll. This probe spontaneously inserts in the bilayer and its fluorescence emission is related to the viscosity of its environment. The fluorescence spectra of Dioll inserted in DS, DP, DM and PO-MMAE liposomes are plotted in Figure 3A. Given the high melting point of DSPC (65 °C), DS-MMAE fluorescence spectra showed a major contribution at 440 nm characteristic of a gel state. In contrast, PO-MMAE was dominated by the 490 nm characteristic of a liquid crystalline state, due to the abundance of POPC with a melting point at 4 °C. A maximum fluorescence emission at 490 nm was obtained for DM-MMAE liposomes, which verified a liquid crystalline membrane state (for DMPC, $T_m = 24$ °C). The two nearly equal contributions for DP-MMAE liposomes indicated a mixture of membrane states in the proximity of the T_m (40 °C).

А



Figure 3. Liposome fluidity varies with liposome composition. (A) Fluorescence emission spectra of Dioll inserted in MMAE-containing liposomes at 37 °C (exc. 390 nm). (B) GP values calculated for each liposome composition as mean \pm SD of at least three independent experiments: top—MMAE-containing liposomes, bottom—liposomes without MMAE.

The generalized polarization (GP) parameter can be calculated from the fluorescence emission spectra as described in the Section 3 (Equation (1)) (Figure 3B). At 37 °C, the membranes of the PO-MMAE and DM-MMAE liposomes were in a fluid state, as indicated by the negative GP values of -0.27 ± 0.02 and -0.17 ± 0.01 , respectively. The membranes of the DP-MMAE liposomes reached a more rigid state, as revealed by the higher but still negative GP value of -0.041 ± 0.002 . The membranes of DS-MMAE liposomes were in a rigid state, as shown by the positive GP value of 0.43 ± 0.01 . The fluidity state of the liposomal membranes can thus be controlled by modulating the lipid chain length and the acyl chain unsaturation degree of the PC constituent, and can be efficiently assessed by GP values (Figure 3B). It is noteworthy that the GP values obtained for liposomes containing the DPPT-MMAE derivative were systematically lower than those obtained for liposomes devoid of DPPT-MMAE (Figure 3B, bottom line). This difference can be explained by the presence of the DPPT-MMAE derivative bulky head, which hinders bilayer organization and thus increases solvent mobility in the vicinity of the fluorophore. As a consequence, we can conclude that the MMAE derivative has been successfully enclosed in the liposome membrane.

2.4. Liposome Stability over Time

The size and the polydispersity of liposomes were measured over a period of five weeks. The size distribution histogram is plotted in Figure 4A. All liposome preparations showed an average size between 120 and 160 nm, remaining constant for at least five weeks, with a rather low polydispersity index (PDI) and a typical size distribution showing a single peak.



Figure 4. Monodisperse liposomes show a good stability over five weeks. (**A**) Typical size distribution histograms for PO-MMAE liposomes measured over five weeks. (**B**) Size and PDI of PO-MMAE liposomes over the same time period.

2.5. Liposome-Attached MMAE Effect on PC-3 Prostate Tumor Cells

The efficiency of the MMAE-prepared liposomes against PC-3 prostate cancer cells was tested. We have previously shown that PO-liposomes efficiently deliver a fluorescent lipid (NBD-PE) to PC-3 cell membranes [33]. Therefore, in order to determine the best time-point for viability measurements, we incubated PC-3 cells with PO-MMAE liposomes for 2 h30, 5 h, 24 h and 48 h. After incubation, the cells were washed with PBS and cultured in fresh medium for an additional 72 h to allow the action of the MMAE cytotoxin, which results in reduced microtubule polymerization and arrest of cell cycle progression. After this additional time, the viability of the cells was checked by their ability to metabolize MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and produce formazan crystals. Viable cells with active metabolism convert MTT into formazan, resulting in an absorbance increase at 590 nm. Dead cells lose this ability and therefore show no signal. The measured absorbance at 590 nm is proportional to the number of viable cells. After 2 h30 incubation, PO-MMAE did not affect PC-3 viability (Figure 5). The DPPT-MMAE derivative dissolved in DMSO, used as control, also had a limited efficiency on PC-3 at this point (80% viability maintained). After 5 h incubation, the PO-MMAE liposomes showed a strong effect on the cell viability comparable to that of the soluble derivative. The same percentage of residual viability was obtained after 24 h and 48 h incubation. Therefore, we can conclude that PO-MMAE liposomes delivered the active compound to PC-3, and that the best contact time between cells and liposomes was 5 h.



Figure 5. Effect of PO-MMAE liposomes on PC-3 cell viability. Grey—DPPT-MMAE derivative in DMSO, red, PO-MMAE liposomes. DPPT-MMAE derivative concentration was of 100 nM in cell culture medium. Viability is expressed as a % of the untreated cell controls. Results are expressed as mean \pm SD out of three independent replicates. *** p < 0.001 Student *t*-test.

48h00

24h00

2.6. Selectivity of Liposomes

2h30

5h00

Viability (%)

0

The selectivity of liposomes towards a target cell type was checked at the selected time point (5 h). PO, DM, DP and DS-MMAE liposomes were incubated with PC-3 and LNCaP prostate tumor cell lines and fibroblast NIH-3T3 cell line (Figure 6A). The effect on cell viability strongly depended on the liposome type. PO-MMAE and DM-MMAE showed reduced efficacy on fibroblasts, while cell viability was very significantly (p < 0.001) reduced by more than 50% after incubation with DP and DS-MMAE liposomes. In the case of tumor cell lines, the opposite effect was recorded, with PO-MMAE inducing a strong decrease in cell viability of over 60% for LNCaP and over 50% for PC-3 cells (p < 0.001). DM, DP or DS-MMAE liposomes had small or no effect on tumor cell lines. As control, a free DPPT-MMAE derivative in DMSO was administered to cells in the same conditions (Figure 6B). The viability generally decreased for all cell lines tested, yet a strong variability was recorded between assays, thus confirming that direct administration of the derivative is not suitable. As shown in Figure 6C, the PO-MMAE liposomes were selectively taken-up by LNCaP and PC-3 cells (p < 0.001 for both PC-3 and LNCaP vs. NIH-3T3 cells). PO-MMAE induced a strong decrease in PC-3 and LNCaP cells for concentrations as low as $25 \ \mu g$ lipids/mL corresponding to 10 nM in MMAE (Figure 6D).

To gain access to the inside of the liposome–PC-3 cell interaction mechanisms, fluorescent PO liposomes were used instead of MMAE-containing liposomes. These liposomes were not toxic to the cells and allowed us to follow the liposome internalization. As shown in Figure 6E, after 5 h incubation of PC-3 cells with PO liposomes containing the fluorescent lipid NBD-PE, the fluorescence was located at the cell periphery, indicating that the fluorophore remained at the level of the plasma membrane. At this point, we cannot conclude whether liposomes adhere to the PC-3 cells or whether they fuse with the plasma membrane. When liposomes containing calcein in the inner compartment were used, fluorescence was present in the cytosol, indicating that calcein was released in the cytosol, which led us to conclude that liposome–cell interaction was based on membrane fusion between liposome bilayer and plasma membrane, leading to the release of calcein in the cytosol and to the diffusion of NBD-PE in cell membrane. Due to its lipophilic nature, MMAE-DPPT derivative is expected to equally diffuse in the plasma membrane (Figure 6F) where it can be degraded by tumor overexpressed glucuronidases or other cellular elements to release MMAE (Figure 6F).



Figure 6. Selectivity of MMAE-liposomes towards fibroblast and tumor cell lines. (**A**) PO, DM, DP and DS-MMAE liposomes were incubated with NIH-3T3, LNCaP and PC-3 cell lines and viability was measured using the MTT test. Results are plotted as the normalized viability against the highest value recorded. Percentage of viability were calculated against viability in cell culture medium. *** p < 0.001, ** p < 0.01 and * p < 0.05, Student test. (**B**) As controls, cells were incubated with DPPT-MMAE derivative in DMSO at 100 nM final concentration. (**C**) PO-MMAE liposomes induce a strong decrease in the viability of PC-3 and LNCaP tumor cells but not in that of NIH-3T3 cells. (**D**) PC-3, LNCaP and NIH-3T3 viability decrease as function of PO-MMAE liposome concentration. (**E**) Interaction between PC-3 cells and NBD-PE or calcein-containing liposomes resulting in different fluorescence distribution patterns. (**F**) Putative action mechanism: fusion of PO-liposomes with PC-3 cell membrane results in DPPT-MMAE accumulation at the membrane and degradation by glucuronidases or other cellular parameters to obtain toxic effects.

As described in the introductory part, accumulating literature data show that cancer cell lines have modified membrane composition with a general tendency to an increased membrane fluidity. Fibroblasts are expected to be globally more rigid, and DP and DS formulation more prone to fuse with the membranes of the NIH-3T3 cells, whereas PC-3 and LNCaP cells are metastatic tumor cell line and globally more fluid. Therefore, fluid PO-MMAE liposomes are readily taken-up by the cells.

The cellular uptake of MMAE-liposomes depended on liposome fluidity and PO-MMAE preparation may constitute an interesting drug delivery candidate as liposomes are taken-up only by tumor cells. Membrane fluidity is one of the key parameters for membrane fusion, since it determines the mobility of the lipids, proteins and water molecules that cooperate in the reorganization and the assembly required and induced by the membrane fusion [53,54]. Membrane lipid composition, and consequently membrane physicochemical state, is closely linked to pathologies, especially in the case of cancers where a higher unsaturation of acyl chains is associated with an elevated membrane fluidity and metastasis aggressiveness [23,55]. In view of in vivo administration, its stability in body fluids still needs to be assessed. We have shown in a previous report that liposomes were stable in cell culture media supplemented with fetal calf serum [33]. Liposomal membrane fluidity also influences pharmacokinetic properties of liposomal carriers and thus, systemic circulation. Studies on two animal models, rodent and zebra fish [56,57] revealed that plasma protein association to fluid liposomes was much lower than to rigid ones. Liposomes with low melting point (fluid liposomes) had longer circulation times and were globally more stable in the blood. Several liposome formulations are currently used clinically or in phase I to III trials, thanks to controllable pharmacokinetic and pharmacodynamic properties that improved bioavailability and limited toxicity. Among them, Myocet® liposomes are about 150 to 250 nm in size and contain cholesterol and egg phosphatidylcholine, and are non-PEGylated. Altogether, these findings make liposomes interesting drug carriers, as liposome composition can be easily tuned to promote liposome fusion with the target membrane and ensure drug selectivity, which may represent a cost-effective alternative to antibody-drug conjugates.

3. Materials and Methods

Lipids and polycarbonate membranes were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, Penicillin/Streptomycin, Phosphate Buffered Saline (PBS) composed of 10 mm phosphate, 137 mm NaCl and 2.7 mm KCL, pH 7.4, DiMethyl SulfOxide (DMSO), para-formaldehyde (PFA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.1. Synthesis of DPPT-MMAE Derivative

To obtain a MMAE lipid derivative, the drug-linker platform (Figure 1, compound a), previously described in [45] was grafted onto 1,2-DiPalmitoyl-sn-glycero-3-PhosphoThioethanol, DPPT (Figure 1, compound b) (Avanti Polar Lipids, Alabaster, AL, USA). The synthesis of the drug-linker platform that included the monomethyl auristatin E (MMAE) cytotoxin, a glucuronide trigger, a self-immolative linker, an auto-hydrolysable maleimide-based bioconjugation head and a polysarcosine unit (MAL-glucu-MMAE-PSAR18) was described elsewhere [45]. In this report, 0.9 mg DPPT solubilized in 500 µL chloroform were incubated in a glass vial with 5 µL trimethylamine and 76 µL MAL-glucu-MMAE-PSAR18 (12 mM) under shaking. The reaction advancement was checked by flushing the reaction medium on HPLC C18 preparative column (Agilent EC-120 C18 Poroshell 3 \times 50 mm, 2.7 µm, Agilent, Santa Clara, CA, USA). Mobile phase A consisted of 0.1% TFA in water, whereas mobile phase B consisted of 100% methanol. Separation was carried out using an elution gradient from 20% to 90% solvent B for 5 min followed by 95% solvent for 7 min, under a flow rate of 0.8 mL/min at 30 °C. Elution was followed by UV detection (214 nm). The unbound MMAE peak totally disappeared after 30 min. The product was then purified on 30 g HPLC C18 preparative column SNAP Biotage (Biotage, Uppsala, Sweden) on Teledyne Isco Rf150 system (Teledyne ISCO, Lincoln, NE, USA) under the same elution conditions. Fractions of interest were pooled and methanol was evaporated. The dry residue was exposed to phosphorous pentoxyde for 3 h. The dry residue was weighted and the product was identified using Q-TOF mass spectroscopy, with a yield of 60%.

3.2. Liposome Preparation

Liposomes were prepared using the thin film hydration method. Briefly, lipids dissolved in chloroform with a total lipid mass of 5 mg were mixed in a round flask. The solvent was dried under vacuum at 50 °C on a rotatory evaporator. The lipid film obtained was hydrated with 1 mL of sterile PBS, while stirring and heated above the lipid melting point. This resulted in the formation of MultiLamellar Vesicles (MLVs) with various sizes and number of layers. Six freeze–thaw cycles in liquid nitrogen were then applied to the prepared liposomes in order to burst the MLVs into Large Unilamellar Vesicles (LUVs). The LUVs size was defined by extrusion through a porous membrane with a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA). Liposomes were heated above their phasetransition temperature (Tm), extruded through a 400 nm and then, a 100 nm pore diameter polycarbonate membrane using a MiniExtruder (Avanti Polar Lipids, Alabaster, AL, USA). The final liposome solution was stored at 4 °C for 4 weeks, without further extrusion.

MMAE-derivative was added to the lipid mixture in chloroform prior to drying. Liposomes containing a final concentration of 5 μ M MMAE-derivative were prepared. This corresponded to 0.12% of the total lipid mass and a lipid/MMAE derivative molar ratio of 2500:1, at a molar percentage of 0.04%. Liposomes contained 20 molar % of fusogenic lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 79.06% of phosphatidyl-choline as follows 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-glycero-3-phosphocholine (DPPC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), for DS, DP, DM and PO-MMAE preparations, respectively. Detailed liposome composition is given in Table 1.

3.3. Liposome Characterization

The membrane fluidity of liposomes was assessed using an in-house Laurdan-derivative probe sensitive to the membrane polarity (Dioll) [34]. Liposomes at a concentration of 0.1 g/L were incubated with the probe at 0.2 µm for 15 min, then the fluorescence emission spectrum was recorded on a FP-8500 spectrofluorometer (JASCO Applied Science, Halifax, Canada) with emission and excitation slits set at 2.5 nm. Spectra were recorded from 400 nm to 600 nm at 37 °C, with an excitation λ_{max} set at 390 nm. The Generalized Polarization (GP) parameter was calculated as indicated on Equation (1), where I₄₄₀ is the fluorescence emission intensity at 440 nm (gel phase) and I₄₉₀ is the fluorescence emission intensity at 490 nm (liquid crystalline phase). Results were expressed as mean ± standard deviation of three independent experiments.

(

$$GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$$
(1)

Liposomes hydrodynamic size distribution and surface charge were analyzed using Malvern Zetasizer[®] Nano ZS (Malvern Instruments S.A., Worcestershire, UK). Z-average diameter (the intensity weighted mean hydrodynamic size) and polydispersity index (PDI) were determined by Dynamic Light Scattering (DLS) at a concentration of 0.15 mg/mL. Analyses were carried out at 25 °C with an angle of detection of 173°. The zeta potential values were obtained by measuring the electrophoretic mobility of liposomes in dispersion. The stability of the particles was investigated by following the size and PDI of the preparations 1 week, 2 weeks, 3 weeks and 5 weeks after preparation. Results were expressed as mean \pm standard deviation of three independent liposome preparations. Liposome PDI results were expressed as the mean PDI of the preparations, and were measured concurrently with liposome size on three independent liposomes' preparations.

3.4. Cell Culture

NIH-3T3 mouse embryonic fibroblast cells [58] LNCaP and PC-3 human cell lines were used as in vitro models. LNCaP is a hormone-sensitive cell line obtained from a lymph node metastasis derived from a prostate tumor [59]. PC-3 cell line was isolated from a vertebral metastasis stemming from a prostate tumor and entirely composed of

carcinoma cells [60]. Cell lines were purchased from ATCC (Manassas, VA, USA). NIH-3T3, LNCaP, and PC-3 cells were cultured in RPMI medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. All cells were cultured in a humidified incubator at 37 °C with 5% CO₂. After standard trypsinization, 6 × 104 cells/cm² for LNCaP, 3 × 104 cells/cm² for PC-3 and NIH-3T3 were seeded in 24-well plates and incubated overnight.

Liposomes were added in the culture medium after 1-night incubation at a final concentration of 0.25 mg/mL which corresponded to a MMAE-derivative concentration of 100 nM, unless otherwise stated After the indicated incubation time, plates were washed with PBS and cultured in fresh medium for an additional 72 h.

The number of adherent viable cells was assessed using the MTT assay, which is based on the reaction of a colorless tetrazolium salt with cellular reductases to form purple formazan crystals. MTT was added at a final concentration of 0.125 g/L. The plate was further incubated for 3 h at 37 °C, after which the culture medium was removed and the formed formazan crystals were dissolved in 1 mL of DMSO. After 20 min incubation, the absorbance of the plate was measured at 570 nm. Absorbance measurements were conducted on an Infinite-M200 pro Plate reader (TECAN, Männedorf, Switzerland). Results were corrected from the absorbance at 590 nm obtained in presence of 10% Triton corresponding to 0% viability and expressed as a percentage relative to an untreated control corresponding to 100% viability. Results were expressed as mean \pm standard deviation of three independent experiments.

3.5. Fluorescence Microscopy Experiments

PC-3 cells were plated overnight in 96-well plates. The amount of cells per well was chosen to ensure 80% surface coverage prior to liposome addition. NBD-PE or calcein PO fluorescent liposomes were added at a final concentration of 0.25 g/L, and the plate was further incubated for 5 h at 37 °C. The plates were rinsed 3 times with PBS, fixed with PFA 3.7% in PBS for 10 min and then, rinsed 3 more times with PBS. Finally, the plates were visualized using an AxioObserverZ.1 (Zeiss, Oberkochen, Germany) epifluorescence microscope. NBD-PE was added to lipid mixture prior to liposome preparation at 2 molar %. Calcein (500 μ M) was dissolved in PBS and was used to resuspend the lipid dry film. The excess of calcein was removed from calcein-loaded liposomes through exclusion chromatography on PD-10 Desalting Columns (GE Healthcare, Chicago, IL, USA).

4. Conclusions

To summarize, following the model of ADC, an MMAE warhead was grafted onto fusogenic liposomes made up of phosphatidylcholines of different chain lengths and fusogenic lipid DOPE. The prepared liposomes were monodispersed and stable for several weeks. A range of membrane fluidity was obtained according to the liposome composition, as attested by fluorescence spectroscopy with a polarity sensitive probe. Only the fluid liposomes made of 80% POPC were taken-up by PC-3 and LNCaP cells. PO-MMAE had a small effect on fibroblast NIH-3T3 cells, which only interacted with rigid DP or DS-MMAE liposomes. This opens the perspective of an alternative targeted delivery of MMAE, based on liposomal membrane fluidity, with PO-liposomes as promising candidate for the delivery of MMAE or other drugs as they selectively target tumor against nontumor cells.

Author Contributions: Conceptualization, O.M., A.G.-E.; investigation, A.A., X.W., J.B., A.B., L.G., C.D. and V.A.; writing—original draft preparation O.M., A.A. and X.W.; writing—review and editing A.G.-E., G.L., B.J., E.P. and T.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by University Claude Beranard Lyon 1, CNRS (prematuration program) and the Technology Transfer Accelerator Office Lyon-Saint-Etienne Pulsalys.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Article Carrier–Tumor Cell Membrane Interactions for Optimized Delivery of a Promising Drug, 4(*RS*)-4-F_{4t}-Neuroprostane

Ariana Abawi¹, Céline Thomann¹, Giovanna Lollo², Thierry Granjon¹, Emma Petiot¹, Anna Bérot¹, Camille Oger³, Valérie Bultel-Poncé³, Alexandre Guy³, Jean-Marie Galano³, Thierry Durand³, Agnès Girard-Egrot¹ and Ofelia Maniti^{1,*}

- ¹ Institute of Molecular and Supramolecular Chemistry and Biochemistry, ICBMS UMR 5246, University Lyon, Université Lyon 1, CNRS, F-69622 Lyon, France; ariana.abawi@etu.univ-lyon1.fr (A.A.); celine.thomann@univ-lyon1.fr (C.T.); thierry.granjon@univ-lyon1.fr (T.G.); emma.petiot@univ-lyon1.fr (E.P.); anna.brt@hotmail.fr (A.B.); agnes.girard-egrot@univ-lyon1.fr (A.G.-E.)
- ² Laboratoire d'Automatique, de Génie des Procédés et de Génie Pharmaceutique, LAGEPP UMR 5007, University Lyon, Université Lyon 1, CNRS, F-69622 Lyon, France; giovanna.lollo@univ-lyon1.fr
- ³ Pôle Chimie Balard Recherche, Institut des Biomolécules Max Mousseron, IBMM, UMR 5247, Université de Montpellier, CNRS, ENSCM, F-34293 Montpellier, France; camille.oger@umontpellier.fr (C.O.); alexandre.guy@umontpellier.fr (A.G.); jean-marie.galano@umontpellier.fr (J.-M.G.); thierry.durand@umontpellier.fr (T.D.)
- * Correspondence: ofelia.maniti@univ-lyon1.fr; Tel.: +33-(0)4-72-44-82-14

Abstract: Nanomedicines engineered to deliver molecules with therapeutic potentials, overcoming drawbacks such as poor solubility, toxicity or a short half-life, are targeted towards their cellular destination either passively or through various elements of cell membranes. The differences in the physicochemical properties of the cell membrane between tumor and nontumor cells have been reported, but they are not systematically used for drug delivery purposes. Thus, in this study, a new approach based on a match between the liposome compositions, i.e., membrane fluidity, to selectively interact with the targeted cell membrane was used. Lipid-based carriers of two different fluidities were designed and used to deliver 4(RS)-4-F_{4t}-Neuroprostane (F_{4t}-NeuroP), a potential antitumor molecule derived from docosahexaenoic acid (DHA). Based on its hydrophobic character, F4t-NeuroP was added to the lipid mixture prior to liposome formation, a protocol that yielded over 80% encapsulation efficiency in both rigid and fluid liposomes. The presence of the active molecule did not modify the liposome size but increased the liposome negative charge and the liposome membrane fluidity, which suggested that the active molecule was accommodated in the lipid membrane. F_{4t}-NeuroP integration in liposomes with a fluid character allowed for the selective targeting of the metastatic prostate cell line PC-3 vs. fibroblast controls. A significant decrease in viability (40%) was observed for the PC-3 cancer line in the presence of F_{4t} -NeuroP fluid liposomes, whereas rigid F4t-NeuroP liposomes did not alter the PC-3 cell viability. These findings demonstrate that liposomes encapsulating F4t-NeuroP or other related molecules may be an interesting model of drug carriers based on membrane fluidity.

Keywords: liposomes; drug delivery; membrane fluidity; 4(RS)-4-F4t-Neuroprostane

1. Introduction

1.1. Liposomes in Cancer Treatment

The latest sglobal data on cancer burden published by the World Health Organization show that in 2020, 10 million deaths were caused by cancer, which equals nearly one in six deaths. According to the International Agency for Research on Cancer (IARC), one of five people worldwide develop cancer during their lifetime. This alarming scenario highlights the need to find more effective treatments.



Citation: Abawi, A.; Thomann, C.; Lollo, G.; Granjon, T.; Petiot, E.; Bérot, A.; Oger, C.; Bultel-Poncé, V.; Guy, A.; Galano, J.-M.; et al. Carrier–Tumor Cell Membrane Interactions for Optimized Delivery of a Promising Drug, 4(*RS*)-4-F_{4t}-Neuroprostane. *Pharmaceutics* 2023, *15*, 2739. https://doi.org/10.3390/ pharmaceutics15122739

Academic Editors: Nejat Düzgüneş and Maria Camilla Bergonzi

Received: 27 October 2023 Revised: 13 November 2023 Accepted: 24 November 2023 Published: 7 December 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). One major clinical concern is the lack of specificity of chemotherapeutic drugs [1]. Conventional chemotherapies have prominent side effects since molecules target not only tumors but also healthy tissues or induce systemic effects. At the end of the 19th century, the term magic bullet, introduced by Paul Ehrlich, led to the establishment of a new generation of cancer treatments. Originally, the concept was based on the selective targeting of a bacterium without side effects on other organisms caused by antitoxins or antibodies [2]. Over time, this concept led to the use of nanotransporters to selectively target cancer cells and allow a controlled release of active molecules. Among them, one can count inorganic nanoparticles (carbon nanotubes, iron oxide, mesoporous silica, gold nanoparticles, etc.), polymeric nanoparticles [3], dendrimers [4], micelles [5] or various lipid nanoparticles [6,7].

In this context, liposomes are one of the most studied drug delivery systems in the last 50 years. Their biocompatibility, biodegradability and low toxicity make them the ideal candidates for anticancer treatments. Their amphiphilic character allows for the encapsulation of a wide range of drugs: lipophilic molecules are located in the lipid bilayer, whereas hydrophilic molecules are dissolved in aqueous space [8]. The encapsulation of drugs in liposomes enhanced the therapeutic windows of different agents, reducing their pharmacokinetic and pharmacodynamic alterations [9]. Moreover, the lack of selectivity in vivo of some antitumoral drugs precludes their use in clinics [10]. Thus, their encapsulation considerably reduces undesirable side effects.

In addition, loaded drugs can be protected against inactivation, hydrolysis, early dilution in body fluids or environmental changes (temperature, pH) that could occur in vivo [11]. Since the approval of the first liposomal anticancer treatment, Doxil/Caelyx, in 1995 [3], liposomes have been considered one of the most successful nanoparticles in clinical cancer care as several liposomal preparations reached the market, such as Vyxeos in 2017, a prescription medicine used to treat acute myeloid leukemia [12], or Onivyde in 2015, for irinotecan delivery to the pancreas. Over the past three years, three liposomal products have been authorized by the FDA (U.S. Food and Drug Administration) and EMA (European Medicines Agency): Thermodox in 2021, thermally sensitive liposomes for the post-tumor ablation delivery of doxorubicin; Exparel in 2020, a local infiltration liposome indicated for post-surgical analgesia; and Arikayce in 2020, an oral inhalation treatment used for lung disease [13]. Even though the largest single application of lipid nanoparticles (LNPs), including liposomes, in drug delivery is cancer treatment, LNPs are also used to treat hormonal, respiratory or fungal diseases [14]. The last few years have been marked by the development of successful LNPs as delivery vehicles for nucleic acids in the two recently approved COVID-19 vaccines, Pfizer/BioNTech and Moderna [2].

1.2. Targeting Strategies: Significance of Membrane Fluidity

In the most currently used preparations, liposome accumulation in tumors is reached by passive targeting, but strategies based on the active targeting of tumors have also come to light. Cellular targets are receptors that are overexpressed in different cancer cells. That is why the surface of liposomes has been further ligated with small molecules, vitamins, proteins, antibodies, etc. [15]. Several targeted liposomal preparations have gone up to phase I or II clinical trials [16], such as transferin-targeted liposomes (MBP-426 from Mebiopharm or SGT-53 from SynergeneTherapeutics), HER-2-targeted liposomes (MM-302 from Merimack or C225_ILS-DOX from University Hospital, Basel) or GAHtargeted liposomes (MCC-465 from Mitsubishi Tanabe Pharma). Although clinical trials were discontinued for many, they are still ongoing for MBP-426 and C225_ILS-DOX. Yet, among the various immunoliposome types, only one preparation, Onivyde [14], is currently used in clinics. The scarce number of clinically available active targeting examples is mainly due to expensive antibody grafting strategies. Therefore, smaller molecules were proposed as active targeting strategies, such as RGD peptides [17] or folate [18], and some potential carriers are under clinical trials [19].

The process of tumorigenesis and tumor development is accompanied by many metabolic modifications. Several studies indicate that tumorigenesis is often accompanied by changes in membrane composition, which in turn determine membrane mechanical properties, i.e., membrane fluidity. Indeed, the relationship that may exist between the metastatic potential of cells and the alteration of their membrane fluidity has been evoked for many years [20]. In the case of breast cancer, for instance, cell malignancy was correlated with an increase in membrane fluidity (an increase in fluidity in the membrane of MT3 breast cancer cells correlates with enhanced cell adhesion in vitro and increased lung metastasis in NOD/SCID mice). This increase in the membrane fluidity of cancer cells can be explained by the alteration of the cell lipid metabolism. Cell membrane compaction is modulated by the presence of certain lipids, such as sphingolipids. The expression of *ceramide synthase-6* genes has been shown to decrease in tumor cells in contrast to non-tumor cells [21]. In the case of prostate cells, the membrane was less stiff, less viscous and thus more prone to deformation than that of the control cell lipe [22].

Although differences in the physicochemical properties of cell membranes between tumor and non-tumor cells have been reported over the years [21,23–25], they are not systematically used for the optimization of drug delivery strategies. In a previous study [26], we showed that targeting cancer cells based on the physical compatibility between cells and liposomes is a promising alternative targeting strategy. By modulating the lipid composition of liposomes, we associated their membrane fluidity state with that of the plasma membrane of targeted tumor cells. This match in membrane properties favors fusion processes between the liposome and the targeted membranes. Liposomes of controlled membrane fluidity were successfully used to deliver Monomethyl Auristatin E (MMAE) to the prostate tumor PC-3 cell line [27].

1.3. Oxylipins and Their Potential as Chemotherapeutics

In addition to the selective delivery of drugs to tumors, effective agents to treat this disease still need to be found. Natural products and their derivatives [28] have been used in cancer chemotherapy for over 50 years. No less than 49% of a total of 175 small-molecule anticancer drugs used in chemotherapy in Western countries over a 70-year period were obtained directly or derived from natural products [29].

Among the natural products found in our everyday diet, omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) play important roles in human health as key precursors of many oxygenated metabolites called oxylipins [30]. PUFAs were first discovered in olive oil. Since then, numerous studies have shown the presence of these compounds and their derivatives in plants, cereals, rice and cocoa.

Oxylipins can be obtained through two pathways: an enzymatic pathway based on the catalysis of arachidonic acid (AA) by cyclooxygenase (COX), which leads to the formation of prostaglandins (PGs), or a non-enzymatic pathway initiated by free radicals. Discovered in 1990, non-enzymatic oxygenated PUFAs (NEO-PUFAs) have various effects in several biological mechanisms [30], suggesting medical potential. Due to the structure of these compounds being similar to that of PGs, they were named isoprostanoids (IsoPs). IsoPs are formed in the lipid membrane from phospholipids, and the molecules generated are racemic with a configuration of the two side chains, mainly *cis* [30]. The IsoP class includes molecules such as phytoprostanes (PhytoPs), neuroprostanes and isoprostanes. The NEO-PUFAs can be regarded as mediators of physiological and pathophysiological processes, including vasoconstriction, anti-arrhythmia, neuroprotection [30] and immunological responses [31].

More recently, it has been demonstrated that some PhytoPs reduce the viability of the MCF-7 and MDA-MB-231 breast cancer cell lines [28]. PhytoPs also possess activities such as anti-inflammatory and cell death-promoting activities and are also able to induce apoptosis in leukemia, Jurkat T-cells [32] and microglial cells [31].

In this study, we evaluated the cytotoxic effect of 12 free isoprostanoids, for which antitumor potential has been suggested but not validated yet. Among the 12 free isoprostanoids, one can find PhytoPs derived from α -linolenic acid (ALA), neuroprostanes (NeuroPs) derived from docosapentaenoic acid (DPA ω 3) or docosahexaenoic acid (DHA),

and isoprostanes derived from arachidonic acid (AA). As the most promising cytotoxic effect was obtained for 4(RS)-4-F_{4t}-neuroprostane (here abbreviated as F_{4t}-NeuroP), this compound was encapsulated in liposomes to target cancer cells using the membranefluidity-based targeting approach previously described [26,27] from the perspective of the use of oxylipin in antitumor treatments for prostate cancer. The advantage of using this molecule is that the 4- and 20-series NeuroPs are the most abundant form of NeuroPs and are less prone to oxidation when compared with other PUFAs. Moreover, F4t-NeuroP has some anticancer properties. F4t-NeuroP showed antiproliferation effects on the human breast cancer cell line (MDA-MB-231), while no inhibitory effect was observed on a human mammary epithelial normal cell line (MCF-10A) [33]. The use of F4t-NeuroP in this study led to a good encapsulation rate (over 80%) of molecules inside the liposome and a notable cytotoxicity effect of the fluid liposome PO- F_{4t} -NeuroP on the PC-3 metastatic prostate cell line. Altogether, in addition to their therapeutic potential, members of the NEO-PUFA family with antitumor activities may be efficiently encapsulated in selective carriers. Conventional chemotherapies have prominent side effects due to the non-specific targeting of cancer cells, which can affect healthy surrounding tissues but also induce systemic effects. The encapsulation of NEO-PUFA in liposomes may enhance the therapeutic window, increase efficacy and considerably reduce undesirable side effects.

2. Materials and Methods

The materials used for liposome preparation (lipids, polycarbonate membranes, extruder and syringes) were purchased from Avanti Polar Lipids (Alabaster, Al, USA). The materials used for the cell culture (Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, phosphate buffered saline (PBS) pH 7.4), dimethyl sulfoxide (DMSO), calcein, paraformaldehyde (PFA) and resazurine) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1. Synthesis of F_{4t} -NeuroP

The total synthesis of F_{4t} -NeuroP was performed according to our published strategies [34,35].

The synthesis is summarized in Scheme 1. Starting from commercially available 1,3cyclooctadiene 1, the key bicyclic intermediate 2 was obtained in 5 steps, giving an 18% yield. The introduction of α and ω chains was performed using regioselective protections/deprotections, oxidations and Wittig elongations. The final step was the saponification of the methyl esters in the presence of lithium hydroxide (LiOH) to obtain the free acid. 4(*RS*)-4-F_{4t}-NeuroP was obtained from intermediate 2 in 16 steps with a 9% yield.



Scheme 1. Synthesis of 4(RS)-4-F_{4t}-NeuroP; details of the synthesis steps are provided in references [32,33].

2.2. Liposome Preparation

Liposomes were prepared using the thin-film hydration method, followed by freeze/thaw cycles and extrusion. Then, 10 mg of lipids dissolved in chloroform were mixed in a round flask. Chloroform was evaporated under vacuum conditions on a rotatory evaporator at 50 °C to obtain a uniform lipid film, which was subsequently hydrated with 1 mL of PBS (10 mM phosphate, 137 mM NaCl and 2.7 mM KCl, pH 7.4) under stirring at 50 °C. To burst the MultiLamellar Vesicles (MLVs) formed, six freeze–thaw cycles in liquid nitrogen were applied to form Large Unilamellar Vesicles (LUVs). The LUVs were extruded through a 400 nm, followed by a 100 nm porous membrane with a Mini-Extruder (Avanti Polar

Lipids, Alabaster, AL, USA) while being heated above their phase transition temperature (T_m) . The liposomes (10 mg/mL) were stored at 4 °C for 4 weeks without further extrusion.

F_{4t}-NeuroP was added to the lipid mixture in chloroform prior to drying. Two liposomes with different membrane fluidities named PO (fluid) and DP (rigid) containing a final concentration of 200 μ M F_{4t}-NeuroP were prepared. This corresponded to 0.7% of the total lipid mass with a lipid / F_{4t} -NeuroP molar ratio of 69:1 at a molar percentage of 1.5%. PO liposomes were composed of POPC:DOPE:F4t-NeuroP (8:2:0.7 mole%), and DP liposomes were composed of DPPC:DOPE:F_{4t}-NeuroP (8:2:0.7 mole%). The detailed liposome composition is given in Table 1. As the study is based on liposome cell interactions according to membrane fluidity, the test includes two liposomes with different physicochemical compositions: DP composed of long acyl chains (16:0), which do not allow movement within the membrane, and PO composed of acyl chains with unsaturations (16:0–18:1), which cause curvature and increase the membrane fluidity. Indeed, the physicochemical properties of liposomes are dictated by various acyl chain lengths and unsaturation. Long acyl chains are associated with a rigid membrane, and the presence of unsaturation increases fluidity. In a previous study [26], we showed that the interaction of the liposomes DO and PO's preparations (fluid) with prostate cell lines, including PC-3, was modulated by their membrane fluidity. Conversely, none of the metastatic cell lines showed an interaction with the DP or DS preparations (rigid). Based on these results, we decided to use the PO and DP liposomes as carriers of opposite fluidity for the delivery of F_{4t} -NeuroP and to check whether the match between carrier and target cell membrane fluidity permits the selective delivery of the active principle. Such biocompatible, biodegradable carriers offer a cost-effective targeting strategy with respect to antibody or molecule grafting.

Table 1. Lipids used for liposome preparation: name, fatty acid composition, structure and phase transition temperatures (melting temperature (Tm)).

Molar%	Liposome Preparation	Acyl Chain Composition	Lipid Name and Structure	T _m	
Main lipid	РО	16:0-18:1 PC	POPC 1-palmitoyl-2-oleoyl-glycero-3- phosphocholine	−4 °C	
(80%)	DP	16:0 PC	DPPC 1,2-dipalmitoyl-glycero-3- phosphocholine	41 °C	
Fusogenic lipid (20%)	All preparations	18:1 (Δ9-Cis) PE	DOPE 1,2-dioleoyl-sn-glycero-3- phosphoethanolamine	-16 °C	
4(RS)-4-F4t- Neuroprostane (0.7% on total lipid mass)	In two preparations		HO CO2H	/	

Excess/free F_{4t} -NeuroP was removed using steric exclusion chromatography (SEC, PD-10 columns, SephadexTM G-25 M, GE Healthcare (Limonest, France). In brief, the column was equilibrated with PBS, and then 1 mL of liposome preparation was added, and the samples were eluted with PBS. As F_{4t} -NeuroP showed a maximum absorbance at 240 nm, the absorbance was measured at this λ for each fraction, and the amount of F_{4t} -NeuroP was estimated against an F_{4t} -NeuroP standard curve.

Fractions of 1 mL were recovered, and the 240 nm absorbance was measured. As the liposomal solution exhibited a strong diffusion, the amount of encapsulated F_{4t} -NeuroP was estimated as the total F_{4t} -NeuroP added minus the non-encapsulated F_{4t} -NeuroP. All measurements were performed in triplicate. The encapsulation efficiency (EE) and drug loading (DL) were calculated following Equations (1) and (2).

$$EE(\%) = \frac{Total \ F4t - NeuroP \ concentration - F4t - NeuroP \ free \ molecule}{Total \ F4t - NeuroP \ concentration} \times 100$$
(1)

$$DL(\%) = \frac{Amount \ of \ F4t - NeuroP \ in \ liposomes}{Amount \ of \ liposomes} \times 100$$
(2)

2.3. Physico-Chemical Characterization of Blank and F_{4t}-NeuroP-Loaded Liposomes

The membrane fluidity of liposomes was measured using radiometric probes and fluorescence spectroscopy on a FP-8500 spectrofluorimeter (JASCO Applied Science, Halifax, Canada). Liposomes at a concentration of 0.1 g/L were incubated with the probe Dioll at 0.4 μ M for 1 h. The fluorescence emission spectrum was recorded from 400 nm to 600 nm with an excitation wavelength of 390 nm. Emission and excitation slits were set at 2.5 nm. Experiments were performed at 37 °C. The Generalized Polarization (GP) parameter was calculated [36,37], as indicated in Equation (3),

$$GP = (I_{440} - I_{490}) / (I_{440} + I_{490})$$
(3)

where I_{440} is the fluorescence emission intensity at 440 nm (gel phase) and I_{490} is the fluorescence emission intensity at 490 nm (liquid crystalline phase).

The Z-average diameter (the intensity-weighted mean hydrodynamic size) and polydispersity index (PDI) of each liposome preparation were determined for each preparation with and without F_{4t} -NeuroP. The liposomes were diluted at a concentration of 0.1 mg/mL in PBS. The analyses were carried out at 25 °C with an angle of detection of 173°.

The surface charge of the liposomes was obtained by measuring the ζ potential values obtained from the electrophoretic mobility of the liposomes in dispersion using Malvern Zetasizer[®] Nano ZS (Malvern Instruments S.A., Worcestershire, UK). The liposome preparation was diluted to 1/10 with water to decrease the solvent ionic force and ensure that the solvent used does not interfere with the measurement of the liposome charge.

The results were expressed as the mean \pm standard deviation of three independent liposome preparations.

2.4. Cell Culture

The PC-3 cell line was purchased from ATCC (Manassas, VA, USA). This cell line was originally isolated from a vertebral metastasis stemming from a prostate tumor and entirely composed of carcinoma cells [38]. The fibroblasts used are primary cells from the dermis, and more specifically, foreskin cells. This cell line was purchased from the hospital cell and tissue bank (HCL, Lyon, France). Fibroblasts and PC-3 cells were cultured in a DMEM medium supplemented with 10% (v/v) FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. PC-3 cells were used because of their high metastatic potential (grade IV prostate cancer), and fibroblasts were used because they are considered a classical control line, making them a valuable model of healthy tissue. All cells were cultured in a humidified incubator at 37 °C with 5% CO₂. After standard trypsinization, 78,000 cells of PC-3 and 25,000 cells of fibroblast per well (500 µL) were seeded in 24-well plates and incubated overnight before treatment with the different drug candidates. These cell densities were chosen to obtain a surface coverage of 80% after 72 h of culture. The cell passages for optimal conditions for culture are P5 for fibroblast and P15 for PC-3.

For cytotoxicity experiments with free isoprostanoids, a concentration of 100μ M of each of the 12 molecules was added to the cell culture medium. Tests with liposomes

encapsulating F_{4t} -NeuroP were performed with a final concentration of 50 μ M F_{4t} -NeuroP in the medium. This corresponded to a lipid concentration of 2.5 mg/mL.

After 5 h of contact with the different molecules, the plates were washed with PBS and cultured in a fresh medium. The plates were then incubated again for 72 h at 37 $^{\circ}$ C in a humidified incubator with 5% CO₂ before performing the cell viability measurements.

2.5. Cell Viability Assay

The cell viability measurements were performed using the resazurin assay. This method is based on the assessment of the metabolic capacity of the cell population after drug exposure. It is based on the conversion of Alamar blue (resazurin) to a resorufin compound in living cells. Resazurin can pass through the cell membrane into the cell, where it is reduced and transformed into a pink, fluorescent compound that is called resorufin. Dead cells that cannot reduce resazurin because they lack metabolic activity will not be able to generate a fluorescent signal [39]. This viability assay is a rapid and sensitive measurement of the metabolic activity of cells. The compound used for this viability test, Alamar blue, does not degrade exposed cells. The advantage of using this non-toxic compound is that it allows the cells to be re-cultured or other tests to be carried out in parallel [40].

A culture medium containing resazurin at a concentration of 0.03 mg/mL was prepared and preheated at 37 °C. The culture medium was removed, and the plates were rinsed with warm PBS. Then, 300 μ L of a warm resazurin medium was added and incubated at 37 °C, with 5% CO₂ for 40 min. Finally, in a 96-well black-bottom plate, 200 μ L of each solution was added, and the fluorescence (excitation at 550 nm and emission at 590 nm) was read on an Infinite-M200 pro Plate reader (TECAN, Männedorf, Switzerland). The activity of the cells treated with Triton corresponded to 0% viability, and the untreated control corresponded to 100% viability. The results were expressed as the mean \pm standard deviation of three independent experiments, and the percentage of viability was calculated as indicated in Equation (4).

$$\% \text{ cells viability} = \frac{IntensityF_{Sample} - IntensityF_{Triton}}{IntensityF_{Control} - IntensityF_{Triton}}$$
(4)

2.6. Statistical Analysis

All tests were performed in triplicate (n = 3). Thus, from the data, we calculated the standard deviation (SD) corresponding to the average amount of variability in the dataset. The results are expressed as the mean +/- SD of three independent replicates.

Student's *t* test was also carried out to compare the means of two samples. *** p < 0.001; ** p < 0.01; and p < 0.05.

3. Results

3.1. Cytotoxic Effect of 12 Free Isoprostanoids

First, a comparative study on several isoprostanoids was performed to identify effective antitumor molecules on different cancer cell lines. In previous studies, several classes of oxylipins derived from different polyunsaturated fatty acids may have been attributed with potential antitumor activity. For instance, PhytoPs have revealed their cytotoxic effect on breast cancer cells [28]. In the present report, we carried out experiments on 12 isoprostanoids, including PhytoPs (Figure 1A), NeuroPs derived from docosepentaenoic acid (Figure 1B), NeuroPs derived from docosahexaenoic acid (Figure 1C) and IsoPs (Figure 1D), and measured their cytotoxic effect on PC-3, a highly aggressive prostate cancer line, and fibroblasts, a non-tumoral cell line (Figure 1E). (A) : -<u>Phytoprostanes from α -linolenic acid (ALA)</u>:



42

2

Fibroblast PC-3

P25 996

PPA

0

control



3 20 $\langle \rangle$

NA S

The isoprostanoid sensitivity of the different cell lines was measured by estimating cell metabolic activity using a resazurin assay as a marker of cell viability. Cells were exposed for 5 h to a concentration of 100 μ M of each isoprostanoid dissolved in DMSO. After 5 h, the cells were washed with PBS to remove all liposomes that had not fused with the cells and then incubated again for 72 h to allow the drug to act on the cell metabolism. Then, the medium was removed, and the cells were incubated with a fresh medium containing resazurin. Depending on the percentage of metabolically active cells, resazurin is transformed into a blue-colored compound, resorufin. The absorbance was measured, and a cell viability % was calculated with respect to the control.

The presence of isoprostanoids did not affect the viability of the fibroblasts, except for the N2 sample (approximately a 40% decrease in viability). A decrease of at least 30% of the viability of the PC-3 prostate cancer cells was observed in the presence of all the molecules tested. The most significant decrease in PC-3 cell viability was observed in the presence of the N6 molecule: over a 70% loss in viability for the cancer line. N6, further denoted as F4t-NeuroP, was selected for liposome encapsulation. F4t-NeuroP is a derivative of docosahexaenoic acid (DHA). Over the past few years, DHA has been shown to be implicated in numerous biological mechanisms and have cardioprotective

or anti-arrhythmic properties. Moreover, it has already been established that F4t-NeuroP is implicated in molecular mechanisms such as the protection of the ryanodine receptor, a form of intracellular calcium channels implicated, for example, in colorectal cancer metastasis [41], or induces antiproliferative effects in the human breast cancer cell line (MDA-MB-231) [28].

3.2. Encapsulation of F4t-NeuroP in Liposomes

Once the cytotoxic activity of F4t-NeuroP was evaluated in its soluble form, this compound was encapsulated in liposomes of a distinct membrane fluidity.

Membrane fluidity is related to the motional capacity of membrane components. This is dictated by lipid chains and their capacity to interact with each other. Therefore, the membrane fluidity was modulated according to the nature of the acyl chains (length) and/or the number of unsaturations of the phospholipids that constituted the liposome membranes. Based on previously published studies [26,27], two liposome samples with different membrane fluidities were prepared: a rigid one composed of 1,2-dipalmitoyl-snglycero-3-phosphatidylcholine (DPPC), further denoted as DP, and a fluid one composed of 1-pamitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), further denoted as PO. DPPC, which possess long saturated acyl chains (16:0), forms rather rigid membranes at 37 °C. The order/disordered transition temperature for DPPC membranes is 40 °C. This means that at the working temperature, membranes composed of DPPC are in a rigid/ordered state. On the other hand, PO liposomes are composed of 80% POPC, which contains one saturated (C16:0) and one unsaturated (C18:1) acyl chain. The cis double bond induces chain torsion and limits the possibility of interactions between lipid molecules in the bilayer. Therefore, the order/disordered transition temperature for POPC membranes is 4 °C, and at 37 °C, the liposome membrane becomes fluid. In addition, 20% of a fusogenic lipid was added to promote inverted hexagonal phase intermediates that favor membrane fusion, as described in the Materials and Methods Section 2.

F4t-NeuroP was dissolved in chloroform and added to the lipid mixture at a final concentration of 200 μ M for a lipid concentration of 10 mg/mL (lipid/drug molar ratio of 69:1). The chloroform was evaporated, and the lipid-F4t-NeuroP film was resuspended in PBS. The liposomes were prepared as described in the Materials and Methods section with a conventional freeze/thaw protocol, followed by an extrusion at 100 nm. The different liposome compositions are listed in Table 1 (Materials and Methods). Free F4t-NeuroP was removed with SEC.

The UV spectrum of the F4t-NeuroP dissolved in PBS showed an absorbance shoulder at 240 nm (Figure 2A). Therefore, the absorbance of the SEC elution fractions was measured at this wavelength, and the F4t-NeuroP elution profile was plotted (Figure 2B). A major elution peak was obtained at around a 3 mL elution volume, which roughly corresponded to the column void volume. This peak was attributed to the elution of the liposomecontaining fraction. The free F4t-NeuroP was eluted in 5 to 10 mL fractions. Using a standard calibration curve of the F4t-NeuroP dissolved in the buffer, we calculated the F4t-NeuroP concentration of each sample. Since the liposome-containing fractions presented a high turbidity due to liposome particle light diffusion, the encapsulation efficiency (%) was calculated by subtracting the total amount of F4t-NeuroP added before the liposome preparation from the free F4t-NeuroP (fractions 5 to 10).

The results show approximately 85% and 86% encapsulation efficiency for the samples PO-F4t-NeuroP and DP-F4t-NeuroP, respectively (Figure 2C).



Figure 2. Absorbance spectrum of F4t-NeuroP in the UV region (**A**). Elution profile of PO and DP F4t-NeuroP from SEC column: absorbance at 240 nm vs. elution volume (**B**). Encapsulated and free F4t-NeuroP concentrations (**C**).

3.3. Liposome Characterization

To assess the physico-chemical properties of the liposomal suspension for in vivo administration, the size and the polydispersity of the liposomes containing or not containing F4t-NeuroP were measured via dynamic light scattering (Figure 3A).



Figure 3. Liposome average size, polydispersity index (PDI) (**A**) and zeta potential (**B**) in the absence or presence of F4t-NeuroP. Results are expressed as mean \pm SD of three replicates.

The F4t-NeuroP liposomes had a diameter of about 150 nm with a PdI varying between 0.05 and 0.2. These results suggest that the size of the PO and DP liposomes was uniform, close to the extrusion size, and that the preparations are monodisperse with a PdI < 0.2. No significant change in the liposome diameter was observed with or without F4t-NeuroP. In terms of charge, the addition of F4t-NeuroP increased the overall charge of both liposome preparations, -24 mV for PO-F4t-NeuoP and -27 mV for DP-F4t-NeuroP (Figure 3B). This can be explained by the insertion of F4t-NeuroP into the bilayer of the liposomes, exposing its negative charge to the solvent.

3.4. Liposome Membrane Fluidity Assessment

As previously reported, the interaction of liposomes with cancer lines can be modulated according to the carrier's membrane fluidity. To check if the addition of the F_{4t}-NeuroP derivative alters the liposome membrane fluidity, this parameter was measured using a fluorescent probe, namely Dioll, that is spontaneously inserted in the bilayer, and its fluorescence emission is related to the change in viscosity of its environment. The fluorescence emission spectra (from 400 to 600 nm) of the liposomal preparations containing F_{4t}-NeuroP obtained with Dioll at 37 °C with an λ ex of 390 nm are shown in Figure 4A.



Figure 4. Evaluation of liposome membrane fluidity. Fluorescence emission spectra of Dioll inserted in liposomes containing or not containing F_{4t} -NeuroP at 37 °C (λ exc of 390 nm) (**A**). GP formula (**B**) and values calculated for each liposome composition: top—liposomes without F4t-NeuroP, bottom— F_{4t} -NeuroP-containing liposomes (**C**).

For the PO fluid liposomes, the Dioll spectrum has a predominant peak at 490 nm, characteristic of a liquid crystalline state. In contrast, the rigid DP liposomes exhibit a Dioll spectrum with a predominant peak at 440 nm. The presence of F_{4t} -NeuroP changes this profile with an increase in the fluid phase contribution. This modification is particularly important for the DP- F_{4t} -NeuroP preparation. In this case, two peaks (440 and 490 nm) of nearly equal contributions are observed.

The GP parameter, as the numerical index of membrane fluidity, was calculated from the fluorescence emission spectra described above (Figure 4B). Both the PO and PO- F4t-NeuroP liposomes have membranes in a fluid state, as shown by the negative GP values of -0.18 and -0.20, respectively. The DP liposomes have a high GP of 0.4 in the absence of F_{4t}-NeuroP, characteristic of a rigid membrane state. This value strongly decreases for the DP- F_{4t}-NeuroP preparation with a GP of 0.01.

A comparison of these GP values with those of the liposomes lacking F_{4t} -NeuroP shows that the addition of F_{4t} -NeuroP increases the overall fluidity of liposomes, with the GP value being lower in the presence of F_{4t} -NeuroP than in its absence, with a most prominent effect on the rigid DP liposomes. It can be assumed that F_{4t} -NeuroP is inserted into the membrane of liposomes between the acyl chains, thus decreasing their ordering capacity. Since the active molecule is capable of modifying the liposome fluidity, it is important to measure it in a systematic way.

3.5. Cytotoxicity Effect of F_{4t} -NeuroP Liposomes on PC-3 Prostate Tumor Cells

Finally, the effect of F_{4t} -NeuroP encapsulated in liposomes was evaluated by a test of cytotoxicity on a prostate cancer cell line using membrane fluidity as a physicochemical parameter. The cells used were of the PC-3 human prostate cell line derived from bone



metastasis and of high tumor aggressiveness. In parallel, a fibroblast cell line was used as a non-tumoral control. The results are presented in Figure 5.

Figure 5. Cellular viability of fibroblasts and PC-3 prostate cancer lines (grade IV bone metastasis) in the presence of 50 μ M of F4t-NeuroP and in the presence of 50 μ M of liposomes—F_{4t}-NeuroP. Results are expressed as mean \pm SD of three independent replicates. *** *p* < 0.001; ** *p* < 0.01; Student test.

The inhibitory effect of free F_{4t} -NeuroP was tested at a concentration of 50 μ M in DMSO on the fibroblast and PC-3 lines. The viability of the PC-3 cancer lines decreased by 25% in the presence of 50 μ M of DMSO with respect to the untreated control. Conversely, the fibroblast viability was not affected by the presence of F_{4t} -NeuroP.

The effect of the PO and DP- F_{4t} -NeuroP encapsulated on the PC-3 and fibroblast cell lines was tested at a final concentration of 50 μ M of F_{4t} -NeuroP. The effect on cell viability is highly dependent on the type of liposome and, more especially, on the liposomal membrane fluidity. Rigid DP- F_{4t} -NeuroP liposomes have a reduced efficiency on the cancer cell line (80% of viability remaining) and a higher efficiency on fibroblasts (65% of viability remaining). For fluid liposomes, PO- F_{4t} -NeuroP liposomes have the opposite behavior, with 60% remaining viability for PC-3 and 120% for fibroblasts. These results are consistent with previous studies revealing that liposomes with fluid membranes fuse more easily with cancer cells [27]. Moreover, the encapsulation of F_{4t} -NeuroP in the PO liposomes further increased the effect on cell metabolic activity with respect to the free molecules at a similar concentration of 50 μ M.

4. Discussion

In vivo, most oxylipins are biosynthesized by enzymes such as cyclooxygenase. Yet, in 1990, a new class of oxylipins was described, namely the non-enzymatically oxygenated polyunsaturated fatty acids (NEO-PUFAs) [42]. NEO-PUFAs are mediators of physiological and pathophysiological processes such as vasoconstriction, anti-arrhythmic processes and cell proliferation.

The therapeutic potential of oxylipins has been the subject of numerous studies, and applications have been found in the treatment of Parkinson's disease (Apokyn[®]), hereditary tyrosinemia (Orphadin[®]) and cancers [43]. In the present work, 12 non-enzymatically oxidized oxylipins, termed isoprostanoids, were tested to study their potential anticancer activity. Their effect on cell metabolic activity was tested on the highly aggressive PC-3 cancer cell line. All the molecules tested showed an effect on the cancer cell metabolic activity. Therefore, this class of oxylipins may have therapeutic potential either alone or in conjunction with other chemostatics [28]. Of note, all the isoprostanoids tested, except one, show little effect on fibroblasts, which may suggest some extent of tumor selectivity.

 F_{4t} -NeuroP was the molecule with the most important inhibitory action on PC-3. Based on the concept of the membrane physicochemical properties of liposomes as a means for

specific drug delivery, we encapsulated F_{4t}-NeuroP in two liposome samples of opposite membrane fluidities, the PO fluid and DP rigid samples.

The use of liposomes as drug delivery nanosystems allows for the encapsulation of a wide range of molecules (hydrophobic and hydrophilic) with therapeutic potential activities. There are two modes for the incorporation of molecules into liposomes: a so-called passive encapsulation, where the hydrophilic molecules are dissolved in the aqueous phase used to hydrate the lipid film and the hydrophobic molecules are dissolved in the organic solvent; and an active encapsulation, where the use of a pH gradient is sometimes necessary to encapsulate hydrophilic molecules with protonable chemical functions [44]. F_{4t}-NeuroP was introduced in the lipid mixture prior to solvent evaporation. After the liposome formation process and the removal of non-encapsulated molecules, an evaluation of the encapsulation efficacy was performed. Good encapsulation yields were systematically obtained: 85% of the active molecule was systematically associated with the liposomal fraction, whatever the phospholipid composition of the liposomes.

Before testing the inhibitory action of the liposomes loaded with F_{4t} -NeuroP, these liposomes were characterized in terms of their size, PdI, zeta potential and membrane fluidity with a view to their use for in vivo administration. The addition of F_{4t} -NeuroP does not influence the average size of the liposomes or their dispersity. In terms of charge, the addition of F_{4t} -NeuroP increases the overall negative charge of the liposomes. Of note, F_{4t} -NeuroP possesses a negative charge at a pH of 7.4 due to the presence of a carboxylate group (Figure 6). By inserting it into the liposome membrane, F_{4t} -NeuroP probably gives them negative charges. We can therefore assume that F_{4t} -NeuroP is able to be accommodated in the membrane of the liposome between lipids.





Figure 6. Structure of F_{4t} -NeuroP (**A**). Schematic representation of a liposome encapsulating neuroprostane (**B**).

This assumption is further supported by the increased membrane fluidity of the liposomes prepared in the presence of F_{4t} -NeuroP-containing preparations. This increase, limited in the case of fluid PO preparation, is particularly important in the case of rigid DP preparation. In this case, the insertion of F_{4t} -NeuroP between lipids most probably hinders chain ordering in the DP membrane of liposomes, resulting in increased membrane fluidity.

It is important to note that the presence of charges in liposomes (positive or negative) reduces the aggregation phenomena between liposomes thanks to the increase in the electrostatic repulsive forces between them. Moreover, the charge of liposomes has a preponderant impact on their recognition by opsonins in the bloodstream. Particles with too-high positive or negative charges will be eliminated more quickly than uncharged particles [45]. It is thus necessary to obtain a composition sufficiently charged to be stable but not excessively charged to avoid in vivo recognition by opsonins and rapid clearance from the body. Therefore, the moderate negative charge of PO-F_{4t}-NeuroP liposomes is highly compatibility with an in vivo application.

The resazurin viability assays showed the specificity of the action of $PO-F_{4t}$ -NeuroP on PC-3 cells, with a remaining viability of 40%. This notable effect confirms that liposomes with fluid membranes specifically fuse with cancer cells [26,27]. According to the literature,

cancer cells have a modified metabolism compared to normal cells. Indeed, tumor cells present an alteration of their lipid profile [21] that is expressed by an increase in unsaturated fatty acid levels and thus makes the cell membrane more fluid [46]. Thus, fibroblasts are expected to be globally more rigid, whereas PC-3 cells have a metastatic tumor cell line and are globally more fluid. Membrane fluidity determines mobility of the lipids, proteins and water molecules that cooperate in the reorganization and assembly required and induced by the membrane fusion [47,48]. Thus, modulating the lipid composition of liposomes could be an effective and specific therapeutic agent to target cancer cells and to exploit the general tendency toward an increased membrane fluidity in tumor cells. The correlation in membrane fluidity between the liposomes and the targeted cancer cell will facilitate membrane fusion and thus the release of the molecule of interest into the target cell.

At this point, it is difficult to assess the cellular target of the molecules. Cancer cells can exhibit properties different from fibroblasts or other non-tumor cells such as proliferative signaling, an evasion of growth suppressors, a resistance to apoptosis, replicative immortality, copious angiogenesis, active invasion and metastasis. Several parented molecules target apoptotic mechanisms, but a further analysis is needed to reveal oxylipin cellular targets.

Alternatively, several studies show that prostate cancer cells and PC-3 cells, in particular, show different lipid profiles [49,50]. Moreover, in a previous study, we showed that the membrane and cell viscosity of PC-3 were different from those of the non-tumor control [22]. We can argue that the PC-3 membrane may be more loosely packed than that of fibroblasts. Therefore, hydrophobic molecules such as F4t-NeuroP may enter the PC-3 membrane, but not that of fibroblasts, and thus exert a differential action.

This hypothesis is supported by the experimental results from Figure 4 in the manuscript, which show an effect of F_{4t} -NeuroP on fibroblasts presented to cells in its encapsulated DP form, a rigid type of liposomes that are uptaken by this type of cell.

This intrinsic specificity may be increased by the use of an appropriate delivery system. In this report, the selective drug delivery is based on the correlation between the fluidity of the cell and that of the liposome membrane. The similarity in membrane fluidity between the PO liposomes and PC-3 cancer cells may facilitate membrane fusion and preferential targeting by the fluid liposomes.

Altogether, by showing a cytotoxic effect on PC-3, a highly aggressive tumor line, F_{4t} -NeuroP may open the way for new derivatives of therapeutic interest. This family of molecules may provide a structural basis for the conception of new antitumor drugs. In the present report, we show that F_{4t} -NeuroP can be easily encapsulated in liposomes, with the PO- F_{4t} -NeuroP liposome representing a promising strategy to promote specific drug delivery and potentiate antitumor activity. PO- F_{4t} -NeuroP liposomes constitute an interesting example of drug carriers, with low manufacturing costs, simple preparation protocols and a proven specificity of action on cancer cells. Based on these encouraging results, studies on biodistribution, stability and tumor accumulation, as well as storage stability and the drug release rate, must be carried out to confirm the translational potential of the preparation of these liposomes.

Author Contributions: Conceptualization, O.M., A.G.-E., T.D. and A.A.; investigation, A.A., C.T., G.L., A.B., C.O., V.B.-P. and A.G.; writing—original draft preparation O.M. and A.A.; writing—review and editing A.G.-E., O.M., T.D., J.-M.G., T.G. and E.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data can be shared up on request.

Conflicts of Interest: The authors declare no conflict of interest.

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Biochimie xxx (xxxx) xxx

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Tailor-made vincristine-liposomes for tumor targeting

Ariana Abawi ^a, Ana-Maria Trunfio-Sfarghiu ^b, Céline Thomann ^a, Emma Petiot ^a, Giovanna Lollo ^c, Thierry Granjon ^a, Agnès Girard-Egrot ^{a, **}, Ofelia Maniti ^{a, *}

a Institute of Molecular and Supramolecular Chemistry and Biochemistry, ICBMS UMR 5246, Univ. Lyon, University Lyon 1, CNRS, 69622, Lyon, France

^b University of Lyon, INSA de Lyon, LaMCoS, CNRS, UMR 5259, 69261, Lyon, France

^c Laboratoire D'Automatique, de Génie des Procédés et de Génie Pharmaceutique, LAGEPP UMR 5007, University Lyon 1, CNRS, 69622, Lyon, France

ARTICLE INFO

Article history: Received 31 May 2024 Received in revised form 22 July 2024 Accepted 30 July 2024 Available online xxx

Handling Editor: C. Forest

ABSTRACT

To ensure selective targeting based on membrane fluidity and physico-chemical compatibility between the biological membrane of the target cell and the lipid membrane of the liposomes carriers. Lipid-based carriers as liposomes with varying membrane fluidities were designed for delivering vincristine, an antitumor compound derived from Madagascar's periwinkle. Liposomes, loaded with vincristine, were tested on prostate, colon, and breast cancer cell lines alongside non-tumor controls. Results showed that vincristine-loaded liposomes with fluid membranes significantly decreased the viability of cancer cell lines compared to controls. Confocal microscopy revealed the intracellular release of vincristine, evidenced by disrupted mitosis-specific labeling of actin filaments in metastatic prostate cell lines. This highlights the crucial role of membrane fluidity in the development of lipid-based drug carriers, offering a promising and cost-effective option for targeting cancer cells as an alternative to conventional strategies.

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1. Introduction

According to estimates from the World Health Organization, the second most common cause of death worldwide is cancer. The International Agency for Research on Cancer (IARC) estimated that 19.3 million new cases of cancer and nearly 10 million cancer deaths occurred in 2020. Most commonly diagnosed cancer is female breast cancer, which recently surpassed lung cancer, with an estimated 2.3 million new cases (11.7 %) followed by lung (11.4 %), colorectal (10 %), prostate (7.3 %), and stomach (5.6 %) cancer [1]. Cancer is caused by an anarchic proliferation of cells which rapidly progress and reach an uncontrollable stage after onset [2]. Various therapeutic options have emerged in the past century for treating cancer, such as surgery, chemotherapy, radiation, immuno, hormone or gene therapy. These treatments used as the first-line

treatment option for most cancers, have their limitations. Most of them fail to control metastatic tumors that have spread to distant organs. The non-specific targeting of cancer cells by drugs leads to various side-effects on healthy tissues [3]. In addition, some anticancer drugs, encounter drawbacks as pharmacokinetic and pharmacodynamic alterations [4], poor aqueous solubility, severe toxicity to normal cells or development of multiple drug resistances [5,6]. Alternative therapeutic approaches are therefore needed.

Nanocarriers or nanostructured systems have long been known as Smart Drug Delivery System for targeting a specific sites [7]. They are designed to achieve the desired drug concentration limiting side effects arising from off-target action [8], to improve drug solubility and prolong their plasma half-life and to reach tumor tissue bypassing the immune system's recognition [3]. The use of nanoparticles (NPs) is more advantageous than the administration of free drug, as it allows, for example, the functionalization of the vector surface [9] and the delivery of several drugs at the same time in order to enhance therapeutic responses [10]. In addition to the therapeutic aspect, recent studies have highlighted the ability of NPs to diagnose tumors as an imaging agent [11,12].

Over the course of the last half-century, different types of organic and inorganic nanocarriers have been successfully introduced for the treatment of cancer, pain and infectious diseases

https://doi.org/10.1016/j.biochi.2024.07.017

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Please cite this article as: A. Abawi, A.-M. Trunfio-Sfarghiu, C. Thomann *et al.*, Tailor-made vincristine-liposomes for tumor targeting, Biochimie, https://doi.org/10.1016/j.biochi.2024.07.017



^{*} Corresponding author.

^{**} Corresponding author.

E-mail addresses: ariana.abawi@etu.univ-lyon1.fr (A. Abawi), ana-maria. sfarghiu@insa-lyon.fr (A.-M. Trunfio-Sfarghiu), celine.thomann@univ-lyon1.fr (C. Thomann), emma.petiot@univ-lyon1.fr (E. Petiot), giovanna.lollo@univ-lyon1.fr (G. Lollo), thierry.granjon@univ-lyon1.fr (T. Granjon), agnes.girard-egrot@univlyon1.fr (A. Girard-Egrot), ofelia.maniti@univ-lyon1.fr (O. Maniti).

[11,13]. The eight most reported nanocarriers are: (i) liposomes, (ii) micelles, (iii) dendrimers, (iv) meso-porous silica nanoparticles, (v) gold nanoparticles, (vi) super paramagnetic iron oxide nanoparticles, (vii) carbon nanotubes and (vii) quantum dots [14]. Liposomes are the longest-serving nanocarriers for drug delivery purposes [12].

Liposome are vesicles composed by a bilayer lipid membrane, which contain natural or synthesized phospholipids. These types of vectors are suitable for drug delivery due to their amphiphilic nature: hydrophilic internal aqueous cavity and hydrophobic membrane [15]. In 1971, Gregory Gregordians and Brenda Ryman were the first to highlight the drug carrier potential of liposomes when they successfully entrapped *Aspergillus niger* amyloglucosidase in liposomes [16].

The first liposome-based treatment was Doxil® (OrthoBiotech), which encapsulates doxorubicin and was approved by US Food and Drug Administration (FDA) in 1995. Doxil has been used for the treatment of Kaposi's sarcoma an ovarian cancer [17]. Subsequently, a number of other liposomal formulations have been introduced in the clinics. The best-known approved anti-cancer formulations include Depocyt® (Cytarabine/Ara-C), DaunoXome® (Daunorubicin), Mepacet® (Mifamurtide) and Onivyde® (Irinotecan) [18]. Application of liposome formulation are not limited to anti-cancer therapies: Amphotec® (fungal diseases), Exparel® (analgesia), Inflexal® (viral vaccines) [4]. All these carriers currently used, clinically reach the target site passively (targeting both normal and diseased cells). To increase the accumulation of liposome drugs in tumors active targeting strategies have been proposed [19]. This can be achieved by targeting overexpressed cancer surface receptors or molecules in the tumor microenvironment [20]. Moieties grafted onto liposome surface include antibodies, proteins, peptides, vitamins, aptamers, etc. Long time considered the "third" generation of liposomes, antibodies-grafted liposomes have shown certain limitations due to the complexity of their design and their high production costs.

In this study, we utilized the membrane fluidity of liposomes as a potential strategy for targeting cancer cells to achieve specific drug release. Biological membranes are composed of lipids and proteins that enable cell compartmentalization and are responsible for many important cellular functions [21]. This is why many pathologies result in an abnormal lipid composition of cell membranes, which modifies their dynamics [22]. Dysregulation of lipid metabolism in cancer cells frequently leads to disruption of membrane compaction, influencing parameters such as membrane fluidity.

The relationship between the metastatic potential of cells and the alteration of their membrane fluidity has been suggested for many years. Work carried out by Dr Nakazawa since the 1970's has shown that the percentage of unsaturated fatty acids promoting membrane fluidity was higher in metastatic lesions than in primary lesions [23]. In prostate cancer cells, we previously showed that the membrane is less stiff, less viscous and thus, more prone to deformation than in the control cell line [24]. The modification in membrane fluidity may also be due to altered cholesterol levels. Cholesterol is an essential structural component of membrane that has been shown to be functionally important in tumorigenesis [25]. Differences in the fluidity membrane of purified plasma membrane of leukemic cells and normal thymocyte by fluorescence polarization, due by low contents of cholesterol and sphingomyelin in contrast of the high amount of unsaturated fatty acyl chains were previously reported [26]. Reduced expression of the ceramide synthase-6 enzyme involved in sphingolipid synthesis for instance induces a decrease in the compaction of tumor cell membranes [27].

By modulating the lipid composition of liposomes to match their

membrane fluidity with that of the tumor plasma membrane they efficiently target prostate tumor cells being internalized through membrane fusion mechanisms [28]. Liposomes of controlled membranes fluidity were successfully used to deliver Monomethyl Auristatin E (MMAE) and 4 (*RS*)-4-F_{4t}-Neuroprostane to the prostate tumor cell line PC-3 [29,30]. Other reports have focused on the specific targeting of tumor cells using hybrid liposomes, with the ability to modulate their membrane fluidity [31,32].

In this report, we encapsulated vincristine in liposomes of distinct membrane fluidity, evaluated by the fluorescence of radiometric fluorophore. This vinca alkaloid is derived from the Madagascan periwinkle plant Catharanthus roseus, known as Vinca rosea [33]. This molecule was purified in the late 1950s and early 1960s by Svoboda et al. This drug stops mitosis by inhibiting tubulin polymerization [34]. Vincristine binds tubulin and prevents its polymerization into microtubules [35]. The absence of microtubules disrupts chromosome segregation by the mitotic spindle triggering apoptosis This cytotoxic molecule has extensive antitumor activity, on various tumor cell lines [34] including leukemia, lung, colon ovarian, prostate, breast carcinomas [NCI Cancer Screen Data for NSC 67574 (vincristine)]. Its first approval by the U.S. Food and Drug Administration (FDA) dates back to 1963 for the treatment of acute leukemia in children. The efficacy of vincristine depends on its concentration and duration of exposure [36]. However, its use is associated with peripheral neuropathy representing a significant side effect. The therapeutic effect of vincristine can be enhanced by encapsulating it in appropriately designed liposomal systems [33] to decrease the drug toxicity [37]. In this study, vincristine was encapsulated in three types liposomes of different membrane fluidity, assed using an in-house synthesized fluorophore, Dioll, to specifically target cancer cells. The use of vincristine in this study resulted in a high encapsulation rate (greater than 65 %) of the molecule within the liposome and notably, the fluid liposome containing vincristine showed significant cytotoxic effects on prostate, colon and breast, tumor cell lines while sparing the viability of non-tumor cells.

2. Materials and methods

2.1. Materials

Materials used for liposome preparation including lipids, polycarbonate membranes, extruder and syringes, were obtained from Avanti Polar Lipids (Alabaster, Al, USA). Vincristine was purchased from Chemodex (St Gallen, Switzerland). All materials needed for cell culture including Fetal Bovine Serum (FBS), Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, Mesenchymal Stem Cell Growth (MSC-GRO) medium, Eagle's Minimum Essential Medium (EMEM), Minimum Essential Medium (MEM), phosphate buffered saline (PBS) solution, dimethyl sulfoxide (DMSO), paraformaldehyde (PFA), citric acid, sodium hydrogen phosphate and resazurin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The materials used for labeling of cellular actin filaments, Alexa Fluor 546 Phalloidin and 4',6diamidino-2-phenylindole (DAPI) were purchased from Thermofisher (Waltham, Massachusetts, USA).

2.2. Liposome preparation

The physicochemical properties of liposomes were modulated according to their composition in natural glycerophospholipids. We prepared 3 different compositions of liposomes with opposite membrane fluidity. From the most rigid to the most fluid: DP composed of 80 % (1,2-dipalmitoyl-glycero-3-phosphocholine) (DPPC) and 20% of fusogenic lipid DOPE (1,2-dioleoyl-sn-glycero-3-

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phosphoethanolamine), DO of 80 % DOPC (1,2-Dioleoyl-*sn*-glycero-3-phosphocholine) and 20 % DOPE, and PO of 80 % POPC (1palmitoyl-2-oleoyl-*glycero*-3-phosphocholine) and of 20 % DOPE.

The liposome preparation protocol was based on the hydration of a lipid thin-film method. In brief, phospholipids were solubilized in chloroform at a lipid concentration of 5 mg/mL. The volume in the flask was adjusted to a minimum of 500 µL to obtain a homogeneous distribution of the lipid film. The chloroform was then evaporated using a rotary evaporator until a uniform film was obtained. Evaporation was carried out under vacuum, with a rotating evaporator in a water bath set at a temperature above the lipid phase transition temperature (Tm). The uniform lipid film obtained, was hydrated with 1 mL of PBS (10 mM phosphate, 137 mM NaCl and 2.7 mM KCL, pH 7.4) and vigorously agitated. To break the MultiLamellar Vesicles (MLVs) formed, a physical treatment was applied to liposomes This treatment consists in a succession of 6 freeze/thaw cycles in liquid nitrogen to weaken the various bilayers and then obtain Large Unilamellar Vesicles (LUVs). The LUVs sizes were adjusted using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA) by extruding 21 times through a 400 nm, followed by a 100 nm porous membrane. The liposomes, were stored at 4 °C for 4 weeks without further extrusion.

2.2.1. Preparation of liposomes encapsulating vincristine

The entrapment of vincristine in liposomes was realized at a molar ratio of 66:1. For vincristine loading, liposomes were prepared in 300 mM citrate buffer pH 4 instead of PBS. Vincristine was encapsulated in performed liposomes using the pH gradient-dependent remote loading technique [38]. Liposomes were mixed with a commercially available solution of 100 μ M vincristine at pH 7 (Chemodex, Switzerland). Then, 1M – Na₂HPO was added and all samples were incubated at 60 °C for 10 min with intermittent mixing.

Excess/free vincristine was removed from liposome preparation by using a size exclusion chromatography (SEC, PD-10 columns, SephadexTM G-25 M, GE Healthcare (Limonest, France)). The column was first rinsed and equilibrated with 10 mL PBS. Then 1 mL of vincristine/liposome mixture was loaded and eluted with PBS. A succession of 10 fractions was collected. As vincristine showed a maximum absorbance at 295 nm, the absorbance was measured at this wave length for each fraction, and the amount of vincristine was estimated relative to vincristine standard curve. The encapsulation ratio of vincristine was determined from the amount of free (non-encapsulated) molecule. All measurements were performed in triplicate. The encapsulation efficiency (EE) and the drug loading (DL) were calculated according equations (1) and (2). zeta potential (charge) and fluidity in order to examine the potential influence of these parameters on their effectiveness.

2.3.1. Size and zeta potential analysis of blank and vincristineloaded liposomes

Size and size distribution of liposomes were performed using dynamic light scattering (DLS) with Zetasizer Nano-S (Malvern, England). All liposome preparations were diluted in distilled water at a concentration of 0.1 g/L prior to the measurements. The Z-average diameter (*i.e.* the intensity-weighted mean hydrodynamic size) and poly-dispersity index (PDI) were determined for each preparation with and without vincristine. The analyses were carried out at 25 °C with an angle of detection of 173°.

The ζ potential values, deduced from the electrophoretic mobility of the liposomes were also measured using the Malvern Zetasizer. Samples were diluted to 1:10 with water to decrease the solvent ionic force and ensure that the solvent used did not interfere with the measurement of liposome charge.

The results were expressed as the mean±standard deviation (SD) of three independent liposome preparations.

2.3.2. Fluidity characterization of blank and vincristine loaded liposomes

The membrane fluidity of liposomes was measured using an inhouse synthesized fluorescence probe, named Dioll, with a FP-8500 spectrofluorometer (JASCO Applied Science, Halifax, Canada).

Dioll was added to the liposome suspension (0.1 mg/mL) at a final concentration of 0.4 μ M. The fluorescence emission spectrum was recorded from 400 nm to 600 nm with an excitation wavelength of 390 nm. Experiments were performed at 37 °C. A numerical index of membrane fluidity named Generalized Polarization (GP) was then calculated, according to equation (3):

$$GP = (I_{440} - I_{490})/(I_{440} + I_{490})$$
(3)

Where I_{440} was the fluorescence emission intensity at 440 nm (characteristic of gel phase) and I_{490} was the fluorescence emission intensity at 490 nm (characteristic of liquid crystalline phase).

The results were expressed as the mean±SD of three independent liposome preparations.

2.4. Cell culture

Fibroblasts, prostate cell lines including WPMY-1, CAF-prostate, LNCaP, PC-3, colon line including HT-29, CAF-colon and breast line including HS578T and BT-20 were used as in vitro models. Fibroblasts, primary cells from foreskin, WPMY-1, healthy prostate

$$EE(\%) = \frac{Total \ vincristine \ concentration - free \ vincristine \ concentration}{Total \ vincristine \ concentration} \times 100$$

$$DL(\%) = \frac{Amount of vincristine in liposomes}{Amount of lipids} \times 100$$
 (2)

2.3. Liposomes characterization

Characterization of liposomes were carried out in term of size,

stroma fibroblasts from a 54-year-old male patient and PC-3, bone metastasis cells from a 62-year-old male patient were cultured using Dulbecco's modified Eagle medium (DMEM) (Sigma, Germany). CAF-prostate and CAF-colon, cancer-associated fibroblasts, were cultured using mesenchymal stem cells growth medium (MSC-GRO) (Vitro Biopharma, USA). LNCaP, lymph node metastasis cells and HT-29, colorectal adenocarcinoma cells were cultured using Roswell Park Memorial Institute medium (RPMI) (Thermo-Fisher, USA). HS578T and BT-20, breast carcinoma cells, were

cultured using Minimum Essential Medium (MEM) (ThermoFisher, USA).

All culture mediums were supplemented with 10 % Fetal Bovine Serum (FBS) (Sigma, Germany) except MEM which is supplemented with 5 % FBS.

All cell lines were cultured in a humidified incubator at 37 °C with 5 % CO_2 . Once the cells reached 80 % confluence, the cells were detached with trypsin, centrifuged and seeded into 24-well plates and then, incubated overnight before treatment with the different liposome compositions. Different tests to evaluate the cytotoxic effects of vincristine-loaded liposomes, including "cell actin filament labeling" and "resazurin cell viability assay" are detailed below.

The origin and culture conditions of all cells lines are given in Table 1.

2.4.1. Cell actin filament labeling

PC-3 cells were fixed with 3.7 % paraformaldehyde for 15 min at room temperature and permeabilized with 0.1 % Triton X-100 for 15 min. Actin filament was stained using Alexa Fluor 546 Phalloidin (ThermoFisher) at room temperature for 1 h, and then cells were stained with DAPI for 5 min at 37 °C. Images were collected with a Zeiss explorer confocal microscope composed of an LSM700 confocal head. Image processing was performed using Image J software developed by the National Institute of Health.

2.4.2. Resazurin cell viability assay

Cell viability measurements were performed using the resazurin assay (Alamar Blue). Living cells have the capacity to metabolically reduce via mitochondrial reductases, resazurin a nonfluorescent dye, to the strongly-fluorescent dye resorufin.

A culture medium containing resazurin at a concentration of 0.03 mg/mL was prepared and preheated at 37 °C. After removing the culture medium, the plates were rinsed with warm PBS. Then warm resazurin medium was added and incubated at 37 °C, with 5 % CO₂ for 40 min. Finally, in a 96-well black-bottom plate, 200 μ L of each solution was added, and the fluorescence (excitation at 550 nm and emission at 590 nm) was read on a Berthold plate reader (LB942, Tristar 5). The activity of Triton-treated cells corresponded to 0 % viability, and the untreated control corresponded to 100 % viability. Results were expressed as the mean±standard deviation of three independent experiments, and the percentage of viability was calculated as indicated in equation (4).

$$\% cells viability = \frac{IntensityF_{sample} - IntensityF_{Triton}}{IntensityF_{Control} - IntensityF_{Triton}}$$
(4)

2.5. Statistical analysis

Results were expressed as the mean±SD over at least three independent experiments, each performed in triplicate.

Student's t-test was also realized to compare the mean of two samples. ***p < 0.001, **p < 0.01 and p < 0.05.

3. Results

3.1. Cytotoxic effect of free vincristine

A comparative analysis was conducted to evaluate the efficacy of vincristine as a potential antitumor agent across on different cancer cell lines prior to encapsulation. To this end, it is important to consider that lower concentrations of the cell targeting drug would be more favorable for the perspective of intravenous injection. Consequently, we conducted an initial cell targeting assay at 10 nM, 5 nM, and 1 nM to explore the vincristine's activity at nanomolar concentrations.

The sensitivity of different cell lines to vincristine was assessed by measuring cellular metabolic activity using a resazurin assay as an indicator of cell viability. Cells were exposed to a concentration of 10 nM, 5 nM or 1 nM of vincristine dissolved in sterile PBS. After a 72 h exposure period, to allow the drug to exert its effects on cell metabolism, the fluorescence of resorufin produced was measured, and cell viability percentage was calculated relative to the control (Fig. 1).

Free vincristine exhibited notable effects towards breast cancer cell lines, inducing a decrease of approximately 70 % in viability for HS578T and BT20 cells at a concentration of 10 nM. Varying degrees of targeting of colon cancer cell lines were also observed; while there was a moderate effect on colon cancer-associated fibroblasts (CAFs) with a 20 % reduction in viability at 10 nM, the HT29 line experiences a more pronounced response with a 70 % decrease in viability at the same concentration.

Concerning prostate cells, vincristine demonstrates significant toxicity towards the non-tumor WPMY-1 line, yielding a substantial 70 % decrease in viability, as well as prostate CAFs, exhibiting an 80 % decrease in viability. However, metastatic prostate cell lines such as LNCaP and PC-3 show a slightly less pronounced reduction in viability (around 50 %), although still significant. Additionally, a notable 30 % decrease in viability is observed for fibroblasts.

These findings on the targeting efficacy of free vincristine across various cell lines lay the groundwork for investigating whether encapsulating this drug in liposomes can lead to enhanced targeting of cancer cells.

3.2. Encapsulation of vincristine in liposomes

After assessing the cytotoxic activity of vincristine in its soluble form, we encapsulated this compound within liposomes featuring

Table 1

Cells lines used for the evaluation of the cytotoxic effect of liposomes-vincristine, with name, origin, culture medium, sending rate and passage number.

	Control lines	Prostate lines				Colon lines		Breast lines	
Name Origin	Fibroblast	WPMY-1 Epithelial cells from healthy prostate stroma	CAF Fibroblast associated cancer	<i>LNCaP</i> Lymph node metastasis	<i>PC-3</i> Bone metastasis of grade IV adenocarcinoma	HT-29 Colorectal adenocarcinoma	CAF Fibroblast associated cancer	HS578T Mammary carcinoma Claudin- Low	<i>BT-20</i> Mammary carcinoma Basal-like
Culture medium	DMEM(+)	DMEM(+)	MSC Gro	RPMI	DMEM(+)	RPMI	MSC Gro	MEM(+)	MEM(+)
(cell/cm ²)	15 000	15 000	10 000	000 04	78 000	10 000	10 000	15 000	15 000

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Fig. 1. Cell viability of fibroblast, prostate, colon and breast lines in presence of 10 nM, 5 nM and 1 nM of vincristine. Results are expressed as mean±SD of three independent replicates.

distinct membrane fluidities. As explained above membrane fluidity is intricately linked to the mobility of membrane components, governed by the lipid chains and their ability to interact. To modulate membrane fluidity, we manipulated the acyl chains' nature (length) and/or the number of unsaturations on the fatty acids composing the liposomal membranes. Based on previously published research [28–30], we prepared three distinct liposome samples with varying membrane fluidities: one with rigid composition containing 80 % 1,2-dipalmitoyl-sn-glycero-3phosphatidylcholine (DPPC), denoted as DP, one with an intermediate fluidity containing 80 % 1-palmitoyl-2-oleoyl-glycero-3phosphocholine (POPC), denoted as PO, and another with a fluid composition consisting of 80 % 1,2-dioleoyl-sn-glycero-3phosphatidylcholine (DOPC), denoted as DO (Table 2). All liposomes contained 20 % DOPE. DP, characterized by long saturated acyl chains (16:0), forms rigid membranes at 37 °C, with a transition temperature from ordered to disordered states at 41 °C. This implies that DP membranes are rigid at typical working temperatures. Conversely, PO and DO liposomes are composed with one saturated (C16:0) and one unsaturated (C18:1) acyl chain and two unsaturated (C18:2) respectively. The presence of a *cis* double bond induces chain torsion, restricting interactions between lipid molecules in the bilayer. Consequently, the phase transition temperatures from ordered to disordered states for PO and DO membrane are lowered to -4 °C and -17 °C, resulting in fluidity at 37 °C.

Vincristine was added to liposomes at a final concentration of 100 mM and the pH was increased to 7 b y addition of phosphate buffer. Free vincristine was then removed by size-exclusion chromatography (SEC).

The absorbance of SEC elution fractions was measured at 297 nm [39], and the elution profile of vincristine was plotted (Fig. 2A). A first elution peak was observed at an elution volume of approximatively 5 mL for the DO and DP samples, corresponding to the void volume of the column and the elution of the fraction

Table 2

Composition of liposomes prepared: molar percentage, fatty acid composition, name, structure and phase transition temperature (Tm, melting temperature).

קר			
71	16:0 PC	1,2-dipalmitoyl-glycero-3-phosphocholine	41 °C
		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	
0	16:0-18:1 PC	1-palmitoyl-2-oleoly-glycero-3-phosphocholine	$-4 ^\circ C$
		~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~	
00	18:1 PC	1,2-dioleoyl-glycero-3-phosphatidylcholine	−17 °C
		John of the for the	
All preparations	18:1 PE	1,2-dioleoyl-glycero-3-phosphoethanolamine	
		Contraction of the second seco	
n three preparations			
	O O Il preparations a three preparations	0 16:0–18:1 PC 0 18:1 PC Il preparations 18:1 PE	$ \begin{array}{c} & & & \\ & & \\ & & \\ 0 & 16:0-18:1 \ PC & 1-palmitoyl-2-oleoly-glycero-3-phosphocholine \\ & & \\ & & \\ & & \\ & \\ 0 & 18:1 \ PC & 1,2-dioleoyl-glycero-3-phosphatidylcholine \\ & & \\ & & \\ & & \\ & \\ & \\ & \\ & \\ & $

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В	Encapsulated vincristine concentration		Free vincristine concentration		Encapsulation Efficiency EE%	Standard deviation (SD)	Drug Ioading DL%
	mg/mL	μM	mg/mL	μM			
DO-vincristine	0.07	80	0.016	19	80%	0.5	0.2%
PO-vincristine	0.06	69	0.03	31	69%	0.92	0.4%
DP-vincristine	0,07	81	0,015	19	80%	0.16	0,2%

Fig. 2. Elution profile of DO, PO and DP vincristine from SEC column (absorbance at 295 nm) (A). Encapsulated and free vincristine concentration (B).

containing the excluded liposomes. For PO sample, this major peak is observed at a 4 mL elution volume. A second peak, corresponding to the elution of free vincristine not encapsulated within the liposomes, appears at an elution volume of approximatively 8 mL for PO sample, and approximatively 9 mL for DO and DP samples. The higher proportion of free vincristine observed for the PO sample can be explained either by lower encapsulation of vincristine when the medium is acidic, or by an escape of the molecule after addition of the basic buffer.

Using a standard calibration curve obtained by measuring the absorbance of free vincristine dissolved in the PBS, the vincristine concentration of each sample was calculated. Due to the high turbidity observed in liposome-containing fractions caused by light diffusion from liposome particles, the encapsulation efficiency (EE %; eq. (1)) was determined by subtracting free vincristine concentration determined from (fractions 7 to 10 for DO and DP samples and from fraction 8 for PO sample) from the total concentration of vincristine added before liposome preparation. The results indicated encapsulation efficiencies of approximately 80 % for DO-vincristine and DP-vincristine samples, and 69 % for PO-vincristine (Fig. 2B).

3.3. Physicochemical characterization of liposomes

The hydrodynamic diameter and the zeta potential of liposomes loaded with vincristine were compared to empty liposomes, to assess whether the addition of vincristine affects their physicochemical properties and stability.

The mean diameter of both vincristine-containing and empty liposomes was consistently around 125 nm (Fig. 3A). This suggests that the presence of vincristine does not significantly alter liposome size. Notably, this size closely matches the intended extrusion size

of 100 nm, indicating precise manufacturing processes. Furthermore, it is noteworthy that whatever the lipid composition of the liposomes, a consistent average diameter is obtained. This observation suggests that the size of liposomes remains unaffected by variations in lipid composition, with different phospholipids primarily influencing the bilayer fluidity and organization [40].

Furthermore, the Polydispersity Index (PDI) values for all preparations were below 0.3, indicating a relatively homogeneous dispersion of liposomes. A PDI value below 0.3 on a scale from 0 to 1 signifies high homogeneity within the particle population, while higher PDI values may suggest the presence of multiple populations [41]. This uniform dispersion is crucial for ensuring consistent behavior and performance of the liposomal formulations. The PDI serves as an indicator of the heterogeneity of the sample size distribution. It can be used to distinguish between monodisperse (narrow distribution) and polydisperse (broad distribution) size distributions.

Regarding the particle charge, we observed that the overall charge of liposomes remained approximately -12 mV whatever the composition (Fig. 3B). This indicates that the addition of vincristine did not significantly influence the charge of the liposomes. Maintaining a stable, slightly negatively charged surface is generally advantageous for the performance of liposomal drug delivery systems.

Globally, the DLS and ζ -potential experiments show that vincristine insertion does not modify the physicochemical properties of liposomes.

3.4. Liposome membrane fluidity assessment

As mentioned in the introduction, the interaction between liposomes and cancer cell lines can be influenced by the fluidity of

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Fig. 3. Liposome average size, polydispersity index (PDI) (A) and zeta potential (B) in the absence or presence of vincristine. Results are expressed as mean±SD of three replicates.

the liposome membrane [28–30]. To investigate whether the addition of vincristine affects the fluidity of the liposome membrane, this parameter was assessed using a ratiometric fluorescent probe called Dioll, which spontaneously incorporates into the bilayer. Fluorescent emission spectra, ranging from 400 to 600 nm, of the liposomal preparation containing vincristine, obtained using Dioll at 37 °C with an excitation wavelength of 390 nm, are shown in Fig. 4A.

We compared samples with and without vincristine to assess the potential influence of the molecule on liposomes membrane fluidity. The fluid liposomes, DO and PO, exhibited a Dioll spectrum with a predominant peak at 490 nm, characteristic of a liquid crystalline state. In contrast, the rigid liposome DP showed a Dioll spectrum with a predominant peak at 440 nm. The presence of vincristine induced a slide decrease of the Dioll profile of the liposomes, indicating that vincristine has low impact on the stiffness or fluidity profile of the liposome membrane. This conclusion is further supported by the GP parameter, which is a numerical index of membrane fluidity calculated from fluorescence emission spectra. Both DO and PO samples, along with their respective counterparts containing vincristine, exhibited membranes in a fluid state, as evidenced by the negative GP values: -0.21 (DO), -0.23 (DO-vincristine), -0.18 (PO), and -0.20 (PO-vincristine). Conversely, DP liposomes exhibited a high GP of 0.40 (without vincristine) and 0.35 (with vincristine), characteristic of a rigid membrane state (Fig. 4B).

3.5. Cytotoxicity effect of vincristine liposomes on tumor cells

The impact of vincristine encapsulated in liposomes of different membrane fluidities on tumor and control cell lines was evaluated as follows: on fibroblasts as a control and on prostate, colon and breast lines encompassing both non-tumoral and tumoral cells. The results are presented in Fig. 5.

Cells were exposed to 10 nM liposomal vincristine for 5 h, followed by washing with PBS to remove any liposomes not fused with the cells, and then reincubated for 72 h to allow the drug to act on cell metabolism. Resazurin, which converts to a blue-colored compound, resorufin in the presence of metabolically active cells, was used for absorbance measurements. The percentage of cell viability was calculated relative to the control. The 5-h exposure of liposome-vincristine was based on a previous study that investigated the incorporation time of fluorescent liposomes into cells [28].

The viability of all cancer cell lines, regardless of tissue origin, decreased by 70 % for LNCaP and PC-3 in the presence of DO-vincristine and PO-vincristine fluid liposomes, by 50 % for HT29 and 55 % for HS578T and BT20. Fluid liposomes did not affect the viability of non-tumoral cell lines, with approximately 100 % remaining viability for fibroblasts and non-tumor WPMY-1 cell line. Of note, free vincristine induced a 30 % and 70 % decrease, for these two cell types, respectively (Fig. 1).

Notably, a more limited decrease, of 30 %, in viability was



Fig. 4. Evaluation of liposome membrane fluidity. Fluorescence emission spectra of Dioll inserted in liposomes containing or not vincristine at 37 °C (λexc of 390 nm) (A). GP formula (B) and values calculated for each liposome composition (C).

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Fig. 5. Cellular viability of fibroblast, prostate, colon and breast lines in presence of 10 nM of liposomes-vincristine (DO, PO and DP). Results are expressed as mean±SD of three independent replicates.

observed for CAF cell lines (prostate and colon) in the presence of fluid liposomes. CAF cell lines play a crucial role in the tumor microenvironment. These fibroblasts are found in the stroma surrounding cancer cells and contribute to tumor progression and metastasis through various mechanisms, including extracellular matrix remodeling, support of cancer cell survival and proliferation [42]. In the provided study, CAF cell lines exhibited only a modest decrease in viability in the presence of fluid liposomes containing vincristine. This observation suggests that CAFs may exhibit differential sensitivity to vincristine and fluid liposomes compared to other cancer cell types.

In parallel, rigid liposomes, DP-vincristine had no effect on the viability of tumoral lines, irrespective of tissue origin, but reduced the viability of fibroblasts (20 % remaining viability).

These results are in agreement with previous studies indicating that liposomes with fluid membranes exhibit increased fusion with prostate cancer cells [29,30]. This study demonstrates that the targeting of cancer cells mediated by liposomes based on membrane fluidity may represent a more generalized mechanism.

When examining the viability outcomes following the encapsulation of vincristine in liposomes and comparing them with the cell targeting effects of free vincristine, several observations emerge. Encapsulation of vincristine improved its efficacy, particularly against prostate cancer cell lines such as LNCaP and PC-3. For instance, a 70 % reduction in PC-3 viability is evident with POvincristine encapsulation, whereas only a 40 % reduction is observed with free vincristine. Similarly, for LNCaP cells, viability decreased from 70 % to 50 % when the drug was encapsulated in DO or PO liposomes.

Moreover, a significant finding from the comparison of viability outcomes pre- and post-encapsulation of vincristine is the limited impact on control cell lines (Fibroblasts and WPMY-1) when vincristine is encapsulated in fluid DO and PO liposomes, which are designed to target cancer cell lines. This highlights the specific targeting of cancer cells, a phenomenon that is not evident when the molecule is administered in its free form.

3.6. Inhibition of mitosis by vincristine

Vincristine is a widely used chemotherapeutic agent known for its potent inhibition of cell proliferation [35]. Its mechanism of action involves halting mitosis and disrupting the polymerization of microtubules, which play vital roles in cell division and intracellular transport. Several studies have suggested that vincristine binds to tubulin proteins, thereby preventing microtubule formation and polymerization [43].

As part of our investigation into the mechanism of action of vincristine-loaded liposomes in cells, we observed a decrease in cancer cells viability in its presence. To elucidate this mechanism, we utilized phalloidin staining to visualize actin structures and investigated the effects of liposome-vincristine on cell division.

As vincristine disrupts the mitotic spindle apparatus during cell division, we hypothesized that its inhibition of mitosis at the metaphase stage would arrest the cell cycle (Fig. 6A). Consequently, this arrest would prevent the formation of the actin-myosin contractile ring required for cytokinesis, thus hindering the division of mother cells into daughter cells.

Our experimental approach involved labeling cells with phalloidin and observing the presence or absence of actin filaments.

The study was conducted using the cell model, PC-3 prostate bone metastasis cells, where we tested three different conditions: free vincristine to demonstrate the molecule's action without liposomes, DP-vincristine and PO-vincristine to assess potential differences in the action of rigid and fluid liposomes, as observed in previous cytotoxicity studies (Fig. 6B).

Actin filaments were not visible in the presence of 10 nM vincristine after 72 h incubation cells with vincristine. A disparity in action between rigid and fluid liposomes was observed. The fluid liposomes, PO-vincristine, showed a specific action on PC-3 cells, resulting in the absence of actin filaments. Conversely, the rigid liposomes, DP-vincristine, showed no action on the PC-3 cancer line. The absence of actin filaments in cells treated with free vincristine or liposome-encapsulated vincristine indicates an inhibition of mitosis, confirming the efficacy of vincristine in preventing cell division. These results, consistent with the expected action of vincristine to disrupt cell division, thus provide compelling evidence that it is possible to target specifically cancer cells based on the membrane fluidity of liposomes used as drug carriers.

4. Discussion

The present study investigated the cytotoxic potential of





Fig. 6. Inhibition of mitosis by vincristine by labelling actin filament of PC-3 cells lines. Principe of inhibition of mitosis by vincristine (A). Actin labeled with phalloidin Alexa Fluor 488 in PC-3 bone metastasis prostate cells line in presence of free vincristine, DP-vincristine or PO-vincristine (B).

liposomal vincristine on various cancer cell lines with membrane fluidity as a targeting parameter to assess vincristine release in cancer cells. This approach underscores the importance of understanding the mechanism of drug release and its impact on cancer cell viability, potentially providing insights into new therapeutic strategies to combat cancer.

The efficacy of vincristine is dependent on its concentration and exposure time, as observed in previous studies [36]. Despite its potential as a chemotherapy agent for targeting cancer cells, vincristine's full therapeutic potential is hindered by dose-dependent neurotoxicity. This neurotoxicity is characterized by increased nerve excitability leading to axonal degeneration [44]. Encapsulating vincristine in liposomes presents an attractive approach to enhance its therapeutic efficacy while minimizing off-target effects and its toxicity to healthy tissues.

As explained in the introduction the development of nanocarriers has allowed for the enhancement of traditional therapies [19]. Among nanocarriers, liposomes have been widely studied and used in targeted drug delivery systems due to their relatively high loading capacities whether for hydrophobic or hydrophilic molecules. However, despite rapid progress in this field, many-based drug delivery systems suffer from inadequate loading capacity [45,46].

One of the primary objectives of liposomal drug development research programs is to improve the specificity of encapsulated drugs. Indeed, the lack of specificity of a drug is clearly the reason for the failure of many drug development programs, particularly if pharmaceutical agents affect not only diseased tissues or cells, but also healthy tissues [37]. The second goal of advancing encapsulated drug development research is to enhance the loading capacity through the development of "remote loading", leveraging techniques such as transmembrane pH gradients. In this study, we have used the "proton gradient method" to encapsulate vincristine with a high loading efficiency in liposomes with different membrane fluidities to improve the efficacy and the specific targeting of vincristine on various cancer cells with the aim to achieve a new cancer therapy.

This pH gradient method, facilitates the uptake and retention of weakly basic therapeutic drugs within preformed vesicles [47]. This is one of the most efficient methods for loading drugs into liposomes as it involves a two-step process. Initially, liposomes are formed in an acidic environment, typically using a citrate buffer with pH of 4. Then, the external medium is exchanged with a neutral buffer at pH 7. This allows the drug in its neutral form to diffuse inside the vesicles, driven by the proton concentration gradient. Once inside, the drug is protonated by the acidic citrate buffer, converting it into its charged form, which thus remains trapped in the vesicle. By carefully balancing the buffer capacity and the amount of external drug, it is possible to achieve almost complete uptake of the drug with an excellent drug loading (DL). Indeed, this method has been applied for the development of liposomal formulations with high encapsulation levels and outstanding retention of drugs such as amphotericin B for antifungal treatment [48] or doxorubicin [49] and vincristine [50] for cancer therapy. In our study, the use of this method resulted in successful encapsulation yields, with over 60 % of vincristine effectively encapsulated in all three liposome compositions. Notably, encapsulation rates reached 80 % for DO and DPvincristine, and 69 % for PO-vincristine. This demonstrates the effectiveness of our approach in successfully incorporating vincristine into liposomes of different membrane fluidities, which is crucial to improve the therapeutic potential of these nanocarriers for cancer treatment.

Before evaluating the inhibitory effects of vincristine-loaded

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liposomes, we carried out a complete characterization of these liposomes. This involved the assessment of their charge (zeta potential), size, polydispersity index (PDI), and membrane fluidity. This characterization was essential to determine their suitability for potential in vivo delivery, thereby ensuring their stability, biocompatibility, and efficacy as drug delivery vehicles for cancer therapy.

All samples tested possess both a negative surface potential (from phosphate) and a positive potential (from the amine group of choline). They are therefore by definition zwitterionic. It is commonly accepted in the scientific literature that a ζ -potential ranging between -10 and +10 mV is considered relatively neutral [51]. The ζ -potential value obtained (-12 mV for all the preparations) indicates that our liposomal preparations are slightly negative.

The negative value obtained can be explained by the fact that the distribution of charges on the liposome membrane is not homogeneous. Indeed, the negative charge of phosphate is more deeply buried than the positive charge of choline, which is more accessible to buffer counterions. This creates a charge gradient at the polar heads of the lipids, which could be responsible for repulsive forces between the liposomes and hence the absence of aggregations.

The presence of a charge in liposomes, whether positive or negative, serves to reduce the aggregation phenomena between liposomes by reinforcing the repulsive electrostatics forces [52]. In addition, the charge of liposomes considerably influences their recognition by opsonins present in the bloodstream. Liposomes with excessively high positive or negative charges are more likely to be cleared quickly than neutral particles [53]. A cationic liposome is more easily recognized by opsonins, whose role is to identify non-self-molecules and facilitate their phagocytosis. One study demonstrated that negative charges on the surface of liposome membrane could completely suppress or even reverse the opsonization process [54]. Optimal preparation therefore entails obtaining a composition that is sufficiently charged but not excessively to maintain stability and avoid recognition by opsonins.

Physicochemical characterization of liposomes is of significant importance due to the dual roles that size and charge play in controlling the pharmacokinetics of liposomal drug formulations [4]. Research indicates that the size of liposomes is a critical factor in their targeting mechanisms. Larger liposomes tend to be taken up by phagocytes, while smaller liposomes (<300 nm) can be effectively penetrate tumor tissue, benefiting from increased permeability and retention [55]. Moreover, larger liposomes are more prone to be cleared quickly than smaller ones [33]. It is therefore imperative to measure this parameter before administering a liposomal formulation.

Additionally, it becomes imperative to characterize the fluidity of the liposomal preparation. This aspect of characterization is essential for understanding the dynamic behavior of liposomes, particularly with regard to their ability to interact with cell membranes and efficiently deliver their encapsulated drug cargo.

As previously demonstrated in our group [30], encapsulation of a drug could alter the membrane fluidity of the liposomes. The advantage of encapsulating a hydrophilic active molecule like vincristine is that it inserts into the aqueous core of the liposomes rather than into the bilayer. This avoids disrupting the interactions between phospholipids inside the membrane and therefore, modifying its fluidity. Insertion of molecules into the liposome membrane can induce spacing between the acyl chains that disrupts the interactions between them, which are then freer to move, thereby increasing the overall fluidity of the membrane. Therefore, it becomes crucial to systematically measure membrane fluidity to assess any changes induced by the presence of the drug. In our study, the incorporation of vincristine seems to induce a subtle increase in membrane fluidity of the three types of liposomes studied. However, the measured fluidity remains consistent with the composition of the liposomes: DP liposomes maintain the highest rigidity, DO liposomes exhibit the highest fluidity, and PO liposomes remain to an intermediate level of fluidity. This result highlights the distinctive membrane properties of each liposome composition, which could influence their effectiveness as drug delivery systems in cancer treatment.

Vincristine was efficiently delivered to cancer cell lines by fluid DO and PO liposomes as shown by the decrease in viability of all cancer cell lines upon exposure, regardless of tissue origin. Notably, viability decreased by approximately 70 % for LNCaP and PC-3, 50 % for HT29, and around 55 % for HS578T and BT20. In contrast, the viability of non-tumoral cell lines was not affected, with approximately 100 % viability for fibroblasts and WPMY-1. This result reveals the possibility of specifically targeting cancer cell using a fluid liposomal formulation of vincristine. 100 % viability for fibroblasts and WPMY-1.

An intermediate effect was recorded for prostate and colon cancer-associated fibroblasts (CAFs), which exhibited a moderate decrease in viability of only 30 % in the presence of fluid liposomes. This difference in sensitivity compared to other cancer cells lines could be explained, for example, by factors such as different levels of expression of drugs targets within the line. Furthermore, the presence of CAFs in the tumor microenvironment can modulate the response of cancer cells induced by therapeutics interventions. CAFs secrete factors that can promote the survival of cancer cells. such as angiogenesis [56], which can have an impact on the effectiveness of the therapeutic formulation administered, and therefore in our case, of the liposomal formulation. Finally, the difference in membrane fluidity between the membranes of DO and PO liposomes and those of CAFs could also influence the viability results obtained. Liposomes with fluid membranes may exhibit enhanced fusion with cancer cells, but may not interact as effectively with CAFs due to differences in membrane properties.

That being said, the results of the cytotoxicity tests that we have obtained in this present work confirm that there is indeed a correlation between the membrane fluidity of liposomes and those of cancer cells which can be used selectively to allow targeted drug delivery.

Finally, a mechanistic study of the mode of action vincristine was carried out by labeling the actin filaments of PC-3 cancer cells. Composed of two multi-ringed structures, vindoline and catharanthine, vincristine interacts with B-tubulin in a region known as the vinca domain. This interaction prevents the formation of spindle microtubules, thereby disabling the cellular mechanism of chromosomes alignment and movement [57,58]. Consequently, disruption of mitosis by vincristine inhibits the division and growth of cancer cells. Treated cells are unable to progress through the cell cycle, resulting in prolonged metaphase arrest, which triggers a cellular response leading to apoptosis or programmed cell death. The inability of cells to complete mitosis due to microtubule disruption triggers a cascade of events ultimately resulting in cell death. In our study, the labeling of actin filaments allowed us to discern the selective action of liposomal vincristine. We demonstrated that fluid liposomes, specifically PO-vincristine, targeted selectively PC-3 cells, while rigid liposomes such as DP-vincristine did not.

In general, both fluid liposomes containing vincristine, DOvincristine and PO-vincristine demonstrated a considerable impact on the viability of tumor cells compared to the control group. Notably, the fluid liposome formulation of PO-vincristine showed a significant disruption in mitosis specifically in the PC-3 cancer cell line, an effect not observed with rigid liposomes, DP-

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vincristine. In summary, the encapsulation of vincristine within liposomes with tailored membrane fluidity presents a promising approach to enhance its effectiveness, reduce toxicity, and amplify its therapeutic benefits in cancer treatment.

Statement

All authors have approved the final article.

Financial support

We acknoledge funding from University Claude Bernard Lyon 1, CNRS and the French Ministry of Higher Education, Research and Innovation.

CRediT authorship contribution statement

Ariana Abawi: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. Ana-Maria Trunfio-Sfarghiu: Methodology. Céline Thomann: Methodology. Emma Petiot: Methodology. Giovanna Lollo: Methodology. Thierry Granjon: Methodology. Agnès Girard-Egrot: Validation, Supervision, Funding acquisition, Conceptualization. Ofelia Maniti: Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Acknowledgements

We acknowledge funding from University Claude Bernard Lyon 1, CNRS and the French Minstry of Higher Education, Research and Innovation.

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