Toxicology Reports 2 (2015) 377–383

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Short communication

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) increases Carmofur stability and *in vitro* antiproliferative effect

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ARTICLE INFO

Article history:
Received 21 October 2014
Received in revised form 9 December 2014
Accepted 6 January 2015
Available online 28 January 2015

Chemical compounds studied in this article:
1,2-Dimyristoyl-sn-glycero-3-phosphocholine (PubChem CID: 5459377)
1-Hexylcarbamoyl-5-fluorouracil (PubChem CID: 2577)
5-Fluorouracil (PubChem CID: 3385)

Keywords:
Carmofur
Cytotoxicity
DMPC
Drug delivery
5-Fluorouracil
^{1}H NMR

ABSTRACT

Addition of DMPC considerably inhibits the degradation of Carmofur in neutral phosphate buffer solutions and this drug becomes less influenced by pH. Carmofur stabilization at neutral pH caused by DMPC addition for *in vitro* studies was characterized and monitored by ^{1}H NMR. Antiproliferative activity studies on various tumor cell lines showed considerable increase of Carmofur ability to prevent tumor cell growth, when it is added as a mixture with DMPC. This technique opens a way for Carmofur drug delivery in neutral and basic media.

1. Introduction

Carmofur (1-hexylcarbamoyl-5-fluorouracil, HCFU, Mifurol) is one of the masked forms of 5-fluorouracil widely used as an antineoplastic agent. This drug, in its oral form, has been used as adjuvant chemotherapy for curatively resected colorectal cancer patients. Trials have confirmed that the drug is effective on patients with this cancer type, extending their survival [1,2]. The Carmofur molecule contains an active core of 5-fluorouracil and hexylcarbamoyl substituent as a transport form, and it helps 5-FU to penetrate into the cell. In the last years the interest in encapsulation of 5-fluorouracil derivatives in liposomal formulations was increased [3]. Liposomal 5-FU microspheres are potent in the treatment of liver metastases increasing the intratumoral concentration of 5-FU up to 2203 times [4]. Recently, experimental research concerning antitumor activity of Carmofur water solubilized by lactic acid oligomer-grafted pullulan nanogels has been reported. Nanogel with Carmofur showed a strong suppressive effect of tumor cells grown, in contrast

Abbreviations: DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; Carmofur, 1-hexylcarbamoyl-5-fluorouracil; 5-Fu, 5-fluorouracil; NMR, nuclear magnetic resonance.

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http://dx.doi.org/10.1016/j.toxrep.2015.01.009

to the Carmofur solution [5]. Treatment of colorectal cancer using a combination of liposomal irinotecan and 5-fluorouracil leads to exposition time prolongation and improves antitumor activity [6]. The main disadvantages of Carmofur is its low solubility; moreover, this drug relatively easily hydrolyzes under physiological conditions with pH > 7.0. Therefore the scope for use of this drug is significantly narrowed. However, nanogels of HCFU have been formulated previously by cross-linked polymeric micelles of N-isopropylacrylamide and N-vinylpyrrolidone and higher accumulation of HCFU in brain was reported [7,8]. As well as Carmofur loaded pluronic polymeric micelles were produced and characterized [9].

Our experience with the chemistry of nucleosides [10,11] and antitumor activity studies [12–14] inspired us to investigate the stability of Carmofur (**1**) in aqueous phosphate buffer solutions at pH from 5.0 to 8.0 and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) influence on Carmofur stability monitored by ^{1}H NMR.

2. Materials and methods

Carmofur was purchased from TCI, DMPC was purchased from NOF Corporation (Tokyo, Japan) and used without further purification. ^{1}H NMR spectra of **1** were run on a Varian-Mercury BB 400 MHz spectrometer at 298 K, with a standard pulse sequence WET for residual water signal suppression. The spectral parameters were: 4695 Hz spectral width, 16K complex data points, number of scans 128, acquisition time 3.5 s, digital resolution 0.007 Hz and relaxation delay 2 s. The acquired FIDs were processed with zero-filling to 32 K.

2.1. Stability studies by NMR

For integration the C_6H signals of **1** (**1A** + **1B**) was used. The samples were prepared in phosphate buffers in D_2O at pH 5.0–8.0; the difference of pH values in D_2O and H_2O water buffers were less than 0.4. Each of the 8 experiments with a specific concentration of HCFU was single point. 86.87 mg (0.338 M) of HCFU was dissolved in 1 ml of chloroform-d_1 ($CDCl_3$). The sample was stored in the fridge at 4 °C in the container with a cap-sung. For the sample preparation 15 μl of solution of CHFU in $CDCl_3$ were taken by the gamiltanian syringe and added to the vial with buffer liposomes. Then the sample was thoroughly shaken and put into the NMR spectrometer. The temperature inside the NMR spectrometer was 25 °C and it was controled by the integrated thermometer. Each spectrum was acquired with t_{2max} = 3.49 s, the relaxation delay was 2 s, and the number of scans was 128. The chemical shifts of the hydrogen atoms of **1** are presented in ppm and referred to the residual signals of $CDCl_3$ δ = 7.26 and D_2O δ = 4.5 (^{1}H) ppm. ^{1}H NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.87 (3H, t, J = 7.2, C$\underline{H}_3$), 1.73–1.28 (6H, m, 3C$\underline{H}_2$), 1.59 (2H, m, J = 7.2, C$\underline{H}_2$), 3.37 (2H, td, J = 7.2, J = 5.1, NC$\underline{H}_2$), 8.42 (1H, d, J = 6.9, =C$\underline{H}$), 8.91 (1H, br s, N$\underline{H}$), 8.96 (1H, t, J = 5.1, N$\underline{H}$).

2.2. DMPC vesicles preparation for NMR studies

DMPC has been dissolved in buffer/water and then sonicated by Ultrasonic Processor Cole Parmer© CPX130 with 30% of power for 5 min with on/off cycle of 10 s. Then to the 800 μl of the vesicle's solution in the 5 mm NMR tube 15 μl of **1** dissolved in $CDCl_3$ with concentration 0.337 M has been added. The resulting mixture was then immediately inserted into spectrometer.

2.3. Computational details

All HF calculations were performed in Jaguar 8.0 (release 515) from Schrödinger [15]. The basis set was 6-311G**++. Geometries were optimized in the gas phase, and energies in solvent were calculated using the Poisson–Boltzmann finite-element (PBF) [16,17] implicit solvation model at the optimized gas phase geometries.

2.4. Preparation of DMPC vesicles with *1* for in vitro studies (*3*)

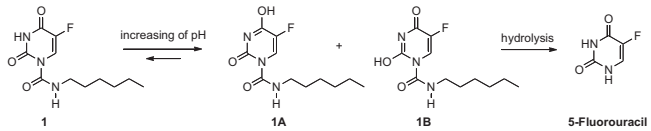
Carmofur (26 mg, 0.1 mmol) and DMPC (0.334 g, 0.5 mmol) were dissolved in 25 ml of chloroform and evaporated on rotatory evaporator under reduced pressure with slow rotation without any heating. Then additional portion of chloroform was added and the procedure was repeated for 5 times.

2.5. In vitro cytotoxicity assay

Monolayer tumor cell line: HT-1080 (human connective tissue fibrosarcoma), C6 (rat glial tumor), SH-SY-5Y (human neuroblastoma), MH-22A (mouse hepatoma), MDA-MB-435s (human melanoma), MCF-7 (human breast adenocarcinoma, estrogen-positive), and normal cell lines: H9C2 (rat myocardium) and NIH 3T3 (Mouse Swiss Albino embryo fibroblasts) were grown in standard medium Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, "Sigma"). About 1–2 × 10^4 cells/well (depending on line nature) were placed in 96-well plates immediately after compounds (250, 50, 10, 2, 0.4 and 0.08 μM) were added to the wells. The control cells without test compounds were cultured on separate plate. The plates were incubated for 72 h, at 37 °C, 5% CO_2. The number of surviving cells was determined using MTT (thiazolyl blue tetrazolium bromide), and OD was read λ = 540 nm. The IC_{50} was calculated using program Graph Pad Prism® 3.0, r < 0.05. The control cells without test compounds were cultured on separate plate and an appropriate volume of control buffer was added to control cells. Each assay was conducted in triplicate.

2.6. Standard operating procedure (SOP) for the Balb/c 3T3 neutral red uptake cytotoxicity test – a test for basal cytotoxicity

The SOP was submitted in 1992 to INVITTOX, where it is still available as Protocol No. 46 (NRU Assay). The NRU cytotoxicity assay procedure is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red, a supra-vital dye. The basal cytotoxicity is to be used to predict starting doses for *in vivo* acute oral LD_{50} values in rodent. Using the 3T3



Scheme 1. Tautomeric forms of **1**.

cell line the borderline concentration, relevant to the highest tolerated dose, is determined for each material. NIH 3T3 (normal mouse fibroblasts) cells were grown in DMEM medium supplemented with 10% FBS. Before compounds (1000, 316, 100, 31, 10, 3, 1 μg/ml) were added to the wells 1×10^4 cells/well were placed in 96-well plates for 24 h. The plates were then incubated for 24 h, 37 °C, 5% CO_2. The cells were incubated then with neutral red dye for 4 h and OD was read, λ = 540 nm. This method is alternative to LD_{50} *in vivo*. LD_{50} values (LD_{50} value is amount of the drug that is taken to kill 50% of test animals) was calculated according to the formula: $\log(LD_{50})$ [mmol/kg] = 0.435 × $\log(IC_{50})$ [mmol/l] = 0.625.

3. Results and discussion

Being a potentially tautomeric system **1** can exist in three tautomeric interconverting structures (Scheme 1). Tautomers fall in line with the stability sequence: 2,4-dioxo > 2-hydroxy-4-oxo > 2-oxo-4-hydroxy, and the presence of water does not affect the relative stabilities as found in the gas phase [18]. Experimental data suggest [19], that the dioxo-tautomer of 5-FU is stable in the solid state, gas phase and in aprotic solutions. The same could be extrapolated for 5-fluorouracil fragment in Carmofur (**1**) molecule. According to quantum-chemical calculations of N1-substituted derivative modeling **1** the stability of diketo form **1**, with relative energy (ΔE) equal to 0 kcal/mol, is large enough as compared with the other tautomers **1A** (ΔE = 12.3 kcal/mol) and **1B** (ΔE = 19.1 kcal/mol). It confirms that the dioxo tautomer of **1** is also the only important tautomer in the gas phase and the same result is true for the PBF water solution method. Fig. 1 demonstrates ^{1}H NMR spectra of the solution of **1** in phosphate buffers at different pH values. According to our studies in aqueous phosphate buffer solutions tautomeric forms of **1** are significantly influenced by their pH value. Increase of pH favors the transformation of diketo form to 2- and 4-hydroxy tautomers (**1A** and **1B**).

Being one of the masked forms of 5-fluorouracil, **1** is extremely susceptible to base catalyzed hydrolysis in aqueous solutions to 5-fluorouracil (**1** is the most stable at pH ~ 4) [20]. In the course of the present study, the degradation of Carmofur in water phosphate buffer solutions was found to be rather accelerated by increasing the pH value of the phosphate buffer. Fig. 2 shows the time courses of the degradation of **1** in the phosphate buffers with different pH values. It is apparent that the degradation rate of Carmofur at pH 8 and pH 7 is much faster, whereas the **1** molecule at pH 5 is practically kept unharmed for more than 14 h.

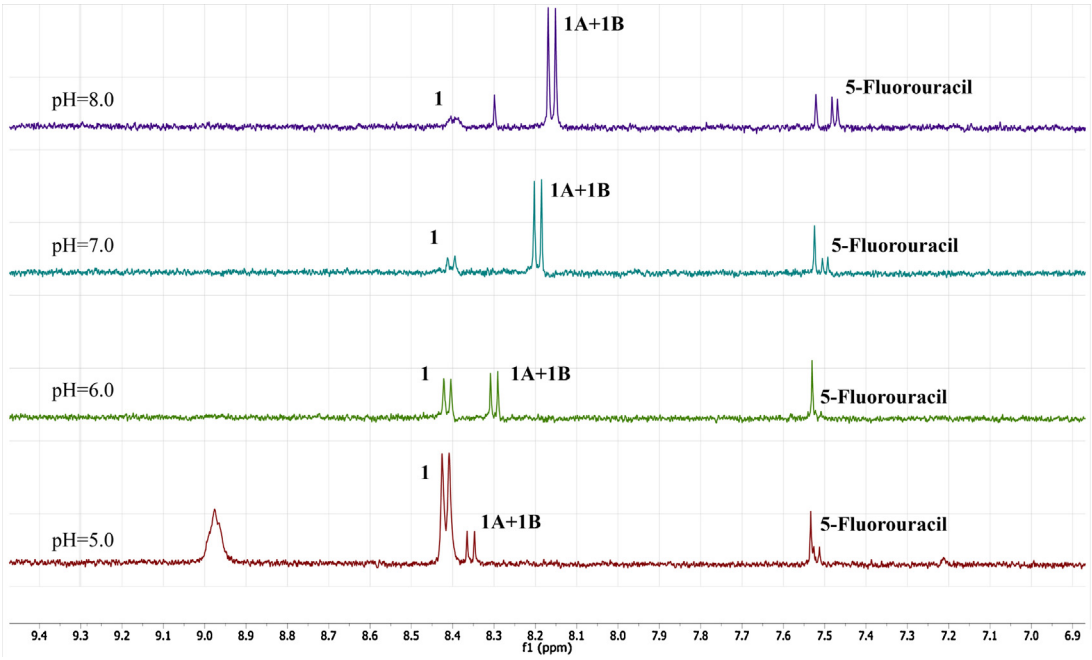


Fig. 1. ^{1}H NMR spectra of the solution of **1** in phosphate buffers at different pH values. The high field shift of C_6H proton signal reflects the changing ratio of **1A** and **1B** forms.

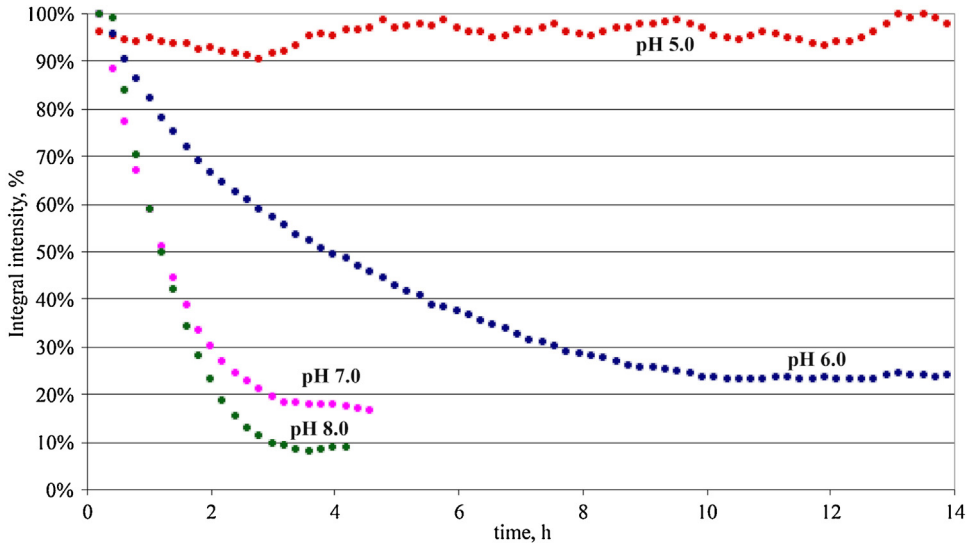


Fig. 2. Visualization of degradation of **1** in aqueous phosphate buffers at different pH values (monitored by ^{1}H NMR). On *y*-axis *C* (mM) denotes the concentration of **1**.

Next, we attempted to stabilize **1** by addition of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). After brief optimization of solubility for the further *in vitro* studies we decided to use 1:5 ratio of **1**:DMPC (**2**). Experiments were made by DMPC emulsification in phosphate buffer solutions in deuterated water and then sonicated by Ultrasonic Processor Cole Parmer© CPX130 with 30% of power for 5 min with on/off cycle of 10 s. Then to the 800 μl of the vesicle's solution in a 5 mm NMR tube 15 μl of **1**, dissolved in $CDCl_3$ with 0.337 M concentration, has been added. The resulting mixture was immediately inserted into spectrometer. Fig. 3 shows the effect of **2** additions on the degradation of **1** in aqueous phosphate buffers. It is apparent that **2** was rather effective in stabilization of **1**. Fig. 3 shows the remaining **1** (in %) after 14 h as a function of pH. The stabilizing effect of **2** increased with decreasing pH and the degradation of **1** was completely prevented even for 2 days.

The *k* values for the hydrolysis reactions of **1** at various pH are presented in Table 1. The degradation rate of **1** can be described in terms of *pseudo*-first-order kinetics. The *k* values obtained indicate that Carmofur is chemically more stable under acidic conditions and its degradation rate grows on going from pH = 5.0 to pH = 8.0.

Addition of DMPC considerably inhibits the degradation of **1** and this drug becomes less influenced by pH. The apparent first order degradation rate constants of **1** in the presence of **2** are summarized in Table 1. A tendency for the inhibition increases with increasing pH value. The greater stabilizing effect is observed for pH = 8.0, where inhibition ratio rises by over 7 times as compared with the pure **1** solution at the same pH. The release of 5-fluorouracil from **1** in

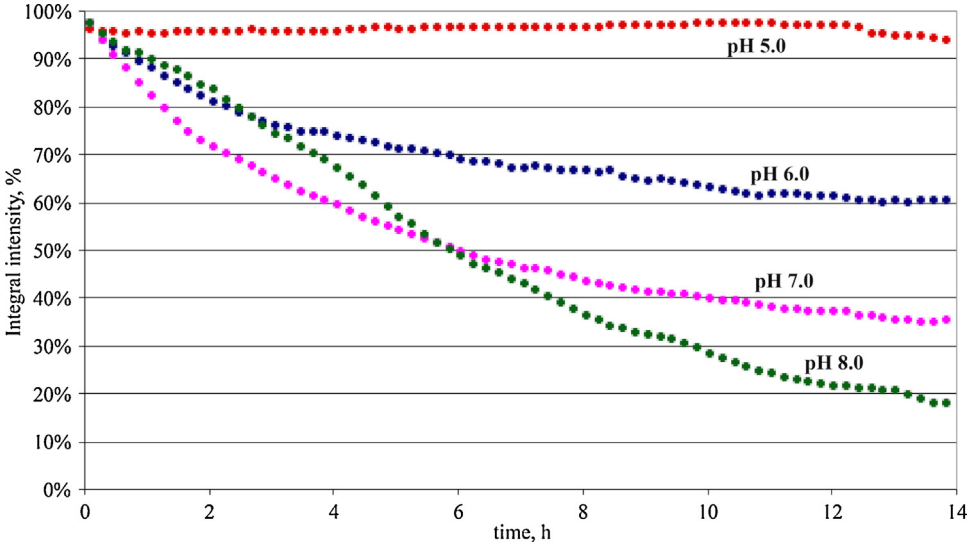


Fig. 3. Degradation of **1** with **2** in phosphate buffers with different pH monitored by ^{1}H NMR. On *y*-axis *C* (mM) denotes the concentration of **1**.

Table 1
K values and inhibition ratios for the hydrolysis reaction of **1** and **3** in phosphate buffers with different pH values.

		pH			
		5.0	6.0	7.0	8.0
Rate constants, K ($\times 10^4$)	**1**	0	0.17	0.54	0.70
	3	0	0.05	0.09	0.09
Inhibition ratio, K_1/K_3		–	3.40	5.96	7.74

the presence of DMPC has been significantly reduced due to that DMPC provides phospholipid environment around the Carmofur molecules incorporated into DMPC nanoparticles.

Although it is known that the lifetime of liposomes in cell culture media is around 200 min [21] the form and size of them is not primary; we decided to check the difference of liposomes made from DMPC (**2**) and the mixture of Carmofur with DMPC (**3**) before *in vitro* tests using phase contrast invert microscope (Nikon Eclipse TE300). Vesicles formed by DMPC in phosphate buffer at pH = 7.2 are with various sizes, besides, liposomes of **3** are more organized and smaller by size (Fig. 4). Since the Carmofur molecule has a lipophilic chain we propose that the inclusion of **1** in general proceeds by intercalation.

In vitro cytotoxicity caused by Carmofur (**1**), DMPC (**2**), Carmofur × 5DMPC (**3**) and 5-fluorouracil was tested on monolayer tumor cell lines: HT-1080 (human connective tissue fibrosarcoma), C6 (rat glial tumor), SH-SY-5Y (human neuroblastoma), MH-22A (mouse hepatoma), MDA-MB-435s (human melanoma), MCF-7 (human breast adenocarcinoma, estrogen-positive), and H9C2 (rat myocardium). Using the NIH 3T3 (mouse fibroblasts) cell line the borderline concentration, relevant to the highest tolerated dose, is determined for each compound. The basal cytotoxicity is to be used to predict starting doses for *in vivo* acute oral LD_{50} values in rodent [22]. The results of these experiments are summarized in Table 2. 5-Fluorouracil interferes with thymidylate synthesis, and has a broad spectrum of activity against solid tumors. However, 5-FU has limitations that include a short biological half-life due to rapid metabolism, incomplete and non-uniform oral absorption due to metabolism by dihydropyrimidine dehydrogenase, toxic side effects on bone marrow and the gastrointestinal tract, and non-selective action against healthy cells [23].

Carmofur exhibits high cytotoxic activity on glioma C6 (IC_{50} = 7 μM) cell line, however combination of Carmofur with 5 molecules of DMPC (**3**) has two times more extended cytotoxic effect on C6 (IC_{50} = 3 μM). Similar results were obtained by comparison of **1** and **3** cytotoxicity on human neuroblastoma SH-SY-5Y cell line, IC_{50} = 76 μM and 22 μM, correspondingly. It should be noted that DMPC showed no cytotoxic effect on these brain tumor cell lines.

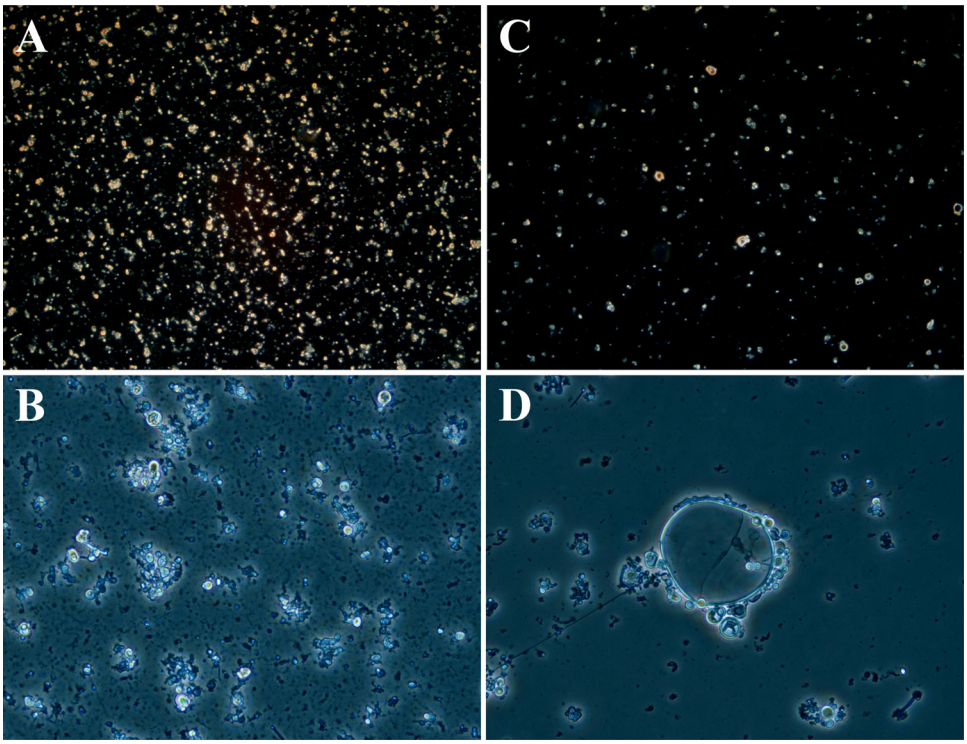


Fig. 4. Representative micrographs of DMPC **2** (A – 10^4; B – 10^7) and Carmofur × 5DMPC **3** (C – 10^4; D – 10^7) taken by contrast invert microscope.

Table 2
In vitro cytotoxicity on monolayer tumor cell lines: HT-1080, C6, SH-SY-5Y, MH-22A, MDA-MB-435s, MCF-7, H9C2, and NIH 3T3 caused by Carmofur (**1**), DMPC (**2**), Carmofur × 5DMPC (**3**) and 5-fluorouracil.

Cell line	(**1**)[a]	(**2**)[a]	(**3**)[a]	5-Fluorouracil[a]
C6	7	[b]	3	–
SHSY-5Y	76	214	22	107
MH-22A	43	[b]	27	–
MDA-MB-435s	213	231	68	31
MCF-7	230	[b]	81	46
HT-1080	67	20	14	14
H9C2	189	[b]	154	–
NIH3T3	184	[b]	45	–
LD_{50} (mg/kg)[c]	489	[b]	589	>2000

[a] IC_{50}, concentration (μM) providing 50% cell killing effect.
[b] No cytotoxic effect.
[c] Starting dose.

Since 5-fluorouracil based drugs usually cannot cross the blood–brain barrier, it may be assumed that the liposomal form **3** can serve as a drug delivery agent for the treatment of brain tumors. Despite 5-fluorouracil without a transport form is useless for *in vivo* procedures, we discovered its cytotoxic effect *in vitro* with a purpose to compare its antiproliferative effect on tumor cells. 5-Fluorouracil showed weak cytotoxic effect on SH-SY-5Y cell line (IC_{50} = 107 μM). Less prominent difference was observed by treatment of hepatoma MH-22A cells with **1** and **3** (IC_{50} = 43 μM for **1**, IC_{50} = 27 μM for **3**). This can be attributed to the high ability to metabolize lipids by all types of hepatic cells; as a result liposomes were destroyed in shorter length of time. Notably, according to received *in vitro* data concerning Carmofur (**1**) ability to prevent growth of human melanoma MDA-MB-435s and estrogen positive human breast adenocarcinoma MCF-7 we can conclude that **1** showed no activity *in vitro*, however, Carmofur was administered to patients in postoperative period with early stage breast cancer [24]. Besides, **3** exhibit medium antiproliferative effect on these breast cancer cell lines (IC_{50} = 68 μM and 81 μM, correspondingly). These data are in agreement with previously reported studies [25]. In the case of human connective tissue fibrosarcoma HT-1080 we discovered good cytotoxic effect caused by **3**, however, DMPC (**2**) surprisingly showed similar effect against HT-1080 cells (IC_{50} = 20 μM). Recent studies demonstrated that primary cardiomyocytes are a valuable tool for studying the metabolic capacity of the heart. H9C2 cell line is a valuable *in vitro* model to study the drug metabolizing enzymes in the heart [26]. Inspection of cytotoxicity of **1** and **3** led us to conclude that both Carmofur and Carmofur × 5DMPC are not cardiotoxic on H9C2. High cytotoxic effect on mouse fibroblasts NIH 3T3 caused by **3** can be explained by expressed phagocytosal properties on this cell line.

4. Conclusions

To sum up, we discovered that the addition of DMPC considerably inhibits the degradation of Carmofur in neutral phosphate buffer solutions and this drug becomes less influenced by pH. Antiproliferative activity studies showed considerable increase of Carmofur ability to prevent tumor cell growth, when it is added as a mixture with DMPC. The most expressed antiproliferative effect has been found against glioma C6 cells (IC_{50} = 3 μM). Similar increasing of cytotoxicity was shown on human neuroblastoma SH-SY-5Y cell line. On contrary to Carmofur, Carmofur × 5DMPC mixture exhibits medium cytotoxic effect against human melanoma MDA-MB-435s and estrogen positive human breast adenocarcinoma MCF-7 cells. This technique opens a way for the drug delivery of Carmofur in neutral and basic media.

Conflict of interest

Authors have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgement

We are grateful for financial support provided by the Latvian Council of Science (447/2012).

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