Supporting information

Inclusion of new 5-fluorouracil amphiphilic derivatives in liposome formulation for cancer treatment

Manuela Petaccia,[a,#] *Maria Condello,* [b,c,#] *Luisa Giansanti,* [a] *Angela La Bella,* [d] *Francesca Leonelli,*[e] *Stefania Meschini,*[b,*] *Denise Gradella Villalva,*[d] *Evelin Pellegrini,*[b] *Francesca Ceccacci,*[f] *Luciano Galantini,*[d] *Giovanna Mancini*[c,*]

[a]*Dipartimento di Scienze Fisiche e Chimiche, Università degli Studi dell'Aquila, Via Vetoio, 67100 Coppito (Aq), Italy*

[b]*Dipartimento Tecnologie e Salute, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy*

[c]*CNR – Istituto di Metodologie Chimiche, Via Salaria km 29.300, 00016 Monterotondo Scalo (RM), Italy*

[d]*Dipartimento di Chimica, Università degli Studi di Roma "Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy*

[e]*Dipartimento di Biologia Vegetale ed Animale, Università degli Studi di Roma "Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy*

[f]*CNR-IMC, Sezione Meccanismi di Reazione c/o Dipartimento di Chimica, Università degli Studi di Roma "Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy*

*** Corresponding author:** Tel: +390690625111; +390649913078; Fax: +390649913629; E-mail address: giovanna.mancini@uniroma1.it

*** Corresponding author:** Tel: +390649902783; Fax: +390649903563; E-mail address: stefania.meschini@iss.it

Content

Experimental

Instrumentation

IR spectra: Shimadzu-FTIR 8400 S infrared spectrophotometer; in cm^{-1}. ^{1}H and ^{13}C spectra (compounds **8**, **9**, **11-13**): Varian Mercury 300 at 300.13 and 75.48 MHz, respectively; δ in ppm relative to the residual solvent peak of $CDCl_3$ at 7.26 and 77.0 ppm for ^{1}H and ^{13}C, respectively. ^{1}H and ^{13}C spectra (compounds **1-3**, **10**): Bruker AVANCE 400 at 400.13 and 100.61 MHz respectively; δ in ppm relative relative to the residual solvent peak of $CDCl_3$ at 7.26 and 77.0 ppm for ^{1}H and ^{13}C, respectively. *J* in Hz. Elemental analyses for C, H, N and S were performed on an EA 1110 CHNS-O instrument. Steady-state fluorescence experiments were carried out on a Fluoromax-4 Horiba-Jobin Yvon spectrofluorimeter.

Materials

DOPC (purity >99%) was purchased from Avanti Polar Lipids (Alabaster, AL). Gemini surfactant **4** was prepared and purified as previously described.[1] Phosphate buffered saline (PBS) tablets (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH=7.4 at 25 °C), Sephadex G-50, NaH, TsCl, 5-FU, **5**, **6** and **7,** Staurosporine (STS), MTT powder were purchased by Sigma Aldrich (St Louis, MO, USA). All solvents and chemicals were used as purchased without further purification. Yields were not optimized. TLC: silica gel 60 F_{254}. Column chromatography (CC): silica gel 60, 70-230 mesh ASTM; aluminium oxide 90 active, neutral (activity I), 70-230 mesh ASTM. Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS), penicillin and streptomycin solutions were purchased by Euroclone (Carmlington, UK).

Chemistry

*General procedures for the synthesis of compounds **8-10**.* The appropriate alcohol compounds **5-7** (1 eq) was dissolved in anhydrous THF (4.5 mL/mmol). The solution was cooled to 0°C and NaH (60% mineral oil, 1.5 eq) was cautiously added under an atmosphere of Ar. After being stirred for 10 min the solution was treated with TsCl (1,5 eq) and the stirring is continued for 14h at r.t. After that time TLC (AcOEt/n-hexane 9/1) indicated the complete disappearance of the

starting material and H_2O was added dropwise at 0°C. The mixture was diluted with Et_2O and the two phases separated. The aqueous phase was thoroughly extracted with Et_2O, the combined org. extracts were washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting residue was purified by CC (SiO_2; AcOEt/n-hexane from 5/5 to 8/2) to afford compounds **8-10**. Compound **8**. Colorless oil. Yield 78%. ^{1}H-NMR (CDCl3): δ = 7.76 (d, *J* = 8.2, 2H), 7.31 (d, *J* = 8.2, 2H), 4.12 (t, *J* = 4.8, 2H), 3.69-3.47 (m, 22H), 3.40 (t, *J* = 6.8, 2H), 2.41 (s, 3H), 1.53 (quint, *J* = 6.7, 2H), 1.33-1.14 (m, 18H), 0.84 (t, *J* = 6.9, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 144.8, 133,0, 129.8, 128.0, 71.6, 70.8, 70.63, 70.59, 70.5, 70.1, 69.3, 68.7, 31.9, 29.70, 29.66, 29.65, 29.5, 29.4, 26.1, 22.7, 21.7, 14.2. Compound **9**. Colorless oil. Yield 72%. ^{1}H-NMR ($CDCl_3$): δ = 7.79 (d, *J* = 8.3, 2H), 7.33 (d, *J* = 8.0, 2H), 4.12 (t, *J* = 4.8, 2H), 3.72-3.52 (m, 26H), 3.43 (t, *J* = 6.8, 2H), 2.44 (s, 3H), 1.56 (quint, *J* = 7.1, 2H), 1.38-1.17 (m, 18H), 0.87 (t, *J* = 6.9, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 144.9, 133,2, 129.9, 128.1, 71.7, 70.9, 70.74, 70.70, 70.6, 70.2, 69.3, 68.8, 32.0, 29.77, 29.76, 29.75, 29.72, 29.6, 29.4, 26.2, 22.8, 21.7, 14.2. Compound **10**. Colorless oil. Yield 56%. ^{1}H-NMR ($CDCl_3$): δ = 7.77 (d, *J* = 8.4, 2H), 7.32 (d, *J* = 8.0, 2H), 4.13 (t, *J* = 4.1, 2H), 3.70-3.50 (m, 30H), 3.42 (t, *J* = 6.8, 2H), 2.40 (s, 3H), 1.54 (quint, *J* = 7.0, 2H), 1.34-1.14 (m, 18H), 0.85 (t, *J* = 7.0, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 144.8, 133,1, 129.9, 128.0, 71.6, 70.8, 70.65, 70.60, 70.13, 32.0, 29.72, 29.67, 29.6, 29.4, 26.2, 22.7, 21.7, 14.2.

*General procedures for the synthesis of compounds **11-13***. The appropriate tosylate derivatives **8-9** (1 eq) were dissolved in dry acetone (9 mL/mmol) and NaBr (1.2 eq) was added under an atmosphere of Ar. The solution was stirred at 65°C for 72h until the TLC (AcOEt/n-hexane 6/4) showed the complete disappearance of the starting material. The reaction mixture was allowed to warm to room temperature and the solution was diluted with Et_2O and filtered on a pad of celite. The filtrate was evaporated under reduced pressure and the resulting residue was purified by CC (SiO_2; AcOEt/n-hexane from 3.5/6.5 to 6/4) to afford compounds **11-13**. Compound **11.** Colorless oil. Yield 83%. ^{1}H-NMR ($CDCl_3$): δ = 3.83-3.75 (m, 2H), 3.69-3.59 (m, 18H), 3.59-3.53 (m, 2H), 3.49-3.39 (m, 4H), 1.56 (quint, *J* = 6.6, 2H), 1.36-1.15 (m, 18H), 0.86 (t, *J* = 6.6, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 71.7, 71.3, 70.8, 70.75, 70.72, 70.7, 70.2, 32.0, 30.4, 29.8, 29.75, 29.74, 29.72, 29.6, 29.4, 26.2, 22.8, 14.2. Compound **12**. Colorless oil. Yield 81%. ^{1}H-NMR ($CDCl_3$): δ

= 3.80 (t, *J* = 6.3, 2H), 3.69-3.59 (m, 22H), 3.59-3.54 (m, 2H), 3.45 (dt, *J* = 6.6, 11.7, 4H), 1.56 (quint, *J* = 7.1, 2H), 1.37-1.17 (m, 18H), 0.87 (t, *J* = 6.9, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 71.7, 71.3, 70.8, 70.75, 70.72, 70.7, 70.2, 32.0, 30.4, 29.8, 29.75, 29.73, 29.6, 29.5, 26.2, 22.8, 14.2. Compound **13**. Colorless oil. Yield 82%. ^{1}H-NMR ($CDCl_3$): δ = 3.79 (t, *J* = 6.3, 2H), 3.70-3.58 (m, 26H), 3.58-3.52 (m, 2H), 3.44 (dt, *J* = 6.6, 10.2, 4H), 1.55 (quint, *J* = 6.9, 2H), 1.37-1.14 (m, 18H), 0.86 (t, *J* = 6.7, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 71.6, 71.3, 70.8, 70.73, 70.70, 70.6, 70.1, 32.0, 30.4, 29.8, 29.74, 29.71, 29.6, 29.4, 26.2, 22.8, 14.2.

*General procedure for the synthesis of amphiphile **1-3***. 5 FU (5 eq) was dissolved in dry DMF (20 mL/mmol) at r.t. and anhydrous K_2CO_3 (1 eq) was added. After 15 min the appropriate bromo derivatives **11-13** (1 eq) were added under an atmosphere of Ar and the solution was heated at 80°C while stirring. After 2 h the TLC ($CHCl_3$/MeOH 9.5/0.5) showed the complete disappearance of the starting material. The reaction mixture was allowed to warm to room temperature and the solution was washed with H_2O (4 times), the two phases were separated and the aqueous one extracted with AcOEt. The combined org. extracts were washed with brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting residue was purified by CC (aluminium oxide; $CHCl_3$/MeOH 9.75/0.25) to afford compounds **1-3**. Amphiphile **1**. Colorless oil. Yield 54%. ATR-IR: 1727.3, 1666.5; ^{1}H-NMR ($CDCl_3$): δ = 9.87 (bs, 1H), 7.55 (d, *J* = 5.9, 1H), 3.88 (t, *J* = 5.0, 2H), 3.70 (t, *J* = 3.7, 2H), 3.65-3.57 (m, 18H), 3.57-3.51 (m, 2H), 3.41 (t, J = 6.8, 2H), 1.54 (quint, *J* = 7.1, 2H), 1.33-1.18 (m, 18H), 0.85 (t, *J* = 6.8, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 157.5 (d, *J* = 26.2), 149.8, 140.0 (d, *J* = 235.0), 130.5 (d, *J* = 33.0), 71.6, 70.7, 70.63, 70.62, 70.60, 70.1, 69.0, 48.4, 32.0, 29.72, 29.70, 29.5, 29.4, 26.1, 22.7, 14.2. Elemental analysis calculated (%) for $C_{28}H_{51}FN_2O_8$: C 59.76, H 9.14, N 4.98. Found: C 59.35, H 9.54, N 4.94. Amphiphile **2**. Colorless oil. Yield 56%. ATR-IR: 1701.3, 1684.9; ^{1}H-NMR ($CDCl_3$): δ = 9.66 (bs, 1H), 7.57 (d, *J* = 5.9, 1H), 3.89 (t, *J* = 4.9, 2H), 3.71 (t, *J* = 4.5, 2H), 3.66-3.58 (m, 22H), 3.57-3.53 (m, 2H), 3.42 (t, J = 6.8, 2H), 1.55 (quint, *J* = 7.1, 2H), 1.35-1.18 (m, 18H), 0.86 (t, *J* = 6.9, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 157.5 (d, *J* = 26.2), 149.8, 140.0 (d, *J* = 235.0), 130.6 (d, *J* = 33.0), 71.6, 70.7, 70.62, 70.60, 70.57, 70.1, 69.0, 48.4, 32.0, 29.75, 29.71, 29.70, 29.6, 29.4, 26.1, 22.8, 14.2. Elemental analysis calculated (%) for $C_{30}H_{55}FN_2O_9$: C 59.38,

H 9.14, N 4.62. Found: C 59.86, H 9.52, N 4.64. Amphiphile **3**. Colorless oil. Yield 48%. ATR-IR: 1705.1, 1699.3; ^{1}H-NMR ($CDCl_3$): δ = 9.37 (bs, 1H), 7.56 (d, *J* = 5.9, 1H), 3.89 (t, *J* = 4.9, 2H), 3.71 (t, *J* = 4.6, 2H), 3.69-3.52 (m, 26H), 3.59-3.53 (m, 2H), 3.43 (t, J = 6.8, 2H), 1.56 (quint, *J* = 6.9, 2H), 1.35-1.16 (m, 18H), 0.86 (t, *J* = 6.8, 3 H). ^{13}C-NMR ($CDCl_3$): δ = 157.5 (d, *J* = 26.3), 149.7, 140.0 (d, *J* = 235.0), 130.6 (d, *J* = 32.9), 71.6, 70.7, 70.63, 70.60, 70.58, 70.1, 69.0, 48.4, 32.0, 29.75, 29.70, 29.6, 29.4, 26.2, 22.8, 14.2. Elemental analysis calculated (%) for $C_{32}H_{59}FN_2O_{10}$: C 59.06, H 9.14, N 4.30. Found: C 59.36, H 9.44, N 4.34

Characterization of amphiphile 1-3

Determination of the Krafft point. A 10 mM aqueous solution of each amphiphile was heated to obtain a clear solution that was kept at 4 °C for 12 h. The critical micellar concentration (cmc) of amphiphiles **1-3** was measured at 25 °C by fluorescence spectroscopy measurements following a procedure described in literature.[2] Aqueous solutions (3 mL) of each amphiphile at concentrations between 10^{-6} M and 10^{-4} M were added to a defined amount of pyrene to obtain a 1 μM final concentration of pyrene (prepared from 50 μL of a 67.4 μM pyrene ethanol solution dried by a nitrogen flux). The solutions were kept above 37 °C, under stirring, for 12 h. Emission spectra of the solutions were acquired in the range 350-450 nm (λ_{exc} = 335 nm).

Liposome preparation

A lipid film was prepared on the inside wall of a round-bottom flask by evaporation of $CHCl_3$ solutions containing the proper amount of lipids (DOPC, the amphiphile **1**–**3** and the gemini cationic surfactant **4** at a 8/1/2 molar ratio). The obtained films were stored overnight under reduced pressure (0.4 mbar) and a PBS solution was added to the lipid film to obtain a lipid dispersion of the desired concentration. The solutions were heated at 45 °C, vortex-mixed and freeze-thawed six times from liquid nitrogen to 45 °C. Dispersions were then extruded (10 times) through a 100 nm polycarbonate membrane (Whatman Nucleopore). Extrusions were carried out at 25 °C using a 2.5 mL extruder (Lipex Biomembranes, Vancouver, Canada).

Liposome size determination

The size and the size distribution of the lipid aggregates were characterized by DLS measurements at 25°C. A Malvern Nano Zetasizer apparatus, equipped with a 4 mW HeNe laser source (632.8 nm) was used. In this apparatus, the light scattered by the sample, placed in a thermostated cell-holder, is collected at an angle of 173°. Autocorrelation functions were analyzed by means of the CONTIN algorithm. Decay times were used to determine the distribution of the diffusion coefficients *D* of the particles, which in turn are converted in a distribution of apparent hydrodynamic radii R_H using the Stokes-Einstein relation $R_H=kBT/6\pi\eta D$, where kBT is the thermal energy and η the solvent viscosity. The value of the R_H reported in the manuscript (Table 1) was averaged over several measurements and was obtained from intensity-weighted distributions.

Liposome zeta potential determination

The measurements of the electrophoretic mobility to determine zeta potential were carried out by means of the laser Doppler electrophoresis technique at 25 °C using a MALVERN Zetasizer apparatus equipped with a 5 mW HeNe laser. Analysis of the Doppler shift in the Zetasizer Nano series was done by using phase analysis light scattering (PALS) implemented with M3 (mixed mode measurement). Low applied voltages were used to avoid the risk of effects due to Joule heating. Zeta potential was derived from the electrophoretic mobility data by using the Henry equation under the Smoluchowski approximation.

Cell Cultures

HCT116 cell line was grown as monolayer in RPMI 1640 medium, supplemented with 10% FBS, 1% penicillin (50 U/mL) and streptomycin (50 μg/mL), in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37 °C.

MTT assay

HCT 116 cells were seeded for 24 h in 96-well plates ($4x10^5$ cells) and then treated for 48 h with 5-FU 2 μM, or empty DOPC/**4** (8:2) liposomes (at the same concentration of **1-3** containing

liposomes), or DOPC/**4**/**1-3** (8:2:1) liposomes (at total lipid concentration of 0.2 mM then 100-fold diluted to guarantee 2 μM **1-3** concentration) or DOPC/**1-3** (10:1) liposomes at 0.2 mM total lipid. As positive control, cells were treated with 1 μM STS for 48 h. Cell viability was then assessed by MTT assay.[3] After washing with PBS, cells were incubated with 2 mg/mL MTT solution for 3 h at 37 °C. All samples were lysed by DMSO, and analyzed by a microplate reader (BioRad, California) at 570 nm. Cell viability (%) was calculated as follow: (absorbance mean value of the treated sample/absorbance mean value of the control sample) x 100.

Statistical Analysis

The values shown in Figures 1 represent the averages ± standard deviations of three independent experiments. Student's t test was used for statistical analysis. Differences were considered significant at p values of ≤ 0.05.

References

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Figures

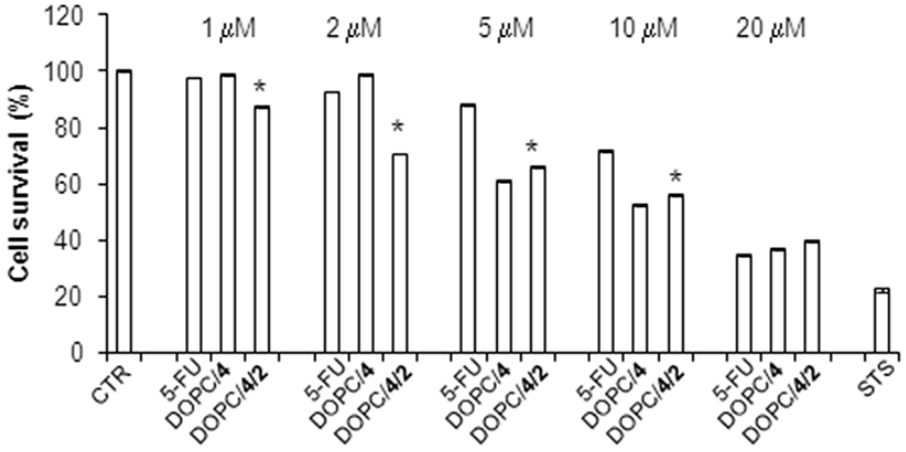

Figure S1. HCT 116 cell viability evaluated by MTT assay after treatment with 5-FU, or DOPC/**4** liposomes, or DOPC/**4**/**2** liposomes at different concentrations of 5-FU and **2** (1, 2, 5, 10 and 20 μM) and total lipids (10, 20, 50, 100 and 200 μM) for 48 h. 1 μM Staurosporine (STS) was used as positive control. The values are averages (*, statistically significant when compared to 5-FU-treated cells).

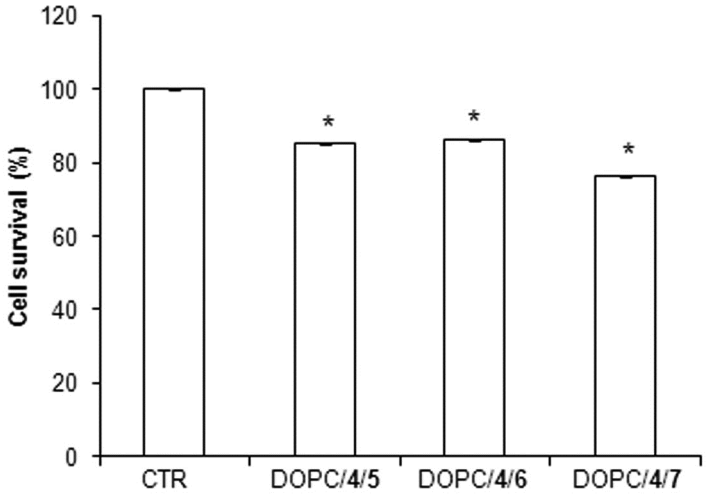

Figure S2. HCT 116 cell viability evaluated by MTT assay after different treatments with DOPC/4/5-7 liposomes after 48 h. The values are averages ± SD (*, statistically significant when compared to control, CTR).