Electronic Supplementary Information (ESI)

Liposomal Formulations for Efficient Delivery of a Novel Highly Potent Pyrimidine-Based Anticancer Drug

Sofia Teixeira [1,2], Débora Ferreira [3,4], Ana Rita O. Rodrigues [2], Ligia R. Rodrigues [3,4], Elisabete M. S. Castanheira [2]*, M. Alice Carvalho [1,]*

[1] Chemistry Centre of University of Minho (CQ-UM), Campus de Gualtar, 4710-057 Braga, Portugal; id9191@uminho.pt (S.T.); mac@quimica.uminho.pt (M.A.C.)
[2] Physics Centre of Minho and Porto Universities (CF-UM-UP) and LaPMET (Laboratory of Physics for Materials and Emergent Technologies), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal; ecoutinho@fisica.uminho.pt (E.M.S.C); ritarodrigues@fisica.uminho.pt (A.R.O.R)
[3] Centre of Biological Engineering (CEB), University of Minho, Gualtar, 4710-057 Braga, Portugal; lrmr@deb.uminho.pt (L.R.R.); deboraferreira@ceb.uminho.pt (D.F.)
[4] LABBELS – Associated Laboratory, Braga, Guimarães, Portugal; lrmr@deb.uminho.pt (L.R.R.); deboraferreira@ceb.uminho.pt (D.F.)
* Correspondence: ecoutinho@fisica.uminho.pt (E.M.S.C); mac@quimica.uminho.pt (M.A.C.)

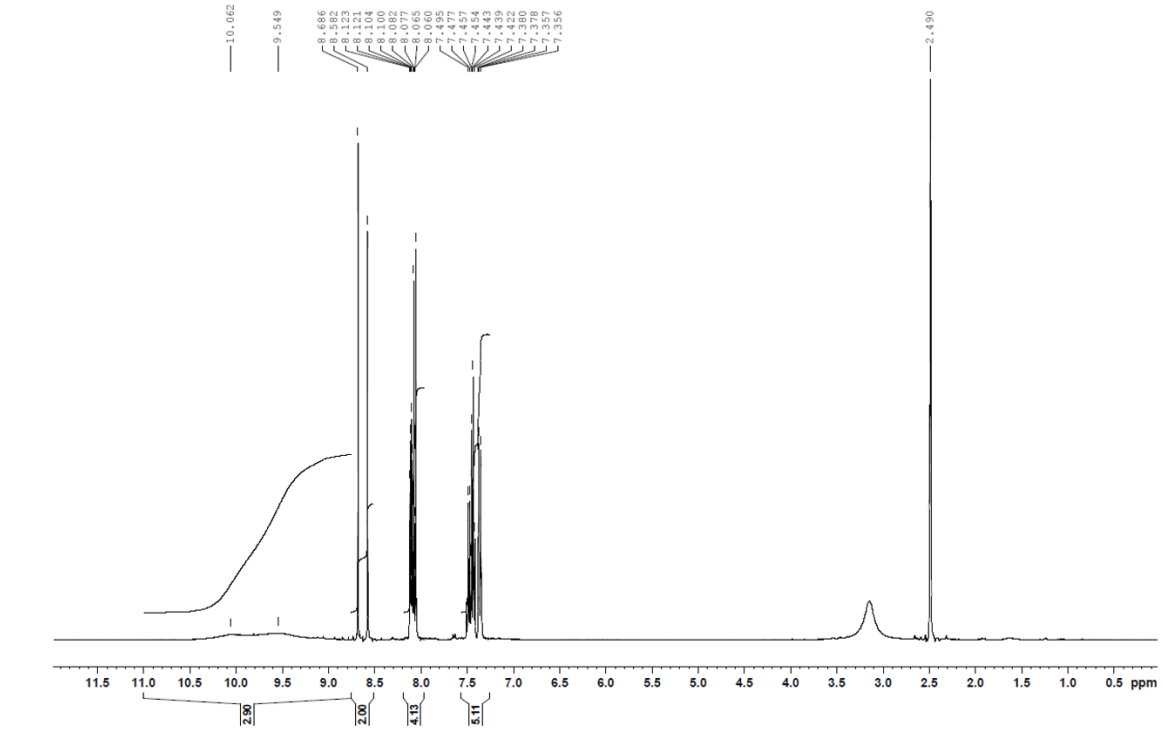
Index

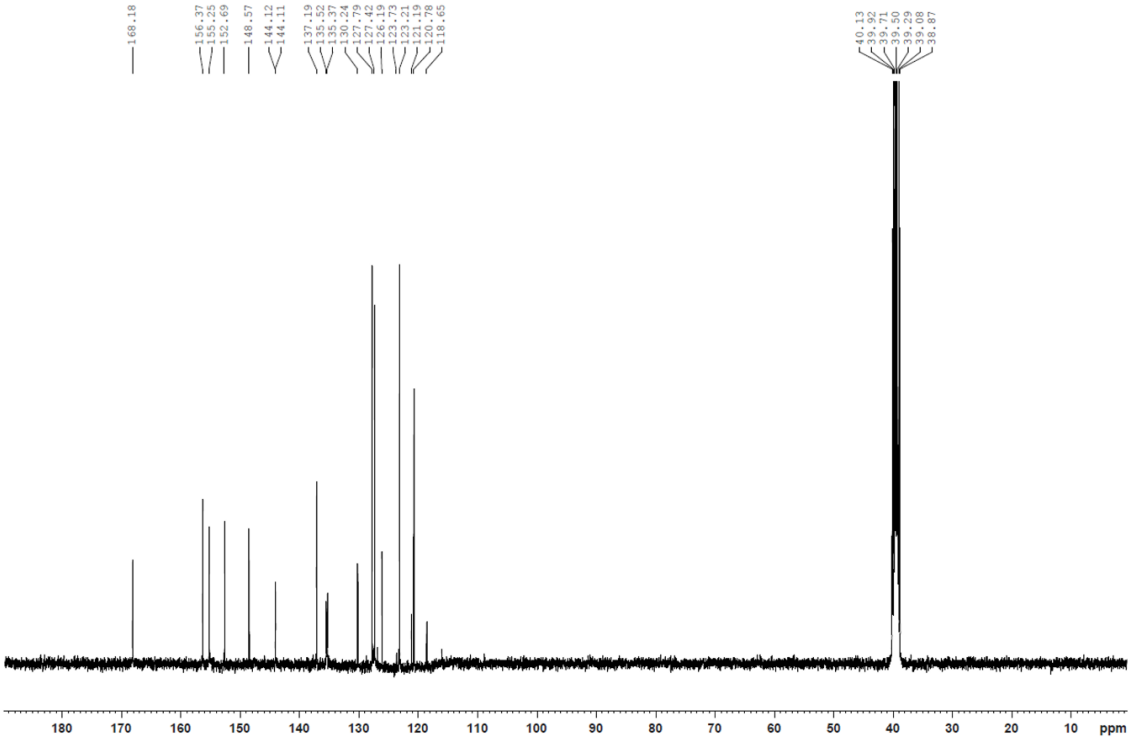
1. Materials and methods …… 2
1.1. ^{1}H and ^{13}C NMR spectra of compounds …….…... 2
1.2. Anticancer activity ……...……. 4
1.2.1. Reagents …….. 4
2. Results …… 4
2.1. PP spectra and calibration curve of fluorescence intensity vs. concentration ……. 4
2.2. Fitting of drug release profiles ……..... 4
2.3. Biological assays ……..…….. 5

1. Materials and methods

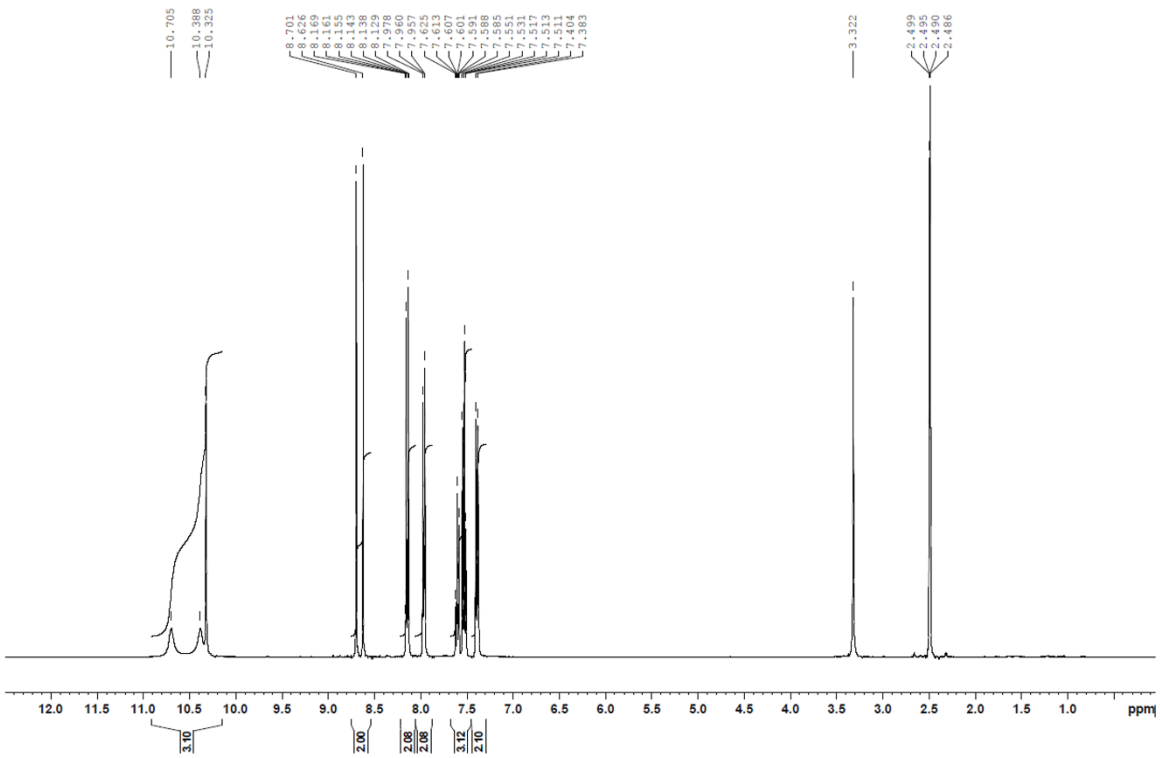
1.1. ^{1}H and ^{13}C NMR spectra of compounds

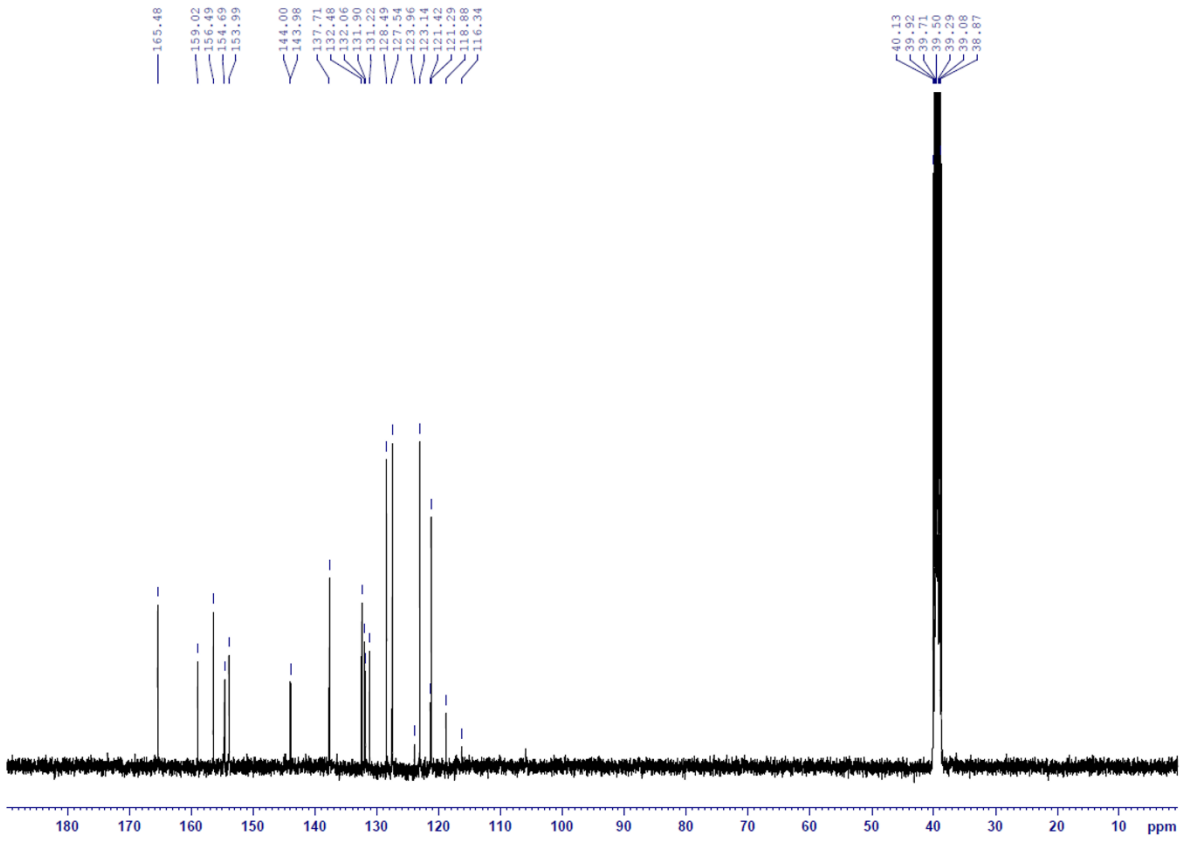
N-(4-imino-8-((4-(trifluoromethoxy)phenyl)amino)pyrimido[5,4-*d*]pyrimidin-3(4*H*)-yl)benzamide **9**





N'-(8-((4-(trifluoromethoxy)phenyl)amino)pyrimido[5,4-*d*]pyrimidin-4-yl)benzohydrazide **PP**





1.2. Anticancer activity

1.2.1. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent and paraformaldehyde (PFA) were acquired from Sigma-Aldrich. 4′,6-Diamidino-2-Phenylindole dihydrochloride (DAPI) and 1,1′- -Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate (CM-DiI Dye) were acquired from Biotium and Invitrogen, respectively. Alexa Fluor 488-Phalloidin was acquired from Molecular Probes. Bovine Serum Albumin (BSA) was purchased from Nzytech. Sodium dodecyl sulfate (SDS) and dimethyl sulfoxide (DMSO) were obtained from Thermo Fisher Scientific.

2. Results

*2.1. **PP** spectra and calibration curve of fluorescence intensity vs. concentration*

Figure S1 shows the absorption and fluorescence spectra of the novel **PP** compound and the calibration curve of fluorescence intensity *vs.* concentration.

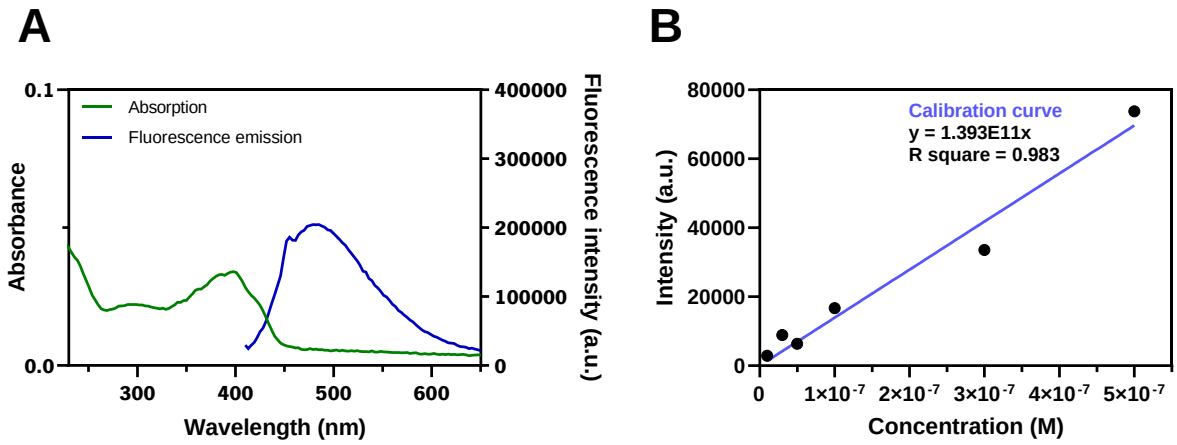


Figure S1. Absorption and fluorescence emission spectra of **PP** compound (10^{-6} M in ethanol) (**A**) and calibration curve of fluorescence intensity of **PP** vs. concentration (**B**).

2.2. Fitting of drug release profiles

The results of the fitting of release profiles to first-order kinetics and Weibull models are displayed in **Table S1**.

Table S1. Parameters obtained by fitting the release profiles to the first-order kinetic model and Weibull model, and the respective coefficients of determination (R^2).

Liposomal formulations	First-order kinetics		Weibull		
	k (min^{-1})	R^2	b	a	R^2
PP-EggPC-L	0.004009	0.914	0.5928	0.0326	0.963
PP-EggPC-Chol-L	0.003876	0.720	0.2954	0.04751	0.968
PP-DPPC-L	0.004873	0.697	0.3419	0.0841	0.944
PP-DPPC-Chol-L	0.003978	0.788	0.3691	0.06816	0.971

2.3. Biological assays

Figure S2 shows that the viability of HCT 116 and MDA-MB-231 cell lines decreased with increasing concentrations of 5-fluorouracil (**5-FU**), demonstrating a dose-dependent effect. **5-FU** showed IC_{50} values of 10.39 ± 0.68 μM for HCT 116 and 183.49 ± 17.13 μM for MDA-MB-231 cells.

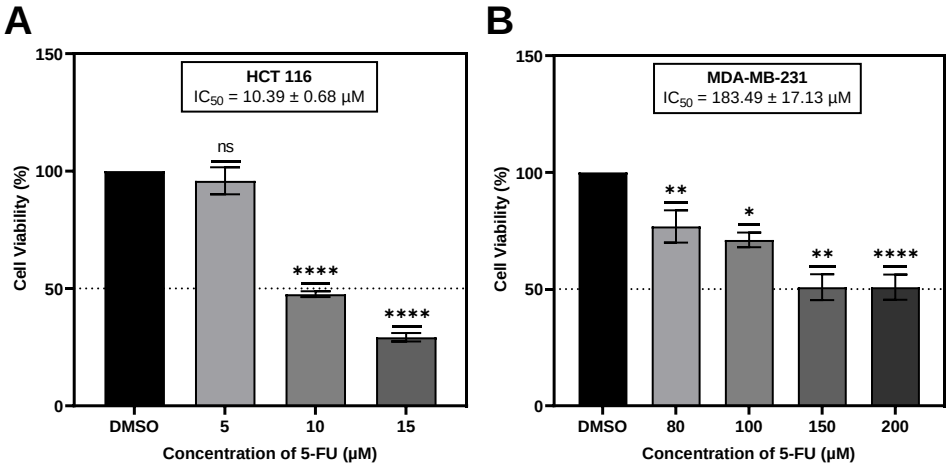


Figure S2. Assessment of the viability of HCT 116 (**A)** and MDA-MB-231 (**B)** cancer cells after 48 hours exposure to **5-FU**. HCT 116 **(A)** exposure to **5-FU** concentrations of 5, 10 and 15 μM over a 48-hour treatment period. **MDA-MB-231 (B, D)** exposure to **5-FU** concentrations of 80, 100, 150 and 200 μM over a 48-hour treatment period. Cell viability was determined using the MTT colorimetric assay, and normalized to DMSO treated cells. The resulting data are presented as IC_{50} (in μM) ± SEM (standard error of the mean) from three independent experiments. One-way ANOVA indicates statistically significant differences to DMSO assessed by Dunnett's post-test, and denoted as follows: ns (non-statistical significance), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

Figure S3 shows the cytotoxic effect of placebo nanoformulations against HCT 116 and MDA-MB-231 cancer cells and BJ-5ta normal cells.

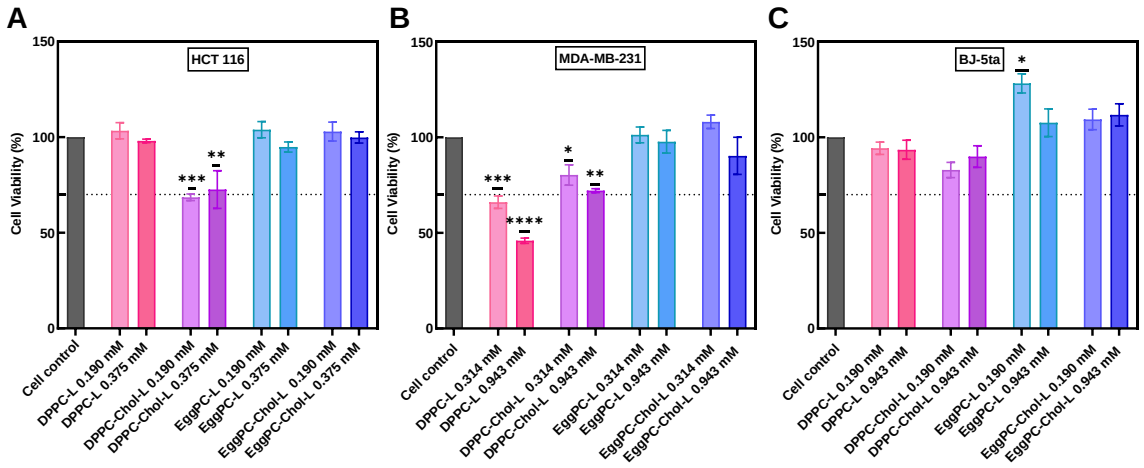


Figure S3. Assessment of the viability of HCT 116 (**A**), MDA-MB-231 (**B**) and BJ-5ta (**C**) after 48 hours exposure to placebo liposomal nanoformulations. HCT 116 (**A**) exposure to placebo liposomal nanoformulations concentrations of 0.190 and 0.375 mM, over a 48-hour treatment period. MDA-MB-231 (**B**) exposure to placebo liposomal nanoformulations concentrations of 0.314 and 0.943 mM, over a 48-hour treatment period. BJ-5ta (**C**) exposure to placebo liposomal nanoformulations concentrations of 0.190 and 0.943 mM, over a 48-hour treatment period. Cell viability was determined using the MTT colorimetric assay, and normalized to untreated cells. One-way ANOVA indicates statistically significant differences to control by Dunnett's post-test, and denoted as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.