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Combinational drug-loaded lipid nanocapsules for the treatment of cancer

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ABSTRACT

The purpose of this study was to investigate the feasibility of an intravenously administered combinational therapy using lipid nanocapsules (LNCs) as a drug delivery carrier for the treatment of different cancers. Therefore, we encapsulated 6 anticancer drugs within LNCs. Their size was approximately 50 nm. Except for oxaliplatin, their encapsulation efficiency, which was measured by different analytical methods, varied between 75% for SN38 to 100% for regorafenib. The *in vitro* studies showed a nonsignificant difference between the cytotoxicity of free and encapsulated drugs and a significant decrease in haemolysis by encapsulation in LNCs. Finally, the *in vivo* experiment showed that a combinational regimen of SN38-LNCs and regorafenib-LNCs abates CT26 murine colorectal cancer growth and increases median survival time.

1. Introduction

Traditional therapies for cancer mainly involve oral or intravenous (IV) administration of cytotoxic molecules that either inhibit tumour growth or lead to tumour shrinkage and regression. Nonetheless, chemotherapy is highly toxic to cancer cells and surrounding healthy tissue and can lead to systemic toxicity that could be lethal for patients (Livshits et al., 2014). Chemotherapy options depend on several factors, such as the cancer type, stage and even the overall health of a patient, due to the side effects of the treatment. The major toxicities of chemotherapeutic drugs have been thoroughly described, and they are dependent on drug pharmacokinetics, biodistribution and genetic factors. For example, the toxicity of oxaliplatin (Oxa) includes severe peripheral neuropathy. To limit the toxicity of the drug, pause and resume treatment regimens have been proposed (Di Francia et al., 2013). Nevertheless, side effects are observed not only with cytotoxic drugs but also with targeted therapy. For example, regorafenib (Reg) is approved for some types of cancer treatments with common side effects, such as fatigue, diarrhoea, high blood pressure, weight loss, abdominal pain and even severe bleeding (Wilhelm et al., 2011).

All of the abovementioned drugs have been used to treat cancer; however, their use is not a panacea because the life of a patient is endangered at the beginning of the treatment period (Livshits et al., 2014). Nanomedicine aims to limit some drawbacks of anticancer drugs by limiting their toxicity, increasing their concentration in tumours and increasing their therapeutic window. Several nanocarriers have been approved for clinical use, mostly including liposomes (Bulbake et al., 2017) and, more recently, polymeric and inorganic nanoparticles (Ventola, 2017).

Lipid nanocapsules (LNCs) are lipid-based nanocarriers that can be prepared with GRAS excipients without the use of toxic solvents, can be easily scaled up, and can encapsulate hydrophobic molecules. Hydrophobic cytotoxic drugs, including paclitaxel, SN38 and etoposide, have been encapsulated within LNCs (Garcion, 2006; Roger et al., 2011; Saliou et al., 2013). Even if LNC formulations have been designed to embody priory hydrophobic molecules, their use is not limited. Hydrophilic molecules can also be incorporated through a simple reverse micelle system, where hydrophobic tails are located within the oily core of LNCs. LNCs-reverse-micelles containing hydrophilic molecules, such as erlotinib hydrochloride and acriflavine hydrochloride, have been previously designed and investigated (Montigaud et al., 2018; Vrignaud et al., 2012).

To date, different formulations of conventional chemotherapy for intravenous administration have been developed including solid lipid nanoparticles, liposomal and polymeric nanoparticles (Patel et al., 2014; Udofot et al., 2016; Wang et al., 2018). This study aimed to assess whether LNCs are suitable nanocarriers for the administration of several types of anticancer drugs and to select a combinational schema to

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Table 1
Mechanism of action, target cancer and partition coefficient (logP) of irinotecan, SN38, oxaliplatin, 5-Fluorouracil, doxorubicin hydrochloride and regorafenib.

Active molecule	Mechanism of action	Target cancer	Side effects	LogP*	References
Irinotecan	Topoisomerase I inhibitor	Colon, small cell lung cancer	Acute toxicity in polymorphism of UGT1A1	2.78	Fujita (2015)
SN38	Topoisomerase I inhibitor	Colon, small cell lung cancer	Acute toxicity in polymorphism of UGT1A1	2.65	Fujita (2015)
Doxorubicin Hydrochloride	Intercalating agent; Topoisomerase II inhibitor	Breast, colorectal cancer	High cardiotoxicity	0.92	Sonowal et al. (2017), Thorn et al. (2011)
Oxaliplatin	Alkylating-like agent	Colorectal cancer	Severe peripheral neuropathy	−0.47	Di Francia et al. (2013)
5-Fluorouracil	Thymidylate synthase inhibitor	Breast, skin, colorectal cancer	Severe systemic toxicity in dihydropyrimidine dehydrogenase deficiency; neutropenia	−0.58	Baek et al. (2006), Latchman et al. (2014)
Regorafenib	Multikinase inhibitor	Metastatic colorectal cancer, hepatocellular carcinoma, gastrointestinal stromal tumor	Fatigue, diarrhea, high blood pressure, weight loss, abdominal pain, severe bleeding	4.49	Wilhelm et al. (2011)

* Log P is estimated with chemaxon calculator.

abate a tumor initiated by colorectal cancer cells. To do so, six anticancer drugs with different pharmacological mechanisms of action and physicochemical properties were investigated (Table 1). We encapsulated conventional chemotherapy comprising 3 hydrophilic drugs (Oxa, Doxorobicin (Dox), and 5-Fluorouracil (5Fu)) and 2 hydrophobic drugs (irinotecan (Iri), and SN38). Furthermore, we encapsulated a multikinase inhibitor Reg, a highly hydrophobic molecule. LNC formulations were characterized to optimize the encapsulation efficiency (EE) of drugs within LNCs. To assess the efficacy of our formulations *in vitro*, without regarding the particular mechanism of each drug, we performed a clonogenic assay. To evaluate the potential haemotoxicity of both free and encapsulated SN38 administered IV, a haemolysis study on human blood was conducted. Finally, the efficacy of the selected drug-loaded LNC formulations administered intravenously was assessed *in vivo*.

2. Materials and methods

2.1. Materials

SN38, irinothecan (Iri), and regorafenib (Reg) were purchased from MedChemExpress (Sweden). Oxa, 5-Fluorouracil (5Fu), and doxorubicin HCl (Dox) were obtained from Chemieliva (China), and 3,3′-dilinoleyloxacarbocyanine perchlorate (DiO) was obtained from Invitrogen (ThermoFisher Scientific, Belgium). Labrafac™ lipophile WL 1349 (caprylic/capric triglycerides), Labrafac PG (propylene glycol caprylate/caprate), Labrafil 1944 CS (oleoyl polyoxyl-6 glyceride), Maisine CC (glycerol monolinoleate) and Transcutol® HP (diethylene glycol monoethyl ether) were gifted by Gattefossé S.A (Saint-Priest, France). Lipoid® S-100 (soybean, approximately 100% phosphatidylcholine) was kindly provided by Lipoid Gmbh (Ludwigshafen, Germany). Solutol® HS15 (a mixture of free polyethylene glycol 660 and polyethylene glycol 660 hydroxystearate), Span 85 (sorbitan trioleate) and Span 80 (sorbitan monooleate) were purchased from Sigma-Aldrich (St. Louis, USA). NaCl was purchased from Prolabo VWR International (Fontenay-sous-Bois, France). HPLC grade solvents such as: Acetonitrile (ACN), acetone, dimethylsulfoxide (DMSO), methanol (MeOH), and tetrahydrofuran (THF) were purchased from Sigma–Aldrich. Culture reagents were obtained from Sigma (St. Louis, USA) and Invitrogen (Merelbeke, Belgium). Cells were purchased from ATCC (Manassas, USA).

2.2. LNC formulation

The composition in weight of the LNCs is shown in Table 2. The percentage of the components is detailed in the Supplementary data (Table S1). Hydrophilic drugs, 5Fu, Dox and Oxa, were encapsulated in reverse micelles (RMs). Span 80 (Dox and Oxa) or Span 85 (5Fu), Transcutol® HP (Oxa), Labrafac PG and water were mixed for at least 3 h. In another vial, Labrafac® PG, Lipoïd® S100, Solutol® HS15, NaCl and water were mixed and underwent 3 heating–cooling cycles. Finally, 500 µl of the reverse micelles was added after the third cycle at 85 °C, and cold water was then added immediately upon the appearance of the phase inversion temperature (PIT).

The formulations of lipid nanocapsules loaded with SN38 or Iri were adapted from Roger et al., with some changes to adjust the lipid nanocapsules for IV administration. Briefly, Labrafil® M 1944 CS, Labrafac®, Lipoïd® S100, Solutol® HS15, NaCl, and 0.1 N NaOH were mixed and heated to 80 °C, and Iri or SN38, which was previously solubilized in Transcutol® HP, was then added. After 3 heating–cooling cycles (80–50 °C), cold 0.05 N HCl was added at one degree below the PIT. The formulations were stirred magnetically for 5 min at room temperature and filtered through a 0.22 µm PVDF filter.

Reg was solubilized in Transcutol® HP at a concentration of 66.6 mg/ml under heating. It was then mixed with Labrafac WL 1349, Lipoïd® S100, Solutol® HS15, NaCl and water, and the solution

Table 2
Composition of the LNC formulations.

Components (mg)		Blank LNCs	Blank-RM-LNCs	Iri-LNCs	SN38-LNCs	Dox-RM-LNCs	Oxa-LNCs	5Fu- RM-LNCs	Reg-LNCs
Active substance				10	1	3.57	0.7	1.64	4
Reverse micelles	Spa n 85	–	–	–	–	–	–	71.4	
	Span 80	–	71.4	–	–	71.4	71.4	–	
	Labrafac 1349	–	357.1	–	–	357.1	285.7	357.1	
	Transcutol® HP	–	71.4	–	–		71.4		
	H2O	–	71.4	–	–	71.4	71.4	71.4	
Transcutol® HP		350	–	350	200	–		–	60
Labrafac WL 1349		200	275	200	200	275	275	275	190
Labrafil 1944 CS		500	–	500	600	–		–	
Solutol		500	628	500	500	628	628	628	242
Lipoid		75	–	75	75	–		–	21
NaCl		35	45.8	35	35	45.8	28.8	28.8	30
0.1 N NaOH		1000		1000	1000	–		–	–
Water		–	667	–	–	667	667	667	690
Cold 0.05 N HCl		1500		1500	1500	–		–	
Cold water			1500	–	–	1500	1650	1650	1570

underwent 3 heating–cooling cycles. Finally, cold water was added at the PIT.

DIO was first dissolved in chloroform before mixing at a final concentration of 0.22 mg/ml in Labrafac Wl 1349. The rest of the ingredients were mixed similar to the blank-RM-LNCs formulation, and cold water was added at the PIT.

2.3. LNC characterization

2.3.1. Size, zeta potential and PDI

The average nanoparticle size, zeta potential and polydispersity index (PDI) of the drug-loaded LNCs were measured by using a Malvern Zetasizer NanoZS (Malvern Instruments, UK). The size and PDI were evaluated using dynamic light scattering measurements, while the zeta potential was evaluated by laser Doppler velocimetry. Each sample was diluted to 1/100 (v/v) in MilliQ water (Merck-Millipore, Germany) (N = 3, n = 3).

2.3.2. LNC drug loading and encapsulation efficiency

2.3.2.1. Ultrafiltration. Free molecules were separated from the encapsulated molecules using a Vivaspin 6 tube (10 kDa). Iri-LNCs, SN38-LNCs and Reg-LNCs were diluted with water (1:10 v/v), and the solution was then centrifuged at $4.000 \times g$ for 15 min at room temperature. Then, dilution at 1:10 v/v in MeOH:THF:DMSO (50/45/5) (v/v) for Iri-LNCs, SN38-LNCs and in MeOH for Reg-LNCs was used to breakdown the LNCs and solubilize the free and encapsulated drugs (considered as the total drug). Then, both of the solutions were filtered and quantified by HPLC-UV.

5Fu-RM-LNCs were diluted with water (1:50 v/v), and the solution was then centrifuged at $4.000 \times g$ for 15 min at room temperature. Then, EtOH was used to release 5Fu from the LNC pellet (1:50 v/v). The supernatant and pellets were analysed by HPLC-UV.

Free Oxa was also isolated from the encapsulates by ultrafiltration using a Vivaspin 6 tube (1:10 v/v). The filtrate (free Oxa) and total Oxa-LNCs were incubated with concentrated HNO_3 at 60 °C for 30 min for decomposition of the organic matrix.

2.3.2.2. Dialysis. Dialysis was performed to separate free Dox from the encapsulated Dox. Briefly, 1 ml of Dox-LNCs was inserted into a float-a-lyzer (3.5–5 kDa), and 500 ml of water was changed thrice over 24 h. All free drug passed through the membrane towards the water. After dialysis, the remaining formulations were diluted in EtOH (1:50) and then analysed by fluorescence.

2.3.2.3. Analytical methods. Chromatographic analysis of Iri, SN38, 5Fu and Reg was carried out using a Shimadzu 20A HPLC instrument with an SPD-20A UV detector. Different mobile phases and modes of detection for each drug are show in Table 3.

Samples containing OXA were analysed by ICP-MS to evaluate the concentration of platinum (^{195}Pt) in the formulations. The samples were further diluted 100 times with 1% HNO_3, and 0.5% HCl solution containing ^{193}Ir served as an internal standard. Pt was analysed using no gas mode.

The encapsulation efficiency (EE) of the drugs in the LNCs was calculated by dividing the concentration of the encapsulated drug by the total concentration of the drug in the formulation.

$$EE\% = \frac{[drug]_{total} - [drug]_{free}}{[drug]_{total}} \times 100 = \frac{[drug]_{encapsulated}}{[drug]_{total}}$$

Drug loading was evaluated as the ratio between the encapsulated amount of drugs and the amount of the oil components in the formulations (Labrafac, Lipoid) (w/w).

$$\text{Drug loading} = \frac{\text{amount of encapsulated drug (mg)}}{\text{amount of oil components (g)}}$$

Table 3
Analytical methods used to evaluate the EE and drug loading of each LNC formulation.

Formulations	Analytical method	Mobile phases	Columns	Mode	Detection system	Retention time (min)	LOQ (μg/ml)
Iri-LNCs	HPLC-UV	A: 0.1% (v/v) formic acid B: 0.1% (v/v) formic acid in methanol	Nucleodur C_{18} column, 125 × 4 mm, 5 μm particle size, 100 Å pore size	0.01′–10% B, 5.0′– 90% B, 6.0′– 90% B, 8.0′– 10% B, 10.0′–10% B.	378 nm	5.48	19.70
SN38-LNCs	HPLC-UV	A: 0.1% (v/v) formic acid B: 0.1% (v/v) formic acid in methanol	Nucleodur C_{18} column, 125 × 4 mm, 5 μm particle size, 100 Å pore size	0.01′–10% B, 5.0′– 90% B, 6.0′– 90% B, 8.0′– 10% B, 10.0′–10% B.	378 nm	6.50	9.58
5Fu-LNCs	HPLC-UV	A: H_2O B: methanol	Nucleosil C_{18} column, 250 × 4.6 mm, 5 μm particle size, 300 Å pore size	90% A, 10% B.	255 nm	2.5	39
Dox-LNCs	Fluorescence	–	–	–	Exc. 480 Em. 560	–	2.89
Oxa-LNCs	ICP-MS	–	–	No gas	–	–	0.08
Reg-LNCs	HPLC-UV	A: H_2O B: methanol C: acetonitrile	Sunfire C18 column, 150 × 4.6 mm, 3.5 μm particle size, 100 Å pore size	20% A, 10% B, 70%C.	261 nm	3.60	22.13

The limit of quantification (LOQ) was estimated using the following equation: LOQ = (10 × sd/S) where sd is the standard deviation and S the slope of the calibration curve.

2.4. In vitro studies

2.4.1. Cell lines and culture

HT29 and SW480 human colon cancer cells, CT26 mouse colon cancer cells and MCF-7 human breast cancer cells were used for the experiments. The colon cancer cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS, Gibco, USA), 100 mU/ml penicillin, 100 μg/ml streptomycin, 2 μM glutamine and 2.5 μg/ml Plasmocin™ prophylactic in a 5% CO_2 atmosphere at 37 °C. MCF-7 cells were cultured in minimum essential medium supplemented with 5% FBS, 100 mU/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate, 10 ng/ml human epidermal growth factor (EGF), 10 μg/ml insulin and 2.5 μg/ml Plasmocin™ prophylactic in a 5% CO_2 atmosphere at 37 °C.

2.4.2. Clonogenic assay

For the clonogenic assay, 0.2×10^6 cells were seeded in 6-well plates and incubated overnight. The cells were then treated with different concentrations of the free drug and drug-loaded LNCs. After 48 h of drug incubation, the growth medium was discarded, and the cells were trypsinized. For each condition, 1000 cells were seeded into new 6-well plates with fresh medium and incubated for 7 days. Clones formed following the incubation were fixed and stained with a 0.5% (w/v) crystal violet solution in ethanol (50% v/v), acetic acid (5%) and water 45%v/v. The IC_{50} was determined using curves generated by GraphPad Prism software. Untreated cells correspond to 100% viability, and 0% viability corresponds to a sufficiently toxic drug concentration that obstructs cells from making clones.

2.5. Cell uptake

2.5.1. Flow cytometry

MCF7 cells were seeded in a 24-well plate at a density of 0.15×10^6 cells. Twenty-four hours after seeding, the medium was replaced with medium containing 5 μg/ml Dox and Dox LNCs, and the cells were incubated for 0 min, 15 min, 30 min, 1 h, 2 h, 3 h, and 4 h. Then, the medium was discarded, and the cells were trypsinized and washed twice with PBS-BSA 0.5% EDTA 1 mM. Next, the cells were transferred to a 96-well Costar FACS well plate and analysed immediately by FACS.

2.5.2. Confocal microscopy

In parallel, 40.000 MCF7 cells were seeded in an 8 μ-slide plate (IBidi, Germany). The cells were incubated with 5 μg/ml of Dox or Dox LNCs, 2 mg/ml of Dio-RM-LNCs (DIO 1 mg/g of Labrafac) or 2 mg/ml of blank-RM-LNCs. After incubation, the medium was discarded, and the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Rhodamine phalloidin (RP) (Thermo Fisher) (0.13 v/v in PBS with 0.05% Triton) was added to stain the cytoskeleton, and finally, cell nuclei were stained with 2 μg/ml DAPI in PBS. Between each step, the cells were washed twice with PBS. Then, the cells were examined using a fluorescence microscope (Axio Imager 2, Zeiss) with 405 nm (DAPI, blue), 555 nm (RP, green), and 488 nm (Dox or Dio, red) excitation filters.

2.6. Haemolysis studies

An assay for the haemolytic effect of the free and encapsulated SN38 was performed. Fresh human blood 0-RhD^+ was centrifuged at 500 × *g* for 5 min to separate the buffy coat and plasma from the erythrocytes. Then, the erythrocytes were washed with PBS and centrifuged at 500 × *g* for 5 min. A 5% (v/v) solution of erythrocytes in PBS was prepared and incubated with different concentrations of free or

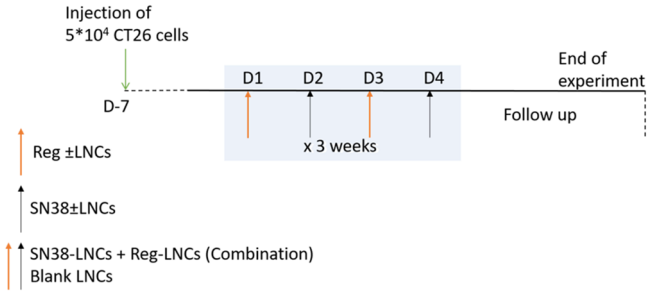

Fig. 1. Treatment protocol. Seven days after CT26 inoculation, Reg (10 mg/kg) and SN38 (1 mg/kg) were alternately administered twice a week for 3 weeks. The free form was administered IP, the encapsulated form and the Blank LNCs were administered IV.

encapsulated SN38 (5 to 50.5 µg/ml) for one hour at room temperature. Triton (1%) was added as a positive control. After incubation, the suspensions were centrifuged at 500 × *g* for 5 min, and the supernatants were transferred to a 96-well plate to measure haemoglobin release according to the absorbance at a wavelength of 545 nm. Haemolysis was calculated as 0% from the absorbance of the untreated erythrocytes and as 100% from the absorbance of the erythrocytes treated with Triton, which completely lysed the cells.

2.7. In vivo anti-tumour efficacy study

The *in vivo* experiments were performed under the guidelines of the Belgian national regulation, with approval from the animal care ethical committee of the Université Catholique de Louvain (2017/UCL/MD/034).

Five-week-old female Balb/C mice were obtained from Janvier (France). The mice were anaesthetized with 13 mg/kg and 100 mg/kg xylazine and ketamine, respectively, and injected subcutaneously with 5×10^4 CT26 cells. At day 8 following tumour inoculation, when the tumours were palpable (approximately 25 mm^3), the mice were randomized and divided into the following 7 groups: group 1: untreated (N = 6); group 2: blank LNCs (N = 6); group 3: SN38 (N = 7); group 4: SN38 LNCs (N = 6); group 5: Reg (N = 7); group 6: Reg LNCs (N = 6); and group 7: combination of SN38-LNCs and Reg-LNCs (N = 6). Finally, the mice were treated with 3 weekly cycles of two doses of SN38 at days 1 and 3 and/or two doses of Reg at days 2 and 4, as presented in Fig. 1. The doses administered to the mice were 1 mg/kg SN38 and 10 mg/kg Reg. The tumour volume was measured with a calliper and calculated with the following equation: length * width * height. During the experiments, the mice were monitored daily, and the body weight and tumours were measured every 2 days. The mice were sacrificed when one of the following endpoints was reached: change in behaviour, clinical sign of distress, 20% weight loss, presence of an ulcerated necrotic tumour or a tumour volume greater than 1500 mm^3.

2.8. Statistical analysis

The results are presented as the mean ± standard deviation (SD) of at least three replicates per experiment. GraphPad Prism software was used to conduct the statistical analysis, and p-values < 0.05 were considered statistically significant (*p < 0.05, **p < 0.01 and ***p < 0.001). For these experiments, n is the number of replicates for each experiment. Survival curves were compared using a Mantel-Cox (log-rank) test.

3. Results and discussion

3.1. Formulation and characterization of the anticancer drug-loaded LNCs

LNCs were prepared using the phase inversion-based technique (Heurtault et al., 2002). Their composition is shown in Table 2, and their physicochemical characterization is shown in Table 4.

Hydrophilic drugs (Dox, 5Fu, and Oxa) could not be encapsulated directly within the LNCs due to their hydrophilicity. Therefore, a previous encapsulation into RM was required. The drugs had to be entrapped in the aqueous phase of the RM that were subsequently included into the oily core of the LNCs. Therefore, the drugs were mixed with surfactants (Span 80, Span 85, Tween 80, and Tween 85) and different oils (Labrafac Lipophile WL 1349, Labrafac PG). A higher EE was obtained with the combination of Span 80 or Tween 85 and Labrafac WL 1349 and with the combination of Tween 85 and Labrafac PG for Dox (Supplementary data, Fig. S1B). Additionally, a higher EE was obtained with the combination of Span 85 or Tween 85 and Labrafac Lipophile WL 1349 and with the combination of Tween 85 and Labrafac PG for 5FU (Supplementary data, Fig. S1C). For Dox and 5FU, Span 80 and Span 85 were used, respectively. The EE of Dox was up to 91.2% with a high loading of 5.9 mg/g. 5Fu-LNCs had an EE of 62.4% with a drug loading of 1.58 mg/g, while Oxa had a poor EE of 22.5% with a low drug loading of 0.2 mg/g.

Iri or SN38 was solubilized in Transcutol® HP prior to the formulation process. As described by Roger et al., an alkaline environment can enhance the solubility of SN38 due to the ionization of the carboxylate group and avoid precipitation during the heating–cooling procedure. After the last heating cycle, a cold acidic solution was added at one degree below the PIT, and the drugs were entrapped in the LNCs in their active lactone forms (Roger et al., 2011). Indeed, the use of alkaline and acidic conditions during the formulation process significantly increased the EE and drug loading of Iri and SN38 (Supplementary data, Fig. S1A). Although the entrapment of SN38 was not significantly different between our two formulations, we excluded the formulation with the citrate buffer for the *in vivo* studies because it is a Ca^{+2} chelator and can lead to lethal hypocalcaemia (Vagianos et al., 1990). We encapsulated Iri into nanocapsules with an EE of 78.6% and a high drug loading of 8.2 mg/g, and its metabolite SN38 had an EE of 76.7% and a drug loading of 0.53 mg/g. Although the drug loading of SN38 was less than that of Iri, we used SN38 for the subsequent studies because the potency of SN38 is 1000 times greater than its parent drug (Pangilinan et al., 2008).

Table 4
Physicochemical characterization of each LNC formulation. Each formulation was replicated at least 3 times (mean ± SD, N = 3).

Formulations	Size (nm)	PdI	ζ potential (mV)	EE (%)	Drug loading (mg/g)
Blank-LNCs	56.0 ± 0.8	0.05 ± 0.01	0.4 ± 0.2	–	–
Blank-RM-LNCs	45.4 ± 0.1	0.06 ± 0.01	−3.8 ± 0.2	–	–
Iri-LNCs	54.0 ± 3.0	0.10 ± 0.05	0.7 ± 0.2	78.6 ± 6.2	8.2 ± 0.1
SN38-LNCs	48.1 ± 0.3	0.08 ± 0.01	0.5 ± 0.2	76.7 ± 7.2	0.5 ± 0.1
Dox-RM-LNCs	49.7 ± 0.3	0.10 ± 0.01	−0.25 ± 0.04	91.2 ± 1.1	5.9 ± 0.2
Oxa-RM-LNCs	51.6 ± 0.8	0.06 ± 0.02	−2.7 ± 0.1	23.0 ± 2.0	0.21 ± 0.01
5Fu-RM-LNCs	42.1 ± 0.3	0.09 ± 0.03	−2.2 ± 0.2	62.4 ± 3.3	1.8 ± 0.1
Reg-LNCs	39.1 ± 1.4	0.09 ± 0.03	−4.6 ± 1.6	99.9 ± 0.4	14.3 ± 1.0

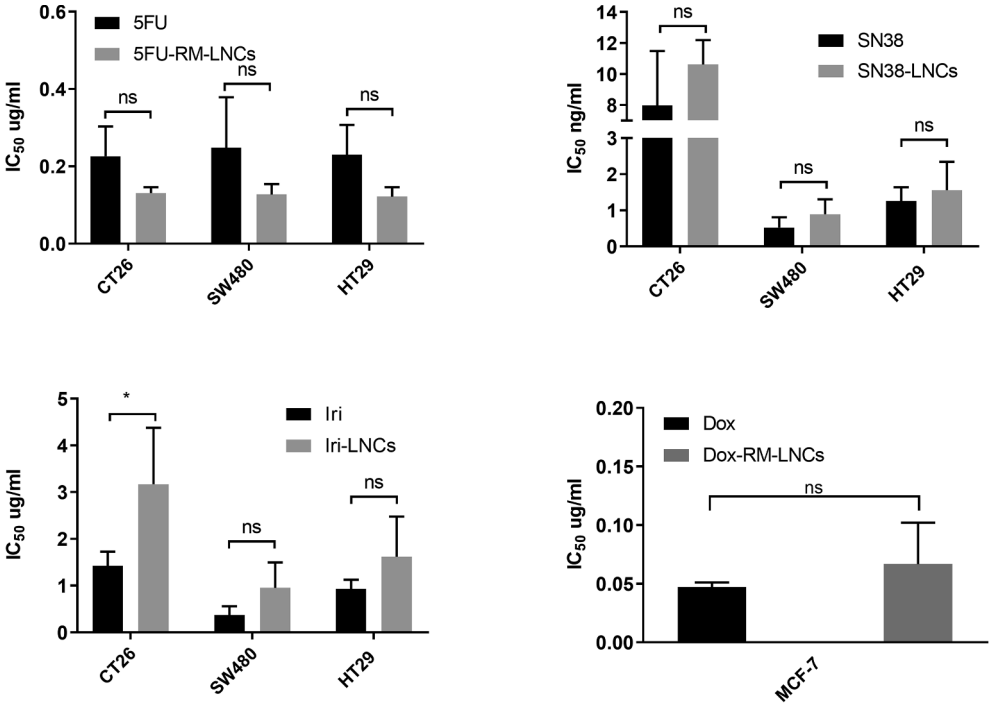

Fig. 2. Clonogenic cytotoxicity assay. The data are presented as the IC_{50} of different free drugs vs. drug-loaded LNCs; A) Iri vs. Iri-LNCs B) SN38 vs. SN38-LNCs, C) 5Fu vs. 5Fu-RM-LNCs in CT26 (murine), SW480, and HT29 (human) colorectal cancer cell lines and D) Dox vs. Dox-RM-LNCs in the MCF7 human breast cancer cell line. Mean ± SD, N = 3, and n = 2.

Because Reg was not soluble in different oils (< 1 mg/ml in Maisine CC, Labrafac PG, Labrafac WL 1349, Labrafil 1944, and Peceol), we solubilized Reg in Transcutol® HP at a concentration of 66.6 mg/ml under heating. Consequently, a high EE of 99.4% with a drug loading of 14.3 mg/g were obtained.

The physicochemical properties of the LNC formulations are shown in Table 3. Blank LNCs had a mean diameter of 57.9 nm, were monodisperse (PdI = 0,03) and a neutral charge (ζ = 0.81 mV), while blank-RM-LNCs were slightly smaller (45 nm), monodisperse (PdI = 0,06) and slightly negatively charged (ζ = −3.8 mV).

The entrapment of drugs did not lead to a significant change in the colloidal properties of LNCs, where the concentration of the surfactant and the oil remained stable.

3.2. Clonogenic assay

Since chemotherapeutic drugs induce tumour regression through several mechanisms, including apoptosis and DNA damage-induced senescence, we evaluated their efficacy through a clonogenicity assay, which is a well-adapted long-term assay that is not sensitive to the particular mechanism of each drug. Cytotoxicity data obtained for the drug-loaded LNCs and free drugs are shown in Fig. 2. Notably, since Iri and 5Fu are used for the treatment of colon cancer and anthracycline is used for the treatment of breast tumour, we used corresponding cell line equivalents to analyse these different compounds. Drug-loaded LNCs were slightly less toxic than the free drug, but the difference failed to reach statistical significance. This could be explained by the nature and size of the molecules. Lipophilic molecules that are less than 1 kDa can easily penetrate mammalian cells via diffusion, unlike formulations such as LNCs, which achieve internalization through endocytosis (Mosquera et al., 2018; Paillard et al., 2010). Furthermore, hydrophilic drugs poorly diffuse through membranes, parasitizing membrane transport proteins that facilitate passage naturally. For example, 5Fu is an antimetabolite that exploits the same transporter as uracil to enter cells (Longley et al., 2003). Encapsulated 5Fu tended to be more toxic than its free form, perhaps as a result of faster cell entry, faster efflux of the free drug, and even drug resistance (Gu et al., 2012; Wohlhueter et al., 1980). The only significant difference was observed in the CT26 murine colorectal cell line, where free Iri was more toxic than the encapsulated drug. Moreover, blank LNCs were not toxic below a concentration of 1 mg/ml (Supplementary data, Fig. S2).

In conclusion, LNCs did not alter the cytotoxicity effect against cancer cell lines because there was no significant difference between the IC_{50} of the free and encapsulated drugs. Clonogenic assays indicated that SN38-LNCs were one of the most potent formulations against CRC cell lines and were cytotoxic at low concentrations. Therefore, this formulation was selected for the *in vivo* experiments.

3.3. Cell uptake

To investigate cell uptake of the formulations, we used LNCs loaded with Dox, which is a fluorescent cytotoxic molecule. Free and encapsulated Dox uptake by MCF7 cells was analysed by flow cytometry (Fig. 3A) and confocal microscopy (Fig. 3B). Dox penetrated cells in the first 15 min. The percentage of cells positive for Dox plateaued at approximately 85% for both free and encapsulated Dox from 60 to 240 min.

To visualize the cellular uptake of Dox, MCF7 cells were treated with 5 µg/ml of Dox or Dox-RM-LNCs corresponding to 2 mg/ml of LNC, 2 mg/ml DIO-RM-LNCs and 2 mg/ml of blank-RM-LNCs serving as a negative control.

For the control cells treated with blank LNCs, there was no fluorescence signal (Supplementary data, Fig. S3). Free and encapsulated Dox were located in the cytoplasm of the cells after 1 h. After 2 h, both free and encapsulated Dox were observed inside the nuclei of the cells. After incubation of the cells with LNCs loaded with the lipophilic fluorescence dye DIO, we observed that DIO-LNCs were primarily located in the cytoplasm after 1, 2 and 4 h.

3.4. Haemolysis studies

Because SN38 induces higher haemotoxicity (Combes et al., 2000) among the investigated chemotherapeutics, we compared the haemolytic effect of the free drug versus the encapsulated drug to determine whether encapsulation in LNCs can protect red blood cells. As shown in Fig. 4, free SN38 was significantly more haemolytic than the

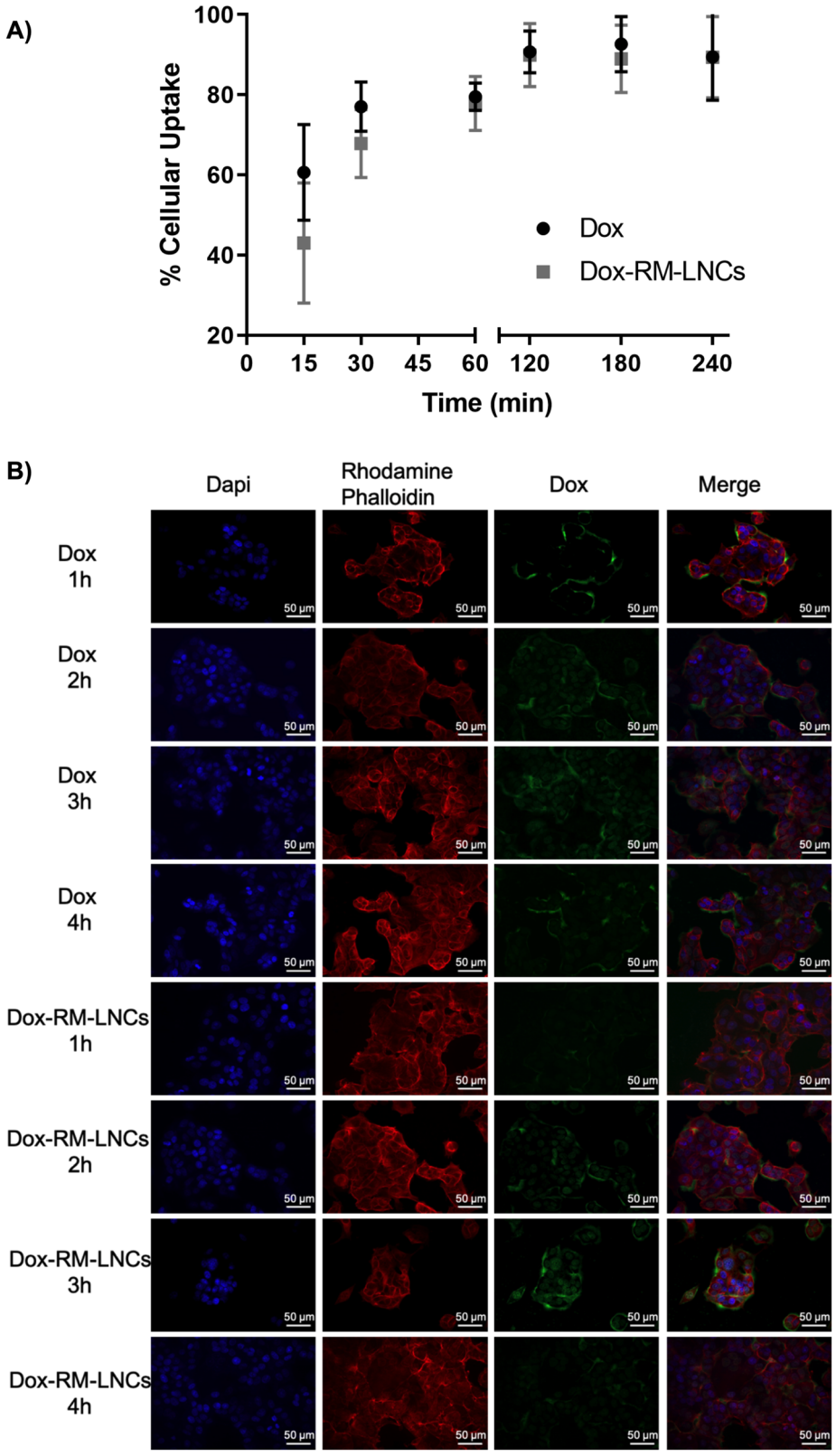

Fig. 3. Cellular uptake of Dox and Dox-RM-LNCs after different hours of incubation. A) Percentage of Dox uptake by MCF-7 (excitation: 480 nm emission: 590 nm). B) MCF-7 cells treated with Dox and Dox-RM-LNCs for 4 h.

encapsulated SN38, and the latter showed a similar haemolytic effect as the blank LNCs. Increasing the dose resulted in increased haemolysis. The nugatory haemolytic effect of the encapsulated drug suggests that the formulation could be suitable for IV administration.

3.5. *In vivo anti-tumour efficacy*

To investigate whether the drug-loaded LNC treatment is suitable for the treatment of colorectal cancer, we carried out an *in vivo*

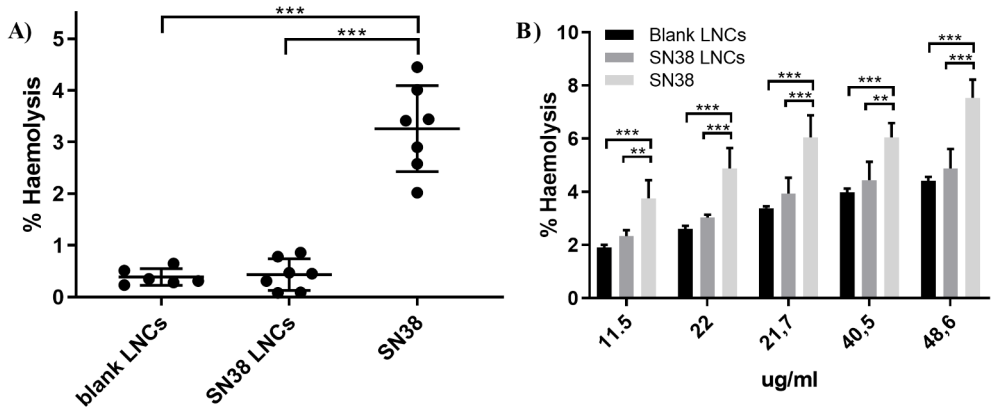

Fig. 4. Haemolytic activity of blank LNCs (2 mg/ml), SN38-LNCs and SN38 (4.8 μg/ml). B) Haemolytic activity of blank LNCs, SN38 LNCs and SN38 using different concentrations. Mean ± SD, N = 3, and n = 2.

experiment using CT26 cells. Among the anticancer drugs, we chose SN38 because we obtained the best results from the cytotoxic assays, and we chose Reg as an adjuvant therapy. Established tumours are highly heterogeneous and include cells that resist cytotoxic drugs and cancer stem cells that may be dormant and escape treatment and re-initiate tumours. Therefore, a combination of our drug-loaded LNCs with different drugs targeting those resistant cells may be the best strategy to eliminate cancer.

The treatment began when the tumours were palpable. Mice were treated with 3 weekly cycles of blank LNCs, 2 doses of SN38 and/or 2 doses of Reg. Slower tumour growth was observed in all the groups compared to the untreated mice (Fig. 5A), but they failed to reach statistical significance except for the group receiving the combined treatment, where the tumour growth was delayed. The individual tumour growth curves are shown in the Supplementary data, Fig. S5. The combined treatment significantly reduced overall tumour growth

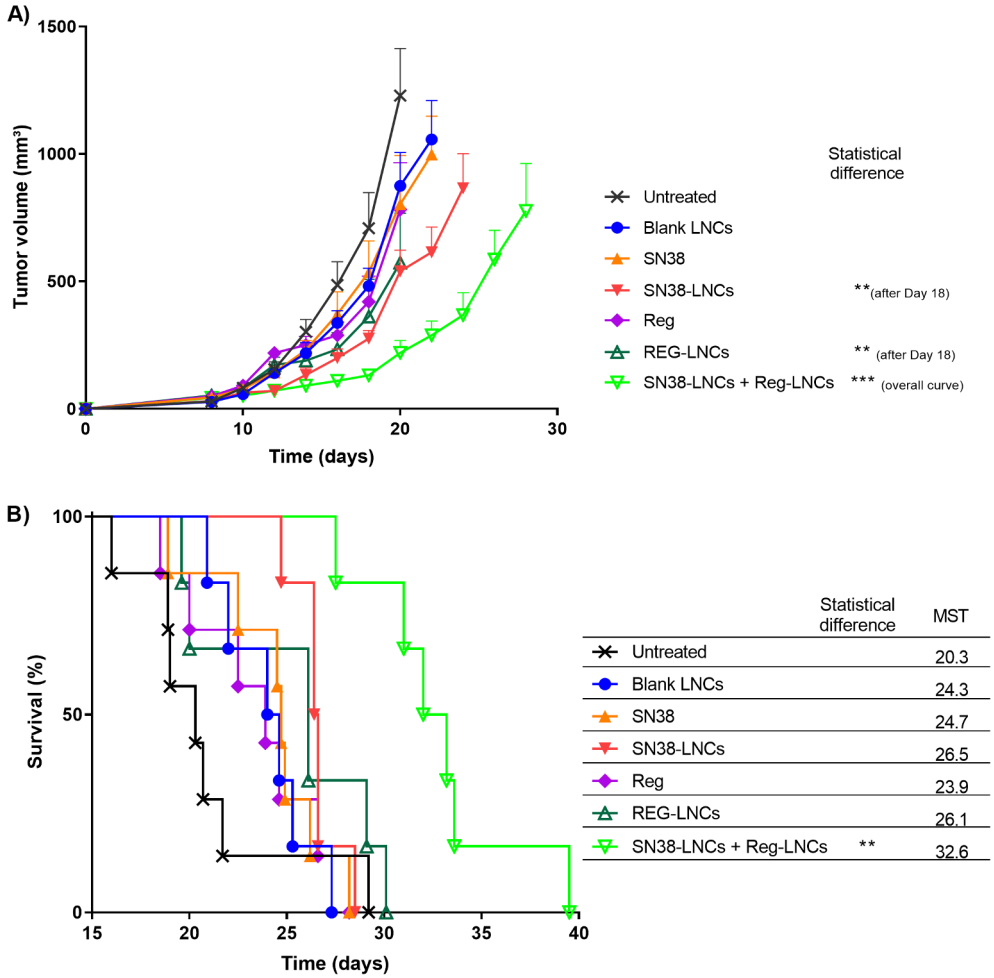

Fig. 5. Anti-tumour efficacy studies in a subcutaneous CT26 model. Administered drug dose(s): 1 mg/kg SN38 and 10 mg/kg Reg. A) Tumour volume (mm^3) as a function of time (mean ± SEM). Statistical analysis (two-way ANOVA) refers to comparisons with the control group. B) Kaplan-Meier survival curves for all animal groups. Statistical analysis refers to the SN38-LNCs + Reg-LNCs Mantel-Cox test.

compared to the untreated ($p < 0.001$), blank LNC ($p < 0.001$), free SN38 ($p < 0.001$), free Reg ($p < 0.001$), SN38 ($p < 0.05$) and Reg LNC ($p < 0.05$) groups. Moreover, the SN38-LNC- and Reg-LNC-treated groups showed significantly decreased tumour growth at day 18 compared to the untreated group ($p < 0.05$).

To assess the efficacy of the treatments on mouse survival, a Kaplan-Meier curve was generated (Fig. 5B). The combined therapy significantly extended the median survival time (MST) (32.6 days) compared to the other groups. No other treatment significantly prolonged the MST. In addition to its activity as a multi-kinase inhibitor, Reg is also an inhibitor of UGT1A9 and UGT1A1 (Schultheis et al., 2013), which are key enzymes for the metabolism of SN38 to an inactive water-soluble glucuroconjugate (SN38-G) (Fan et al., 2017). Inhibiting the UGT1A1 enzyme results in an increased concentration of SN38. Nonetheless, particular toxicity was not observed, as indicated by the mice that were observed daily, and their weight was stable during the treatment (Supplementary data, Fig. S4). Furthermore, a significant decrease in tumour growth was only observed in the combined treatment group without any undesirable side effects, suggesting that LNCs protect against systemic drug toxicity or that the concentration of SN38 was not high enough to provoke systemic toxicity.

4. Conclusion

We encapsulated six different cytotoxic agents in LNCs (Table 1) and evaluated whether those drug-loaded LNCs can be used for the treatment of colorectal cancer. Modifications of the formulations were carried out to achieve the production of drug-loaded LNCs with high EE and drug loading (Table 4). The *in vitro* studies showed that the IC50 of SN38 was less than that of the other drugs (Fig. 2). This result justified the selection of SN38 for the *in vivo* preclinical proof of concept experiments. Although the drug loading of Iri-LNCs was greater than that of SN38-LNCs, Iri is primarily metabolized in the liver by carboxylesterases to its metabolite SN38; thus, the direct administration of the active molecule was advantageous. Then, we demonstrated that SN38 encapsulation within LNCs reduced drug-induced haemolysis (Fig. 4), suggesting that SN38-loaded LNCs are safer for intravenous administration than free SN38 without significantly modifying the toxicity against various cancer cells (Fig. 2).

To overcome the problem of cells being resistant to a specific treatment, a combined therapy using SN38- and Reg-loaded LNCs was administered for intravenous therapy. Compared to the untreated group, SN38-LNCs and Reg-LNCs succeeded in significantly delaying tumour growth, which was observed beginning on day 18. Furthermore, the combined treatment group showed a significant decrease in tumour growth and an improved survival outcome (Fig. 5). Although the combined treatment did not completely eradicate the tumour, a higher dose or even a differently scheduled regimen could be more efficient, and the combined treatment could be further improved.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2019.118588.

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