Oncology

Pharmacokinetic, biodistribution and therapeutic efficacy of 5-fluorouracil-loaded pH-sensitive PEGylated liposomal nanoparticles in HCT-116 tumor bearing mouse

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The objective of the study was to investigate the pharmacokinetics and efficacy of 5-FU entrapped pH-sensitive liposomal nanoparticles with surface-modified anti-epidermal growth factor receptor (EGFR) antibody (pHLNps-5-FU) delivery system. Cytotoxicity of 5-FU and pHLNps-5-FU was determined in vitro against HCT-116 cells. The biodistribution and pharmacokinetic parameters of the administered 5-FU and pHLNps-5-FU as well as efficacy of 5-FU and pHLNps-5-FU were determined in HCT-116 subcutaneous mouse model. Mean size of pHLNp–5-FU was 164.3 ± 8.4 nm with entrapment efficiency (E.E) of 54.17%. While cytotoxicity of 5-FU and pHLNps-5-FU showed a strong dose-dependent, pHLNps-5-FU proved to be more effective (2-3 fold high) than that of 5-FU against HCT-116 cells. Pharmacokinetic study showed a prolonged plasma circulation of pHLNps-5-FU and a more significant body exposure while accumulation of pHLNps-5-FU in tumor was significantly higher than that of free 5-FU. Further, the efficacy of pHLNps-5-FU, was greater than free 5-FU at equivalent 5-FU dose. The study suggests that pHLNps may be an effective drug delivery system to enhance the anticancer activity of 5-FU against colorectal tumor growth.

Colorectal cancer | liposomes | 5-FU | Nanoparticles | pharmacokinetics

Introduction

Among men and women in United States, colorectal cancer (CRC) is the third most common cause of cancer death and the third most commonly diagnosed cancer, with nearly 137,700 new cases estimated to be diagnosed in 2015 [1]. The lifetime risk of developing CRC is about 4.5% of the entire population based on 2010-2012 data [2]. Treatment of CRC could be improved by delivering greater amount of chemotherapy drugs to the tumor while avoiding normal or healthy tissues. For chemotherapy drugs, cytotoxic effect on healthy tissues is usually dose-limiting factor which ultimately impedes the efficacy of chemotherapy due to a poor efficacy/toxicity balance.

5-Fluorouracil (5-FU), an antimetabolite of the pyrimidine analogue type, remains the gold standard for chemotherapy of CRC, despite its poor response rate of 20% when used as monotherapy or improved the response rate to merely 45% when used in combination with other antitumor drugs [3]. Also, due to its high rate of metabolism in the liver and blood, where more than 80% of 5-FU is metabolized by enzyme dihydropyrimidine dehydrogenase (DPD) to dihydrofluorouracil [4], maintenance of a therapeutic plasma concentration requires the continuous administration of high doses which may lead to severe toxicity (such as hematological and gastrointestinal adverse side effects) if the drug concentration exceeds a critical limit [5-8].

By encapsulating 5-FU in a nanocarrier, the dihydropyrimidine dehydrogenase attack on 5-FU could be reduced or avoided and efficacy of 5-FU could significantly be improved while its associated toxicity would be greatly reduced.

Nanocarriers, such as liposomes [9-11] and polymeric nanoparticles [12] have all been reported to improve the therapeutic efficacy of anticancer drugs by enhancing local drug accumulation, improving drug bioavailability, and prolonging systemic circulation [13] provided that nanocarrier remains intact during transport, and reasonably protected from metabolism and reticuloendothelial system (RES) through PEGylation [14]. In addition, nanocarriers provide a platform for incorporating high amount of drug. By enhanced permeability and retention (EPR) effect, nanocarriers could preferentially deliver high payload of drugs into tumor tissue and improve therapeutic efficacy of anticancer drug [15].

We formulated pH-sensitive liposomal nanoparticle (pHLNP) as cancer drug delivery system for 5-FU. The pH-sensitive liposomal nanoparticle was composed of cholesteryl hemisuccinate (CHEMS), Tween 20, cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (DSPE-PEG_{2000}). CHEMS has the ability to self-assemble into bilayers in basic and neutral (pH 7) aqueous environment but becomes unstable and fuse at acidic pH [16]. With respect to pH-sensitive liposomal nanoparticle, the ionization state of CHEMS dictates the phase behavior, and thus the fusogenic behavior of the lipid ensemble. Moreover, under the acidic environment that is usually found in tumors, the CHEMS component becomes unstable within the liposomal nanoparticle leading to break and release of 5-FU.

In the present study, we hypothesized that pHLNP-5-FU would be bioavailable and increase tumor response to 5-FU in subcutaneous mouse model of HCT-116-induced colorectal cancer. Our *in vitro* and *in vivo* anticancer studies revealed significant inhibitory effect on cancer growth in the pHLNP-5-FU-treated animals than those treated with free 5-FU. Pharmacokinetic and biodistribution studies also revealed that pHLNP-5-FU possessed prolonged blood circulation time (increased 5-FU half-life), higher area under the plasma drug concentration-time curve (AUC), and increased 5-FU tumor accumulation. This demonstrates that pHLNP could be a better anticancer delivery system for 5-FU and improve the therapeutic index of 5-FU.

Conflict of interest: No conflicts declared

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Materials and Methods

Materials: All the chemicals including 5-FU and reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Cholesterylhemisuccinate (CHEMS) 1,2-dioleoyl-3- Cholesterol (CH), Tween 20 and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol)-2000] (DSPE-PEG_{2000}) lipids were all purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Colorectal cancer HCT-116 cells were obtained from American Type Culture Collection (ATCC); Female athymic nude (Nu/Nu) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Preparation of 5-FU loaded pH-sensitive liposomal nanoparticles

Based on our previous studies on pH sensitive liposomes as anticancer drug delivery system [17], we prepared pHLNp using CHEMS, CH, Tween 20, DSPE-PEG_{2000} according to the molar ratio indicated in Table 1. Based on the molar ratio as shown in table 1, respective amounts of CHEMS, CH, Tween 20, DSPE-PEG_{2000} were weighed and placed in round bottom flasks containing chloroform. The lipids were mixed thoroughly until a homogenous solution was obtained, and afterwards removed the chloroform by passing nitrogen gas through inner side of the flask in a fume hood. The thin film obtained was further dried under vacuum overnight to remove any residuals. The dried film was then hydrated at a temperature above the transition temperature of the lipid (60 ^{o}C) with 2 ml of phosphate buffer solution (PBS) pH 7.4 containing 19 µM of 5-FU. The hydrated film was then vortexed for 1min and bath sonicated for 5 min. The resulting multi-laminar liposomal vesicles were then downsized further by extruding through a 200 nm polycarbonate filter membrane. The free 5-FU was finally removed by using 1000 kdaltons vivaspin (15 ml) concentrator tube at 5,000 rpm for 10 min at room temperature. The final product (the retained formulation) was further coated with anti-EGFR antibody through electrostatic interaction. Briefly, 50 µL (1 mg/mL) of anti-EGFR antibody (cetuximab) was added to 2 ml containing 5mg of the final product and stirred at 20 rpm overnight at 4 ^{o}C (cold room). The anti-EGFR antibody coated pHLNp-5-FU was purified using precoated 1% albumin concentrator vivaspin tube with 1000 kdaltons molecular weight cut-off at 5,000 rpm for 10 min at 4^{o}C. The filtrate was analyzed for free or unbound anti-EGFR antibody using BCA protein assay kit. The purified anti-EGFR antibody coated pHLNp-5-FU was lyophilized using mannitol (5% w/w) as cryo-protectant.

Characterization of pH-sensitive liposomal nanoparticles

Size measurement: The particle size and zeta potential of the blank carrier (pHLNp) and pHLNp-5-FU were determined by dynamic light scattering using a zeta potential/Particle sizer instrument (NICOMP™ 380 ZLS). All measurements were performed in triplicate and the results reported in mean diameter ± SEM.

Entrapment efficiency (EE %): To determine the amount of entrapped 5-FU in pHLNp, 10 mg of lyophilized pHLNp-5-FU was suspended in 2ml of PBS (pH-7.4) after which 100 µL of 30% Triton X-100 was added for the sole purpose of disrupting the liposomal carrier, pHLNp. The suspension was gently mixed for 2 min to avoid foaming and then centrifuged at 6,000 rpm at room temperature for 5 min. The supernatant was removed and analyzed for 5-FU using reverse phase high performance liquid chromatography (HPLC). *The reverse phase HPLC*: Mobile phase solution consisted of 95% of 5mM phosphate buffer (pH 5) and 5% of methanol mixture (v/v). The mixture and the samples were filtered through 0.22 µm filter and analyzed using HPLC according to method described [18]. The samples as well as internal standards (injection volume of 20µL) were injected through a XB-C18 column at a flow rate of 1.0 mL/min (250 mm × 4.6 mm; Agilent, Santa Clara, CA) at room temperature. The 5-FU was detected at 270 nm with Waters 996 photodiode array detector (Waters, Columbia Maryland. USA). The entrapment efficiency was calculated based on the equation below:

$$\textbf{Entrapment efficiency}\ (\%) = \frac{\textbf{Amount of drug entrapped in nanoparticles}}{\textbf{Intial amount of drug}}\ \boldsymbol{x}\ \mathbf{100}$$

Cell viability

In vitro cytotoxicity of free 5-FU or pHLNp-5-FU was evaluated by trypan blue assay method. The HCT-116 cells were seeded into 12-well plates at a density of 5 x 10^3 cells per well and cultured in DMEM/F12 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. Cells were then treated with different concentrations of 5-FU or its equivalent in pHLNp-5-FU after they have grown to 75% confluence. After 48 hr, the experiments were terminated and cells were detached, stained with 2% trypan blue and counted with an automated cell counter (Bio-Rad TC-20™). Cell viability (%) relative to the control was determined using the equation below:

$$\textbf{Cell Viability}\ (\%) = \frac{\textbf{Number of cells treated with the nanoparticles}}{\textbf{Number of the untreated cells}}\ \boldsymbol{x}\ \mathbf{100}$$

Animal studies

Efficacy of pHLNp-5-FU on tumor suppression: Female athymic nude (Nu/Nu) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6 to 8 weeks of age with an average weight of 20 – 25g. The animals were housed in a virus-free indoor temperature and controlled barrier environment, and were provided ad libitum access to food and water. All procedures with animals were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Florida A&M University Animal Care and Use Committee. Colorectal HCT-116 cancer cells were cultured in DMEM/F12 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. The cells were incubated in 5% CO_2 and humidified environment of 95% air at 37 °C.

Mice were then inoculated with HCT-116 cells (2.3 x 10^{-6} in 100µl PBS) and injected subcutaneously in the lower right flanks of the mice. Tumors with average volume (150 mm^3) appeared within 2 weeks of inoculation, after which the mice were randomly grouped into 4 groups with 5 mice in a group (control, antiEGFR, 5-FU and pHLNp-5-FU). The mice in each group were

Table 1. Characterization of 5-FU loaded-pH-sensitive liposomal nanoparticle

Formulation	Lipid Composition	Molar Ratio	Mean Particle Size (nm)	Zeta Potential (mV)	E.E (%)
pHLNP	CHEM:CH:TWEEN 20:DSPE-PEG_{2000}	60:20:10:10	136.1 ± 10.2	1.30 ± 0.8	-
pHLNP-5-FU	CHEM:CH:TWEEN 20:DSPE-PEG_{2000}	60:20:10:10	164.0 ± 8.4	1.23 ± 0.2	54.17

pHLNP = Blank nanoparticle, E.E = Entrapment efficiency. Data expressed as mean ± SEM, n =3

treated for 2 weeks with 200 µg antiEGFR antibody (cetuximab), 12.5 mg/kg of free 5-FU or pHLNp-5-FU (5-FU equivalent dose), every other day for 3 times a week (QD x 3) via intraperitoneal (i.p) administration except the control group where saline was administered instead. The i.p injection was preferred to intravenous (IV) injection because we wanted to prevent vascular damage due to the repeated injections (3 injections per week) and also to avoid severe stress on animals, which needed to be restrained if not anesthetized during injection. Tumor size was measured on the first day of treatment and followed every other day for 19 days. Mice were euthanized when tumor volume reached 2 cm^3.

Pharmacokinetic studies: A group of female athymic nude (Nu/Nu) mice were injected intraperitoneally with a single dose of 12.5 mg/kg of free 5-FU and pHLNp-5-FU (at 5-FU equivalent dose). Blood samples were drawn at different time points from the lateral tail vein of mice (1min, 5 min, 10 min, 15 min, 30 min, 1, 2, 4, 8, 12, and 24 hr), placed in heparinized centrifuge tubes and immediately centrifuged at 10,000 rpm for 10 min. The plasma was then stored at -20 ^oC until analysis.

Biodistribution of pHLNp-5-FU: In a separate study, a similar dose to that of the PK study was administered to free 5-FU and pHLNp-5-FU groups, 24 hr post injection, the mice were perfused with normal saline and tissues were collected, rinsed with PBS, and blotted dry on filter paper and weighed.

Plasma and tissue samples preparation: Plasma and tissue samples were prepared for HPLC analysis as described by Wang and his colleagues [18] with minor modifications. Plasma samples were allowed to thaw at room temperature (20–28 ^oC) and 50µL plasma was added to 450 µL Methanol in micro 1.5-mL plastic centrifuge tubes. The tubes were vortex-mixed for 30s and centrifuged at 10,000 rpm for 10 min, after that 200µL of the clear supernatant was diluted with distilled water to 600 µL. Using vivaspin 500 ultrafiltration tube (membrane with 10kDa molecular weight-cut off), 500µL of the diluted supernatant was ultrafiltrated at 5,000 rpm for 10 min at room temperature and 200 µL of the obtained ultrafiltrate was used HPLC analysis.

For tissue sample preparation; tissues after weighing were homogenized in 2mL of distilled water with a probe type sonicator at operational mode of 300 watts in pulse (3s on and 2 s off), on ice-water to keep the temperature under 25^oC. The homogenate was then centrifuged at 10,000 rpm for 10 min. The supernatant was then collected and 200µL of it was deproteinized with methanol and then ultrafiltrated as described above for that of plasma preparation. Two-hundred microliters of the filtrate was then analysis for 5-FU using HPLC.

HPLC analysis: For plasma and tissue calibration standards and quality control samples, untreated mouse plasma samples were spiked with aliquots of known amount of 5-FU prior to the addition of methanol while the supernatant of untreated mouse tissue homogenate was spiked with appropriate aliquots of 5-FU before the deproteinization. The mobile phase comprised a mixture of 5 mM phosphate buffer (pH 5)-methanol (95:5, v/v) and degassed by sonication and filtered through a 0.45 mm millipore membrane filter before use [18]. Sample injection volume was 20µL and pumped through a XB-C18 column at a flow rate of 0.6 mL/min (250 mm × 4.6 mm; Agilent, Santa Clara, CA) at room temperature. 5-FU was detected at 265 nm with Waters 996 photodiode array detector (Waters, Columbia Maryland. USA). The HPLC method provided a good baseline, symmetrical and a sharp peak for 5-FU and a high resolution between 5-FU and the internal standards.

Assay validation and recovery: A calibration curve was prepared using the peak areas of 5-FU against the respective concentrations of 5-FU. Linear regression analysis of the calibration data was performed using the equation $y = mx + c$, where y is the peak area, x is the concentration of 5-FU, and m and c are the slope and intercept, respectively. The recovery of 5-FU from plasma or tissue samples was determined by comparing the peak areas from the samples containing known added 5-FU with those from 5-FU standard solutions reconstituted by adding an appropriate amount of the ultrafiltrate of untreated mouse's plasma or tissue samples.

Statistical analysis

All experiments were performed in triplicates and analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, and CA. USA). The differences between the 5-FU treatment group and that of pHLNp-5-FU were determined using Student's paired t-test and considered significant at $p<0.05$.

Results

Characterization of 5-FU pH-sensitive liposomal nanoparticles

Mean particle size of blank liposomal nanoparticle, pHLNp was 136 ± 10.2 nm, while the mean size of 5-FU-loaded liposomal nanoparticle, pHLNp-5-FU was found to be 164.3 ± 8.4 nm. With our formulation technique, 54.17% (entrapment efficiency (E.E)) of 5-FU was entrapped in pHLNp-5-FU with a final zeta potential value of 1.23 ± 0.8 as shown in Table 1.

Cell viability

Figure 1 shows the effect of increasing concentration of 5-FU and pHLNP-5-FU on the viability of HCT-116 cells. As shown in fig. 1, pHLNP-5-FU significantly (2–3 fold high) inhibited HCT-116 cells growth at all corresponding concentrations compared with that of free 5-FU.

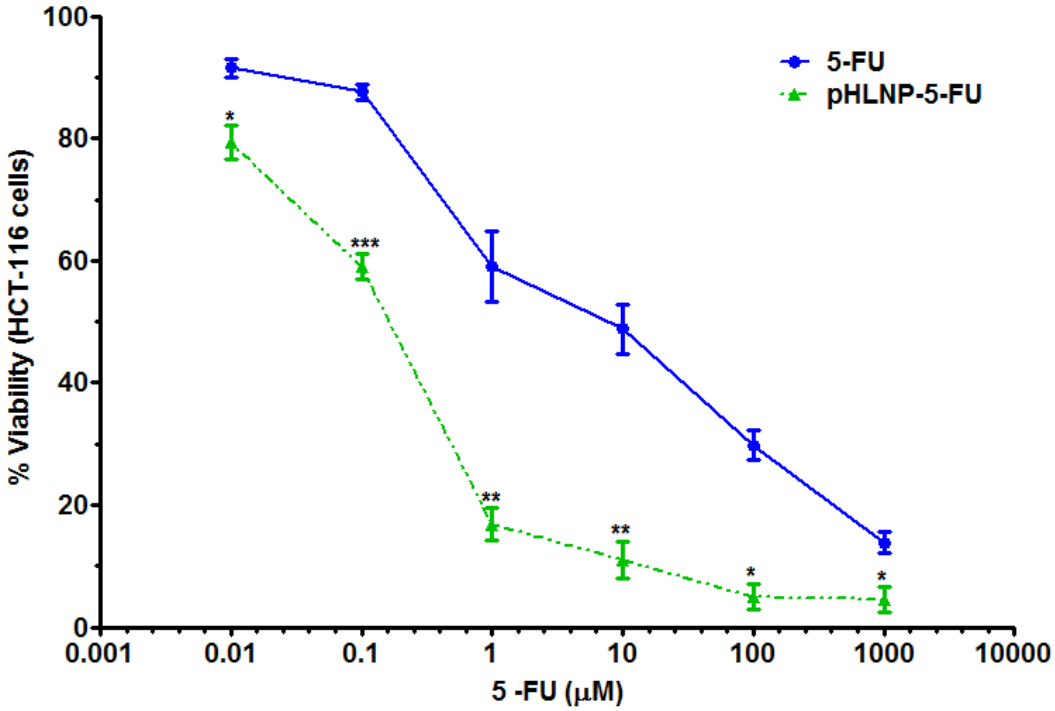

Figure 1. Cytotoxic studies showing the toxicity of 5-FU and pHLNP-5-FU on HCT-116 cells. Data represents mean ± SD, n=3. (*p<0.05, **p<0.01 and ***p<0.001), p-values were calculated by Student's *t*-test.

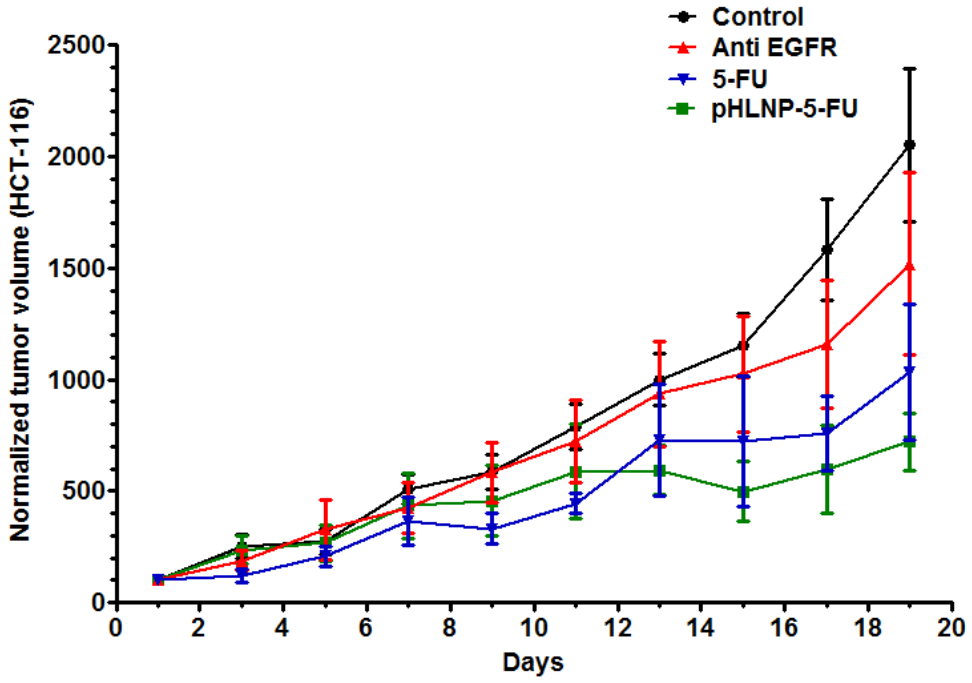

Figure 2. Comparison of anticancer effects of 5-FU, anti-EGFR antibody and pHLNP-5-FU on tumor growth of HCT-116 tumor bearing mice. Data represents mean ± SD, number of mice in each treatment group = 5 (n =5/group).

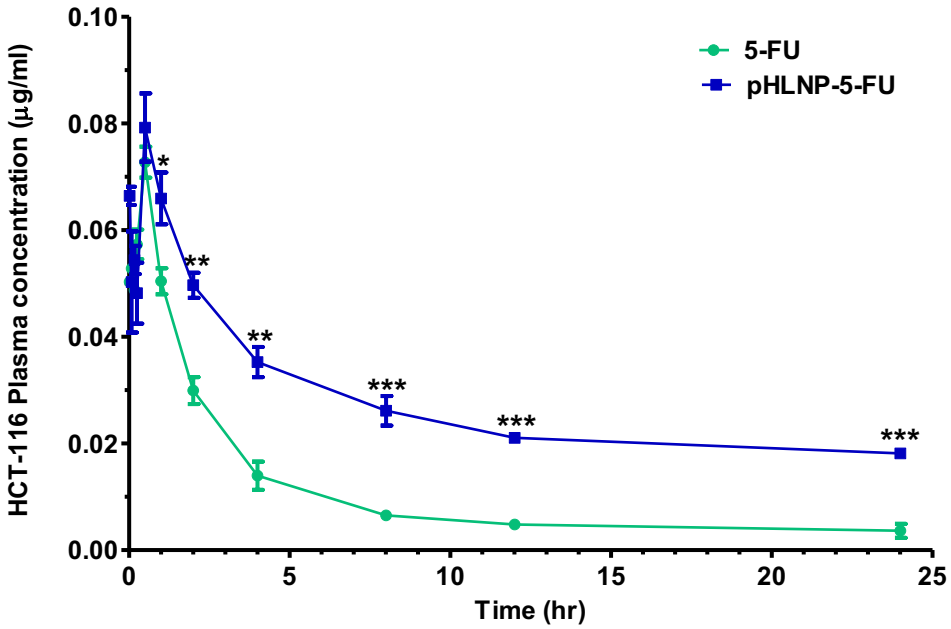

Figure 3. Pharmacokinetic profile of 5-FU and pHLNP-5-FU in HCT-116 in tumor bearing mice. Data represents mean ± SD, number of mice per group = 5. (*p<0.05, **p<0.01 and ***p<0.001), p-value was calculated by Student's *t*-test.

In vivo studies

Efficacy study

Figure 2 shows the effect of free 5-FU and pHLNp-5-FU (at 5-FU equivalent dose) and anti-EGFR antibody on tumor volume/growth. We observed that on the 11th day, normalized tumor volume of free 5-FU treated mice group was 300 mm^3 while the normalized mean tumor volume of pHLNp-5-FU treated mice group was 400 mm^3. However, on the 13th day, mean tumor volume of free 5-FU treated group increased to 700 mm^3 but no appreciable tumor growth was observed for pHLNp-5-FU treated group on the 13th day. Whereas, the mean tumor volume of free 5-FU treated group barely increased between 13th and 17th days, mean tumor volume of pHLNp-5-FU treated group reduced slightly to 300 mm^3 on the 15th day but increased again to 400 mm^3 on the 17th day. At the end of the studies (19th day), the mean tumor volume of pHLNp-5-FU treated group was under 500 mm^3 and that of free 5-FU treated reached 700 mm^3.

Pharmacokinetic and biodistribution studies

Pharmacokinetic parameters obtained in mice given 12.5 mg/kg of free 5-FU and pHLNP-5-FU (at 5-FU equivalent dose) as listed in Table 2. Biphasic decay with an initial phase characterized by rapid decline and this is evident in both 5-FU and pHLNP-5-FU as shown in fig 3. The $t_{1/2}$ of pHLNP-5-FU (1.41 ± 0.50) was 2.7 fold high compared with 5-FU (0.52 ± 0.11). The maximum plasma concentration of 5-FU alone after administering a single i.p. bolus dose of 12 mg/kg was 0.079 µg/ml, 30 min post injection and reduced to 0.009 µg/ml at 24 hr, while that of pHLNp-5-FU at 30 min post injection was 0.081µg/ml and declined to 0.027 µg/ml at 24 h.

Biodistribution**:** Figure 4 shows amount of deposition of free 5-FU or pHLNP-5-FU in various organs. For the brain (Fig. 4-A), there was higher amount of 5-FU deposited (17±1.9 µg/g wet tissue) than pHLNP-5-FU (8.9 ± 0.7 µg/g wet tissue). In figure 4-B, we observed a significant amount of 5-FU in the heart (35.2 ± 2 µg/g) compared to that of pHLNP-5-FU (9.63 ± 0.81µg/g). Although liver received the highest amount of either 5-FU or pHLNP-5-FU compared with all other tissues, the uptake of 5-FU was significantly higher (204.5 ± 5.2 µg/g) compared with pHLNP-5-FU (172.2 ± 10 µg/g). Further, a higher amount of 5-FU was observed in lungs (57.3 ± 3.3 µg/g) which was 2.5 fold high that of pHLNP-5-FU (23.4 ± 2.7 µg/g).

Table 2. Pharmacokinetic profile of 5-FU and pHLNP-5-FU in HCT-116- tumor bearing mouse

Parameter	5-FU	pHLNP-5FU	p-value
AUC(mg/ml hr)	0.15 ± 0.02	0.38 ± 0.05	0.001
MRT(hr)	0.75 ± 0.16	2.04 ± 0.72	0.03
$t_{1/2}$ (hr)	0.52 ± 0.11	1.41 ± 0.50	0.04
K_{10} (1/hr)	1.38 ± 0.37	0.53 ± 0.19	0.01
K_{12} (1/hr)	0.31 ± 0.06	1.49 ± 1.06	ns
K_{21} (1/hr)	1.51 ± 0.80	0.68 ± 0.55	ns
CL (ml/hr)	2184 ± 237	1069 ± 253	0.001
V_d (ml)	1635 ± 279	2054 ± 292	ns

MRT, mean residence time of drug molecules in the body; K_{10}, elimination rate constant from the systemic circulation; K_{12}, first order rate constant for transfer of the drug from the systemic circulation to the periphery tissue; K_{21}, first order rate constant for transfer of the drug from the periphery tissue to the systemic circulation compartment; CL, the volume of plasma in the vascular compartment cleared of drug per unit time by the processes of metabolism and excretion; V_d, apparent volume to which the drug must be distributed in order to reflect the drug concentration attained in body. Data expressed as mean ± S.D, ns = not significant.

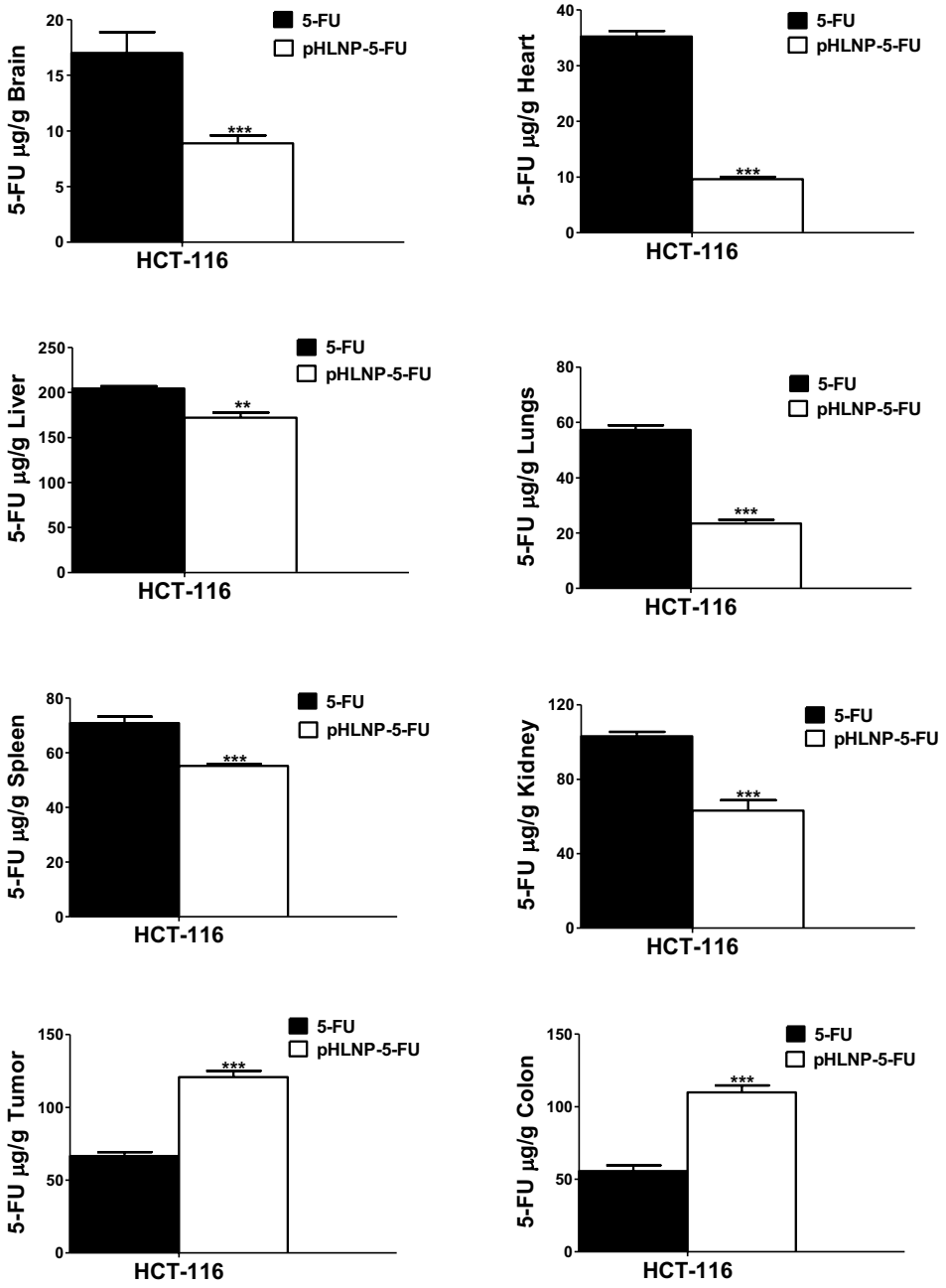

Figure 4. Biodistribution of 5-FU and pHLNp-5-FU at 24 h after i.p injection 12.5 mg/kg of free 5-FU and pHLNp-5-FU (5-FU equivalent dose) into HCT-116 tumor-bearing mice. Data represents mean ± SD, number of mice per group = 5. (*p<0.05, **p<0.01 and ***p<0.001), p-value was calculated by Student's *t*-test.

On the contrary, significant amount of pHLNP-5-FU was accumulated in tumor (Fig.4-G, 120.8 ± 8.4 µg/g), spleen (Fig.4-E, 55.2 ± 1.3 µg/g) and colon (Fig.4-H, 109.8 ± 1.3 µg/g), compared with their corresponding 5-FU values. Interestingly, kidney (103.15 ± 4.6 µg/g) received a significant amount of 5-FU compared to that accumulated by pHLNP-5-FU (63.2 ± 11 µg/g).

Discussion

Pharmaceutical nanocarriers such as liposomes have demonstrated enhanced in vivo stability and efficiency of drugs. The benefits of nano-scaled delivery systems are supported by numerous preclinical and clinical data [19, 20] and several commercialized products such as Myocet®, a doxorubicin liposomal for metastatic breast cancer and Doxil®, a doxorubicin liposomal for ovarian cancer.

For cancer, nanocarriers as anticancer drug delivery systems are generally designed to improve, for example, high drug loading capacity, prolonged systemic circulation, ability of the nanocarrier to accumulate specifically in the required pathological zone, and the nanocarrier's ability to respond to local stimuli such as pH change resulting in accelerated drug release [21].

5-FU, an antimetabolite, exerts its effect through the inhibition of the nucleotide synthetic enzyme thymidylate synthase (TS) by its active metabolite fluorodeoxyuridine monophosphate, which results in thymidylate depletion and finally apoptosis in cancer cells [22, 23], though it still remains the drug of choice in the treatment of colorectal cancer, it's still being plagued by efficacy and delivery issues such rapid systemic elimination, severe adverse effects, and lack of specificity. Furthermore, it's unfavorable pharmacokinetic requires administration of high dose, and imposes on patients a rigorous schedule for reaching the desired therapeutic effect.

To resolve some of these issues, we developed a 5-FU-loaded pH-sensitive PEGylated liposomal nanoparticle with surface-modified with anti-EGFR (pHLNps-5-FU) so as to enhance 5-FU stability in systemic circulation by increasing its half-life, and improve its therapeutic efficacy. One of the unique features of pH-sensitive liposome is its stability at physiological pH (pH 7.4) whilst it undergoes destabilization and acquire fusogenic properties under acidic conditions, thus leading to the release of their aqueous contents [24]. In addition, our liposome nanoparticle which was prepared with CHEMS, cholesterol Tween 20 and DSPE-PEG_{2000} and of size < 200 nm has been reported to have a considerable decrease in leakage of entrapped 5-FU due to the presence of cholesterol [25] and also promote non-specific interactions between the liposome and serum proteins (opsonins) due to the presence of DSPE-PEG_{2000}, thus avoiding liposome clearance by the cells of the RES.

In cytotoxicity study, we assessed the effect of 5FU or pHLNP-5-FU on HCT-116 cells, which possess a doubling time of 24 hr (data not shown). Cells were therefore exposed to the blank liposome, 5-FU or pHLNP-5-FU for 48 hr, a sufficient period to assess inhibition. Though blank liposomes were well tolerated by HCT-116 cells, 5-FU or pHLNP-5-FU demonstrated a dose-dependent inhibitory effect with pHLNP-5-FU showing more significant reduction in cell viability. This is probably due to higher cellular uptake of the liposomal form leading to greater internalization of 5-FU.

Nanocarriers are useful in drug delivery because they can alter the pharmacokinetics and biodistribution of their associated therapeutics. This was observed in our study of free 5-FU and pHLNP-5-FU, where the analysis of pharmacokinetic parameters revealed that $t_{1/2}$, MRT, V_d or $AUC_{0\text{-}24h}$ for pHLNP-5-FU was significantly greater than $t_{1/2}$, MRT, V_d or $AUC_{0\text{-}24h}$ for 5-FU except the C_{max} where both exhibited comparable peaks. This may suggest that no significant biological/barrier restriction was encountered by pHLNP-5-FU with average size of 164.0 ± 8.4nm as both free 5-FU and pHLNP-5-FU (given as i.p injections) appeared to have similar C_{max} and T_{max}. (figure 3). Further, the pharmacokinetic parameters suggest that plasma clearance of free 5-FU was faster than pHLNP-5-FU and that free 5-FU plasma concentration did not significantly contribute to the exposure of the mouse model to the drug especially after 8 hr (figure 3 and Table 2). Overall, as expected, pHLNP exhibited prolonged exposition to 5-FU as compared to free 5-FU drug.

Further, plasma concentration-time curve of free 5-FU and pHLNp-5-FU exhibited biphasic behavior with free 5-FU having a rapid initial distribution compared with pHLNp-5-FU. This pattern of distribution and elimination phases suggests a two compartmental [26].

For biodistribution study, 5-FU delivered to tumor by pHLNp was significantly higher than 5-FU alone and it is important to note this approach of delivery may have led to significant restriction in tumor growth compared to the free drug. The mechanism of delivery by pHLNp could be attributed to higher enhanced permeability and retention (EPR) effect where nanoparticle size carriers are entrapped in solid tumors and retained there at high concentrations for long period of time, whereas low-molecular weight drugs such as 5-FU molecules are not retained but returned to the circulating blood by diffusion[27, 28]. In addition, the existence of tumor's acidic environment largely contributed to the degradation of pH-sensitive pHLNp and accelerated delivery of 5-FU to the tumor interstitium [29].

Conclusion

In this study we developed a suitable pH-sensitive PEGylated liposomal anticancer delivery system for 5-FU to enhance anticancer activity of 5-FU in a HCT-116 subcutaneous mouse model. By protecting 5-FU from metabolism and enzyme attack through PEGylated liposome, and by developing suitable sized pHLNp, it was to able deliver a significant amount of 5-FU to tumor, exhibited a favorable PK characteristics, and significantly inhibited tumor growth in comparison to 5-FU therapy. Putting together, our results indicate that a pHLNp delivery system for 5-FU may be able to effectively reduce adverse side effects of 5-FU and improve the therapeutic index of 5-FU.

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