Cytotoxicity of 5-fluorouracil-loaded pH-sensitive liposomal nanoparticles in colorectal cancer cell lines

Ofonime Udoefo, Kevin Affram, Bridg’ette Israel and Edward Agyare*
Division of Basic Sciences College of Pharmacy and Pharmaceutical Sciences, Florida A&M University, Tallahassee, Florida, USA

Abstract
5-Fluorouracil (5-FU) is widely used in cancer therapy, either alone or in combination with other anti-cancer drugs. However, poor membrane permeability and a short half-life (5-20 min) due to rapid metabolism in the body necessitate the continuous administration of high doses of 5-FU to maintain the minimum therapeutic serum concentration. This is associated with significant side effects and a possibility of severe toxic effects. This study aimed to formulate 5-FU-loaded pH-sensitive liposomal nanoparticles (pHLNps-5-FU) and evaluate 5-FU release characteristics and anti-cancer effect of pHLNps-5-FU. Particle size and zeta potential were determined using a particle size analyzer. The release patterns of pHLNps-5-FU formulations were evaluated at 37°C at pH 3, 5, 6.5, and 7.4, while drug release kinetics of 5-FU from a pHLNps-5-FU formulation were determined at pH 3 and 7.4 at different time points (37°C). Cell viability and clonogenic studies were conducted to evaluate the effectiveness of pHLNps-5-FU against HCT-116 and HT-29 cell lines while cellular uptake of rhodamine-labeled pHLNps-5-FU was determined by flow cytometry and confocal imaging. The average sizes of the pHLNps-5-FU, pHLNps-5-FU and pHLNps-5-FU liposomes were 200 nm ± 9.8 nm, 181.9 nm ± 9.1 nm, and 164.3 nm ± 8.4 nm respectively. In vitro drug release of 5-FU from different pHLNps-5-FU formulations was the highest at pH 3.8. Both cell lines treated with pHLNps-5-FU exhibited reduced viability, two- or three-fold lower than that of 5-FU-treated cells. Flow cytometry and confocal imaging confirmed high uptake of rhodamine-labeled pHLNps-5-FU in both cell lines. The drug release profile of the chosen pHLNps-5-FU formulation was optimal at pH 3 and had the poorest release profile at pH 7.4. The release profile of pHLNps-5-FU showed that 5-FU release was two-fold higher at pH 3 than that at pH 7.4. This study demonstrates that pHLNps3-5-FU may be a potential candidate for the treatment of colorectal cancer.

Introduction
Colorectal cancer (CRC) is the formation of abnormal growths or polyps in tissues that line the colon or rectum. CRC is the third most commonly diagnosed cancer worldwide, and the second most common cause of cancer-related death in the western world [1-5]. It is estimated that 93,090 new cases of colon cancer and 39,610 of rectal cancer will be diagnosed in the year 2015; 49,700 cancer-related deaths are expected to be attributed to colorectal cancer [6].

Apart from radiation, surgery, and biologic therapies (immunotherapy and hormonal therapy), cytotoxic drugs comprise the majority of chemotherapy regimens used clinically for the treatment of cancers [7]. Most chemotherapeutics in current use interfere with cell replication in some manner, either by acting like nucleoside analogs (leading to S-phase arrest), or damaging deoxyribonucleic acid (DNA). As cancer cells undergo rapid cell divisions, they are generally more susceptible to these drugs than normal cells. In addition, cancer cells often lack the ability to recognize and/or repair DNA damage that leads to improper replication of cellular DNA and eventually causes cell death. The negative impact of this therapy is widespread, including chemotherapeutic drug resistance and interference with normal cell division, causing profound drug toxicity [7]. The next-generation targeted drugs may have few side effects since they are designed to target specific factors more accurately, such as the overexpressed receptors or proteins that are barely or never present in normal cells. These drugs are still largely in clinical trial and not available for most patients or they are barely in common clinical use, with a few well-known exceptions (i.e. human epidermal growth factor receptor 2 (HER2)-targeting trastuzumab and various epidermal growth factor receptor (EGFR) inhibitors [7,8].

5-FU is an antimetabolite of the pyrimidine analogue type with a broad spectrum of activity against solid tumors, either alone or in combination with other chemotherapy regimens. Due to its structure, which is a base analogue that mimics both uracil and thymine, 5-FU interferes with nucleoside metabolism by incorporating into ribonucleic acid (RNA) and DNA, leading to cytotoxicity and cell death. Despite its therapeutic efficacy, 5-FU has limitations that include: i) tumor cell resistance; for example, overall response rate of advanced CRC to 5-FU alone is 10-20% while that of 5-FU in combination with other antitumor drugs is 40-45% [9], and ii) short biological half-life (5 to 20 min), which is owing to rapid metabolism in the body; therefore, the maintenance of therapeutic serum concentration often requires continuous administration of high doses, which may lead to severe toxicity [7,10].

These issues can be mitigated by formulating 5-FU in a delivery system that causes accumulation of the drug in tumor regions and increases exposure time in cancer cells. A suitable 5-FU delivery system with these characteristics should have the following properties: a) physical stability; b) small size to allow capillary distribution and uniform perfusion at the desired target site; c) the ability to carry

Correspondence to: Edward Agyare, Division of Basic Sciences, College of Pharmacy and Pharmaceutical Sciences, Florida A and M University, Tallahassee FL. 32301, USA; Tel: (850)-599-3581; E-mail: edward.agyare@famu.edu

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adequate amount of the drug with negligible or low drug leakage. d) the ability to protect 5-FU from degradation, and e) controllable (or predictable) 5-FU release rates from the carrier at the desired target site [11,12].

Recently, the focus of liposomal research has been the development of strategies to increase the ability of liposomes to mediate intracellular delivery of biologically active molecules [13]. This has led to the emergence of liposomes called stealth liposomes (liposomes sterically stabilized with polyethylene glycol (PEG)). Stealth liposomes are more suitable than polymers as a delivery system for 5-FU because they are stable, biocompatible, biodegradable, lack immunogenic response, and overall possess the properties of a good delivery system already described above, while polymers may cause serious toxicity with innate breakdown products. In addition, the Food and Drug Administration (FDA) has previously approved stealth liposomes for the delivery of doxorubicin for the treatment of breast cancer and ovarian cancer [14].

pH-sensitive liposomes are a modified form of stealth liposomes that are stable at physiological pH (pH 7.4) but undergo destabilization under acidic conditions. These are reported to be more efficient in delivering anti-cancer drugs than conventional or long-circulating liposomes owing to their fusogenic property [13]. pH-sensitive stealth liposomes functionalized with an appropriate moity (i.e. an antibody) targeted to receptor expressing cancer cells such as EGFR have been shown to significantly increase the intracellular delivery of their liposomal content [15].

In this study, we describe the development of 5-FU-loaded pH-sensitive liposomal nanoparticles with surface-modified anti-EGFR antibody-conjugated pHLNps-5-FU, and provide an in vitro evaluation of its therapeutic potential for cancer chemotherapy. The in vitro uptake and the cytotoxic activity of pHLNps-5-FU have been analyzed and compared with 5-FU by using HCT-116 and HT-29 cell lines, which are established cell culture models of CRC cancer.

Materials and methods

Materials

All chemicals including 5-FU and reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Dioleoylphosphatidylethanolamine (DOPE), cholesterylhemisuccinate (CHEMS), phosphatidylcholne (L-α-PC), 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP), cholesterol (CH), 1,2-distearylo-sn-glycero-3-phosphoethanolamine-N-[aminopolyethylene glycol]-2000] (DSPE-PEG), and 1,2-distearylo-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)]-2000] (DSPE-PEG-FA) lipids were all obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cancer cell lines, HCT-116 and HT-29, were obtained from American Type Culture Collection (ATCC) in January, 2013.

Preparation of 5-FU loaded pH-sensitive liposomal nanoparticles

Different pH-sensitive liposomal nanoparticles containing 5-FU and pHLNp (pHLNp-5-FU, pHNLNp-5-FU, and pHNLNp-5-FU) were prepared by thin film hydration method [16]. Briefly, varying amounts of lipids were measured in different molar ratios (Table 1) and placed in different round-bottom flasks. The lipids were then dissolved in chloroform and mixed thoroughly. The chloroform was then removed by passing nitrogen gas through the inner side of a 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°C) with 2 ml of phosphate buffer solution (PBS) pH 7.4 containing 19 µM 5-FU. The hydrated film was then vortexed for 1 min and bath sonicated for 5 min. The resulting multi-lamellar liposomal vesicles were then extruded through a 200-nm polycarbonate filter membrane to further reduce the size. The free 5-FU was finally removed by dialysis against PBS for 24 hr using a 12 kDa molecular weight cutoff dialysis tube.

Characterization of pH-sensitive liposomal nanoparticles

Size measurement: The particle size and zeta potential of the blanks, pHLNp, pHNLNp, and pHNLNp, and their corresponding loaded 5-FU, pHLNp-5-FU, pHNLNp-5-FU, and pHNLNp-5-FU were determined by dynamic light scattering using a zeta potential/Particle Sizer Instrument (NICOMP™ 380 ZLS) (Table 1). All measurements were performed in triplicates and the results were reported in mean diameter ± SEM.

Entrapment efficiency (EE %): Ten milligrams of lyophilized pHLNp-5-FU, pHNLNp-5-FU, or pHNLNp-5-FU was suspended in 2 ml of PBS (pH 7.4). The liposomal suspension was disrupted by adding 100 µL of 30% Triton X-100, gently mixed for 2 min, and centrifuged at 6,000 rpm at room temperature for 5 min. The supernatant was collected and analyzed for 5-FU using reverse phase high-performance liquid chromatography (HPLC). The reverse phase HPLC: the mobile phase solution consisting of 95% PBS and 5% of methanol was prepared and filtered according to method described [17]. The internal standard or sample injection volume was 20 µL, which was pumped 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. 5-FU was detected at 270 nm with Waters 996 photodiode array detector (Waters, Columbia Maryland, USA). The entrapment efficiency was calculated according to the following equation:

\[
\text{EE} \%(\%) = \frac{\text{Amount of drug encapsulated in nanoparticles}}{\text{initial amount of drug}} \times 100
\]

FTIR analysis: Based on HPLC analysis, the pHNLp formulation was found to have the highest EE (%) of 5-FU (Table 1). FTIR analysis was further conducted on the pHNLp to confirm the incorporation

Table 1. Characterization of 5-FU loaded-ph-sensitive liposomal nanoparticles.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lipid composition</th>
<th>Molar ratio</th>
<th>Mean particle Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHLNp-5-FU</td>
<td>DOPE:CHEM:DSPE-PEG<del>m</del></td>
<td>50:30:20</td>
<td>193.3 ± 7.7</td>
<td>2.14 ± 1.5</td>
<td>74.4</td>
</tr>
<tr>
<td>pHLNp-5-FU</td>
<td>PC:CHEM:TWEEN 80:DSPE-PEG<del>m</del></td>
<td>40:40:10:10</td>
<td>155.4 ± 8.5</td>
<td>0.59 ± 1.3</td>
<td>-</td>
</tr>
<tr>
<td>pHLNp-5-FU</td>
<td>CHEM:CH:TWEEN20:DSPE-PEG<del>m</del></td>
<td>60:20:10:10</td>
<td>136.1 ± 10.2</td>
<td>1.30 ± 0.8</td>
<td>54.17</td>
</tr>
<tr>
<td>pHNLNp-5-FU</td>
<td>CHEM:CH:TWEEN20:DSPE-PEG<del>m</del></td>
<td>60:20:10:10</td>
<td>164.0 ± 8.4</td>
<td>1.23 ± 0.8</td>
<td>-</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM, n = 3. (Dioleoylphosphatidylethanolamine (DOPE), cholesterylhemisuccinate (CHEMS), cholesterol (CH), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[aminopolyethylene glycol]-2000] (DSPE-PEG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[folate(polyethylene glycol)]-2000] (DSPE-PEG-FA).
of 5-FU. Physical mixture (lyophilized blank pHLNp, and 5-FU), lyophilized blank pHLNp, 5-FU, and lyophilized pHLNp, 5-FU were analyzed in spectra range of 740–4000 cm⁻¹ using FTIR spectrophotometer (PerkinElmer Life and Analytical Sciences, Connecticut, USA). The acquired spectra were then used to determine the presence of 5-FU in pHLNp.

**Evaluation of in-vitro drug release**

5-FU release at different pH values: Buffer solutions of different pH values (3.0, 5.0, 6.5 and 7.4) were prepared and 1 ml of liposomal nanoparticle suspension containing 2.5 mg/ml of pHLNp, 5-FU pHLNp, 5-FU, or pHLNp, 5-FU was placed in a dialysis bag; this was immersed completely in different pH solutions and stirred continuously (100 rpm) for 24 hr at 37°C. After 24 hr, 500 µL of solution was removed from each receiver chamber and analyzed for the presence of 5-FU using reverse phase HPLC as described above.

5-FU release at different time points: A buffer solution of a pH 3.0 was prepared and 1 ml of liposomal nanoparticle suspensions containing 2.5 mg/ml of pHNP, 5-FU pHNP, 5-FU, or pHNP, 5-FU was placed in dialysis bag and immersed completely in solution of pH 3 and stirred at 100 rpm continuously for 24 hr at 37°C. At predetermined time intervals of 1, 5, 10, 15, 30, 60, 120, 240, 480, 720, and 1440 min, 500 µL of receiver solution was sampled out and replaced with equal volume of fresh PBS at 37°C. Amount of 5-FU present in each sampled solution was determined by reverse phase HPLC.

**Cell viability**

The *in vitro* cytotoxicity of free 5-FU, pHLNp, 5-FU pHNP, 5-FU, or pHNP, 5-FU was evaluated using HCT-116 and HT-29 colon cancer cell lines. The HCT-116 and HT-29 cell lines were seeded into 12-well plates at a density of 5 x 10⁴ cells per well and cultured in DMEM/F12 media supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. The cells were treated with different concentrations of 5-FU or its equivalent in pHLNp, 5-FU, or pHNP, 5-FU after they had reached 75% confluence. After 48 hr, the experiments were terminated and the cells were detached, stained with 2% trypan blue, and counted with an automated cell counter (Bio-Rad TC-20™). The cell viability (%) relative to the control was determined.

**Cellular uptake**

**Confocal imaging:** HCT-116 and HT-29 cancer cells were grown in 6-well plates (with cover slips) at a cell density of 2 x 10⁴, for 24 hr at 37°C. The cells were then treated with rhodamine-labeled pHLNPs in growth media (Rho-pHNP, Rho-pHNP, or Rho-pHNP). After 3 hr, Rho-pHNP, Rho-pHNP, or Rho-pHNP, was removed and the cells gently washed twice with PBS (pH 7.4). Next 0.75 µg/ml of 4,6-diamidine-2-phenylindole (DAPI) was added for nuclear staining; finally, the cells were fixed, using 4% paraformaldehyde, then mounted and imaged using Leica SP2 Multiphoton system.

**Lysosomal delivery of Lyso Tracker Red DND-99 by pHLNPs:** HCT-29 and HCT-116 cells were seeded on cover slips at a density of 2 x 10⁴ per well in a 6-well plate. After 24 hr cultivation, cells were incubated with 500 µg/ml of Lucifer yellow-labeled pHNP, 5-FU (LY-pHNP, 5-FU) for 4 hr at 37°C, and then incubated with LysoTracker Red DND-99 (200 nM) for 1 hr. The cells were then washed three times with cold PBS (pH 7.4), fixed using 4% paraformaldehyde, and the cover slips were mounted cell-side down with slides and viewed using a Zeiss LSM 880 Confocal Microscope [18].

**Flowcytometry:** To determine 5-FU-loaded liposomal nanoparticle uptake by the cells, HCT-116 and HT-29 cells were plated onto 6-well plates with a density of 5 x 10⁴ and cultured in growth media until 75% confluence. Cells were incubated with the different rhodamine-labeled liposomes (Rho-pHNP, Rho-pHNP, or Rho-pHNP,) for 24 hr at 37°C. After incubation, the cells were detached from the culture plate with 0.25% trypsin-EDTA solution, washed three times with PBS, and centrifuged at 3,000 rpm for 5 min. Finally, the cells were re-suspended in 500 µL PBS, fixed with 4% paraformaldehyde, and kept on ice until analysis using a BD FACSCanto™ Analyzer and a BD FACSaria™ Cell Sorter (BD Biosciences)

**Colony formation assay**

For colony assay, HCT-116 and HT-29 cell lines were seeded into T-25cm² culture flask at a density of 5 x 10⁵ cells and cultured in DMEM/F12 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% FBS, and 1% penicillin/streptomycin. After the cells reached 75% confluence, they were exposed to different concentrations of free 5-FU and pHLNp, 5-FU. After 48 hr exposure and two treatments, the experiment was terminated, cells harvested, and then re-plated onto 6-well plates at a density of 200, 500, and 1,000 cells per well, and incubated with growth medium. After the control cells reached 75% confluence, the experiment was terminated by fixing and staining the plates with 0.5% crystal violet solution. The stained colonies (fifty per colony) were counted using a Jenco™ Stereomicroscope; plating efficiency (PE) and surviving fraction (SF) were calculated, and a graph of survival curve graph was generated [19].

**Statistical analysis**

All experiments were performed in triplicates and analyzed using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA. USA). The differences between the 5-FU treatment group and that of pHLNp, 5-FU pHLNp, 5-FU or 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 sizes of blank liposomal nanoparticles pHLNp, pHLNp, and pHLNp, 5-FU were 189.3 nm ± 7.7 nm, 155.4 nm ± 8.5 nm, and 136 nm ± 10.2 nm, respectively, while the mean sizes of 5-FU-loaded liposomal nanoparticles; pHLNp, 5-FU, pHLNp, 5-FU and pHLNp, 5-FU were found to be 200 nm ± 9.8 nm, 181.9 nm ± 9.1 nm, and 164.3 nm ± 8.4 nm, respectively (Table 1). Of all the formulations, pHLNp, 5-FU had the highest 5-FU entrapment efficiency (EE) of 54.17%, whereas pHLNp, 5-FU and pHNP, 5-FU showed comparable 5-FU EE of 3.25 and 4.74%, respectively. The zeta potential values of pHLNp, 5-FU, pHNP, 5-FU, and pHNP, 5-FU were 5.16 ± 1.4, 1.98 ± 0.5, and 1.23 ± 0.8, respectively (Table 1).

**FTIR Analysis:** Although FTIR analysis is not confirmatory approach to fully determine entrapment of 5-FU in the liposomal carrier, it could be used to assess the association of 5-FU and the carrier. A careful examination of 5-FU spectrum revealed completely different absorption peaks when compared with blank or empty pHNPs carrier (Figure 1A and 1D). Further, neither 5-FU (Figure 1A) nor blank pHNPs (Figure 1D) absorption peaks were not similar to that of pHLNPs-5-FU (Figure 1B) peaks. The unique features of pHLNPs-5-FU showed a close association between 5-FU and pHNPs carriers. The FTIR spectra of pure 5-FU showed a ~N-H stretch at
3120 cm$^{-1}$ and –C=O stretch at 1644.9 cm$^{-1}$. These same stretches were also seen in the pHLNPs-5-FU sample that was analyzed, therefore confirming the interaction of 5-FU and the liposomal nanocarrier.

**In vitro 5-FU release**

**5-FU release at different pH values:** The percent of 5-FU released decreased as pH increased from 3 to 7.4. This was a general trend observed in all of pHLNPs-5-FU formulations as shown in Figure 2A. However, pHLNPs-5-FU formulation had a much greater release of 5-FU (Figure 2B) compared to pHLNPs-5-FU and pHLNPs-5-FU (Figure 2A). Among all the formulations, pHLNPs-5-FU had the highest release of 5-FU (30%) at pH 3, compared to 5-FU release at pH 7.4 (15%). Further, pHLNPs-5-FU was the most pH-sensitive formulation compared with pHLNPs-CHEM, pHLNPs-5-FU and pHLNPs-5-FU at pH 3.

**5-FU release at different time points:** The cumulative release profile of 5-FU from pHLNPs-5-FU at pH 3 was biphasic, as shown in Figure 3C. There was an initial rapid release of 35% of 5-FU for the first 100 min, followed by the release of an additional 15% over a period of 700 min (from 100 to 800 min). After 800 min, 5-FU release did not significantly increase with increasing time; only 3% of 5-FU was released from 800 min to 1,440 min (Figure 2C).
Cell viability

Figure 3 shows the effect of increasing concentration of 5-FU, pHLNP -5-FU, pHLNP -5-FU, and pHLNP -5-FU on the viability of HCT-116 and HT-29 cancer cell lines. As shown in Figure 3A, pHLNP -5-FU significantly inhibited HT-29 cell growth at all concentrations compared to free 5-FU. Further, pHLNP -5-FU was two- to three-fold more effective than pHLNP -5-FU or pHLNP -5-FU. As shown in Figure 3B, pHLNP -5-FU was the most effective formulation against HCT-116 cell lines, as compared to free 5-FU, pHLNP -5-FU, and pHLNP -5-FU.

For example, 1 µM 5-FU-loaded pHLNP was four-fold more effective in inhibiting HCT-116 cell growth than pHLNP -5-FU or pHLNP -5-FU, and also three-fold more effective than free 5-FU in HCT-116 cell growth inhibition. To measure the effectiveness of free 5-FU and the 3 pHLNPs-5-FU formulations in inhibiting both HT-29 and HCT-116 cells’ growth, we modeled the data obtained from cell viability testing using varying concentrations of pHLNP -5-FU, pHLNP -5-FU, and pHLNP -5-FU to generate half-maximal inhibitory concentrations (IC₅₀) as shown in Figure 4. As expected, pHLNP -5-FU was the most effective formulation, with IC₅₀ values of 0.4114 (µM) on HT-29 cells and 0.2041 (µM) on HCT-116 cells. Conversely, pHLNP -5-FU (IC₅₀ = 6.256 (µM) against HT-29 cells and IC₅₀ = 69.26 (µM) against HCT-116 cells) was found to be the most ineffective formulation to inhibit HCT-116 cells growth while pHLNP -5-FU (IC₅₀ = 8.150 (µM) against HT-29 cells and IC₅₀ = 0.4107 (µM) against HT-116 cells) was found to be least effective formulation against HT-29 cells.

Cellular uptake

Confocal imaging: Cellular uptake of pHLNP formulations was determined by treating both HT-29 and HCT-116 cells for 3 hr at 37°C with DOPE-Rho-conjugated pHLNPs; Rho-pHLNP, Rho-pHLNP, and Rho-pHLNP. Confocal images of HT-29 and HCT-116 cells showed significant uptake of Rho-pHLNP, Rho-pHLNP, and Rho-pHLNP as shown in Figure 5. The merged images clearly showed that majority of internalized nanoparticles were localized in the cell nuclei.

Lysosomal delivery of Red DND-99 by pHLNP: We also investigated the internalization and intracellular fate of pHLNP, by counterstaining with LysoTracker Red DND-99. Figure 6A and 6D show that most of LY-pHLNP -5-FU was taken up by cells (green color). LysoTracker Red DN-99 uptake by the cells was observed as red color (Figure 6B and 6E). The co-localization of LY-pHLNP and LysoTracker Red (Figure 6C & F) was followed and the resulting yellow color shows the accumulation of LY-pHLNP -5-FU in the lysosomes. This was performed for both HT-29 and HCT-116 cell lines.

Flow cytometry: To further confirm the internalization of the formulations by HT-29 and HCT-116 cells, flow cytometry analysis was performed. Figure 7 shows cellular uptake of Rho-pHLNP, Rho-pHLNP, and Rho-pHLNP after the cells were incubated for 24 hours at 37°C. The results show significant uptake of the formulations by both cells, however HCT-116 cells exhibited greater cellular uptake of the formulations compared to HT-29 cells.

Colony assay

The proliferative properties of both HT-29 and HCT-116 cell lines
were assessed after treatment with free 5-FU and pHLPNp-5-FU via a clonogenic assay method. Figure 8A shows a difference in percent survival of free 5-FU or pHLPNp-5-FU-treated HT-29 cells, as the concentrations of both free 5-FU and pHLPNp-5-FU were increased. As concentration increased from 0.01 to 10 µM, pHLPNp-5-FU was able to disrupt the proliferative property of the cancer cell better than free 5-FU. This is further confirmed by the survival curve shown in Figure 8B. Figure 9 shows a reduction of colony formation in HCT-116 cells as the doses of free 5-FU and pHLPNp-5-FU were increased. Figure 9B shows a survival curve with a similar pattern to that in Figure 8A. It also shows that pHLPNp-5-FU was more effective and significantly decreased the percent survival compared to free 5-FU at a concentration range of 0.01-10 µM.

Put together, the results show that pHLPNp could increase the delivery and anti-cancer activity of 5-FU in HT-29 and HCT-116 colorectal cancer cells.

Discussion

5-FU is a low molecular weight drug that acts as a thymidylate synthase inhibitor to block the synthesis of the pyrimidine thymidine, which is essential for DNA replication. It is currently used clinically to treat colorectal cancer either alone or in combination with other anticancer drugs [20]. For optimal therapeutic activity, 5-FU is administered continuously for an extended period of time to cancer patients due to a short half-life. However, due to lack of specificity, long-term therapy with 5-FU may lead to severe cardiotoxicity [21]. The aim of this study was to develop and study pH-sensitive liposomal nanoparticles loaded with 5-FU (pHLPNps-5-FU), with surface-modified anti-EGFR antibody for the following purposes: i) increased 5-FU plasma circulation half-life, ii) increased anti-cancer activity of 5-FU, iii) reduced associated toxicity, and iv) improved specificity. A pH-sensitive liposome is an attractive delivery system due to the fact that the tumor site is relatively acidic compared to the normal tissue site, and the liposome can undergo destabilization to release its aqueous content under acidic condition [13,22].

Three pHLPNps-5-FU formulations (pHLPNp-5-FU, pHLPNp-5-FU, and pHLPNp-5-FU) were prepared and characterized by size, zeta potential, and entrapment efficiency; pHLPNp-5-FU was chosen for...
further study because it had the highest entrapment efficiency (54.17%) (Table 1). This was attributed to the presence of a -CH moiety in the pHLNP formulation, which was necessary to prevent the leakage of encapsulated 5-FU from pHLNP. CH molecules fill in a free space formed due to a kink in the chain of unsaturated lipids that was present in the liposome formulation [23]. In addition, CHEMS, a component of pHLNP, is reported to cause destabilization of the liposomal membrane at acidic pH, which would enhance the deposition of 5-FU at the tumor site [24]. Entrapment of 5-FU in liposomal nanoparticles was also confirmed by FTIR. The FTIR spectrum of pure 5-FU showed a ~N-H stretch at 3120 cm⁻¹ and ~C=O stretch at 1644.9 cm⁻¹ (Figure 1). Similar stretches or absorption peaks were also observed in the pHLNPs-5-FU spectrum suggesting the entrapment or presence of 5-FU in pHLNP. As expected, in vitro release of 5-FU was highest in pHLNP compared to pHLNP and pHLNP. This may be due to the fact that pHLNP has a much higher amount of CHEM lipid compared to pHLNP and pHLNP, which is speculated to cause destabilization of the formulation and improve release of 5-FU under acidic conditions. The rapid disruption of pHLNP and release of 5-FU in the first 100 min at pH of 3 compared to pHLNP, or pHLNP, is attributed to the same reason [25].

The cytotoxicity of 5-FU was compared to pHLNP-5-FU, pHLNP-5-FU and pHLNP-5-FU formulations on HT-29 and HCT-116 cancer cell lines to assess the most effective anti-cancer agent. Based on the viability and clonogenic results, HT-29 and HCT-116 cells were most sensitive to pHLNP-5-FU compared to all other formulations, while pHLNP-5-FU and pHLNP-5-FU were less effective. The low anti-cancer activity of pHLNP-5-FU and pHLNP-5-FU is largely attributed to the low entrapment of 5-FU. Conversely, the high payload and increased delivery of 5-FU by pHLNP, coupled with quick disruption of pHLNP, under acidic conditions may significantly account for the enhanced anti-cancer activity of pHLNP-5-FU. This was further supported by the IC₅₀ results for pHLNP-5-FU, which was the most effective agent against both HT-29 and HCT-116 cells. It was unclear why the IC₅₀ for pHLNP-5-FU was far better than that of 5-FU on HCT-116 cells.

Findings of flow cytometry and confocal studies revealed that the treatment of cells with the Rho-pHLNP, Rho-pHPLNP, and Rho-pHPLNP, led to a comparable level of cell total Rho fluorescence intensity, which clearly indicates that the uptake of pHLNP, pHLNP, and pHLNP, by HT-29 or HCT-116 cells was similar, although HCT-116 uptake of liposomal nanoparticles was slightly higher than that of HT-29 cells. While confocal and flow cytometry studies were conducted to assess the uptake of our formulations by the cells, delivery of pHLNP, to the lysosomal compartment was studied to assess accumulation in the lysosomes. LY-pHLNP, 5-FU was chosen because of a high entrapment efficiency and extremely low IC₅₀ values. The presence of LY-pHLNP, 5-FU in HT-29 or HCT-116 cells was observed by color green, while the lysosome compartment was stained the color red. To determine the accumulation of LY-pHLNP, 5-FU in the lysosome, the two images was merged and the yellow color (combination of green and red colors) was observed (Figure 6), confirming the presence of LY-pHLNP, 5-FU nanoparticles in the lysosome compartment.

In tumors, clonogenic assay measures the ability of individual cells to proliferate to form colonies of at least 50 or more cells, which is a critical metric of cell viability. Only clonogenic cells have the ability to cause recurrence or create metastasis [26]. Based on this, HT-29 and HCT-116 cells were tested for their ability to proliferate after treatment with 5-FU and pHLNP-5-FU. The pHLNP-5-FU and pHLNP-5-FU formulations were not tested due to low 5-FU entrapment and poor inhibition capabilities made them poor candidates. The data suggest that pHLNP-5-FU was more effective in rendering the cells incapable of proliferation compared to 5-FU (Figure 8 and 9). However, in comparing HT-29 and HCT-116 cells, it was evident that pHLNP-5-FU had a much more pronounced effect in disrupting the proliferative property of HCT-116 than that of HT-29.

Conclusion

We have successfully formulated pH-sensitive thermo-sensitive liposomal nanoparticles, of which pHLNP3 was very responsive to pH lower than or equal to 4, exhibiting enhanced release of 5-FU under this condition. The pHLNP, 5-FU nanoparticles exhibited a stronger anti-cancer effect compared to 5-FU against HT-29 and HCT-116 cancer cells. The findings provide strong may evidence in support of a possible therapeutic application of pHLNP, as a drug delivery system for 5-FU, which can overcome some of the limitations of 5-FU currently has, such as poor cell membrane permeability and short half-life. Currently, pHLNP, 5-FU is being studied in an animal model to evaluate in vivo efficacy on tumor growth.

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