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Targeted therapy of colorectal neoplasia with rapamycin in peptide-labeled pegylated octadecyl lithocholate micelles

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Abstract

Many powerful drugs have limited clinical utility because of poor water solubility and high systemic toxicity. Here, we formulated a targeted nanomedicine, rapamycin encapsulated in pegylated octadecyl lithocholate micelles labeled with a new ligand for colorectal neoplasia, LTTHYKL peptide. *CPC;Apc* mice that spontaneously develop colonic adenomas were treated with free rapamycin, plain rapamycin micelles, and peptide-labeled rapamycin micelles via intraperitoneal injection for 35 days. Endoscopy was performed to monitor adenoma regression in vivo. We observed complete adenoma regression at the end of therapy. The mean regression rate for peptide-labeled rapamycin micelles was significantly greater than that for plain rapamycin micelles, $P < 0.01$. On immunohistochemistry, we observed a significant reduction in phospho-S6 but not β -catenin expression and reduced tumor cell proliferation, suggesting greater inhibition of downstream mTOR signaling. We observed significantly reduced renal toxicity for peptide-labeled rapamycin micelles compared to that of free drug, and no other toxicities were found on chemistries. Together, this unique targeted micelle represents a potential therapeutic for colorectal neoplasia with comparable therapeutic efficacy to rapamycin free drug and significantly less systemic toxicity.

Keywords

micelles; targeted therapy; colorectal cancer; rapamycin; endoscopy

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1. Introduction

Worldwide, more than 1.36 million individuals are diagnosed with colorectal cancer (CRC) each year.[1] These numbers are expected to nearly double over the next 20 years, as obesity is increasing in epidemic proportions and more developing countries are adopting Western diets.[2] Surgery is the first choice for therapy in patients with early CRC. Adjuvant chemotherapy, most frequently 5-fluorouracil (5-FU), oxaliplatin, and irinotecan, is commonly administered. Unfortunately, these drugs frequently incur severe side effects, such as diarrhea and neuropathy, that may result in dose reduction and limited effectiveness. [3] After the visible tumor is resected, circulating cancer cells can result in cancer recurrence ~50% of the time.[4] Targeted therapies include monoclonal antibodies[5] and small molecules,[6] and have been FDA-approved for treatment of metastatic CRC. The pharmacokinetic properties of antibodies, including extravasation, diffusion, and penetration, are limited by size and reduce drug accumulation within tumors. Antibodies can also be immunogenic. Effective drugs for treatment of early CRC have also been limited by poor water solubility. New methods that target delivery of powerful chemotherapeutic agents with improved pharmacokinetics and low toxicity are needed.

Encapsulation of drugs in micelles can reduce effective volume of distribution, maximize tumor accumulation, and minimize systemic toxicity. They can be labeled with a targeting moiety to improve cellular internalization. Deoxycholic acid and their derivatives have been used to modify chitosan for use as nanocarriers of cancer therapy.[7] Micelles are particularly promising for packaging hydrophobic agents. By partitioning the drug within its core, micelles can increase drug loading, extend drug release, and improve plasma half-life. [7, 8] These agents form spontaneously by self-assembly and degrade within tumor cells. [7-9] They have better long-term stability compared to other delivery platforms, such as liquid emulsions. Rapamycin is a potent mTOR inhibitor that has low and unpredictable oral bioavailability.[7-9] Parenteral use of this lipophilic drug is limited by poor water solubility (2.6 µg/mL at 25°C)[7-9] and high systemic toxicity. Free rapamycin is highly hydrophobic and dissolves minimally in organic solvents that can be harmful to the liver and kidney. The model drug, rapamycin has physicochemical properties that are well suited for packaging in micelles, and can be developed in a clinically useful formulation.

Peptides are promising for systemic use as ligands that bind preferentially to overexpressed tumor targets and mediate tumor cell internalization. Because of their smaller size compared to antibodies, peptides have improved extravasation, more rapid diffusion, increased tumor penetration, and lower immunogenicity.[10] They can be developed to detect a broad range of molecular targets with high specificity and binding affinity. We have previously shown that peptides can home to colonic neoplasms on diagnostic imaging with minimal risk for toxicity.[11] We used phage display technology with in vivo biopanning to select a panel of candidate peptides that bind specifically to colonic adenomas that form in the *CPC;Apc* mouse model of CRC.[12] This mouse was genetically engineered to somatically delete an *Apc* allele under Cre regulation, and develops adenomas spontaneously.[13] This model is representative of human disease because *Apc* mutations are found in >80% of sporadic colorectal cancers.[14] Here, we aim to demonstrate the effective and safe use of peptide-

labeled pegylated octadecyl lithocholate micelles to encapsulate rapamycin for targeted therapy to induce regression of colonic adenomas.

2. Materials and methods

2.1 Animal model

8-10 month old *CPC;Apc* mice that have been genetically engineered with a Cre regulated somatic deletion in one *Apc* allele were used. These mice spontaneously produce adenomas in the distal colon that range in size from 2-4 mm in diameter at this age. Mice were cared for with approval of the University Committee for Use and Care of Animals (UCUCA) at the University of Michigan. Mice were housed in specific pathogen-free conditions and supplied water *ad libitum* throughout the study.

2.2 Peptide synthesis

NIR peptide synthesis for in vivo peptide selection—A panel of peptides (QPIHPNNM-GGGSK, KCCFPAQ-GGGSK, LTTHYKL-GGGSK, AKPGYLS-GGGSK, YTTTNAS-GGGSK and DNEPIAQ-GGGSK) were synthesized for selection of micelle labeling using solid phase peptide synthesis with standard Fmoc chemistry[15] on a PS3 (Protein Technologies, Inc.) automatic peptide synthesizer. Cy5.5 NHS ester was conjugated at the C-terminus on the side chain of a lysine residue via the GGGSK linker. Resins (~0.03 mmol) were swelled in DMF (1 mL). In a separate tube, Cy5.5 NHS ester (18 mg, 0.03 mmol) was dissolved in DMF (0.6 mL). ~23 μ L of DIEA (46 μ L, 0.26 mmol) was added to both tubes. Cy5.5 solution was added to the resins. The reaction was allowed to stir 2-3 days at RT. The resins were washed and cleaved. The resulting peptides were precipitated in cold diethyl ether. The peptides were then purified using a semi-preparative HPLC (Water Breeze HPLC) with water-acetonitrile gradient mobile phase containing 0.1% trifluoroacetic acid (TFA). The resulting peptides were lyophilized and characterized with an ESI mass spectrometer (Micromass LCT Time-of-Flight mass spectrometer with electrospray). Peptides purity was >95% on analytical HPLC.

Peptide synthesis for micelle conjugation—The LTTHYKL-GGGSK peptide was selected for micelle labeling, and synthesized as described above. A maleimide functional group was conjugated at the C-terminus on the side chain of a lysine residue via a GGGSK linker. Briefly, LTT* resins (~0.06 mmol) were swelled in DMF (1.5 mL). In another tube, 3-maleimidopropionic acid (25 mg, 0.15 mmol) and HOBt (70 mg, 0.46 mmol) were dissolved in DMF (1 mL). DIC (70 μ L, 0.45 mmol) was added and the solution mixture was added to resins after 10 min of activation. The reaction was allowed to stir overnight at RT. Resins were washed, cleaved, and the resulting peptide was precipitated in cold diethyl ether. The peptide was then purified using a semi-preparative HPLC (Water Breeze HPLC) and characterized with an ESI mass spectrometer (Micromass LCT Time-of-Flight mass spectrometer with electrospray). Peptides purity was >95% on analytical HPLC.

2.3 Synthesis of micelles

Octadecyl lithocholate—Lithocholic acid (1.5 g, 4 mmol) and HOBt (1.5 g, 10 mmol) were dissolved in N,N-dimethylformamide (DMF) (12 mL). DIC (1.5 mL, 10 mmol) was

added. After 10 min for activation, octadecyl amine (0.9 g, 3.3 mmol) was added along with dichloromethane (DCM) 4 mL, and the reaction was allowed to stir overnight at room temperature (RT). The resulting product was filtered and vacuum dried (MW 628 Da).

Succinyl octadecyl lithocholate—Octadecyl lithocholate (573 mg, 0.91 mmol) was dissolved in anhydrous DCM (15 mL). Catalytic amount of DMAP was added. Succinic anhydride (90.9 mg, 0.91 mmol) and DIEA (950 μ L, 5.45 mmol) were then added and the reaction was allowed to run overnight at RT. The solvent was evaporated under N₂ and the resulting product was vacuum dried (MW 728 Da).

Pegylated octadecyl lithocholate—Succinyl octadecyl lithocholate (64.2 mg, 0.09 mmol) was dissolved in DCM 2.5 mL and DMF 1 mL. HOBt (41.3 mg, 0.27 mmol) was added, following by adding DIC (50 μ L, 0.27 mmol). Methoxy PEG amine (143 mg, 0.05 mmol) was added after 10 min for activation. The reaction was allowed to stir overnight at 40°C. The solvent was partially removed under N₂ and the resulting product was precipitated in cold diethyl ether, centrifuged, and vacuum dried. Succinyl octadecyl lithocholate was conjugated with thiol PEG amine in the same manner.

2.4 Micelle platform for targeted drug delivery

Maleimide peptide and thiol pegylated octadecyl lithocholate conjugation—

Thiol pegylated octadecyl lithocholate (74.8 mg, 0.02 mmol) and TCEP (9.68 mg, 0.03 mmol) were dissolved in phosphate buffer pH 8.0 (15 mL). Maleimide peptide (0.02 mmol) was added and the reaction was allowed to run overnight at RT. The resulting product was dialyzed against three changes of water (MWCO 1 kDa) and lyophilized.

Micelle preparation—The micelles were prepared by dissolving pegylated octadecyl lithocholate and/or LTT* pegylated octadecyl lithocholate in PBS. Polymers were sonicated ~30 min or until fully dispersed. Rapamycin (18 mg/mL) or coumurin-6 (0.25 mg/mL) ethanolic stock solution was added to the polymer solution. The solution was sonicated for 5 min. The organic solvent was removed by purging with N₂. After removing the organic solvent, the micelle solution was centrifuged at 8000 rpm for 10 min to remove insoluble material. Rapamycin or coumurin-6 micelles in supernatant were separated. For the cell staining, cytotoxicity and in vivo targeted therapy experiments, 0.35, 0.67, and 1.73 mM of polymer was used, respectively.

2.5 Specific binding of micelles to surface of cell panel

SW620, HT29, SW480, DLD1, and CCD841CON cells (5×10^4 cells/mL) were grown on gelatin coated cover slips up to ~80% confluence. The cells were washed with PBS, blocked with 0.5% bovine serum albumin (BSA) for 30 min at 4°C, and washed with PBS. The cells were incubated with coumarin-6 encapsulated micelles (1.3 mg/mL) that have 50% LTT* targeting peptide density for 60 min at 4°C. Cells were washed with PBS three times, fixed with 4% paraformaldehyde (PFA) for 5 min, washed once with PBS, and then mounted on glass slides with Prolong Gold reagent containing DAPI. Fluorescence images were collected on a Leica Inverted SP5X Confocal Microscope System. Fluorescence images were quantified with Matlab software by averaging 3 images for each cell. The fluorescence

intensity from each image was normalized by number of cells using gray scale value of DAPI intensity.

2.6 Competitive binding assay

Specific binding of LTT*-labeled coumarin-6 micelles was validated on competitive inhibition with unlabeled LTT* peptide. This experiment was performed at the same time as that for specific cell surface binding. SW620, HT29, SW480, DLD1, and CCD841CON cells were prepared, as described above. Unlabeled LTT* peptide at 50 and 250 μ M were incubated for 30 min at 4°C. Cells were then incubated with LTT*-labeled coumarin-6 encapsulated micelles (1.3 mg/ mL) for 60 min at 4 °C. The cells were washed with PBS three times, fixed with 4% paraformaldehyde (PFA) for 5 min, washed once with PBS, and then mounted on glass slides with Prolong Gold reagent containing DAPI. Fluorescence images were collected on a Leica Inverted SP5X Confocal Microscope System. Fluorescence images were quantified with Matlab software by averaging 3 images for each cell and unlabeled peptide concentration. The fluorescence intensity from each image was normalized by number of cells using gray scale value of DAPI intensity.

2.7 In vivo imaging of colonic adenomas in *CPC;Apc* mice

We performed in vivo imaging of colon in *CPC;Apc* mice to select the peptide to label the micelles. The mice were placed on a heat pad. Anesthesia was induced and sedation was maintained using inhaled isoflurane mixed with oxygen via a nose cone at a dose of 4% and 1.5%, respectively, and a flow rate of 0.5 L/min. A small animal endoscope (Karl Storz Veterinary Endoscopy) was used to visualize the colon.[19] A stage was used to adjust the position of the mouse and to manipulate the endoscope and calipers. The distal tip of the endoscope was inserted rectally, and advanced. Debris was rinsed away with water. The colon was insufflated with air to keep the lumen open. Adenomas with diameter >1.5 mm were identified, and the location from the anus was recorded. A distance of 0 mm was defined when the endoscope tip touched the adenoma. The distance of the endoscope tip to the adenoma was recorded during withdrawal using a gauge. Videos were recorded, and individual images were exported using Axiovision Lite software. The surface area of the adenoma was quantified using established relationship between distance of endoscope tip to the adenoma and surface area of a grid with $1 \times 1 \text{ mm}^2$ squares.

2.8 Adenoma regression with targeted rapamycin micelle therapy

CPC;Apc mice were randomly divided into four treatment groups ($n=6, 6, 6, 7$, respectively): 1) normal saline solution (NSS), 2) free rapamycin, 3) unlabeled (plain) rapamycin micelles, and 4) LTT*-labeled rapamycin micelles. The number of mice (number of adenomas) at the end of the study were $n=5$ (20), 6 (24), 5 (22), and 7 (21), respectively. Two mice (one in NSS group and one in unlabeled (plain) rapamycin micelle group) died from unexplained cause. Mice in the treatment groups were given NSS, free rapamycin, plain rapamycin micelles, and LTT*-labeled rapamycin micelles at a dose of 5 mg/kg through a daily intraperitoneal (i.p.) injection for 35 days. Mice underwent endoscopy every ~4 days to monitor adenoma regression and were weighed every 2 days for 5 weeks. A total of $n=230$ colonoscopy examinations was performed.

2.9 Safety of micelles

Blood chemistries—After completion of therapy, the *CPC;Apc* mice were euthanized by carbon dioxide (CO₂) overdose. Blood was collected immediately by cardiac puncture, per UCUCA guidelines, and submitted to the Unit for Laboratory Animal Medicine (ULAM) Pathology Cores for Animal Research (PCAR) for evaluation of chemistries.

Necropsy—All tissues (heart, spleen, kidney, liver, and colon) were fixed in phosphate-buffered formalin for 24 hours, paraffin-embedded, and cut in 10 µm sections. Routine histology (H&E) was performed. Renal toxicity was assessed after completion of therapy. Histology (H&E) from representative murine kidney sections (*n*=4) from the different treatment groups were evaluated for tubules with vacuoles on five random fields (400X magnification) for each specimen.

Cytotoxicity—Cytotoxicity was determined by measuring growth inhibition for a panel of cells (SW620, HT29, SW480, DLD1, CCD841CON, and HEK393) on an MTT assay. Cell viability was calculated based on a comparison of untreated cells and those treated with free rapamycin and with plain and LTT*-labeled rapamycin micelles under the same conditions. The panel of cells were harvested and seeded at a density of 5×10³ cells/well in 96-well plates. Cells were cultured for 24 hours prior to adding rapamycin micelles in serial dilution. Free rapamycin at equivalent doses was incubated with cells in the same manner. After 1 day for incubation in culture medium without phenol red, the medium was removed, cells were washed twice with PBS, and MTT solution (100 µL, 0.5 mg/mL) was added to each well. After an additional 4 hours for incubation, MTT solution was removed. Formazan crystals produced by live cells were solubilized in DMSO and the absorbance was measured at 570 nm (test) and 630 nm (reference) using a microplate reader. Cytotoxicity of tested substance was presented as the half maximal inhibitory concentration (IC₅₀). At the concentration, the test substance inhibits cell viability by 50%.

2.10 Immunohistochemistry (IHC) of adenoma regression

Immunohistochemistry was performed using the following dilutions of primary antibodies: mouse anti-total β-catenin (1:600, BD Transduction Laboratories), rabbit anti-Ki67 (1:500, Pierce), and rabbit anti-phospho-S6 (Ser^{235/236}, 1:150, clone 91B2, Cell Signaling Technology) and developed with DAB (3,3' diaminobenzidine) substrate. The integrated optical density was measured from randomly placed IHC-positive areas of cells from 3 boxes (20×20 µm²) for each section using custom Matlab software (Mathworks) to quantify IHC staining.[20] The mean result was determined by averaging 7 images per antibody for each treatment group.

3. Results and discussion

Here, we demonstrate a dramatic therapeutic effect on colorectal adenomas in *CPC;Apc* mice with systemically administered rapamycin encapsulated in micelles labeled with targeting peptides. Micelles were prepared by conjugating hydrophobic octadecyl lithocholate with hydrophilic PEG. We used rapamycin as a model lipophilic drug that partitioned spontaneously into the micelle core. This formulation produced comparable

therapeutic effectiveness to free rapamycin, as shown by complete adenoma regression, with significantly less adverse systemic effects.

3.1 Selection of peptide for micelle labeling

On endoscopy, we measured in vivo fluorescence intensities from binding of a panel of Cy5.5-labeled peptides administered intravenously to colonic adenomas and adjacent normal appearing mucosa. The target-to-background (T/B) ratios are shown, Fig. S1. The highest mean value was measured for the peptide LTTHYKL, hereafter LTT*, which was chosen to label the micelles. The result was significantly greater than that of other peptides, including QPIHPNNM, YTTNKH, and DNEPIAQ, and non-significantly greater than that of KCCFPAQ and AKPGYLS, $*P<0.05$ by Kruskal-Wallis test and Dunn's multiple comparison test.

3.2 Selection of peptide density to coat micelle surface

Micelles that encapsulated coumarin-6 were labeled with LTT* peptide covering an average surface density that included 0%, 25%, 50% and 75%. On microscopy, micelles bind to the surface of HT29 cells (arrows), Fig. S2A-D. The intensity of micelles coated with 50% LTT* was significantly greater than that for 0 and 25% and non-significantly greater than that for 75%, $*P<0.05$ by Kruskal-Wallis test, Fig. S2E. No significant improvement was found at increased density of 75%. Therefore, we labeled the micelles with 50% peptide density. This result can be explained by a multivalency effect whereby increased ligand density improves the likelihood of nanoparticle interaction with cell surface targets.[24]

3.3 Micelle platform for targeted drug delivery

We assembled a hydrophobic section of octadecyl lithocholate (blue) with hydrophilic polyethylene glycol (PEG, black) and LTT* peptide (red) at 50% density, Fig. 1A. Micelles formed by self-assembly from intermolecular forces aggregating the individual components in a predetermined fashion. We prepared micelles with either 50% LTT* and no peptide (plain) for control. On transmission electron microscopy (TEM), the LTT* and plain micelles (arrows) form a spherical geometry, scale bar 200 nm, Fig. 1B,C.

3.4 Micelle Characterization

We measured the hydrodynamic size of the rapamycin micelles on dynamic light scattering (DLS), and found a non-significant difference of 130.1 ± 15.5 and 121.7 ± 22.1 nm for LTT*-labeled and plain rapamycin micelles, respectively, $P=0.62$ by unpaired t-test, Fig. 2A. The size of the micelles measured was consistent with that seen on TEM. Both micelles had a polydispersity index (PDI) of ~ 0.2 . This estimate of size distribution shows that the micelles were relatively monodispersed. The LTT*-labeled and plain rapamycin micelles had a slightly negative mean surface charge (μ eta potential) of -0.01 ± 0.83 and -0.78 ± 0.47 , respectively, and showed a non-significant difference in entrapment efficiency (%EE) for rapamycin of $88.1\pm7.9\%$ and $91.4\pm6.9\%$ ($\sim 13.8\%$ and 15.8% w/w), $P=0.61$ by unpaired t-test, respectively. We measured a rapamycin concentration that ranged from 990-1005 $\mu\text{g/mL}$ in micelle solution, resulting in a ~ 380 -fold improvement in solubility compared to free rapamycin in water ($2.6 \mu\text{g/mL}$).

We measured a critical micelle concentration (CMC) of 18 and 12 μM for LTT* and plain micelles, respectively, on fluorescence probe studies using pyrene, Fig. 2B. At the CMC, micelles form spontaneously and solubilize hydrophobic substances in water. Both LTT* and plain micelles showed a sustained release ratio of rapamycin over time with a half-life of ~ 44 hours, Fig. 2C. Rapamycin release can be described by Fickian diffusion from a sphere,[21] $R^2 = 0.9488$ and 0.9674 , for LTT* and plain micelles, respectively. There was no burst release observed, suggesting that the rapamycin molecules partition predominantly in the micelle core. Chemical stability was assessed at 4°C on reverse phase HPLC. LTT* and plain micelles were found to be stable up to 22 days with a high percentage of rapamycin retention, 81.2% and 91.5%, respectively, Fig. 2D.

3.5 Specific binding of micelles to surface of cell panel

We validated specific binding of LTT*-labeled rapamycin micelles encapsulating the fluorophore coumarin-6 to a panel of human CRC cells, including SW620, HT29, SW480, and DLD1, Fig. S3. On confocal microscopy, strong binding was observed to the surface of all cells, Fig. S3A-D. Minimal signal was observed for normal human colonic CCD841CON (control) cells, Fig. S3. Free LTT* peptide at concentrations of 50 μM , Fig. S3F-J, and 250 μM , Fig. S3K-O, was added to compete with the LTT*-labeled rapamycin micelles containing coumarin-6 for binding. Concentration-dependent reduction in signal was observed, Fig. S4. This result shows that the peptides rather than micelles mediate binding to the cell surface. Minimal signal was observed for plain rapamycin micelles (control), Fig. S3P-T.

3.6 Adenoma regression with targeted rapamycin micelle therapy

Groups of tumor-bearing mice were treated with free rapamycin, LTT*-labeled rapamycin micelles and plain rapamycin micelles via intraperitoneal injection for 35 days and with normal saline solution (NSS). On endoscopy, we observed rapid regression of large (2-4 mm) colonic adenomas (arrows) in response to therapy with LTT*-labeled rapamycin micelles over a 5-week period, Fig. 3A-E. In absence of therapy (NSS), no regression was observed, Fig. 3F-J. The mean regression rate for adenoma treated with LTT*-labeled rapamycin micelles was significantly greater than that for plain rapamycin micelles and NSS, and non-significantly greater than that for free rapamycin, $*P < 0.01$ on linear mixed effects regression analysis, Fig. 3L. Many of the treated adenomas reverted to completely normal histology, Fig. 5D. While both labeled and unlabeled micelles can reach the tumor via an enhanced permeability and retention (EPR) effect, the presence of targeting ligands can increase therapeutic efficacy by mediating micelle internalization in tumor cells.[24] Mice treated with LTT*-labeled rapamycin micelles and free rapamycin gained more weight than mice that received no treatment (NSS) and plain rapamycin micelles, Fig. S6. All mice that received some form of rapamycin therapy showed improvement in physical signs, including greater activity, less rectal bleeding, and shinier hair, within ~ 1 -2 weeks. In vivo monitoring was performed on repetitive endoscopic imaging over 5 weeks of therapy, Fig. 3.

3.7 Safety of micelles

Blood chemistries—Labs performed on blood obtained from euthanized *CPC;Apc* mice after completion of rapamycin therapy are shown, Fig. S5. Although a significant elevation in alkaline phosphatase (ALP) was seen with the LTT*-labeled and plain rapamycin micelles compared to NSS, $*P<0.05$ by Kruskal-Wallis test, these values fell within the normal range (65.5-364.2 U/L) of ALP for mice.

Necropsy—The *CPC;Apc* mice were euthanized after completion of therapy for necropsy, including examination of the heart, spleen, kidney, liver, and colon. Evidence for renal toxicity was assessed on histology (H&E) by counting the number of vacuolated tubules (arrow) per high-power field at 400X magnification, Fig. 4A.[22] We observed significantly reduced renal toxicity in mice treated with rapamycin encapsulated in micelles by comparison to that with free drug. Mice treated with free rapamycin were found to have a significantly greater number of vacuoles per high-power field than mice treated with either LTT*-labeled or plain rapamycin micelles and untreated control mice (NSS), $*P<0.05$ by ANOVA, Fig. 4B. Both peptide-labeled and unlabeled micelles showed significantly fewer vacuolated tubules than that for free rapamycin in kidney on necropsy. Rapamycin therapy is associated with renal toxicity, including distal magnesium wasting, tubular collapse, vacuolization and nephrocalcinosis.[30-32] Our results are consistent with previous studies that show therapy with rapamycin protected by nanoparticles can significantly reduce kidney damage.[22]

Cytotoxicity—We evaluated drug cytotoxicity on a panel of human CRC cells. HEK293 cells were included because of their known mTOR signaling activity.[23] The dose-response curves for cell viability are shown, Fig. 4C-H. The in vitro cytotoxicity studies in CRC cells showed less toxicity (higher IC_{50}) upon exposure to LTT*-labeled rapamycin micelles compared to free rapamycin, Fig. 4. The IC_{50} values show that LTT*-labeled and plain rapamycin micelles can deliver drug in higher concentrations to each group of cells than free rapamycin (R). In addition, polymer conjugates, LTT*-labeled and unlabeled pegylated octadecyl lithocholate without encapsulated rapamycin, did not show any cytotoxicity up to 10 mg/mL.

These results can be explained by a prolonged drug release from the micelles, variations in basal activation of cancer cell signaling, differences in cell sensitivity to the drug, and unexplored drug effects.[27] Other targeting ligands have been developed to improve efficiency of drug delivery to CRC cells in vitro that have not been demonstrated in vivo. An Fab' antibody fragment has been used to coat the surface of liposomes to increase intracellular uptake in EGFR overexpressing cells.[28] A fibronectin-mimetic peptide (PR-b) has been used to label pH sensitive liposomes for internalization in $\mu 5\mu 1$ expressing cells. [29]

3.8 Immunohistochemistry of adenoma regression

Adenomas in the untreated mice (NSS) were found in greater number and with larger tumor volumes on histology (H&E), Fig. 5A. Adenomas in the rapamycin treatment groups showed nearly complete regression after completion of therapy, Fig. 5B-D. The rapamycin

treated adenomas showed more regenerative changes, active colitis, submucosal and mucosal scarring, and smaller tumor volumes. Many lesions treated with the LTT*-labeled rapamycin micelles appeared histologically normal, Fig. 5D. On immunohistochemistry, there was no difference in β -catenin staining between NSS and the rapamycin-treated adenomas, Fig. 5E-H. Expression of phospho-S6 was significantly reduced in all groups of rapamycin treated adenomas compared to NSS, $*P < 0.05$ by ANOVA, Fig. 5I-L. Adenomas treated with LTT*-labeled rapamycin micelles showed a greater reduction in phospho-S6 staining than either plain rapamycin micelles or free rapamycin, $*P < 0.05$ by ANOVA, Fig. 5R, suggesting that greater inhibition of mTOR signaling had occurred. Unremarkable differences in β -catenin expression suggested that this inhibitory effect occurs downstream of β -catenin accumulation. This result is consistent with that found in the study by Fujishita et al.[25], whereas the study of Koehl et al.[26] revealed no difference in accumulation of β -catenin in rapamycin-treated adenomas and normal intestinal epithelium. The rapamycin-treated adenomas showed a significant reduction in Ki67 staining compared to NSS, $*P < 0.05$ by ANOVA, corresponding to decreased tumor cell proliferation, Fig. 5M-P. The optical densities for antibody staining on immunohistochemistry were quantified, Fig. 5Q-S.

4. Conclusion

The results of this study support the use of targeted micelles to encapsulate and deliver hydrophobic anticancer drugs for adjuvant treatment of CRC with reduced systemic toxicity. In addition to rapamycin and possibly other mTOR inhibitors, this methodology may be generalized to encapsulation of other powerful lipophilic drugs, such as doxorubicin, paclitaxel, cyclosporin A, geldanamycin, dipyridamole, and camptothecin, whose widespread clinical use is currently limited by either poor solubility or high toxicity. Additional studies including biodistribution, adverse effects, and off target drug effects are needed before these agents can be safely introduced into the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Micelle platform for targeted drug delivery

A) Structure of rapamycin encapsulated micelle includes octadecyl lithocholate (blue), PEG (black), and LTTHYKL peptide (red) at 50% density. Transmission electron micrograph (TEM) shows morphology of **B)** LTT*-labeled and **C)** plain rapamycin micelles, scale bar 200 nm.

Fig. 2. Micelle characterization

A) Parameters for LTT*-labeled and plain rapamycin micelles, including diameter on dynamic light scattering (DLS), polydispersity index (PDI), zeta (μ) potential (surface charge), and rapamycin entrapment efficiency (%EE). **B)** The ratio of fluorescence intensities at $I_1 = 371$ nm and $I_3 = 383$ nm from pyrene mixed with LTT*-labeled and plain rapamycin micelles were measured to determine a critical micelle concentration (CMC) of 18 and 12 μ M respectively. **C)** LTT*-labeled and plain micelles provide a sustained release ratio for rapamycin over time with a half-life of ~44 hours. **D)** LTT*-labeled and plain micelles retained 81.2% and 91.5% of the encapsulated rapamycin after 22 days.

Fig. 3. Adenoma regression with targeted rapamycin micelle therapy

In vivo images collected with white light endoscopy on days 0, 8, 16, 24, and 32 in *CPC;Apc* mice show **A-E**) rapid regression of colonic adenoma (arrow) with LTT*-labeled rapamycin micelle therapy. **F-J**) No regression was seen with no therapy, NSS (control). **K**) Relative tumor surface area (%) was monitored over time. **L**) The mean adenoma regression rate for LTT*-labeled rapamycin micelles was significantly greater than that for plain rapamycin micelles and NSS, and non-significantly greater than that of free rapamycin, $*P < 0.01$ on linear mixed effects regression analysis. Results are from mice treated with NSS ($n=5$ mice, 20 adenomas), free rapamycin ($n=6$ mice, 24 adenomas), plain rapamycin micelles ($n=5$ mice, 22 adenomas), and LTT*-labeled rapamycin micelles ($n=7$ mice, 21 adenomas).

Fig. 4. Safety of micelles

A) Renal toxicity was assessed on number of vacuolated renal tubules (arrow) seen on histology (H&E). **B)** Significantly fewer vacuoles were seen on histology in mice treated with plain and LTT*-labeled rapamycin micelles compared to those treated with free rapamycin, $*P<0.05$ by ANOVA. **C)** Cytotoxicity assay on cell panel shows that rapamycin encapsulated in either LTT*-labeled or plain rapamycin micelles resulted in significantly higher IC_{50} compared to that for free rapamycin (R).

Fig. 5. Immunohistochemistry of regressed adenomas

A-D) Representative histology (H&E) of regressed adenomas treated with NSS, free rapamycin, plain rapamycin micelles, and LTT*-labeled rapamycin micelles is shown. **E-H)** No significant difference was found in β-catenin staining among the 4 treatment groups, $P=0.18$ by ANOVA. **I-L)** Adenomas treated with LTT*-labeled rapamycin micelles showed greater reduction in phospho-S6 staining than free rapamycin, plain rapamycin micelles and NSS, $*P<0.05$ by ANOVA. **M-P)** Rapamycin-treated adenomas showed a significant reduction in Ki67 staining compared to NSS, $*P<0.05$ by ANOVA. Image analysis of integrated optical density shows differences in antibody staining intensity for **Q)** β-catenin, **R)** phospho-S6, and **S)** Ki67 after 5 weeks of treatment with different rapamycin formulations, $*P<0.05$ by ANOVA.