Colon Carcinomas

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Colon carcinomas themselves comprise over two-thirds of all colorectal cancers with an overall 5-year survival rate of 64%, which rapidly decreases to 14% when the cancer becomes metastatic. Depending on the stage of colon carcinoma at diagnosis, patients can undergo surgery to attempt complete tumor resection or move directly to chemotherapy with one or a combination of drugs. As with most cancers, colon carcinomas do not always respond to chemotherapies, so targeted therapies and immunotherapies have been developed to aid chemotherapy.

We report the development of a local combination therapy for colon carcinoma whereby chemo- and immuno-therapeutic entities are delivered intratumorally to maximize efficacy and minimize off-target side effects. A hydrophobic chemotherapeutic agent, docetaxel (DTX), and cholesterol-modified Toll-like receptor 9 (TLR9) agonist CpG (cho-CpG) oligonucleotide are co-loaded in synthetic HDL (sHDL) nanodiscs. *In vivo* survival analysis of MC-38 tumor-bearing mice treated intratumorally with DTX-sHDL/CpG (median survival; MS = 43 days) showed significant improvement in overall survival compared to mice treated with single agents, free DTX (MS = 23 days, $p < 0.0001$) or DTX-sHDL (MS = 28 days, $p<0.0001$). Two of seven mice treated with DTX-sHDL/CpG experienced complete tumor regression. None of the mice experienced any systemic toxicity as indicated by body weight maintenance and normal serum enzyme and protein levels. In summary, we have demonstrated that chemo- and immuno-therapies can be co-loaded into sHDLs, delivered locally to the tumor, and can be used to improve survival outcomes significantly compared to chemotherapy alone.

Keywords: co-delivery ; chemotherapy ; immunotherapy ; colon carcinoma

1. Formulation and Characterization of DTX-sHDL

The docetaxel-loaded sHDL (DTX-sHDL) was prepared as described previously [1]. Briefly, 22A, egg sphingomyelin (eSM), and DTX were dissolved in acetic acid. The acetic acid solutions of 22A, eSM, and DTX were mixed and freeze-dried for 24 h (mass ratio of 22A: eSM: DTX = 1:2:0.05). The lyophilized powder was rehydrated by PBS (pH 7.4). Three heat-cooling cycles (50 °C, 5 min followed by room temperature for 5 min) were performed to form DTX-sHDL. DTX-sHDL/CpG particles were prepared by incubating DTX-sHDL with CpG-Cholesterol in 10 mM phosphate buffer at room temperature for 2 h. The particle size of DTX-sHDL was analyzed by dynamic laser scattering (DLS). The purity of the DTX-sHDL nanoparticles was evaluated by gel permeation chromatography (GPC) at 220 nm using Tosoh TSK gel G3000SWx 7.8 mm × 30 cm column (Tosoh Bioscience, King of Prussia, PA, USA).

2. In Vitro Uptake Assays

MC-38 cells were cultured in RPMI medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin antibiotics. When cells reached their exponential growth phase, they were trypsinized and plated on 12-well tissue culture plates at 50,000 cells per well to be incubated overnight at 37 °C to allow adherence. HDL was labeled with lipophilic dye DiD at a ratio of 2:1:0.01 eSM:22A:DiD. DiD-labeled sHDL was passed through a desalting column (MWCO 7kDa) to remove free dye molecules prior to use.

Cells were dosed with three different concentrations of DiD-sHDL normalized by 22A concentration to evaluate the effect of the dose on cell uptake. Following dosing, cells were incubated at 37 °C for 3 h and then washed with PBS before analysis by confocal microscopy on a Nikon A1si confocal microscope or by FACS on a CytoFlex cytometer. FlowJo and ImageJ were used for quantitative analysis.

For the block lipid transport-1 (BLT-1) inhibition experiment, cells were pretreated with SR-B1 inhibitor BLT-1 with different concentrations for 1 h. Then, DiD-sHDL was added to each well (final 22A concentration = 10 mcg/mL). The cells were further incubated for 3 h followed by FACS analysis.

3. In Vitro Cytotoxicity Assay

MC-38 cells were cultured in RPMI medium supplemented with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin antibiotics. When cells reached their exponential growth phase, they were trypsinized and plated on 96-well tissue culture plates at 10,000 cells per well to be incubated overnight at 37 °C to allow adherence. Cells were dosed with six different doses of DTX in either free drug form or encapsulated in HDL to test the effect of increasing dose on cell death. Following dosing, cells were incubated at 37 °C for 48 h before analysis using the CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) from Promega, Madison, WI, USA. Absorbance at 490 nm was quantified using a BioTek SynergyNEO spectrophotometer. Negative control wells (without treatment) were considered to have the maximum absorbance at 100% viability, and the viability of other wells was calculated as the ratio of treated well absorbance to untreated well absorbance.

4. Western Blot of SR-B1 Expression on Murine Cancer Cells

Four different murine cancer cell lines–MC-38, B16-F10, CT-26, and 4T1–were cultured, trypsinized, and spun down so that cell pellets could be collected and flash frozen. Cell lysates were prepared and centrifuged at 16,000× g for 20 min at 4 °C. Supernatant was collected and stored on ice to perform total protein quantification by BCA assay. Samples were normalized to 30 µg total protein for loading onto an SDS-PAGE gel. The gel was run, transferred, and incubated with SR-B1 and actin primary antibodies overnight at 4 °C followed by incubation with HRP-conjugated secondary antibody at room temperature. The gel was imaged using a BioRad chemiluminescent imager and analyzed with ImageJ.

5. In Vivo Treatment Using Combination Chemotherapy and Immunotherapy

Thirty-five female C57BL/6 mice aged 7–8 weeks (Charles River Laboratories, Wilmington, MA, USA) were inoculated with 1 million MC-38 cells at a concentration of 10 million cells/mL subcutaneously superior to the right flank. On day 8 after tumor inoculation, mice were split into four groups of seven for treatments. Mice were injected intratumorally with (1) PBS, (2) DTX, (3) DTX-HDL, or (4) DTX-HDL/CpG twice a week at 1 mg/kg DTX and 15 µg CpG for five treatments. Mice were euthanized when tumors surpassed 15 mm in one dimension or ulcerated extensively. Blood samples were taken four days after the final treatments were administered for serum isolation, and analysis of toxicity markers was performed by the In-Vivo Animal Core Animal Diagnostic Laboratory.

References

1. Padma Kadiyala; Dan Li; Fernando M. Nuñez; David Altshuler; Robert Doherty; Rui Kuai; Minzhi Yu; Neha Kamran; Marta Edwards; James J. Moon; et al. High-Density Lipoprotein-Mimicking Nanodiscs for Chemo-immunotherapy against Glioblastoma Multiforme. *ACS Nano* **2019**, *13*, 1365-1384, 10.1021/acsnano.8b06842.