SUPPORTING INFORMATION

Ligand Conjugated Low-density Lipoprotein Nanoparticles for Enhanced Optical Cancer Imaging In Vivo

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MATERIAL AND METHODS

Preparation of DiR-LDL

LDL was isolated from fresh plasma of healthy donors by sequential ultracentrifugation as described previously by Lund-Katz et al. [1]. The lipophilic near infrared dye DIR (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindotricarbocyanine iodide) (ex. 748nm, em. 782nm) was purchased from Molecular Probes (Eugene, OR) and incorporated into the surface of LDL using the method described in the literature [2, 3]. The molar ratio of this NIR dye to apoB-100 protein (DiR:LDL) was approximately 8:1. This is based on the assumption that apoB-100 was the only protein present with one protein per LDL particle.

Synthesis of Folate-N-Hydroxysuccinimide ester (FA–NHS)

Folic acid dihydrate (54mg, 113μmol) was dissolved in 1mL of anhydrous dimethyl sulfoxide (DMSO) followed by the addition of 20μL of triethylamine. Then N-hydroxysuccinimide (20mg, 170μmol) and dicyclohexylcarbodiimide (26mg, 226μmol) were added, and the reaction mixture was kept at room temperature under argon for 40h. After removing by-product dicyclohexylurea by filtration, FA-NHS was precipitated from the concentrated filtrate using a 5-fold volume excess of anhydrous ether. The yellow FA-NHS precipitate was washed several times with anhydrous ether, dried under high vacuum, and stored as a powder.

Preparation of DiR-LDL-FA

The pH of DiR-LDL solutions (5mL) was increased to 9.4 by dialyzing against 0.1M NaH2PO4, 0.1M H3BO3 buffer at 4°C overnight. FA-NHS in anhydrous DMSO was slowly added to this LDL solution (molar ratio of FA-NHS/LDL = 200:1). The reaction mixture was kept at 4°C for 40h, then centrifuged at 500rpm at 4°C to remove any precipitate from degraded LDL and subsequently dialyzed against EDTA buffer (0.3mM EDTA, 0.9% NaCl, pH 7.4) at 4°C overnight. Over the course of the dialysis, the pH of the DiR-LDL-FA solution returned to 7.4, and unreacted FA-NHS starting materials were eliminated. This dialysis procedure was repeated until no evidence of free FA was detectable by UV spectrophotometry. The resulting product, DiR-LDL-FA, was obtained with a yield of 75%. The number of FA molecules conjugated to each LDL nanoparticle was determined by UV spectrophotometry as described previously.

Cell Lines

KB cells (human nasopharyngeal epidermoid carcinoma cells, FR+) [4], HT-1080 cells (human fibrosarcoma cells, FR−) [5], and CHO cells (FR−) [6] were purchased from the American Type Tissue Collection (Manassas, VA). Both KB and HT-1080 cells were cultured in Eagle’s Minimum Essential Medium (MEM) supplemented with 2mM L-glutamine, 17.9mM sodium bicarbonate, 0.1mM non-essential amino acids, 1.0mM sodium pyruvate, and 10% fetal bovine serum (FBS). CHO cells were cultured in Ham's F12K medium with added 2mM L-glutamine, 17.9mM sodium bicarbonate, and 10% FBS. HepG2 cells (human hepatoblastoma G2, LDLR−) [7], a generous gift from Dr. Theo van Berkel’s laboratory at the University of Leiden, Netherlands, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 100U/mL
penicillin-streptomycin. All cells were grown at 37°C in a humidified atmosphere containing 5% CO2.

**Confocal Microscopic Studies**

Cells were grown for three days in four-well Lab-Tek chamber slides (Naperville, IL). After three quick washes with ice-cold phosphate buffered saline (PBS), experiments were initiated with the addition of FA deficient RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 0.38μM of FA conjugated LDL. After 4h incubation at 37°C, cells were washed five times with ice-cold PBS containing 0.8% bovine serum albumin and two times with PBS alone and fixed for 20min with 2% formaldehyde in PBS at room temperature. Chamber slides were then mounted and sealed for confocal microscopic analysis with a Leica TCS SPII laser scanning confocal microscope (Heidelberg, Germany). Filter settings was 488nm for DiR-LDL.

**Experimental Animals and Induction of Tumor Xenografts**

All protocols for animal experiments and maintenance were approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC). Adult female nude mice (average 20g) were permitted ad libitum access to food and water throughout the study. To induce solid tumors, 10^6 KB and HT1080 cells were injected subcutaneously into the respective right and left lower flanks of the nude mice. Five mice with size matched tumors (3-5mm) were used for in vivo imaging.

**In Vivo Near-Infrared Fluorescent (NIRF) Imaging and Biodistribution Studies**

DiR-LDL-FA was administered to mice via tail vein injection at a concentration of 5.8μM. In vivo fluorescence imaging was performed before and various times following the injection of DiR-LDL-FA with a Xenogen IVIS Lumina imaging system (Hopkinton, MA, USA). Mice were anesthetized with ketamine/acepromazine (50/5 mg/kg i.p.) and placed prone into the light-tight chamber of the IVIS imager. Fluorescent images were acquired with following settings: ICG filter (ex=710-760nm, em=810-875nm), stage B, small Binning, fstop/2, lamp high level, and exposure time of 6 sec.

For biodistribution studies, organs/tissue (HT1080 tumor, KB tumor, liver, heart, muscle, kidney) of mice (24h post-injection of DiR-LDL-FA) were harvested, weighed and homogenized in PBS. Homogenates were then combined with a 3-fold excess of a CHCl₃:MeOH (2:1) mixture, and vortexed for 2min. Solutions were subsequently centrifuged at 3,000rpm for 2min. Using a Perkin-Elmer LS-50B spectrofluorometer, the fluorescence intensities of the various samples were measured (ex. 748nm; em. 782nm). A standard curve was generated using the free DiR. Fluorescence signals were normalized for sample weight and dilution factor. Both the NIRF imaging and biodistribution studies were repeated four times to ensure reproducibility.

An additional group of mice were studied to examine the specificity of DiR-LDL-FA in vivo. Mice with dual tumor xenografts were co-injected with DiR-LDL-FA (13 nmol) and excess free FA (30 fold). In vivo NIR fluorescent images were acquired before and at various times post treatment. At the end of this study tumor xenografts were excised and homogenized for semi-quantitative analysis.

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References:
Figure 1: Fluorescence intensity in tissue and tumor extracts 24 hours following injection of DiR-LDL-FA (5.8μM) with and without folic acid inhibition. Results are presented as mean ± standard deviation (N= 3 mice/group). Fluorescence intensity is expressed in arbitrary units relative to muscle tissue.
Figure 2: Comparative real time *in vivo* fluorescence imaging of mice before and after i.v. injection of DiR-LDL-FA (5.8μM) with or without FA inhibition.