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Tricarbocyanine Cholesteryl Laurates Labeled LDL: New Near Infrared Fluorescent Probes (NIRFs) for Monitoring Tumors and Gene Therapy of *Familial hypercholesterolemia*

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Abstract—For monitoring low-density lipoprotein receptors (LDLr) in tumors and in livers of patients with *familial hypercholesterolemia* (FH) treated with gene therapy, a series of tricarbocyanine cholesteryl laurates were synthesized with the cholesteryl laurate moiety serving as the lipid-chelating anchor for low-density lipoprotein (LDL). One of these conjugates, TCL17, was successfully used to label LDL to give a new NIRF, TCL17-LDL. Ex vivo biological studies on an LDLr overexpressing tumor model, human hepatoblastoma G_2 ($HepG_2$), confirmed that this NIRF were internalized selectively by the tumor and detected with high sensitivity by a low-temperature 3-D redox scanner.

LDL plays a central role in the etiology of atherosclerosis[1] and is also a key vehicle for selective delivery of therapeutic agents to a number of tumors that overexpress LDL receptors (LDLr).[2–4] *Familial hypercholesterolemia* (FH) is a genetic disorder caused by mutations in the LDLr gene, resulting in elevated concentrations of LDL-bound cholesterol in blood, leading to premature coronary heart disease.[1] Currently, the only effective treatments for homozygous FH are liver transplantation,[5] removal of LDL by plasma exchange[6] and LDL apheresis.[7] However, these treatments are laborious and in the case of liver transplantation contain risks with both operation and tissue rejection. In recent years gene therapy by viral delivery and expression of the normal LDLr gene in the liver has become a promising new alternative for treating this disease.[8] Both diagnosis of malignancies that overexpress LDLr and monitoring FH patients treated with liver gene therapy require a robust, inexpensive and noninvasive assay system. To date only a few radioactive imaging agents (^{125}I, ^{111}In or ^{68}Ga labeled LDL and ^{14}C labeled cholesterol) fit this category.[1,9,10] In most cases, probes were directly or indirectly affixed to protein residues of Apolipoprotein B-100, the binding ligand to the LDLr. Therefore, the probe/LDL ratio was generally kept low to avoid disrupting the 3-D structure of the recognition protein. As an alternative approach, Urizzi et al.[10] labeled the LDL with indium via a lipid-chelating agent incorporated in the LDL phospholipid monolayer.

Near infrared (NIR) optical imaging is a new, safe, and inexpensive cancer detection modality, which permits noninvasive differentiation of tumor and normal (healthy) tissue based on differences in tissue absorption or fluorescence.[11] Because tissue is relatively transparent to NIR light, target specific NIRF can overcome the small intrinsic contrast between tumors and normal tissue and thus achieve high sensitivity/specificity. Tricarbocyanine dyes have many of the ideal properties of NIR fluorophore, namely long wavelength absorption (750–850 nm), high extinction coefficients (>100,000) and quantum yields.[12] They have proven to be promising contrasting agents for the in vivo NIR imaging of tumors[13] and have been successfully applied as NIRFs for a number of target-specific carriers.[14]

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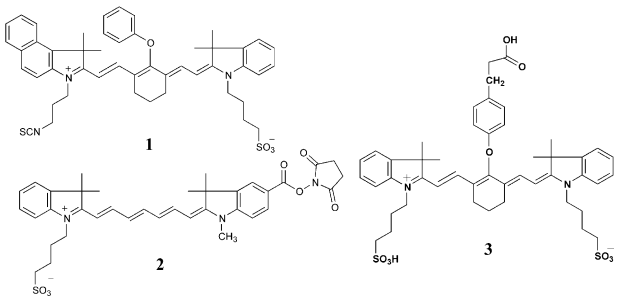


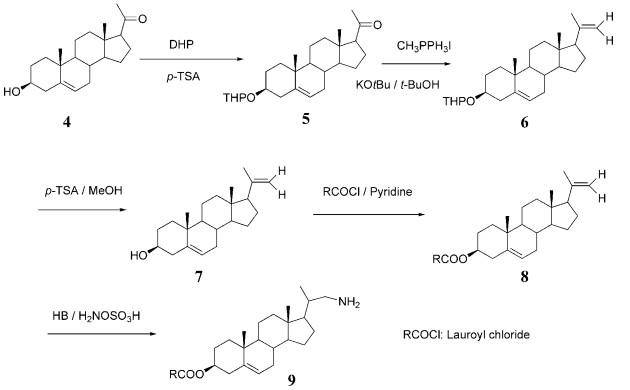
Figure 1. Selected tricarbocyanine dyes containing isothiocyanate, succinimide ester and carboxylic acid groups.

Our strategy for monitoring LDLr in tumors over-expressing LDLr is to incorporate tricarbocyanine cholesteryl laurate within the lipid region of LDL, which could be internalized by tumor cells via LDLr-mediated endocytosis. Accumulation of the NIRF in the target cells provides a facile mechanism for amplification of the NIR detectable signal. Herein, we report the synthesis and preliminary biological evaluation of novel tricarbocyanine cholesteryl laurates as target specific NIRFs for LDLr.

For our studies, three tricarbocyanine dyes with isothiocyanate, succinimide ester and carboxylic acid liners were selected as NIR fluorophores (Fig. 1).[15]

To enable a directed and uniform conjugation of the above three dyes, we prepared a cholesteryl laurate with a primary amine group as the common substrate. As shown in Scheme 1, the 3β-hydroxyl group of pregneolone **4** was first protected as its THP ether analogue by reaction with dihydropyran. The THP ether **5** was then subjected to methylenation by Wittig reaction to form the diene **6**. Upon the removal of THP group of **6** with *p*-TSA as the catalyst, 3β-hydroxypregna-5,20(22)-diene **7** was formed and converted to laurate **8** with lauroyl chloride in pyridine. The in situ hydroboration amination of **8** gave the final product 22-amino-5-pregnen-3β-yl laurate **9**.

Though the coupling reactions between isothiocyanate, succinimide ester, carboxylic acid and primary amine were well known, we soon found that amine **9** was



Scheme 1. Synthesis of cholesteryl laurate with a primary amine group.

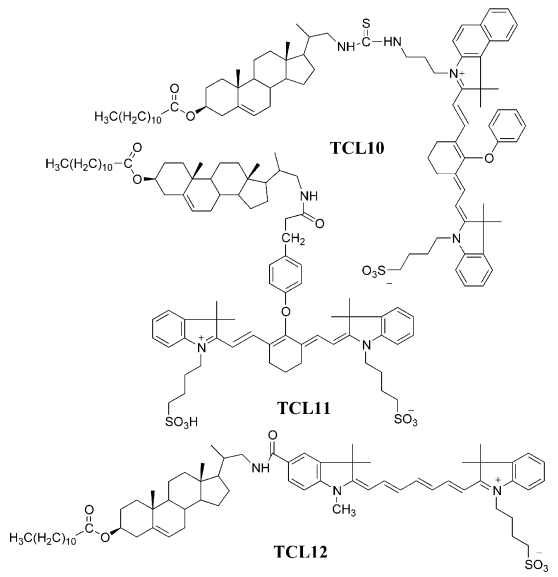
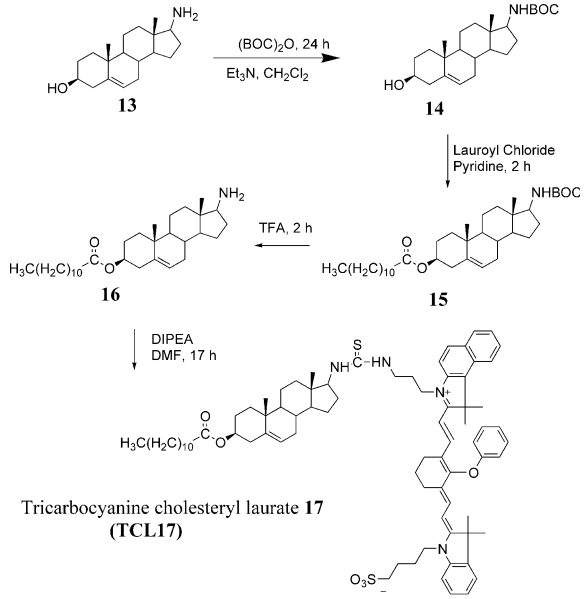


Figure 2. Synthesized tricarbocyanine dye labeled cholesteryl laurates.

extremely unstable[16] and this led to very low yields in the synthesis of tricarbocyanine conjugates.[17] These efforts, however, produced three desired conjugates, **TCL10**–**12** (Fig. 2).[18] Nevertheless, due to the low yield, it is not practical for undertaking systematic in vivo biological evaluation of these new compounds.

In order to solve this problem, we developed an alternative pathway (Scheme 2) for synthesis of a steric hindered and thus more stable cholesteryl laurate amine.

As shown in Scheme 2, a commercially available cholesteryl amine **13**, 5-androsten-17β-amino-3β-ol (Steraloid Inc., Newport, RI), was first Boc-protected (**14**) and esterified with lauroyl chloride to give its corresponding cholesteryl laurate amine **15**. Deprotection of **15** at the C-17 gave the corresponding cholesteryl laurate



Scheme 2. Synthesis of a stable cholesteryl laurate amine **13** and its corresponding tricarbocyanine dye conjugate, **TCL17**.

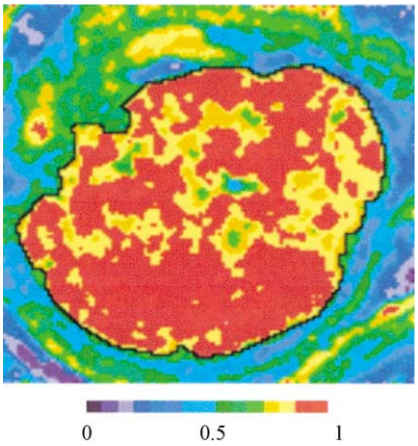


Figure 3. The redox image of $HepG_2$ tumor tissue after TCL17-LDL tail vein injection. The color scale bar reflects the relative fluorescent intensity.

amine **16** in 60% overall yield. Coupling of this amine to the tricarbocyanine dye **1** yielded a new dye conjugate **TCL17** in good yield (40%). ^1H NMR and mass spectroscopy studies confirmed its structure.[18] Subsequently, following the method described by Pitas et al.,[19] **TCL17** was successfully used to label LDL and gave a new NIRF, namely TCL17-LDL.[20]

To confirm the selective delivery of this new NIRF to tumors via LDLr-mediated endocytosis, a low-temperature 3-D redox scanner[21] was used as our NIR fluorescent imager on a LDLr overexpressed tumor model, $HepG_2$. The redox scanner functions by means of automated scanning of surface fluorescence state of the frozen tissue. The scanning process is fully computerized and programs have been developed which allow 3-D reconstruction of the data in terms of 'redox ratio models'. Figure 3 shows a redox image of $HepG_2$ tumor tissue intravenously injected with TCL17-LDL.[22] As shown in this figure, strong fluorescent signal (red region of the image) of this NIRF was detected only inside the tumor tissue, demonstrating that TCL17-LDL was selective internalized into the tumor.

In summary, efforts were made to synthesize a cholesteryl laurate with a primary amine functional group that was stable enough to form its corresponding tricarbocyanine conjugate in a reasonable yield. By using the low-temperature 3-D redox scanner, we have confirmed in a $HepG_2$ tumor model that the selective delivery of TCL17-LDL to tumor tissue via LDLr pathway was achieved. Because of its target specificity and its high sensitivity, this new NIRF should be useful for monitoring LDLr in tumors overexpressing LDLr and in livers of FH patients treated with gene therapy.

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15. Dye **1** (IRD41) and **3** (IRD80) were purchased from LICOR (Lincoln, NB). Dye **2** (NIR97235-NHS ester) was a gift from Shering AG, Germany.
16. Amine **9** was found to be unstable, it decomposed even in very weak basic conditions: (a) pH 9.3 buffer, 2 h; (b) Et_3N in CH_2Cl_2, DMF, THF, CH_3CN and toluene; (c) DMAP, NMM, 4-picoline and 2,6-di-*tert*-butylpyridine in DMF. However, it is relatively stable with diisopropylethyl amine (DIPEA) in DMF.
17. For labeling the isothiocyanate functionality, a slight excess of dye **1** was reacted with amine **9** in DMF with DIPEA for 48 h, yielding the labeled conjugate **10** in 10% yield. Under similar conditions, succinimide ester containing dye **2** reacted with **9** gave small amount of dye conjugate **12**. The coupling reaction between carboxylic acid containing dye **3** and amine **9** using 1:1:1 proportions DCC/HOBT/DIPEA in DMF gave dye conjugate **11** in 10% yield.
18. Spectroscopic data and characterization for selected compounds.

Pregneolone 3-THP ether (**5**): Anal. calcd for $C_{26}H_{40}O_3$: C, 77.95; H, 10.06. Found: C, 77.68; H, 9.88; ^1H NMR ($CDCl_3$) δ 5.35 (1H, 6-H), 4.72 (1H, 2′-H), 3.92 (m, 1H, 6′H), 3.52 (m, 1H, 3-H), 3.51 (m, 1H, 6′-H), 2.12 (s, 3H, 21-H), 1.01 (s, 3H, 19-H), 0.63 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 209.4 (C-20), 141.1 (C-5), 121.2 (C-6), 96.9 (C-1′), 31.8 (C-21), 19.3 (C-19), 13.2 (C-18).

3β(Tetrahydropyan-2′-yl)oxypregna-5,20(22)-diene (**6**): Anal. calcd for $C_{27}H_{42}O_2$: C, 81.35; H, 10.70. Found: C, 81.95; H, 10.72; ^{1}H NMR ($CDCl_3$) δ 5.34 (1H, 6-H), 4.82 (1H, 22-H), 4.69 (2H, 22-H, 2′-H), 3.89 (m, 1H, 6′-H), 3.50 (m, 1H, 3-H), 3.48 (m, 1H, 6′-H), 1.74 (s, 3H, 21-H), 0.99 (s, 3H, 19-H), 0.56 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 145.7 (C-20), 140.9 (C-5), 121.3 (C-6), 110.6 (C-22), 96.9 (C-1), 75.9 (C-3).

3β-Hydroxypregna-5,20(22)-diene (**7**): Anal. calcd for $C_{22}H_{34}O$: C, 84.02; H, 10.90. Found: C, 82.42; H, 10.82; ^{1}H NMR ($CDCl_3$) δ 5.38 (1H, 6-H), 4.87 (1H, 22-H), 4.73 (1H, 22-H), 3.54 (m, 1H, 3-H), 1.78 (s, 3H, 21-H), 1.03 (s, 3H, 19-H), 0.61 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 145.5 (C-20) 140.8 (C-5), 121.5 (C-6), 110.6 (C-22), 71.7 (C-3).

Pregna-5,20(22)-diene-3β-yl laurate (**8**): Anal. calcd for $C_{34}H_{56}O_2$: C, 82.20; H, 11.36. Found: C, 82.43; H, 11.82; ^{1}H NMR ($CDCl_3$) δ 5.40 (1H, 6-H), 4.87 (1H, 22-H), 4.86 (1H, 22-H), 4.73 (m, 1H, 3-H), 1.78 (s, 3H, 21-H), 1.05 (s, 3H, 19-H), 0.60 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 173.2 (C-1′), 145.5 (C-20), 139.7 (C-5), 122.4 (C-6), 110.6 (C-22), 73.5 (C-3).

22-Amino-5-pregnen-3β-yl laurate (**9**): Exact mass calcd for $C_{34}H_{59}NO_2$: 513.4; Found by ESI-MS: 514.4 (MH^+); ^{1}H NMR ($CDCl_3$) δ 5.36 (1H, 6-H), 4.59 (1H, 3-H), 3.54 (2H, 22H), 1.02 (s, 3H, 19-H), 0.64 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 173.3 (CO), 139.7 (C-5), 122.5 (C-6), 73.7 (C-3), 70.6 (C-22).

Tricarbocyanine cholesteryl laurate 10 (**TCL10**) (coupling of isothiocyanate of dye **1** and amine **9**): Exact mass calcd for $C_{82}H_{110}N_4O_6S_2$: 1310.79; Found by ESI-MS: 1311.2 (MH^+).

Tricarbocyanine cholesteryl laurate 11 (**TCL11**) (coupling of carboxylic acid of dye **3** and amine **9**): Exact mass calcd for $C_{81}H_{113}N_3O_{10}S_2$: 1351.79; Found by ESI-MS: 1352.4 (MH^+).

Tricarbocyanine cholesteryl laurate 12 (**TCL12**) (coupling of succinimide ester of dye **2** and amine **9**): Exact mass calcd for $C_{67}H_{95}N_3O_6S$: 1069.69; Found by ESI-MS: 1070.5 (MH^+).

5-Androsten-17β-Boc-amino-3β-ol (**14**): Exact mass calcd for $C_{24}H_{39}NO_3$: 389.29; Found by ESI-MS: 390.4 (MH^+); ^{1}H NMR ($CDCl_3$) δ 5.34 (m, 1H, 6-H), 4.40 (brs, 1H, N-H), 3.53 (m, 2H, 3-H + 17-H), 1.43 (s, 9H, Boc-H), 1.00 (s, 3H, 19-H), 0.67 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 141.1 (C-5), 121.5 (C-6), 71.9 (C-3), 19.6 (C-19), 12.0 (C-18).

5-Androsten-17β-Boc-amino-3β-yl laurate (**15**): Exact mass calcd for $C_{36}H_{61}NO_4$: 571.46; Found by ESI-MS: 572.3 (MH^+); ^{1}H NMR ($CDCl_3$) δ 5.36 (m, 1H, 6-H), 4.61 (m, 1H, 3-H), 4.39 (brs, 1H, N-H), 3.54 (m, 1H, 17-H), 1.02 (s, 3H, 19-H), 0.85 (t, 3H, CH_3 of lauroyl), 0.67 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 173.5 (CO_2), 140.1 (C-5), 122.4 (C-6), 73.8 (C-3), 14.3 (C-19), 12.0 (C-18).

5-Androsten-17β-amino-3β-yl laurate (**16**): Exact mass calcd for $C_{31}H_{53}NO_2$: 471.41; Found by ESI-MS: 472.5 (MH^+); ^{1}H NMR ($CDCl_3$) δ 5.40 (m, 1H, 6-H), 4.61 (m, 1H, 3-H), 2.71 (m, 1H, 17-H), 1.02 (s, 3H, 19-H), 0.87 (t, 3H, CH_3 of lauroyl), 0.68 (s, 3H, 18-H); ^{13}C NMR ($CDCl_3$) δ 173.5 (CO_2), 140.0 (C-5), 122.5 (C-6), 73.8 (C-3), 14.3 (C-19), 11.3 (C-18).

Tricarbocyanine cholesteryl laurate 17 (**TCL17**) (coupling of dye **1** and amine **16**): Exact mass calcd for $C_{79}H_{104}N_4O_6S_2$: 1268.74; Found by ESI-MS: 1269.6 (MH^+) and 1291.6 ($M + Na^+$).

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20. Tricarbocyanine dyes including IRD41 are sensitive to photobleaching. Therefore, aluminum foil was frequently used to exclude ambient light in reactions and LDL reconstitution process to avoid photodegradation of the dye. The resulting TCL17-LDL can be stored at 4 °C in dark for up to four weeks without detectable change in fluorescence and absorption. Meanwhile, the laser power used in ex vivo detection is small (5 mw), we found that photobleaching is relatively insignificant in this case. Chemically, TCL17 is quite stable. It can be easily purified by preparative TLC with 10% methanol in dichloromethane.

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22. $HepG_2$ tumor was inoculated subcutaneously on nude mice with 10×10^6 cells. After the development of the tumor to about 1 cm in diameter, the mice received an intravenously injection of TCL17-LDL. Two hours after the injection, the mice were frozen in the liquid nitrogen under the anesthetization and the tumor tissue was used for the redox scanning.