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A Hydrazino Nicotinamide Derivative of Cholesterol for Radiolabelling Liposomes with ^{99m}Tc

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Summary

Liposomes were labelled with ^{99m}Tc by a new method, via a nicotinyl hydrazine derivative of cholesterol (**7**). The synthesis of the technetium-binding molecule was completed in 5 steps with an overall yield of 18%. Preliminary ^{99m}Tc labelling studies indicate that **7** can be incorporated into distearoylphosphatidyl choline/cholesterol liposomes and retain technetium binding activity.

Keywords: ^{99m}Tc labelling, liposome, hydrazinonicotinic acid, HYNIC, diagnostic imaging.

Introduction

The incorporation of drugs into liposomes for *in vivo* delivery has been shown to facilitate drug delivery by providing improved circulation times, reduced cell-specific toxicity, and tumor-specific partitioning (*1*). We sought to develop a ^{99m}Tc imaging agent that could be incorporated into such liposomes for use in diagnostic imaging of tumors. ^{99m}Tc (γ, 140.5 KeV) is a radionuclide commonly used for diagnostic imaging due to its convenient half-life (6 h), low cost, and ready availability (*2*).

Tc-labelled liposomes have already been used for scintigraphic imaging techniques, albeit with mixed success. Reductive labelling (*3*) of phosphate head groups by exposure to sodium pertechnetate/$SnCl_2$ has been used, although later studies revealed that technetium is only weakly

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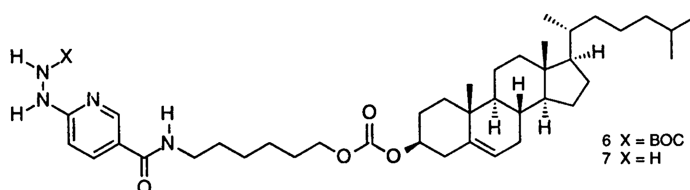
bound to the phospholipid surface and images thus obtained do not necessarily represent intact liposomes in the body (*4*). DTPA-dipalmitoylphosphatidyl-ethanolamine conjugates (*6*) have been used to chelate ^{99m}Tc at the liposome surface; this approach exhibited high labelling efficiency (ca. 100%) but suffered from unacceptable ^{99m} Tc dissociation (50%, 1 hr) in plasma. ^{99m} Tc has also been shown to be irreversibly trapped within liposomes containing internal glutathione using the lipophilic chelator hexamethylpropyleneamine oxime (HMPAO) (*5*,*6*). High (66-90%) labelling efficiency and excellent *in vitro* and *in vivo* stability of liposome preparations was demonstrated using this approach. Tin dioxinate has also been used to ^{99m} Tc-label liposomes with high efficiency, stability in biological fluids, and without formation of tin colloids *(7)*.



The imaging agent described here consists of the ^{99m}Tc-binding moiety 6-hydrazinonicotinic acid (HYNIC) conjugated to cholesterol via a six-carbon alkyl spacer (**7**). 6-hydrazinonicotinic acid was selected as the ^{99m}Tc binding component on the basis of its excellent *in vivo* stability as reported in a study using HYNIC-deriviatized human polyclonal immunoglobulin (IgG) to image bacterial infections in rats (*8,9*). HYNIC was shown to exhibit binding efficiency (90-95%), stability of the ^{99m}Tc-hydrazino complex in the presence of DTPA, L-cysteine, sodium diethylthiocarbamate and low serum dissociation (5%, 5.5 hr). Highly satisfactory images were obtained using this binding agent. We reasoned that if a cholesterol-HYNIC conjugate could be adapted for use in liposomes it could provide an effective means for imaging tumors by taking advantage of the tumor-specific partitioning of certain liposome formulations (*1*). This paper describes the synthesis of a cholesterol-HYNIC conjugate and preliminary ^{99m}Tc labelling studies which indicate such an approach should indeed be feasible.

Synthetic Plan and Labelling Studies

The preparation of cholesterol derivative **7** is outlined in Scheme 1. The amino group of 6-aminohexanol was first protected as the *t*-butyoxy carbamate (BOC) and conjugated to cholesterol chloroformate (**3**) in 65% yield. This material was BOC-deprotected (CF_3COOH/CH_2Cl_2) and

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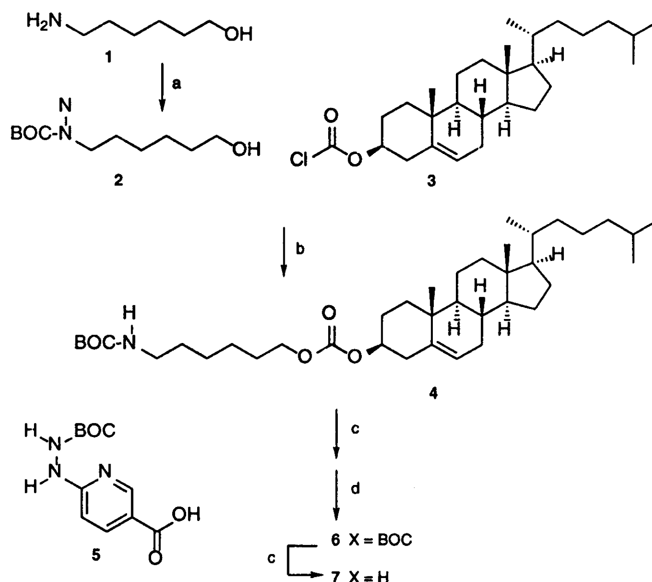
coupled with 6-BOC-hydrazinopyridine-3-carboxylic acid (*9*) using a DCC-HOBT coupling in DMF-CH_2Cl_2 solvent. Conjugate **6** was obtained as a stable, pale yellow crystalline solid.



Scheme 1. Reagents and conditions: (a) $(BOC)_2O/CHCl_3/NaHCO_3$(aq.)/rt; (b) Py/CH_2Cl_2/rt; (c) TFA/CH_2Cl_2/0 °C; (d) **5**, Et_3N/DCC-HOBT/DMF/CH_2Cl_2.

Compound **6** was BOC-deprotected (TFA/CH_2Cl_2) and used in liposome formulations which were labelled with ^{99m}Tc. Liposomes were prepared by probe sonication of hydrated lipid films. The lipid films were made by pipetting aliquots of lipid dissolved in chloroform into glass tubes. The molar lipid composition was 2:1:**X** distearoylphosphatidyl choline (DSPC), cholesterol, and **X**=variable amounts of **7**, respectively. The chloroform was then evaporated at 65 °C under a stream of nitrogen gas, and the film was then placed under a vacuum for at least three hours prior to use. All samples were hydrated in 9% sucrose 25 mM phosphate buffer, pH 7.4. Liposome solutions were exposed to ^{99m}Tc and allowed to stand for 45 minutes. An aliquot was removed and loaded onto a plug of Sephacryl S-400 and washed to collect the void volume containing liposomes. This material was counted (gamma counts at 80-165 KeV) and compared to counts obtained from a portion of the column packing. The fraction incorporated is reported as: (counts eluted/counts eluted + counts bound). Non-specific binding was determined under identical conditions in the absence of **7**.

The results of the assays are shown in Figure 1. The data show reasonable percent incorporation (48-68%, corrected for non-specific binding of 8%) at 3-6 mol% of **7**. These results demonstrate that cholesterol-HYNIC conjugate **7** can bind ^{99m}Tc when sequestered in a liposome and

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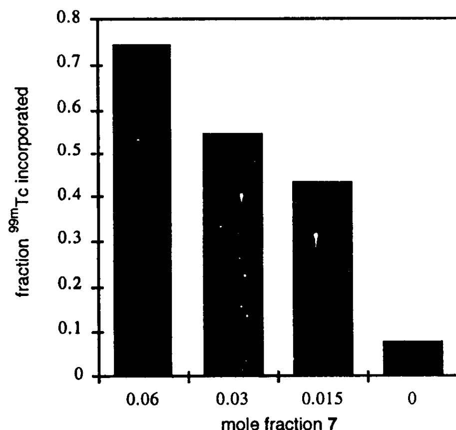

Figure 1: Graph of the fraction of ^{99m}Tc incorporated into several different preparations of DSPC/cholesterol liposomes. **7** is present as different mole fractions of the total lipid content. The control sample (no added **7**) is marked with a zero.

thus provides a new means for radiolabeling liposomes for scintigraphic imaging studies. Based upon this preliminary assay, it appears the 6-hydrazinonicotinic acid moiety can be tethered to cholesterol and incorporated into DSPC/cholesterol liposomes with retention of ^{99m} Tc-binding activity. It remains to be seen if a conjugate such as **7** will function as well as the existing methodology used for labelling liposomes with ^{99m}Tc. More work, both from the standpoint of formulation optimization and *in vitro* testing (plasma, cysteine, and EDTA challenge experiments) will be required to fully evaluate the potential of cholesterol-HYNIC conjugates for radiolabelling liposomes for diagnostic imaging purposes.

Procedure for Preparation of 6

Materials and Methods. Analytical TLC was performed with Merck silica gel 60 F254 precoated glass plates (0.25 mm thickness). Preparative silica gel chromatography employed Merck silica gel 60 (230-400 mesh). All reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Chloroform, hexanes, and ethyl acetate were used as delivered. Pyridine was distilled from calcium hydride and stored over KOH pellets. Methylene chloride was freshly distilled from calcium hydride prior to use. "Anhydrous grade" DMF was used as delivered. Melting points (uncorrected) were obtained on a Fisher-Johns apparatus. 300 MHz ^{1}H NMR spectra were recorded on a General Electric QE-300. Optical rotations were measured with a 1.0 ml/1dm cell using a Jasco DIP-1000 digital polarimeter.

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6-BOC-amino-1-hexanol (2). 6-amino-1-hexanol (1.023 g, 8.7 mmol) was dissolved in chloroform (18 ml) and combined with $NaHCO_3$ (738 mg, 8.8 mmol) in water (13 ml). Di-*tert*-butyl-dicarbonate (1.905 g, 8.7 mmol) was added portionwise to the vigorously stirred suspension, with stirring maintained for 45 minutes at room temperature. The reaction mixture was transferred to a separatory funnel and the product was extracted into chloroform which was washed with water and brine. The organic phase was dried with sodium sulfate, filtered, and concentrated to an oil *in vacuo*. An oily product was obtained (1.789 g, 94% yield) that was sufficiently pure (^{1}H NMR) to be used in the next step without rigorous purification. ^{1}H NMR (300 MHz, $CDCl_3$): 1.2-1.6 ppm (8H, m); 1.4 ppm (9H, s); 1.8 ppm (1H, broad); 3.1 ppm (2H, t); 3.6 ppm (2H, t); 4.5 ppm (1H, broad).

BOC-aminohexanol-cholesterol carbonate 4. Cholesterol chloroformate (3.51 g, 7.8 mmol) was added to a solution of 6-BOC-amino-1-hexanol (1.789 g, 8.2 mmol) in pyridine (20 ml) and methylene chloride (10 ml). The solution was stirred at room temperature for 1.5 hours and quenched by the addition of several ice chips. After 5 minutes of additional stirring, the solvents were removed on a high-vacuum rotary evaporator. The residue was dissolved in a minimum amount of methylene chloride and purified with flash silica gel chromatography, eluting with 2:1 hexane/ether followed by 1:1 hexane/ether, yielding a white solid, m.p. = 145-148 °C (3.18 g, 65% yield). ^{1}H NMR (300 MHz, $CDCl_3$, diagnostic peaks only): 1.4 ppm (9H, s); 4.1 ppm (2H, t); 4.5 ppm (1H, m); 5.4 ppm (1H, d). $[\alpha]_D^{27}$ = -38.7° (*c* 1.25, $CHCl_3$)

Hydrazinonicatinamidohexanol-cholesterol carbonate 6. BOC-aminohexanol-cholesterol carbonate **4** (625 mg, 1.1 mmol) was dissolved in methylene chloride (12 ml) and cooled to 0 °C. Trifluoroacetic acid (5 ml) was added dropwise with stirring. The reaction was stirred for 90 min at 0 °C, after which the reaction was judged complete by TLC analysis. The solvents were removed *in vacuo*, and the residue was twice dried by azeotropic distillation using toluene under reduced pressure. The residue was dissolved in DMF/CH_2Cl_2 (1:1, 6 ml) and converted to the free base by addition of triethylamine (150 µl). 6-BOC-hydrazinopyridine-3-carboxylic acid (279 mg, 1.1 mmol), HOBT (150 mg, 1.1 mmol) and DCC (229 mg, 1.1 mmol) were added portion-wise in succession. The reaction was allowed to stir for 20 hours at room temperature, after which it was filtered through a pad of Celite, washing with ethyl acetate. The filtrate was diluted with additional ethyl acetate and washed with dilute sodium bicarbonate, brine, and then dried with sodium sulfate. The solvents were removed *in vacuo* and the residue was dissolved in a minimum amount of methylene chloride and

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purified on a silica gel column that had been prepared in a slurry of methylene chloride, eluting with 1:1 hexane/ethyl acetate (60 ml), 1:2 hexane/ethyl acetate (80 ml), then pure ethyl acetate (120 ml). The pure fractions were concentrated to give a crystalline yellow solid, m.p. = 134 °C, dec. (304 mg, 37% yield). ^1H NMR (300 MHz, $CDCl_3$, diagnostic peaks only): 1.4 ppm (9H, s); 3.4 ppm (2H, q); 4.1 ppm (2H, t); 4.5 ppm (1H, m); 5.4 ppm (1H, d); 6.7 ppm (1H, d); 8.0 ppm (1H, d); 8.5 ppm (1H, s). $[\alpha]_D^{25}$ = -19.5° (*c* 1.13, $CHCl_3$). HRMS (FAB) calcd for $C_{45}H_{73}N_4O_6$ (M+H): 765.5530 , found 765.5490.

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