Selective Localization of Radioiodinated Alkylphosphocholine Derivatives in Tumors

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We have designed and synthesized two radioiodinated analogs of hexadecylphosphocholine in order to evaluate their tumor imaging potential. 12-(m-[125]Iodophenyl)dodecyl phosphocholine (NM-324) and hexadecyl-2-[N,N-dimethyl-N-(m-[125]Iodobenzyl)-ammonium] ethyl phosphate (NM-326) demonstrated the ability of such compounds to localize in and thereby visualize the Walker 256 tumor in rats. However, the tumor avidity of NM-324 was far superior to NM-326. In addition, NM-324 showed excellent tumor localization in athymic mice bearing subcutaneous human tumors.

Introduction

Various strategies have been employed to achieve selective accumulation of a radiopharmaceutical within tumor cells. A particular problem associated with the currently available agents (e.g. [67Ga]citrate) is poor tumor specificity (Merrick, 1984).

In order to achieve greater tumor specificity, numerous laboratories have explored the ability of tumor antibodies to serve as carriers for various radionuclides (Bejent, 1985; Pietersz et al., 1987; Eger et al., 1987; Woo et al., 1988). Studies in our own laboratory, on the other hand, have focused on drug molecules which have shown an ability to accumulate in tumors to serve as carriers of the radiodiagnostic probe (Counsel1 et al., 1969; Beierwaltes et al., 1968; Huang et al., 1979; Korn et al., 1979).

Several years ago we became interested in phospholipid ethers (PLEs) as a result of some pioneering studies by Fred Snyder and co-workers (Snyder and Wood, 1968, 1969). They found that PLEs, which are normally present in low concentration in tissues, accumulated in tumor cells as opposed to surrounding normal cells. Moreover, they provided evidence that this metabolic difference was at the site involving cleavage of the alkyl ether bond and that tumor cells as opposed to normal cells had very low concentrations of this alkyl cleavage enzyme. This metabolic difference between neoplastic and normal cells suggested a means for selectively targeting drug molecules to tumors.

In addition, analogs of the naturally-occurring alkyl lysophospholipid are known to exhibit antitumor and antimetastatic activities. PLE, such as rac-1-octadecyl-2-methylglycerol-3-phosphocholine (ET-18-OMe) (Fig. 1), have shown a selective cytotoxicity towards a variety of human and murine tumors in both in vivo and in vitro studies (Munder et al., 1981; Berdel et al., 1985). Similar antitumor activity has been shown for less complex structures known as alkylphosphocholines (APC) (Fig. 1) (Scherf et al., 1987; Muschiol et al., 1987). APC were developed to study the structure–activity relationship of alkyl lysophosphocholines as they may represent the minimal structural requirement for antitumor activity for such PLE-like compounds. In addition, hexadecylphosphocholine (He-PC), an APC, has been evaluated in a clinical pilot study in breast cancer patients with skin involvement. Tumor regression was seen when He-PC was applied topically (Unger et al., 1989). Based on these observations, regular phase I trial studies have been started. Despite earlier studies by Snyder and Wood (1968, 1969), the mechanism whereby PLEs accumulate in tumor cells is still unknown.

Based upon what is currently known about the accumulation of PLE-like molecules within cancer cells, a suitably radiiodinated PLE analog would be expected to concentrate within neoplastic cells and thereby allow visualization via scintigraphic imaging. Accordingly, a radiiodinated analog of...
Materials and Methods

Syntheses of m-iodophenyl derivatives of He-PC

Preparation of 12-(m-iodophenyl)dodecylphosphocholine (3). To an ice-cooled solution of 12-(m-iodophenyl)dodecanol (1) (Meyer et al., 1989) (110 mg, 0.28 mmol) in dry Et₂O (2 mL) containing Et₃N (0.05 mL, 0.36 mmol) was added dropwise 2-bromoethyl dichlorophosphate (83 mg, 0.34 mmol) in dry Et₂O (1.5 mL). The mixture was stirred at 0°C for 10 min and was gently refluxed for 3 h. Additional dichlorophosphate (68 mg) and Et₃N (0.04 mL) were added and the refluxing was continued for an additional 3 h. Water (1 mL) was added and the mixture was refluxed for 2 h. After removal of the solvents, the residue was dissolved in CHCl₃. The CHCl₃ was washed with H₂O, 0.1 N HCl and H₂O, dried and evaporated to dryness. The resulting residue was chromatographed on silica gel first with CHCl₃:MeOH (15:1) to remove less polar impurities, then with CHCl₃:MeOH (8:1) to elute pure intermediate 2 (101 mg, 62%) as a glassy solid, which was used for the next step without further purification.

A mixture of 2 (101 mg, 0.18 mmol) and Me₃N (1.3 mL, 45–50% aqueous soln) in CHCl₃:DMF:2-PrOH (3:5:5) (10 mL) was stirred at 60°C for 6 h. After cooling, silver carbonate (63 mg) was added to the mixture and the mixture was heated for 2 h. The silver salts were removed by filtration with Celite the filtrate was concentrated to dryness. The residue was chromatographed on silica gel first with CHCl₃:MeOH:CH₃OH (100:20:5) to remove starting material (23 mg), and then with CHCl₃:MeOH:H₂O (65:25:3) to elute pure 3 (59 mg, 61%) as a white solid. ¹H-NMR δ: 7.52 (1H, s, Ar-2H), 7.49 (1H, d, Ar-4H), 7.12 (1H, d, Ar-6H), 6.99 (1H, t, Ar-5H), 4.29 (2H, bs, -CC₃CH₂N), 3.86–3.75 (4H, m, -C₆H₄OPOCH₃N), 3.39 (9H, s, N(Me)₃), 3.26 (bs, H₂O), 2.52 (2H, t, -CH₂P). Anal. calcd for Cₒ₃H₂₅INO₃P·H₂O: C, 48.34; H, 7.58; N, 2.45. Found: C, 48.09; H, 7.71; N, 2.45.

Preparation of hexadecyl 2-[N,N-dimethyl-N-(m-iodobenzyl)ammonium]ethylphosphosphate (5). To an ice-cooled solution of hexadecanol (500 mg, 2.06 mmol) in dry Et₂O (15 mL) containing Et₃N (0.36 mL, 2.60 mmol) was added dropwise 2-bromoethyl dichlorophosphate (600 mg, 2.48 mmol) in dry Et₂O (10 mL). The mixture was stirred at 0°C for 10 min and was gently refluxed for 4.5 h. After cooling, water (3.5 mL) was added and the mixture was heated at 60°C for 2 h. The solvents were removed under reduced pressure and the resulting residue was dissolved in CHCl₃. The CHCl₃ was washed with H₂O,
Radioiodinated alkylphosphocholine

0.1 N HCl and H2O, dried and evaporated to dryness. The residue was chromatographed on silica gel first with CHCl3:MeOH (15:1) and then with CHCl3:MeOH (8:1) to give pure intermediate 4 (Hansen et al., 1982) (376 mg, 43%) as a white solid, which was used without further purification. A mixture of 4 (100 mg, 0.23 mmol) and N,N-dimethylm-iodobenzylamine (304 mg, 1.16 mmol) in EtOH (5 mL) was refluxed for 3 days and then for 2 h in the presence of Ag2CO3 (83 mg). After removal of the silver salts by filtration, the filtrate was concentrated to dryness under reduced pressure. The resulting residue was chromatographed on silica gel with CHCl3:MeOH:H2O (65:25:4) to give pure 5 (41.5 mg, 29%) as a white solid. 

Radioiodination by iodine exchange

Radioiodination of 3 and 5 with iodine-125 was accomplished by the same method as for the preparation of NM-294 reported previously (Counsel et al., 1990). Briefly, the compound (1-2 mg) to be labeled was treated with Na125I (1.3–3 mCi) in pivalic acid (13 μL) at 160°C for 45 min. The radioiodinated product was purified by silica gel column chromatography (1 × 5 cm). A solvent mixture of CHCl3:MeOH:H2O (65:25:4) was used to elute 3, whereas CHCl3:MeOH (1:1) was used for elution of 5. Radiochemical purity of the isolated product was established by radio-TLC with unlabeled material serving as standard. Radiochemical yields based on TLC of exchange reaction mixture was 66 and 72% for 3 and 5, respectively.

Animals

Female Sprague-Dawley rats, 200–250 g (Charles River, Portage, Mich.) were housed in a temperature and light-controlled room and had free access to food and water. The rats were inoculated with Walker 256 carcinosarcoma cells (1.0 × 10^6 cells) in saline in the left and/or right hindlimb. These animals were used 7–10 days later when the tumor weight averaged 10 g. Althymic nude mice, 20–25 g (Charles River, Wilmington, Del.) were housed in specific pathogen-free rooms and had free access to food and water. The mice were inoculated subcutaneously into the shoulder region with human carcinoma cells (1.0 × 10^5 cells). These animals were used when the tumors were approx. 5 mm in diameter. The time to reach this tumor size varied with the different cell lines. For example, HTB-63 (human melanoma) and NCI-H69 (human small cell carcinoma of the lung) cells required 4–6 weeks for tumor growth whereas, HTB-77 (human ovarian carcinoma) took 10–14 days.

Cell lines and culture conditions

The Walker 256 cell line was provided by Dr James Varani of the Department of Pathology, University of Michigan. The cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 200 units/mL of penicillin, and 0.2 mg/mL of streptomycin. HTB-63 (human melanoma), NCI-H69 (human small cell carcinoma of the lung) and HTB-77 (human ovarian carcinoma) cells were purchased from ATCC (American Type Tissue Culture Collection). HTB-63 cells were maintained in McCoy’s 5a medium plus 10% FBS. NCI-H69 and HTB-77 cells were maintained in RPMI 1640 plus 10% FBS. In addition, medium for the human cell lines contained penicillin/streptomycin (final concentration of 50 μg penicillin/mL and 50 μg streptomycin/mL) and L-glutamine, 200 mM (final concentration of 292 μg/mL). All cells were maintained at 37°C in a humidified atmosphere of 90% N2, 5% CO2 and 5% O2.

Tissue distribution

The radioiodinated compounds were dissolved in 95% EtOH and Tween-20 (0.1 mL/mg of compound) was added. The solvent was evaporated with a stream of nitrogen. Physiological saline was added, and the final traces of solvent were removed by passing nitrogen over the solution until it became clear (in final solution 2–3% Tween). The solubilized radioiodinated compound (15–35 μCi, rats: 0.5–1.0 mL, mice: 0.1 mL) was administered i.v. via a tail vein to tumor-bearing animals. The rats were sacrificed by exsanguination and the mice by decapitation while under ether anesthesia at the various time points. The blood was collected and selected tissues were removed and botted of excess blood. Large organs were minced with scissors. Tissue samples were weighed in cellulose acetate capsules and counted with a Searle 1185 well scintillation counter (85% counting efficiency). The concentration of radioactivity in each tissue was expressed as percentage of administered dose per gram of tissue.

Lipid extraction

Samples of liver, plasma and tumor were homogenized in CHCl3:MeOH by the method of Folch et al. (1957). Briefly, 0.1 g tissue or 0.1 mL plasma and 0.4 mL water were homogenized in three washes of 3 mL each of CHCl3:MeOH (2:1) and the resulting homogenate filtered. The filter paper was air-dried and assayed for radioactivity. The filtrate was adjusted to 10 mL with CHCl3:MeOH, an additional 2 mL H2O was added and centrifuged for 10 min at 800 g. The aqueous and organic phases were separated. The aqueous phase was adjusted to 5 mL with
CHCl₃: MeOH: H₂O (3:48:47) and a 1 mL aliquot was taken for counting. The organic phase was evaporated under nitrogen, then redissolved in 1 mL of benzene. A 0.1 mL aliquot was taken for counting and the remainder was sealed and stored overnight. The benzene fraction was dried, then the compound resuspended in diethyl ether and spotted on a silica gel 60 F-254 thin layer chromatography (TLC) plate (Eastman Kodak, Rochester, N.Y. or Merck, Darmstadt). After allowing the plates to air dry, they were developed in CHCl₃: MeOH: HOAc: H₂O (75:25:8:3) (NM-324) and CHCl₃: MeOH (1:2) (NM-326). After removal from the developing tank, the plates were allowed to air dry, then scanned using a System 200 Imaging Scanner. The radioactivity appearing in the lipid extract is expressed as % of total radioactivity present on the filter, and both in the aqueous and organic phase.

**Gamma-camera imaging of rats and mice**

Scanning of the animals was done with an Ohio Nuclear Mobile Camera with a high sensitivity–low energy collimator. Animals were sedated with 87 mg/kg ketamine and 13 mg/kg xylazine. Images were accumulated at various time points following administration of radiolabeled compound (35–75 μCi).

**Results and Discussion**

12-(m-Iodophenyl)dodecylphosphocholine (3, NM-324) and hexadecyl-2-[N,N-dimethyl-N-(m-iodobenzyl)ammonium] ethylphosphate (5, NM-326) were synthesized as shown in Fig. 3. 12-(m-Iodophenyl)dodecanol (1) was prepared by the procedure reported previously (Meyer et al., 1989). Phosphorylation of both 1 and hexadecanol with 2-bromoethyl dichlorophosphate gave 12-(m-iodophenyl dodecyl-2-bromoethylphosphate (2) and hexadecyl-2-bromoethylphosphate (4) in 62 and 43% yield, respectively. The phosphate (2) was readily converted to the choline phosphate (3) in 62% yield by heating with trimethylamine in CHCl₃: 2-PrOH: DMF (3:5:5) for 6 h. Condensation of the phosphate 4 and M-iodobenzylamine was carried out by refluxing in EtOH for 3 days to give N-iodobenzyl derivative 5 in 29% yield.

To evaluate the potential of NM-324 and NM-326 as tumor localizing agents, scintigraphic images were obtained at various time points following injection as shown in Fig. 4. Administration of NM-324 to a rat bearing a Walker carcinosarcoma in the hind limbs produced excellent images of the tumors. A high background activity was seen in the abdominal area at the early time points with subsequent clearing.

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**Fig. 3. Synthesis of radioiodinated analogs of alkylphosphocholine, NM-324 and NM-326.**
Fig. 4. Gamma-camera scintigraphy at various time periods following administration of NM-324 and NM-326 of rats bearing Walker 256 tumors implanted in the thighs. The dose administered and the time to accumulate 100,000 counts are shown.
NM-324 in athymic mice bearing human tumors

Ovarian carcinoma

Small cell carcinoma

Melanoma

Day 5 Day 8 Day 12

Fig. 7. Gamma-camera scintigraphy of athymic mice bearing human tumors at 5, 8 and 12 days following administration of NM-324. The dose administered was 30 μCi.
Radioiodinated alkylphosphocholine

NM-324 and NM-326 tissue distribution in tumor-bearing rats at 24 h

Fig. 5. Distribution of radioactivity following i.v. administration of radioiodinated analogs in tumor-bearing rats at 24 h. The dose administered was 15 μCi.

NM-326 also produced good images of the tumors, but had even higher background activity. In addition, a comparison of the image acquisition time for the two compounds showed that NM-326, at a dose of 85 μCi, took longer to acquire 100,000 counts than NM-324 at a smaller dose of 58 μCi (Fig. 4). This suggested that the NM-326 was being rapidly cleared from the animal while NM-324 was being retained by the tumor.

Tissue distribution studies with NM-324 in the rat bearing the Walker tumor at 24 h post-injection demonstrated the tumor to accumulate 10.5% of the dose. On the other hand, only 2% of the dose of NM-326 was taken up by the tumor at 24 h (Fig. 5). In addition, NM-326 showed minimal uptake of radioactivity in all tissues. Earlier time points (data not shown) for NM-326 showed high levels of radioactivity in liver, duodenum and kidneys with subsequent clearing by 24 h. Most of the radioactivity appeared to be eliminated shortly after injection. NM-324 also showed (Fig. 6) high levels of radioactivity in the duodenum, kidneys and liver which
Table I. Lipid soluble radioactivity extracted from rat liver, plasma and tumor following i.v. administration of NM-324 and NM-326*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>NM-324</th>
<th>NM-326</th>
</tr>
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<tbody>
<tr>
<td>Liver</td>
<td>73.8 ± 1.4</td>
<td>80.5 ± 1.4</td>
</tr>
<tr>
<td>Plasma</td>
<td>69.2 ± 7.1</td>
<td>72.9 ± 1.8</td>
</tr>
<tr>
<td>Tumor</td>
<td>77.4 ± 1.1</td>
<td>72.5 ± 1.9</td>
</tr>
</tbody>
</table>

| Liver  | 69.9 ± 3.6 | 77.1 ± 3.6 |
| Plasma | 58.1 ± 9.1 | 60.1 ± 9.1 |
| Tumor  | 75.4 ± 1.8 | 72.5 ± 1.9 |

| Liver  | 74.4 ± 3.7 | 76.9 ± 2.8 |
| Plasma | 66.7 ± 13.8 | 66.4 ± 11.4 |
| Tumor  | 64.0 ± 9.1 | 76.9 ± 2.8 |

| Liver  | 84.4 ± 5.9 | — |
| Plasma | 77.1 ± 3.5 | — |
| Tumor  | 78.0 ± 2.1 | — |

| Liver  | 76.7 ± 12.1 | — |
| Plasma | 67.8 ± 2.9 | — |
| Tumor  | 73.1 ± 4.7 | — |

*Percent ± SEM; N = 3-4; tumored animals had Walker 256 carcinosarcoma implanted in thigh.
†N = 1.

In vivo metabolism of NM-324 in tumor-bearing rats at 24 and 120 h

Fig. 8. Thin layer chromatography of parent compound and lipid extracts of liver and tumor from rats bearing a Walker 256 tumor at 24 and 120 h following administration of NM-324. The solvent system used was CHCl₃:MeOH:HOAc:H₂O (75:25:8:3). The origin was at 4.0 cm and the solvent front at 17 cm. Pure radiolabeled compound used for administration was employed as the reference standard.

decayed with time. Clearance of radioactivity from the tumor, however, was much slower with tumor radioactivity peaking at 48 h.

These encouraging results with NM-324 prompted further evaluation in athymic mice bearing subcutaneous human tumors such as the HTB-63 (melanoma), NCI-H69 (small cell carcinoma of the lung) and HTB-77 (ovarian carcinoma). Gamma-camera scintigraphy at 5 days post-injection showed tumor localization with a high background activity. By 8 and 12 days the background activity had cleared from the liver and GI tract leaving clear images of the tumors (Fig. 7). Tissue distribution data obtained on these mice following the last day of scanning showed tissue/blood levels > 25/1 in all three models (data not shown).

Lipid extraction of the liver from tumored rats at 3, 6, 24, 48 h and 5 days (NM-324) and 3, 6 and 24 h (NM-326) demonstrated that most of the radioactivity present was lipid extractable (Table 1). Thin layer chromatography of the organic phases at 24 and 120 h showed that most of the radioactivity remained associated with the parent compound for NM-324 in the liver and tumor (Fig. 8). There was a much lower level of lipid extractable radioactivity from the
plasma suggesting clearance of the radioactivity over time.

With NM-326 the percent of radioactivity associated with the parent compound remained stable over time in the liver and tumor tissue. This coupled with the fact that nondetectable levels were seen in the plasma suggested that the parent compound was cleared from the animal fairly rapidly.

Based on these studies with two structural analogs of APC, it is clear that certain structural features are required for optimal retention of these molecules in tumors. In contrast to NM-324, NM-326 showed poor accumulation in the Walker 256 carcinosarcoma tumor. Basically these compounds differ only by the placement of the iodophenyl marker moiety. Placement of this group on the choline portion of the molecule markedly altered the ability to localize in the tumor. Other investigators have shown that alteration of this portion of the molecule either by replacing with a phosphoinositol group (Noseda et al., 1987) or by lengthening the carbon chain (Schick et al., 1987) causes a decrease in tumor cytotoxicity. In summary, radioiodinated analogs of APC represent a new class of agents whose excellent tumor uptake suggests that they may have potential clinical use not only as noninvasive tumor imaging agents but also as candidates for tumor therapy.

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