[^{125}I/^{127}I/^{131}I]Iodorhodamine: Synthesis, Cellular Localization, and Biodistribution in Athymic Mice Bearing Human Tumor Xenografts and Comparison with [^{99m}Tc]Hexakis(2-methoxyisobutylisonitrile)

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The synthesis of halogenated rhodamine (Rh) derivatives was carried out by controlling the stoichiometry of the halogenating agents, bromine and iodine monochloride. In the no-carrier-added synthesis of radioiodinated rhodamine 123, direct labeling of rhodamine 123 (Rh 123) with Na^{125}I/Na^{131}I required the presence of the oxidant peracetic acid. ^{125}I/^{131}I-Rh 123 was synthesized in modest yields (40−45%). HPLC purification separated Rh 123 from its mono- and diiodo derivatives. Monohalogenation of Rh 123 did not alter the compound's ability to permeate viable cells and localize in mitochondria. ^{125}I/^{131}I-Rh 123 was stable in serum in vitro but rapidly metabolized after intravenous injection into mice. Consequently, scintigraphy and biodistribution data reveal poor targeting of subcutaneously growing human tumor xenografts. The results are compared to those obtained following the administration of [^{99m}Tc]hexakis(2-methoxyisobutylisonitrile) which also did not image human tumor xenografts in nude mice.

Introduction

Rhodamine 123 (methyl *o*-(6-amino-3-imino-3*H*-xanthen-9-yl)benzoate monochloride, Rh 123, Scheme 1, **1**), a lipophilic, permeant cationic fluorescent dye, has been used extensively as a mitochondrial stain for studying cellular functions.[1] In vitro studies have shown that mitochondria of muscle and a variety of carcinoma cells retain Rh 123 for prolonged periods (2−5 days), whereas most normal cells release it within 1−16 h.[2,3] In addition, Rh 123 is relatively nontoxic in healthy cells, but displays selective toxicity in certain carcinoma cells in vitro[4−6] and in vivo,[7] and prolongs the survival of mice bearing experimentally induced Ehrlichs ascites tumor or MB49 bladder carcinoma, but not that of mice bearing L1210 leukemia, P388 leukemia, B16 melanoma, or Lewis lung carcinoma.[7] This selectivity of action reflects differences in P-glycoprotein (Pgp) expression in various cell lines, as the compound is a Pgp substrate.[8]

These observations have prompted researchers to radioiodinate Rh 123 and evaluate its tumor-targeting potential in vivo.[9,10] However, since the synthesis of nonradioactive [^{127}I]iodorhodamine 123 (I-Rh 123) has not been reported, the radiolabeled chemical derived from direct electrophilic radioiodination of Rh 123 has not been authenticated. In fact, Kinsey et al. found that electrophilic radioiodination of Rh 123 using iodogen and chloramine T resulted in formation of the iodide salt wherein the radioiodine was not covalently bound to the xanthene ring of the dye,[11] not surprising since electrophilic iodination would be greatly inhibited by the presence of the delocalized positive charge on the ring. In an effort to circumvent this problem, these investigators radioiodinated the reduced form of Rh 123, i.e., dihydrorhodamine 123, but found that it did not target carcinoma and other tumors in vivo in nude mice.[11]

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In our current studies, we have attempted to address the following two questions: (i) did the radioiodination of Rh 123 reported heretofore[9,10,12] lead to the synthesis of a covalently labeled iodo derivative of Rh 123 or a radioiodide salt of Rh 123, and (ii) is the electrophilic radioiodination of Rh 123 possible? We describe, herein, the synthesis and characterization of nonradioactive I-Rh 123 and its cellular localization in vitro and the no-carrier-added synthesis of ^{125}I-Rh 123 and its biodistribution in athymic mice bearing human tumor xenografts. In addition, we have compared the in vivo data with that obtained after the administration of hexakis(2-methoxyisobutylisonitrile)technetium(I) (^{99m}Tc-MIBI), an agent that is currently used in the clinic to image myocardial disease and certain tumors.[13−17] ^{99m}Tc-MIBI had been previously reported by Piwnica-Worms et al. to accumulate, as demonstrated by scintigraphy, in one of the animal tumors used in our studies.[18]

Results and Discussion

Rhodamine 123, a cationic mitochondrial stain of viable mammalian cells, has 10−20-fold greater accumulation in certain types of cancer cells than in normal cells.[19] Consequently, there have been several attempts to radiolabel this molecule. However, despite reported syntheses of radioiodinated derivatives of Rh 123,[9,10,12] this radiopharmaceutical has not found significant application in tumor imaging and other modalities. One major limitation has been lack of a facile no-carrier-added synthesis of the radiochemical. Another is the lack of chemical evidence of its formation during electrophilic iodination of Rh 123 with iodogen or chloramine T.[11] Whether the radiolabel is truly covalently bound or is an ionic iodide salt can only be demonstrated by comparison with authentic iodo analogues of Rh 123. Another important issue is whether

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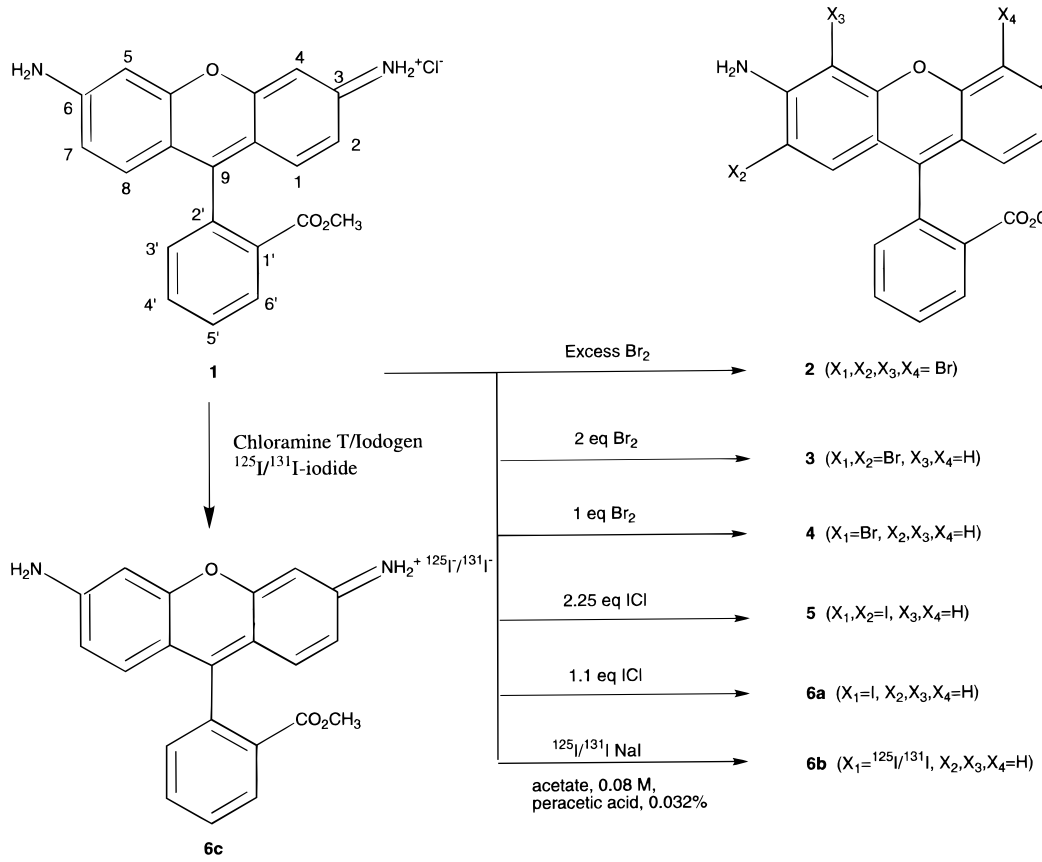
Scheme 1. Synthesis of Halogenated Rh 123



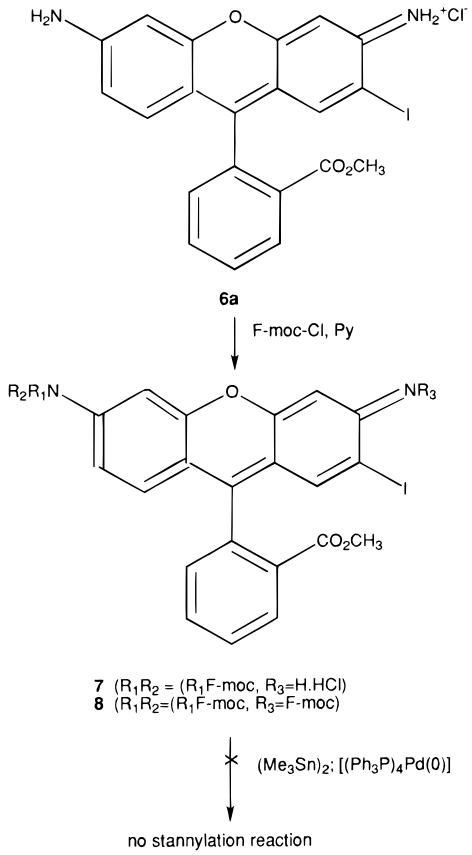
the halogenated derivatives show the same propensity to bind to mitochondria as rhodamine 123. Groups such as iodine on rhodamine 123 introduce to the compound not only steric bulk (on the order of a methyl group) but also hydrophobicity, and this may have some bearing on the intracellular localization of I-Rh 123.

Halogenation of Rh 123 may potentially result in mono-, di-, tri-, and tetrasubstituted Rh 123 (see Scheme 1). Wang et al.[20] reported the formation of tetrabromorhodamine 123 upon reaction of rhodamine with excess bromine. By controlling the stoichiometry of bromine and the rate of its addition, we synthesized monobromo (**4**), dibromo (**3**), and tetrabromo (**2**) derivatives and characterized them by NMR and HRMS. We then employed iodine monochloride and used the same approach to synthesize and characterize mono- and diiodorhodamine 123 (**6a** and **5**, respectively). Tetraiodorhodamine 123 could not be prepared.

Our initial approach to the synthesis of ^{125}I/^{131}I-Rh 123 was via iododestannylation of trimethylstannylrhodamine 123. Our attempts to stannylate I-Rh 123 using (i) hexamethylditin in the presence of tetrakis(triphenylphosphine)palladium or (ii) tributylstannyl chloride in *n*-butyllithium did not succeed in furnishing the desired product. Since stannylation reactions are somewhat retarded or inhibited by the presence of amino or imino groups,[21] we then hypothesized that blocking these groups with a suitable protective group would facilitate the stannylation reaction. Accordingly, using either 1 or 2 equiv of F-moc chloride, a mono- (**7**) or di- (**8**) F-moc derivative was prepared (Scheme 2). The F-moc group was selected because the basic conditions required for its deprotection were those in which the trimethylstannyl group would be stable. Despite the protection of amino groups by F-moc, the stannylation reaction did not proceed, compelling us to focus on direct radioiodination of Rh 123 with a suitable oxidizing agent.

Our efforts to prepare I-Rh 123 using the conventional iodinating agents iodogen or chloramine T resulted in the formation of iodide salts of Rh 123 (**6c**) wherein chloride was exchanged for iodide.[11] In searching for a nonhalogenated oxidizing agent for radioiodination, we selected peracetic acid. The parameters of radiolabeling including the buffer and its strength and pH, time, and temperature were optimized, and ^{125}I/^{131}I-Rh 123 (**6b**) was obtained in 40-45% yield (Scheme 1). The R_f of the radiochemical **6b** on TLC and its retention time on HPLC matched those of authentic nonradioactive I-Rh 123 (**6a**) prepared as shown in Scheme 1. A reversed-phase C_{18} TLC system (mobile phase of methanol−0.085 M phosphate buffer−acetic acid, 3:7:2) was effective in differentiating the radioiodide salt of Rh 123 (**6c**, R_f = 0.38) from covalently labeled I-Rh 123 (**6b**, R_f = 0.22). During optimization of radiolabeling, unsuitable conditions always led to the formation of the radioiodide salt which had the same R_f as Rh 123 (**1**). When covalent radiohalogenation resulted, however, the R_f of the radioactive spot was always superimposable on the fluorescent spot of authentic I-Rh 123 (**6a**). In addition, a mixture of Rh 123, I-Rh 123, and I_2-Rh 123 separated well on the C_{18} column HPLC (Figure 1). This approach proved very useful for the separation of ^{125}I-Rh 123 and ^{131}I-Rh 123 from excess Rh 123, free iodide, and peracetic acid (the oxidizing agent).

Scheme 2. F-moc Protection and Stannylation of I-Rh 123



The fluorescence microscopy of KB cells shows that I-Rh 123 localizes within the cytoplasm (Figure 2, right) in a manner very similar to that of Rh 123 (Figure 2, left). The hollow region seen in the center of cells, more clearly with I-Rh 123 than Rh 123, attests to the fact that these chemicals do not enter the cell nucleus but remain within the cytoplasm. The corresponding photomicrograph of cells under conditions of phase contrast (not shown) also reveals that the observed fluorescence emanates, in fact, from within the cytoplasm. On the other hand, Br_2-, Br_4-, and I_2-Rh 123 do not seem to permeate cells under these conditions.

The cell-uptake findings prompted us to evaluate ^{131}I-Rh 123 as a possible imaging agent for mitochondria-rich tissues such as tumors and the myocardium. Prior to these in vivo studies, we examined the in vitro serum stability of ^{125}I-Rh by incubating (37 °C, up to 24 h) the radiochemical with freshly isolated human or murine serum. TLC demonstrated that ^{125}I-Rh is very stable as <4% of free radioiodide was formed and no breakdown products were observed. The diagnostic potential of ^{131}I-Rh 123 was then tested in nude mice bearing subcutaneous tumors of human origin, namely, the epidermoid carcinoma KB, and the results were compared with those obtained after the intravenous administration of ^{99m}Tc-MIBI, a radiopharmaceutical that is currently used in the clinic in the scintigraphic detection of myocardial disease, certain tumors, and other lesions.[13−17] To this end, dynamic images were collected over 1 h, and the cumulated data were analyzed for tumor localization. However, neither ^{131}I-Rh nor ^{99m}Tc-MIBI was able to image these lesions in tumor-bearing mice (Figure 3). The inability of ^{99m}Tc-MIBI to detect subcutaneous KB tumors is in disagreement with the scintigraphic images published by Piwnica-Worms et al.[18]

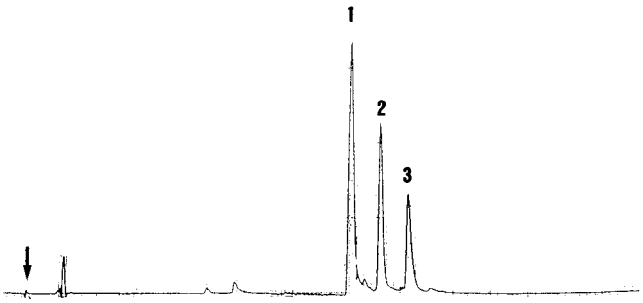


Figure 1. HPLC chromatogram of a mixture of Rh 123 (1), I-Rh 123 (2), and I_2-Rh 123 (3). Retention times were 15.95, 18.0, and 19.4 min, respectively.

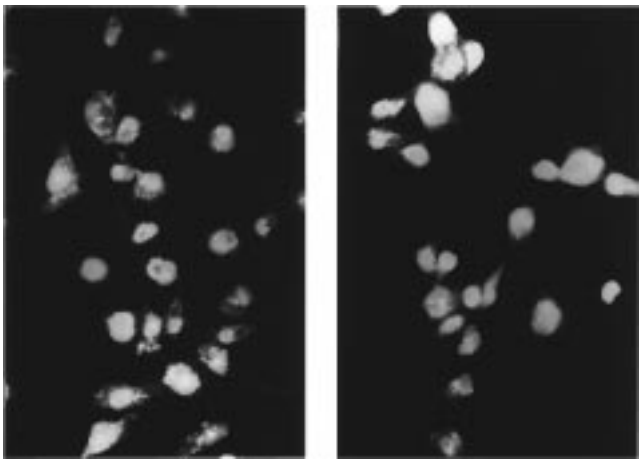


Figure 2. Fluorescence micrograph of KB cells stained with I-Rh 123 (left) and Rh 123 (right). Brightly lit areas are mitochondria within cytoplasm. Note hollow areas in center of cells (in the left micrograph) that indicate absence of dye in the nucleus.

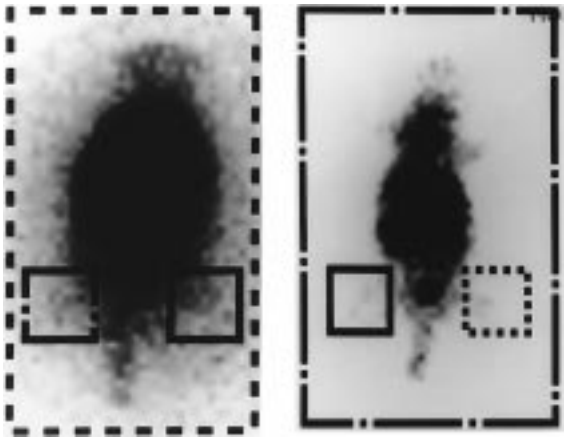


Figure 3. Gamma camera images of mice bearing implanted KB carcinoma. Animals bearing ~l-cm diameter tumors in left flank were injected intravenously with either 15 μCi of ^{131}I-Rh 123 (left) or 75 μCi of ^{99m}Tc-MIBI (right). Tumor-bearing region (small square) was located by placing a radioactive source directly over left flank. For comparison, similar square on right flank represented region without tumor. FOM: for ^{131}I-Rh 123, 0.50; for ^{99m}Tc-MIBI, 0.33.

In an attempt to understand the mechanism(s) underlying these negative results, the biodistribution of ^{125}I-Rh and ^{99m}Tc-MIBI was studied in nude mice

Table 1. Biodistribution of ^{125}I-Rh and ^{99m}Tc-MIBI in Athymic Mice Bearing Human Tumor Xenografts

agent	tumor	time after injection	percentage injected dose per gram					ratios			
			tumor (T)	blood (B)	muscle (M)	heart (H)	lung (L)	T:B	T:M	H:B	H:L
^{125}I-Rh	KB[a]	1 h	1.67 ± 0.24	4.6 ± 0.6	1.44 ± 0.38	2.56 ± 0.83	3.23 ± 0.78	0.36 ± 0.07	1.2 ± 0.4	0.6 ± 0.2	0.8 ± 0.3
	LS174T[b]	2 h	0.59 ± 0.06	1.74 ± 0.42	0.36 ± 0.09	0.6 ± 0.09	0.91 ± 0.09	0.34 ± 0.08	1.6 ± 0.4	0.5 ± 0.1	0.7 ± 0.1
	TE671[b]	2 h	1.1 ± 0.43	1.74 ± 0.42	0.36 ± 0.09	0.6 ± 0.09	0.91 ± 0.09	0.63 ± 0.28	3.1 ± 0.9	0.5 ± 0.1	0.7 ± 0.1
^{99m}Tc-MIBI	KB[a]	1 h	0.65 ± 0.19	0.17 ± 0.04	2.74 ± 0.51	8.13 ± 2.13	1.82 ± 0.37	3.8 ± 1.4	0.24 ± 0.08	48 ± 17	4.5 ± 1.4
	LS174T[b]	2 h	0.52 ± 0.20	0.13 ± 0.11	1.22 ± 0.56	6.0 ± 2.4	0.5 ± 0.1	4 ± 2	0.43 ± 0.25	46 ± 22	12 ± 6
	TE671[b]	2 h	1.1 ± 0.3	0.13 ± 0.11	1.22 ± 0.56	6.0 ± 2.4	0.5 ± 0.1	8.5 ± 3.7	0.90 ± 0.47	46 ± 22	12 ± 6

[a] $n = 4$. [b] $n = 5$.

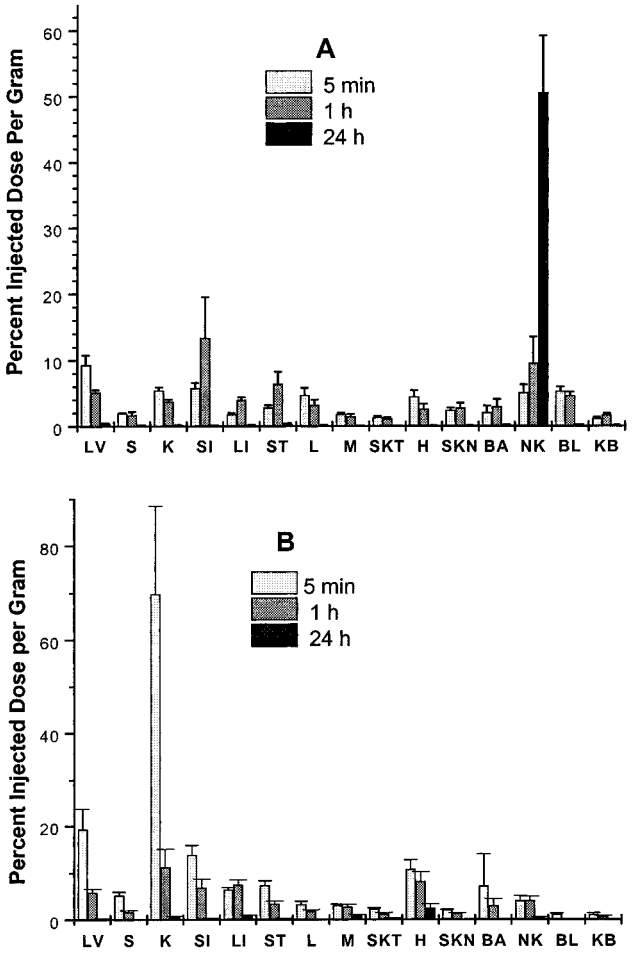


Figure 4. Biodistribution profile of ^{125}I-Rh (A) and ^{99m}Tc-MIBI (B) in KB-tumor-bearing athymic nude mice ($n = 4$). Key: LV, liver; S, spleen; K, kidney; SI, small intestine; LI, large intestine; ST, stomach; L, lungs; M, muscle; SKT, skeleton; H, heart; SKN, skin; BA, bladder; NK, neck contents; BL, blood; KB, tumor.

bearing KB, LS174T, and TE671 human tumors. The percentage injected dose per gram of tissue (% ID/g) as a function of time for the two radiochemicals is shown in Figure 4. Here again, uptake of either agent by the tumors was not particularly high (0.5−1.7% ID/g), and tumor-to-normal-tissue ratios were not impressive. While tumor-to-blood ratios for ^{99m}Tc-MIBI were clearly higher than those obtained with ^{125}I-Rh (Table 1), the reverse was true for tumor-to-muscle ratios, thus explaining our inability to scintigraphically detect the tumors with either radiochemical. That ^{99m}Tc-MIBI is an agent of choice for heart imaging was seen from its high heart uptake (6−8.1% ID/g) and its high heart-to-blood (46−48) and heart-to-lung (4.5−12) ratios 1−2 h post-injection (Table 1).

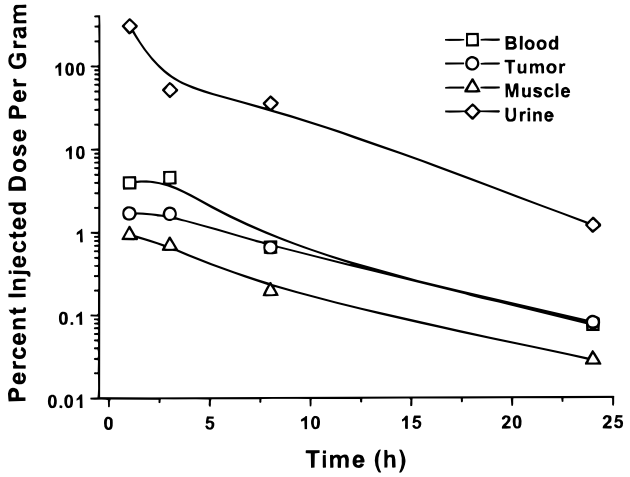
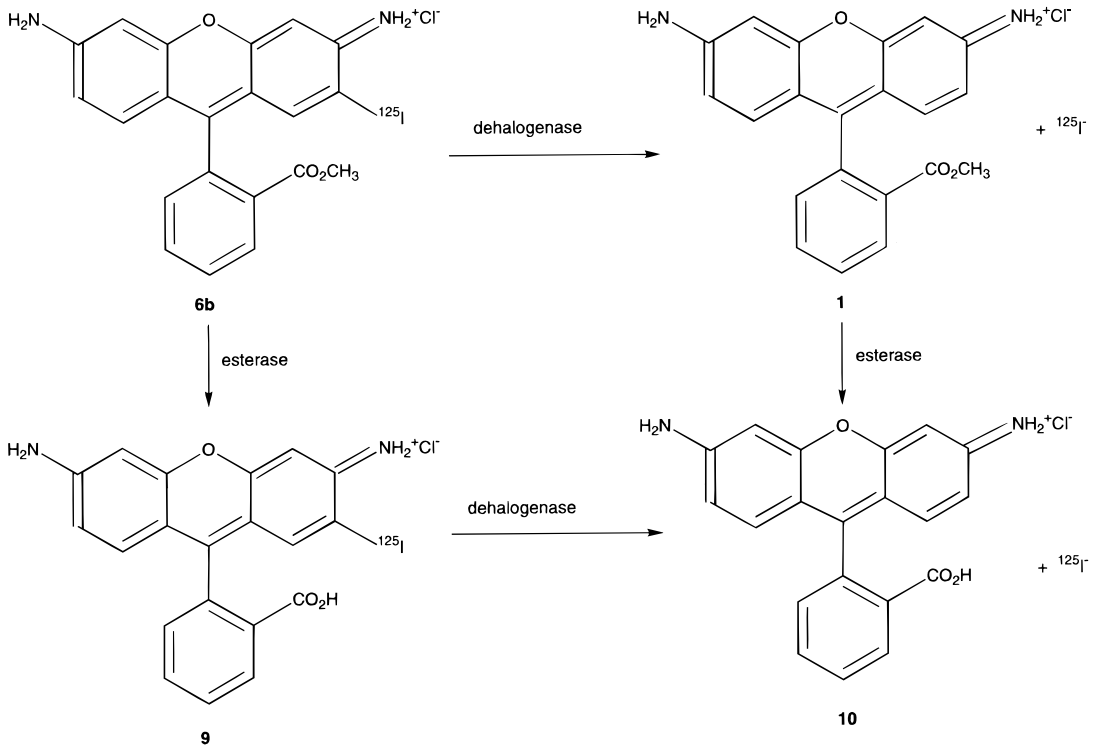


Figure 5. Pharmacokinetics of activity in blood (□), tumor (○), muscle (△), and urine (◇) following administration of ^{125}I-Rh 123. Note that 1 h after uptake, activity from all tissues cleared essentially at the same rate. Error bars cannot be seen as standard deviations range between 9% and 12% of mean.

The absence of ^{125}I-Rh accumulation in the heart (heart-to-lung ratio of <1, Table 1) contrasts with the expectation that, being a mitochondria-specific agent, it should have targeted mitochondria-rich heart muscle. However, the pharmacokinetics of ^{125}I-Rh 123 (Figure 5) reveals that the clearance of radioactivity from the tumor, muscle, and urine parallels that of blood. The poor localization of ^{125}I-Rh 123 in tumor and heart muscle could thus be a consequence of its in vivo metabolism into one or more radioactive moieties whose in vivo behavior is different from that of a mitochondrial-avid reagent. In fact, the high accumulation of radioactivity in the neck region (i.e., thyroid) is a clear indication of in vivo deiodination of ^{125}I-Rh 123 and thyroid uptake of radioiodide (Figure 4). To identify the metabolic product(s), ^{125}I-Rh 123 was injected into mice, blood was drawn at various times, and the plasma was analyzed by C_{18} TLC. These results (Figure 6) indicate that ^{125}I-Rh 123 rapidly disappears from blood (half-life < 10 min) and that this is accompanied by the simultaneous appearance of free radioiodide and ^{125}I-Rh 110 (**9**), the former arising from the in vivo deiodination of ^{125}I-Rh 123 by dehalogenases and the latter (i.e., ^{125}I-Rh 110) from esterase-induced hydrolysis (Scheme 3). Being electrically neutral, ^{125}I-Rh 110, similar to Rh 110,[1] does not permeate cells and therefore will not bind to mitochondria, thus explaining the poor targeting of the radiochemical in tumors and in heart muscle. Furthermore, the data show that the testing of the in vitro serum stability of a chemical does not necessarily reflect its in vivo characteristics. If future work on the synthesis of radiolabeled Rh 123 analogues

Scheme 3. Metabolism of ^{125}I-Rh 123 in KB-Bearing Athymic Mice[a]



[a] ^{125}I-Rh 110 is formed as a consequence of enzymatic hydrolysis of ^{125}I-Rh 123 and radioiodide from deiodination of ^{125}I-Rh 123 and ^{125}I-Rh 110.

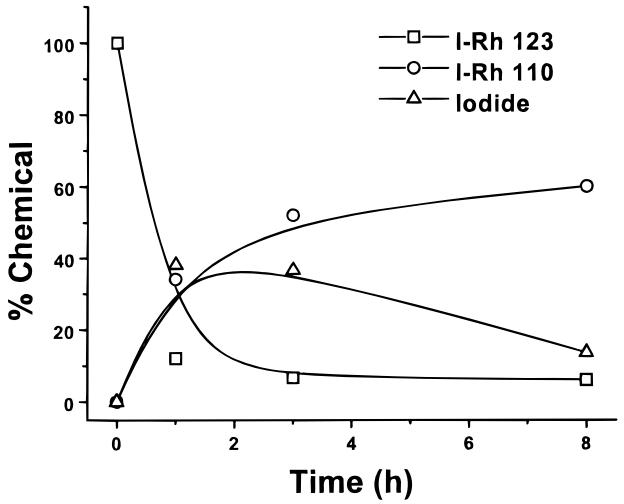


Figure 6. Time course of formation of the metabolites ^{125}I-Rh 110 (○) and free radioiodide (△) after administration of ^{125}I-Rh 123 (□). Error bars cannot be seen as standard deviations range between 10% and 12% of mean.

is undertaken, it is essential that the molecule resist deiodination and ester hydrolysis and be tested for its stability in vivo as well as in vitro.

Experimental Section

Reagents and chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI). Rhodamine 123 and rhodamine 110 were purchased from Fisher Scientific (Pittsburgh, PA). Carrier-free Na^{125}I (0.63 TBq/mg) and Na^{131}I (0.44 TBq/mg) were obtained from Amersham Corp. (Arlington Heights, IL). [^{99m}Tc]Sestamibi (^{99m}Tc-MIBI) was prepared from a Cardiolite kit (DuPont Merck Pharmaceuticals). Melting points were determined on a Fisher-Johns melting point apparatus (Pittsburgh, PA) and are uncorrected. NMR spectra were obtained on a Varian XL-500 instrument. Electron impact (EI) and direct chemical ionization fast atom bombardment (DCI/FAB; H) mass spectra were recorded on a ZAB 7070 mass spectrometer at the University of Illinois, Urbana. The UV spectra were recorded on a Hitachi UV/vis spectrophotometer (Lambda 3B), and fluorescence spectra were obtained from a Perkin-Elmer fluorescence spectrophotometer (LS-50B). Analytical TLC was carried out on either silica gel plates (Baker-flex, IB2-F; J. T. Baker Inc., Phillipsburg, NJ) or C_{18} TLC plates (Uniplate RPSF; Analtech, Newark, DE), and preparative TLC purification on silica plates (20 × 20 cm, 1000 μm; Analtech) was employed. Silica gel plates were run in a mobile phase consisting of methylene chloride−benzene−methanol−triethylamine (2:2:1:0.04) and C_{18} plates in a mixture of methanol−0.085 M phosphate buffer−acetonitrile−acetic acid (3:7:2:0.05). The plates were examined under UV and exposed to X-ray film, and autoradiographs were developed for the visualization of radioactivity.

Methyl *o*-(2,4,5,7-Tetrabromo-6-amino-3-imino-3*H*-xanthen-9-yl)benzoate Monochloride, Tetrabromorhodamine 123 (2). To a suspension of Rh 123 (100 mg, 0.262 mmol) in 1.5 mL of ethanol was added bromine (328 mg, 105 μL, 2.05 mmol) dropwise at room temperature. The reaction mixture was stirred for 2 h. Bromination was complete as judged by the appearance of a new nonpolar spot on TLC. The red precipitate was filtered and washed with cold ethanol (4 mL). The solid was dissolved in methanol−methylene chloride (1:1) and purified by preparative TLC in methanol−methylene chloride (1:1). The solvent was evaporated, and the chemical was crystallized from ethyl acetate−hexane (yield 104 mg, 56.45%): mp 185−188 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.58 (s, 3 H, CO_2Me), 4.1 (bs, 2 H, NH_2), 7.023−7.071 (m, 2 H, H-1, H-8), 7.455 (d, 1 H, J = 8 Hz, H-6′), 7.837 (t, 1 H, J = 8 Hz, H-4′), 7.890 (t, 1 H, J = 8 Hz, H-5′), 8.253 (d, 1 H, J = 8 Hz, H-3′), 9.45 (bs, 2 H, NH_2); HRMS calcd for $C_{21}H_{13}N_2O_3Br_4$ 660.761 870, found 660.763 600 $(M - Cl)^+$.

Methyl *o*-(2,7-Dibromo-6-amino-3-imino-3*H*-xanthen-9-yl)benzoate Monochloride, Dibromorhodamine 123 (3). To a suspension of Rh 123 (100 mg, 0.262 mmol) in 1.5 mL of ethanol was added bromine (83.74 mg, 26.99 μL, 0.524 mmol)

dropwise at −40 °C. The reaction mixture was stirred for 2 h. Bromination was complete as judged by TLC. The solvent was removed under reduced pressure, and the compound was purified by preparative TLC (yield 76%): mp 220 °C dec; ^{1}H NMR (DMSO-d_6) δ 3.585 (s, 3 H, CO_2Me), 3.91 (bs, 2 H, NH_2), 7.039 (s, 4 H, H-1,4,5,8), 7.45 (d, 1 H, J = 8 Hz, H-6′), 7.833 (t, 1 H, J = 8 Hz, H-4′), 7.907 (t, 1 H, J = 8 Hz, H-5′), 8.247 (d, 1 H, J = 8 Hz, H-3′), 9.094 (bs, 2 H, NH_2); HRMS calcd for $C_{21}H_{15}N_2O_3Br_2$ 500.944 940, found 500.946 000 $(M - Cl)^+$.

Methyl *o*-(2-Bromo-6-amino-3-imino-3*H*-xanthen-9-yl)-benzoate Monochloride, Monobromorhodamine 123 (4). To a suspension of Rh 123 (100 mg, 0.262 mmol) in 1.5 mL of ethanol in an ice bath was added bromine (41.87 mg, 14 μL, 0.262 mmol) in ethanol (200 μL). The reaction mixture was stirred for 1 h, the solvent removed under reduced pressure, and the crude reaction mixture purified by preparative TLC. To obtain the proper resolution, the plates were run three times (yield 62%): ^{1}H NMR (DMSO-d_6) δ 3.60 (s, 3 H, CO_2Me), 3.91 (bs, 2 H, NH_2), 6.94 (m, 5 H, H-1,2,4,5,8), 7.45 (d, 1 H, J = 8 Hz, H-6′), 7.80 (t, 1 H, J = 8 Hz, H-4′), 7.88 (t, 1 H, J = 8 Hz, H-5′), 8.23 (d, 1 H, J = 8 Hz, H-3′), 8.5 (bs, 2 H, NH_2); HRMS calcd for $C_{21}H_{16}N_2O_3Br$ 423.034 429, found 423.035 700 $(M - Cl)^+$.

Methyl *o*-(2,7-Diiodo-6-amino-3-imino-3*H*-xanthen-9-yl)benzoate Monochloride, Diiodorhodamine 123 (5). To a suspension of Rh 123 (76 mg, 0.2 mmol) in 10 mL of dry THF was added iodine monochloride (72 mg, 450 μL, 0.44 mmol) dropwise at −40 °C. The reaction mixture was allowed to warm to room temperature and stirred for 5 h. The reaction mixture showed 40% conversion to I-Rh 123. The solvent was removed under reduced pressure, and the crude reaction mixture was purified by preparative TLC (yield 76%):^{1}H NMR (DMSO-d_6) δ 3.58 (s, 3 H, CO_2Me), 6.980 (m, 4 H, H-1,4,5,8), 7.45 (d, 1 H, J = 8 Hz, H-6′), 7.818 (t, 1 H, J = 8 Hz, H-4′), 7.891 (t, 1 H, J = 8 Hz, H-5′), 8.24 (d, 1 H, J = 8 Hz, H-3′), 8.70 (bs, 2 H, NH_2), 10.20 (bs, 2 H, NH_2); HRMS calcd for $C_{21}H_{15}N_2O_3I_2$ 596.917 222, found 596.918 200 $(M - Cl)^+$.

Methyl *o*-(2-Iodo-6-amino-3-imino-3*H*-xanthen-9-yl)-benzoate Monochloride, Monoiodorhodamine 123 (6a), and (2-Iodo-6-amino-3-imino-3*H*-xanthen-9-yl)benzoic Acid Monochloride, Monoiodorhodamine 110 (9). To a suspension of Rh 123 (76 mg, 0.2 mmol) in 10 mL of dry THF was added iodine monochloride (32 mg, 200 μL, 0.2 mmol) dropwise at −40 °C. The reaction mixture was allowed to warm to room temperature and stirred for 5 h. The reaction mixture showed 40% conversion to I-Rh 123. The solvent was removed under reduced pressure, and the crude reaction mixture was purified by preparative TLC. The nonpolar band was scraped and eluted with methylene chloride−methanol (1:1). The solvent was removed and the compound dried in vacuo (yield 34%): mp 165 °C; ^{1}H NMR (DMSO-d_6) δ 3.60 (s, 3 H, CO_2Me), 6.921 (m, 5 H, H-1,4,5,7,8), 7.458 (d, 1 H, J = 8 Hz, H-6′), 7.801 (t, 1 H, J = 8 Hz, H-4′), 7.875 (t, 1 H, J = 8 Hz, H-5′), 8.20 (d, 1 H, J = 8 Hz, H-3′), 8.70 (bs, 2 H, NH_2), 10.20 (bs, 2 H, NH_2); HRMS calcd for $C_{21}H_{16}N_2O_3I$ 471.020 570, found 471.022 100 $(M - Cl)^+$.

Similarly Rh 110 was iodinated to form monoiodo-Rh 110 (**9**; yield 41%): mp 182−184 °C; ^{1}H NMR (DMSO-d_6) δ 6.95−6.96 (m, 5 H, H-1,4,5,7,8), 7.481 (d, 1 H, J = 8 Hz, H-6′), 7.852 (t, 1 H, J = 8 Hz, H-4′), 7.903 (t, 1 H, J = 8 Hz, H-5′), 8.28 (d, 1 H, J = 8 Hz, H-3′), 8.82−9.05 (bm, 4 H, NH_2).

^{125}I-Rh 123 (6b) and ^{125}I-Rh 110 (9). To 20 μL of Rh 123 solution (1 mg/mL in 0.04 M acetate buffer, pH 5.0) placed in a screw-cap vial was added 50 μL of aqueous peracetic acid (0.032%) followed by 0.5−1 mCi of $Na^{125}I/Na^{131}I$ solution in aqueous sodium hydroxide. After vortex mixing and incubation at ambient temperature for 1 h, analysis by C_{18} TLC revealed the reaction was approximately 80% complete. The mixture was extracted with dichloromethane (3 × 1 mL), and the organic phase was washed with 10% sodium bisulfite, water, and brine. The solution was evaporated; the solid was dissolved in 50 μL of ethanol, and the sample was diluted to 1 mL with water and then passed through a C_{18} Sep-Pak cartridge preconditioned with water, methanol, and water. Successive elutions from water to methanol furnished the product in 50% aqueous methanol in 40−45% yield and over 98% chemical and radiochemical purity. Alternatively, the reaction mixture following a 1-h incubation was directly injected into the HPLC column under the conditions described below, and the product was eluted at 18.0 min. If there was any carryover of unlabeled Rh 123, the eluate was evaporated, redissolved in dichloromethane, and washed with water to remove excess Rh 123. Final purity of the radiochemical was checked by silica gel and C_{18} TLC by cospotting with authentic I-Rh 123.

The same procedure was followed for radioiodination of Rh 110. ^{125}I-Rh 110 was isolated in 40% yield after HPLC purification (retention time: 10.1 min for Rh 110 and 11.4 min for ^{125}I-Rh 110).

Di(fluoromethoxycarbonyl)iodorhodamine 123 (8). I-Rh 123 (38 mg, 0.1 mmol) was suspended in dry methylene chloride (15 mL) under argon atmosphere. The mixture was cooled in ice, and diisopropylethylamine (400 μL) was added, followed by F-moc-Cl (0.24 mmol) in portions. The reaction mixture was stirred for 4 h. TLC showed 95% conversion from starting material to an orange-yellow nonpolar spot. The crude reaction mixture was purified by preparative TLC (yield 70%): ^{1}H NMR ($CDCl_3$) δ 3.85 (s, 3 H, CO_2Me), 6.34 (d, J = 7 Hz, 1 H), 6.534 (m, 2 H), 6.665 (d, J = 7.5 Hz, 1 H), 6.787 (s, 1 H), 7.15 (d, J = 7.5 Hz, 1 H), 7.261 (s, 2 H), 7.331 (m, 5 H), 7.417 (t, J = 8 Hz, 4 H), 7.637 (m, 16 H), 7.78 (d, J = 8 Hz, 4 H), 8.01 (d, J = 8 Hz, 1 H), 8.05 (d, J = 8 Hz, 1 H). Using 1.1 equiv of F-moc-Cl, the mono-F-moc derivative **7** was obtained.

Attempted Stannylation of F-moc Derivatives 7 and 8. A solution of **7** or **8** (0.05 mmol), hexamethylditin (25 mg, 0.075 mmol), and tetrakis(triphenylphosphine)palladium(0) (5 mg, 0.0045 mmol) in 5 mL of anhydrous dioxane was heated under gentle reflux in a nitrogen atmosphere for 12 h. TLC analysis revealed the formation of multiple decomposition products, and the expected stannylation product could not be isolated.

High-Performance Liquid Chromatographic Separation of Rh 123, I-Rh 123, I_2-Rh 123, Rh 110, and I-Rh 110. A reversed-phase separation was accomplished using a mobile phase of 0.02 M phosphate buffer, pH 7 (mobile phase A), and acetonitrile (mobile phase B) by adaptation of the procedure of Banes et al.[22] A linear gradient of 10−80% B in 15 min on a Spheri-5 RP-8 column (100 × 4.6 mm, 5 μm; Applied Biosystems, Foster City, CA) with a precolumn (30 × 4.6 mm) at a flow rate of 2 mL/min provided satisfactory separation. A Waters 510 pump and 486 absorbance detector were employed. The retention times for Rh 123, I-Rh 123, I_2-Rh 123, Rh 110, and I-Rh 110 were 15.94, 18.0, 19.4, 10.1, and 11.4 min, respectively.

Uptake of I-Rh 123 in V79, KB, LS174T, and TE671 Cells. Cells ($2-4 \times 10^4$) were grown for 24 h in Eagle's minimum essential medium (MEM; Gibco BRL, Life Technologies, Inc., Gaithersburg, MD), supplemented with 100 mM nonessential amino acids, 2 mM L-glutamine, and 5% fetal calf serum, in Leighton tubes and then incubated for 1 h in the presence of 15 μg of either Rh 123 or I-Rh 123 containing 15 μg of 2-deoxyglucose. The slides were rinsed with phosphate-buffered saline (PBS), fixed with 4% neutral buffered formalin, and dehydrated with an increasing gradient of ethyl alcohol and finally with butanol. The cells were examined under a fluorescence microscope (Nikon, Labophot-2) using a G2A filter, and photomicrographs were recorded on 35-mm Kodak ASA 400 film.

Biodistribution of ^{125}I-Rh 123 and ^{99m}Tc-MIBI in Athymic Mice Bearing KB, LS174T, and TE671 Tumors. KB (human epidermoid carcinoma), LS174T (human colonic adenocarcinoma), and TE671 (human medulloblastoma) cells, all from ATCC, were maintained as monolayers in Eagle's MEM as described above. To provide single-cell suspensions for injection, the cells were trypsinized and gently pipetted, and the suspension was repeatedly passed through a syringe with a 22-gauge needle. Athymic Nu/Nu female mice (4−5 weeks old; Harlan Sprague−Dawley, Indianapolis, IN) were injected

subcutaneously in the flank with 10^6 cells/0.1 mL of PBS/mouse, and the tumors were allowed to grow to approximately 1 cm in diameter (0.3-g weight, 8−20 days). Each mouse then received an intravenous injection via a lateral tail vein of either 5 μCi of ^{125}I-Rh 123 or 25 μCi of ^{99m}Tc-MIBI in 0.1 mL of saline. The mice were killed at various intervals, and organs/tissues were dissected and counted in a gamma counter. Based on the injected activity and its percentage found in each organ/tissue, the percentage injected dose per gram of tissue/organ (% ID/g) and tumor-to-nontumor ratios (T/NT) were determined.

Scintigraphy of Mice following Administration of ^{131}I-Rh 123 and ^{99m}Tc-MIBI. The KB-tumor-bearing animals were anesthetized with a mixture of ketamine (50 mg/kg) and xylazine (7.5 mg/kg) given intraperitoneally as a saline solution (0.2 mL) and were positioned over a gamma camera (GE-Starcam; LEAP collimator). Either 15 μCi of ^{131}I-Rh 123 or 75 μCi of ^{99m}Tc-MIBI was then injected as a bolus via a lateral tail vein. Sequential planar images were collected at 60-s intervals up to 1 h. Each image was corrected online for camera nonuniformity with a 300-million count flood and stored at a digital resolution of 64 × 64. Images were corrected for decay and no attenuation or scatter correction was used. The percentage activity in the tumor-bearing flank was determined as a ratio of counts accumulated in the tumor-containing box over those of the entire mouse, and this was compared to the corresponding value in the nontumor-bearing flank. Based on these values, figure of merit (FOM) calculations were performed to determine the significance of tumor uptake of radioactivity.[23]

In Vitro Stability and in Vivo Metabolism of ^{125}I-Rh 123. To determine the in vitro stability of ^{125}I-Rh 123, 0.5 mL of mouse or human plasma was placed in a microfuge vial, 20 μCi of a solution of ^{125}I-Rh 123 was added, and after vortex-mixing, the capped vials were kept in an incubator at 37 °C. At various intervals from 0 to 24 h, samples (3 μL) were withdrawn, mixed with 5 ng each of nonradioactive Rh 123, I-Rh 123, Rh 110, and I-Rh 110, and spotted on a C_{18} TLC plate.

For in vivo metabolism assessment, 20 μCi of ^{125}I-Rh 123 was injected in KB-bearing mice via a tail vein and the blood was collected at various intervals by cardiac puncture. The radioactive plasma along with the above standard chemicals was spotted on C_{18} TLC plates. The plates were run in a mobile phase consisting of 0.085 M potassium phosphate−methanol−acetonitrile (7:3:3). The nonradioactive chemicals on the plate were visualized by fluorescence and the radioactive chemicals by autoradiography (18-h exposure time). Under these conditions the R_f values for Rh 123, I-Rh 123, Rh 110, I-Rh 110, and free iodide (I^-) were 0.29, 0.17, 0.64, 0.60, and 0.98, respectively. On the basis of the radioactivity (cpm) found in each grid that displayed fluorescence of a respective chemical, the in vitro as well as the in vivo profiles were determined. Plasma samples were also analyzed by HPLC (see above), and the data were in agreement with the C_{18} TLC results (data not shown).

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