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'3+1' Mixed-Ligand Oxotechnetium(V) Complexes with Affinity for Melanoma: Synthesis and Evaluation in Vitro and in Vivo

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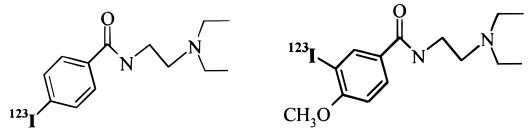
'3+1' Mixed-ligand [^{99m}Tc]oxotechnetium complexes with affinity for melanoma were synthesized in a one-pot reaction. Complexation of technetium-99m with a mixture of *N*-R(3-azapentane-1,5-dithiol) [R = Me, Pr, Bn, $Et_2N(CH_2)_2$] and *N*-(2-dialkylamino)ethanethiol [alkyl = X = Et, Bu, morpholinyl] using Sn^{2+} as the reducing agent resulted in the formation of '3+1' mixed-ligand technetium-99m complexes [$TcO(SN(R)S)(SNX_2)$] in high radiochemical yield (60−98%). In vitro uptake studies in B16 murine melanoma cells indicated a moderate tumor-cell accumulation (40%) of compound **1** [R = Me, X = Et] and a higher accumulation (69%) of compound **2** [R = Me, X = Bu] after a 60-min incubation. In vivo evaluation of compounds **1**−**6** in the C57Bl6/B16 mouse melanoma model demonstrated tumor localization. Compound **2** displayed the highest accumulation with up to 5% ID/g at 60 min after injection. In vivo, **2** also showed a low blood-pool activity and high melanoma/spleen (4.3) and melanoma/lung (1.9) ratios at 1 h. These results suggest that small technetium-99m complexes could be useful as potential melanoma-imaging agents.

Introduction

Increase in the incidence of skin cancer is of great concern.[1,2] Nearly all deaths caused by skin malignancies result from malignant melanoma. The significant mortality of this disease is caused by the high proliferation rate of melanoma cells and the early occurrence of metastases. The choice of treatment depends on the timely detection of the melanoma and any associated metastases. While positron emission tomography (PET) using 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG), an ^{18}F-radiolabeled glucose analogue, has been successfully used for melanoma imaging,[3−5] a ^{99m}Tc-labeled single-photon-emission computed tomography (SPECT) radiopharmaceutical with affinity for melanoma may provide a cost-effective means of early detection and diagnosis with widespread availability.

Previous attempts to image melanoma with radiolabeled monoclonal antibodies met with little success.[6,7] Subsequent use of simpler radiolabeled molecules, including radioiodinated amino acids[8,9] and nucleic acids,[10,11] as false precursors in the melanin formation cycle either displayed insufficient localization in tumors, resulting in low tumor-to-nontumor ratios,[8−11] or possessed poor pharmacokinetics.[8,11] More promising results were obtained recently with ^{99m}Tc-labeled α-melanotropin peptides.[12,13] Tumor-uptake and biodistribution studies with these peptide-based radioconjugates generated favorable results, indicating that these labeled peptides may be useful for in vivo melanoma scintigraphy. However, the search for nonpeptidic small molecules that possess high affinity for melanoma continues.



[^{123}I]BZA [^{123}I]IMBA

Figure 1. Iodine-123-labeled *N*-(2-diethylaminoethyl)-4-iodobenzamide ([^{123}I]BZA) and *N*-(2-diethylaminoethyl)-3-iodo-4-methoxybenzamide ([^{123}I]IMBA).

In this regard, high tumor uptake was recently obtained with ^{123}I-labeled *N*-(2-diethylaminoethyl)-4-iodobenzamide ([^{123}I]BZA) and *N*-(2-diethylaminoethyl)-3-iodo-4-methoxybenzamide ([^{123}I]IMBA).[11,12] The chemical structures of BZA and IMBA are shown in Figure 1. In vivo investigations with subcutaneously transplanted B16 melanoma cells in C57Bl6 mice showed uptake values ranging from 5% to 20% injected dose/g (ID/g) tumor.[14,15] Subsequent human clinical trials also indicated adequate uptake by melanoma and good scintigraphic images.[14,16] While recent reports suggest that the uptake is nonsaturable and may be related to the formation of melanin within the melanosome,[17] structurally similar benzamides containing a piperidinyl substituent have also been shown to possess σ-receptor affinity.[18,19] Although such radioiodinated benzamides have entered phase I clinical trials[14] for the diagnosis

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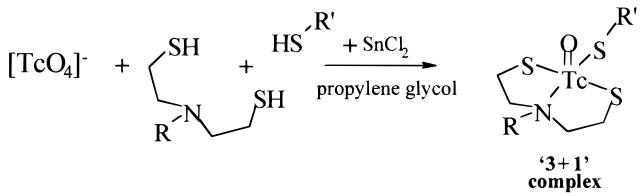
Scheme 1. Preparation and Numbering Scheme of '3+1' Mixed-Ligand Complexes



'3+1' complex	R	R'	yield(%)
1	CH_3	$(CH_2)_2N(C_2H_5)_2$	98
2	CH_3	$(CH_2)_2N(n\text{-}C_4H_9)_2$	98
3	CH_3	$(CH_2)_2N$O	92
4	$n\text{-}C_3H_7$	$(CH_2)_2N(C_4H_9)_2$	98
5	$C_6H_5\text{-}CH_2$	$(CH_2)_2N(C_2H_5)_2$	80
6	$(CH_2)_2N(C_2H_5)_2$	CH_3	60

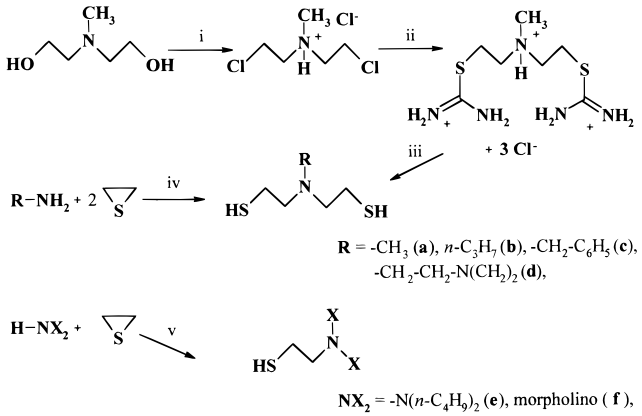
of malignant melanoma, their routine clinical use may be hampered by the associated disadvantages of iodine-123, i.e., lack of routine availability and low cost-effectiveness.

The most widely used isotope in clinical nuclear medicine, technetium-99m, possesses ideal characteristics ($t_{1/2}$ = 6.02 h, 140 keV monoenergetic γ-emission) for nuclear medicine imaging and is available on demand via the ^{99}Mo−^{99m}Tc generator system. These properties have led to the search for small technetium-99m complexes possessing high affinity for melanoma. Initial attempts to replace the radioiodinated benzamides by a series of technetium-99m complexes containing the *N*-(2-diethylaminoethyl)benzamide structural element gave melanoma-uptake values with a maximum of 1.6% ID/g tumor (1 h postinjection).[20−22] While tumor uptake was low, the values suggest that further refinement in the molecular structure might lead to a higher tumor affinity and in vivo uptake. Following this hypothesis, we envisioned that removal of the aromatic ring and concomitant integration of the oxotechnetium core with the *N*-(2-diethylaminoethyl) part of the pharmacophore might enhance the melanoma affinity of these compounds compared with the previously described complexes possessing the entire aromatic structure element.[20−22] Various *N*-alkyl-substituted (SN(R)S) [R = Me, Pr, Bn, $Et_2N(CH_2)_2$]-3-azapentane-1,5-dithiols as tridentate chelating agents and *N*-(2-dialkylamino)-ethanethiols [X = Et, Bu; NX_2 = morpholinyl] as monodentate pharmacophores were evaluated with regard to developing a small nonpeptidic technetium-99m complex with affinity for melanoma.

Results and Discussion

Chemistry. The '3+1' mixed-ligand technetium-99m complex is generally described as an entity containing a tridentate ligand and a monodentate ligand surrounding an oxotechnetium core (Scheme 1). These complexes are quite versatile in that the substitution of the monodentate ligand allows for easy manipulation of the physicochemical properties and a simple radiolabeling

Scheme 2. Preparation of Tridentate Ligands SN(Me)S (**a**) and SN(R)S [(R = *n*-C_3H_7 (**b**), CH_2-C_6H_5 (**c**), CH_2-CH_2-$N(CH_2)_2$ (**d**)] and Monodentate SNX_2 Ligands [NX_2 = $N(C_4H_9)_2$ (**e**), morpholinyl (**f**)][a]



[a] (i) $CHCl_3$, $SOCl_2$, 60 °C; (ii) $S{=}C(NH_2)_2$, 100 °C, 30 min; (iii) NaOH (diluted); (iv, v) toluene, autoclave, 25 °C/2 h, 80 °C/8 h.

Table 1. RP-HPLC Comparison of Technetium-99m Complexes with Analogous '3+1' Mixed-Ligand Rhenium Complexes

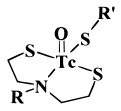
	t_R (min)			t_R (min)	
complex	Re	Tc	complex	Re	Tc
1	5.9	6.1	**4**	17.5	16.6
2	7.1	7.0	**5**	8.9	10.0
3	4.1	4.4	**6**	3.7	3.8

procedure with high radiochemical yield of the final complex.[23] Utilizing the flexibility inherent in this approach, we synthesized compounds **1**−**6** as described in Scheme 1 to obtain a series of '3+1' technetium-99m complexes.

The required tridentate ligands containing the SNS metal coordinating donor atoms were prepared as described in Scheme 2. The monodentate ligands, where X = Bu or NX_2 = morpholinyl (complexes **2**−**4),** were synthesized by autoclaving in a high-pressure reaction vessel the appropriate secondary amine with ethylene sulfide in argon-saturated toluene (Scheme 2), while *N*-(2-diethylamino)ethanethiol (monodentate ligand for complexes **1** and **5)** and methanethiol (monodentate ligand for complex **6**) were available from commercial sources.

Replacement of the benzamido portion of the BZA (Figure 1) by the metal chelate unit [TcO(SNS)(S)] was made possible by using the '3+1' approach. Complexes **1**−**6** were obtained by mixing [^{99m}Tc]pertechnetate, the tridentate ligand, and the monodentate ligand with Sn^{2+} as reducing agent and heating the reaction mixture at 45 °C for 20 min. This synthesis was based on an optimized protocol,[23] which was essential for obtaining high radiochemical yields of '3+1' technetium complexes. In a typical no-carrier-added technetium-99m synthesis, a 1:10 ratio of tridentate ligand:monodentate ligand was always maintained. The resulting technetium complexes were purified by RP-HPLC, and the radiochemical yields ranged between 60% and 98% (Scheme 2). Since the final concentration of the technetium-99m complexes was generally in the nanomole to picomole range, the identification of these complexes was based on chromatographic comparisons with previously characterized analogous nonradioactive rhenium complexes (Table 1).[24]

Table 2. pK_a and Lipophilicity Data for Prepared Compounds



'3+1' complex	R	R′	pK_a	$D_{(pH\ 7.4)}$	log $D_{(pH\ 7.4)}$	P	log P
1	$-CH_3$	$-(CH_2)_2$-N$(C_2H_5)_2$	9.7	1.0	0	47.0	1.7
2	$-CH_3$	$-(CH_2)_2$-N$(C_4H_9)_2$	9.9	10.0	1.0	3210	3.5
3	$-CH_3$	$-(CH_2)_2$-N-morpholino	7.2	9.0	1.0	14.0	1.2
4	$-C_3H_7$	$-(CH_2)_2$-N$(C_4H_9)_2$	9.9	14.0	1.1	4987	3.7
5	$-CH_2$-C_6H_5	$-(CH_2)_2$-N$(C_2H_5)_2$	9.6	9.0	0.95	4468	3.6
6	$-(CH_2)_2$-N$(C_2H_5)_2$	$-CH_3$	8.3	43.0	1.6	530	2.7

Lipophilicity and pK_a. Since lipid partitioning and transport of the complexes across cell membranes are governed not only by lipophilicity of the complex but also by basicity of the pendant tertiary amine, which can be protonated at physiologic pH, measurements were made to determine partition coefficients of the unprotonated free amine (log P) as well as the partition coefficient at physiologic pH (log $D_{(pH\ 7.4)}$).[25,26] This provides a composite parameter that is a function of both lipophilicity and pK_a. The pK_a of complexes **1**−**6** were also determined using HPLC techniques[25,26] and are presented in Table 2.

As complex **1** contains the *N*,*N*′-diethylamino group, the observed pK_a of 9.7 is consistent with >98% of the complex being protonated at the physiologic pH of 7.4 and the observed log $D_{(pH\ 7.4)}$ value of 0. The presence of a more lipophilic *N*,*N*′-dibutylamino group in complex **2** leads to a slightly higher pK_a (9.9) and a significantly higher log $D_{(pH\ 7.4)}$ (1.0). The replacement of the dialkylamine moiety by a morpholino group in complex **3** decreases the pK_a of the complex to 7.2 but maintains a log $D_{(pH\ 7.4)}$ comparable to that of **2**. Thus, roughly 50% of complex **3** is unprotonated and neutral at pH 7.4.

To maintain the pK_a of **2** while altering the lipophilicity (log $D_{(pH\ 7.4)}$) of the complex, compound **4** was synthesized, which carries the same monodentate ligand as **2** but a more lipophilic substituent in the tridentate part of the molecule, thus yielding a pK_a of 9.9 and a log $D_{(pH\ 7.4)}$ of 1.1. In an analogous manner, the pK_a of complex **5** (9.6) is very similar to that of complex **1,** but due to the introduction of a benzyl group in the tridentate portion, **5** is more lipophilic than **1** and comparable with **2** and **3**. Additional modification of the pK_a and lipophilicity has been achieved by altering the position of the disubstituted amino group from the monodentate portion of the complex to the tridentate side as in complex **6**. Here the pK_a of the disubstituted amine is tempered by being attached at the β-position to the metal-coordinating nitrogen in the tridentate ligand. This results in both a lower lipophilicity (log P) and a lower pK_a (8.3) relative to **1** and **5**. The lowering of the pK_a consequently results in a significantly higher log $D_{(pH\ 7.4)}$ as less of the amine is protonated.

Melanoma-Uptake Studies. To evaluate whether the complexes possess melanoma affinity, initial in vivo tumor-uptake studies of the technetium-99m complexes were performed in the murine C57Bl6/B16 mouse tumor model.[15] The tumor uptake of complexes **1**−**6** (Figure 2) and the biodistribution of these complexes (Table 3) are calculated as percentage injected dose per gram (% ID/g). In addition, the melanoma/organ ratios are also

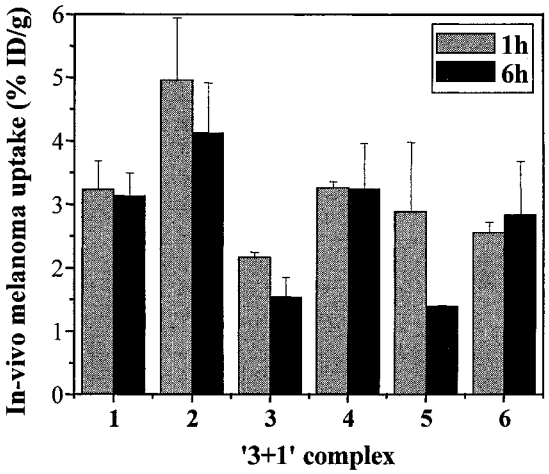


Figure 2. In vivo melanoma uptake (% ID/g) of complexes **1**−**6** at 1 and 6 h postinjection in C57/B16 mice with palpable hind limb B16 melanoma nodules.

summarized for organs of special interest, including the lung, liver, and spleen, since these are the likely sites for the occurrence of metastases.

Compound **1** shows a 1-h tumor uptake of 3.2% ID/g and a very slow washout from the tumor (0.1%) between 1 and 6 h postinjection (pi). Although uptake in the spleen and lung approximates that in the tumor at 1 h, washout from these organs relative to the tumor is faster and results in a tumor/spleen ratio of 4.5 and a tumor/lung ratio of 1.6 at 6 h pi.

The highest melanoma uptake of 5.0% ID/g is attained with compound **2** at 1 h pi. Even at this time point, compared with complex **1,** a high tumor/blood ratio (3.9), a significantly higher tumor/spleen ratio (4.3), and a slightly higher tumor/lung ratio (1.9) are observed. Over 6 h the tumor/blood ratio increases to 4.2 and the tumor/lung ratio to 3.1; however, the melanoma/spleen ratio drops to 3.2. Although there is a significantly faster washout from the tumor over 6 h (0.8%) compared with **1**, the tumor uptake remains high relative to nontarget organs.

Complexes **3**−**6** all display lower tumor uptake and lower tumor/nontumor ratios with no significant improvement over a 6-h period relative to complex **2** (Table 3). Due to the lipophilic nature of these complexes, the liver uptake of all compounds investigated is in the range of 7% (**6**) to 43% (**2**).

To elucidate some of the parameters necessary for melanoma targeting of these '3+1' complexes, correlation of tumor uptake with molecular parameters, including lipophilicity, pK_a, and position of the pharma-

Table 3. Biodistribution and Tumor/Nontumor Ratios of Compounds **1**−**6** at 1 and 6 h Postinjection

	1		**2**		**3**		**4**		**5**		**6**	
organ	1 h[a]	6 h	1 h	6 h	1 h	6 h	1 h	6 h	1 h	6 h	1 h	6 h
blood	2.08[b]	1.35	1.26	0.98	2.36	1.44	1.34	1.44	3.28	1.30	3.49	2.05
	(0.41)[c]	(0.23)	(0.33)	(0.54)	(0.52)	(0.32)	(0.41)	(0.24)	(0.57)	(0.33)	(0.93)	(0.30)
heart	1.84	0.84	0.66	0.46	1.26	0.83	1.17	0.73	2.09	0.86	1.74	1.15
	(1.11)	(0.11)	(0.06)	(0.12)	(0.21)	(0.09)	(0.05)	(0.08)	(0.41)	(0.07)	(0.25)	(0.23)
lung	2.73	1.96	2.60	1.35	5.75	2.91	3.27	2.07	13.2	5.50	5.73	3.40
	(1.32)	(0.31)	(0.32)	(0.52)	(0.75)	(0.36)	(0.25)	(0.38)	(2.46)	(0.48)	(0.59)	(0.50)
spleen	3.44	0.70	1.15	1.30	4.02	1.53	3.35	1.25	6.04	1.44	2.39	1.66
	(1.04)	(0.27)	(0.08)	(1.00)	(1.22)	(0.21)	(1.34)	(0.51)	(1.47)	(0.73)	(0.22)	(0.21)
liver	27.3	18.7	43.3	23.0	19.4	11.3	35.3	17.7	16.6	7.23	7.11	6.10
	(3.12)	(2.72)	(4.00)	(5.80)	(2.37)	(1.74)	(3.65)	(3.46)	(3.49)	(1.18)	(0.49)	(1.10)
kidney	17.9	17.9	30.4	19.3	16.0	13.3	15.3	11.7	24.0	12.9	13.0	10.1
	(1.40)	(1.60)	(2.24)	(4.56)	(0.79)	(1.43)	(2.33)	(2.20)	(1.80)	(0.15)	(1.08)	(1.59)
muscle	0.57	0.39	0.24	0.20	0.64	0.26	0.53	0.26	0.57	0.26	0.78	0.39
	(0.02)	(0.10)	(0.04)	(0.07)	(0.10)	(0.01)	(0.07)	(0.04)	(0.08)	(0.02)	(0.21)	(0.11)
brain	0.18	0.14	0.08	0.07	0.20	0.12	0.13	0.08	0.32	0.13	0.24	0.15
	(0.08)	(0.10)	(0.01)	(0.01)	(0.11)	(0.02)	(0.01)	(0.01)	(0.06)	(0.03)	(0.05)	(0.01)
melanoma	3.23	3.13	4.95	4.12	2.16	1.53	3.26	3.23	2.88	1.39	2.55	2.83
	(0.46)	(0.37)	(1.00)	(0.80)	(0.09)	(0.32)	(0.10)	(0.74)	(1.10)	(0.02)	(0.17)	(0.85)
melanoma/blood	1.6	2.3	3.9	4.2	<1	1.1	2.4	2.2	<1	1.1	<1	1.4
melanoma/spleen	<1	4.5	4.3	3.2	<1	1.0	<1	2.6	<1	<1	1.1	1.7
melanoma/lung	1.2	1.6	1.9	3.1	<1	<1	1.0	1.6	<1	<1	<1	<1
melanoma/liver	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1

[a] $n = 3$ animals per time point. [b] Values represent % ID/g wet tissue. [c] Standard deviation in parentheses.

Table 4. Molecular Mass and MS and ^{1}H NMR Data for Prepared Ligands

ligand	MW (g/mol)	MS data[a] m/z $[M + H]^+$	^{1}H NMR δ
SN(Me)S (**a**)	151	152	2.83−2.84 (s, 1H, *H*S-CH$_2$-), 2.84−2.87 (m, 2H, -S-C*H*$_2$-), 2.86−2.87 (s, 3H, N-C*H*$_3$), 3.30−3.35 (m, 2H, N-C*H*$_2$-CH$_2$-)
SN(Pr)S (**b**)	179	180	0.84−0.87 (t, 3H, -CH$_2$-C*H*$_3$), 1.39−1.46 (sext, 2H, -CH$_2$-C*H*$_2$-CH$_3$), 1.68−1.73 (s, 2H, *H*S-CH$_2$-), 2.34−2.37 (t, 2H, CH$_3$-CH$_2$-C*H*$_2$-N), 2.53−2.56 (t, 4H, N-CH$_2$-C*H*$_2$-S), 2.58−2.61 (t, 4H, N-C*H*$_2$-CH$_2$-S)
SN(Bn)S (**c**)	227	228	2.57−2.62 (m, 4H, S-C*H*$_2$-CH$_2$-), 2.68−2.74 (m, 4H, N-C*H*$_2$-CH$_2$-), 3.61−3.64 (s, 2H, aryl-C*H*$_2$-N), 7.30−7.35 (m, 5H, aryl)
SN(NR$_2$)S (**d**)	212	213	1.02 (t, -CH$_2$-C*H*$_3$(ethyl)), 1.87 (s, SH), 2.6 (m, (-C*H*$_2$)$_2$ + -C*H*$_2$-CH$_3$(ethyl))[29]
SNBu$_2$ (**e**)	189	190	0.84−0.92 (t, 6H, -(CH$_2$-C*H*$_3$)$_2$), 1.28−1.38 (sext, 2H, -CH$_2$-C*H*$_2$-CH$_3$), 1.55−1.64 (m, 2H, -CH$_2$-C*H*$_2$-CH$_2$-CH$_3$), 2.74−2.77 (m, 2H, -S-C*H*$_2$-CH$_2$), 3.30−3.80 (m, 4H, -N(C*H*$_2$)$_2$-CH$_2$), 3.22−3.26 (m, 2H, -CH$_2$-C*H*$_2$-NR$_2$)
S-morpholinyl (**f**)	147	148	1.73−1.84 (s, 1H, *H*S-CH$_2$-), 2.41−2.48 (m, 4H, -N<(C*H*$_2$-CH$_2$)$_2$>O), 2.53−2.58 (m, 2H, HS-C*H*$_2$-CH$_2$-), 2.59−2.66 (m, 2H, -S-CH$_2$-C*H*$_2$-N<), 3.67−3.73 (m, 4H, -N<(CH$_2$-C*H*$_2$)$_2$>O)

[a] Ionization technique: fast atom bombardment (FAB) in glycerol matrix and positive polarity.

cophore, was undertaken. Complexes **2** and **4** both contain the same amine-bearing pharmacophore in the monodentate ligand portion of the complex. This results in both complexes having identical pK_a values with differing lipophilicity. If there is a simple correlation between tumor uptake and lipophilicity, the slightly more lipophilic complex **4** should have a slightly higher or comparable accumulation. In fact, a lower melanoma uptake is observed for **4**. On the other hand, compound **5** also displays a lower melanoma uptake compared with complex **2**, even though the log $D_{(pH\ 7.4)}$ for both is equivalent. Thus, a simple correlation between melanoma uptake and lipophilicity (log $D_{(pH\ 7.4)}$) does not seem to exist. This is consistent with earlier reports on a series of radioiodinated benzamide derivatives.[14] The presence of a β-oxygen atom in the cyclic amine (**3**) or a variation of the pharmacophore position in the molecule (**6**) leads to a less basic tertiary amine nitrogen and results in decreased melanoma uptake. Thus a more constrained molecule caused by either the cyclic amine (**3**) or by positioning on the tridentate ligand (**6**) and a pK_a lower than 9.7 cause decreased melanoma uptake in this class of compounds.

As mentioned above, the highest melanoma uptake is obtained with compound **2**. Unlike compound **1**, this complex possesses a more basic amine in the side chain and a higher lipophilicity caused by the *N*-dibutyl group. A further increase in lipophilicity, however, does not improve in vivo tumor uptake (see **4,** Tables 2 and 3). Although it is not clear what other parameters or structural features may affect the in vivo tumor uptake, complex **2** possesses a basic amine group with a pK_a of 9.9 and a stronger H-bond donor character at physiologic pH which seem to favor high in vivo tumor uptake.

In Vitro Tumor-Uptake Studies. To gain a better understanding of the tumor-uptake mechanisms, compounds **1** and **2** were further examined in vitro in the B16 melanoma and in an amelanotic C3H 10T1/2 fibroblast cell line. The B16 melanoma tumor-cell-uptake kinetics of compound **2** at 37 °C reveal that the complex is readily taken up and 68% of the complex is cell-associated in 60 min (Figure 3a). Consistent with the in vivo results, compound **1** displays a significantly lower uptake at 37 °C with 38% total uptake in 60 min (Figure 3c). To differentiate the active and passive components of the uptake, experiments were performed at 4 °C. Under these conditions a 65% reduction in the total cell uptake of **1** (Figure 3c) and a 30% reduction in that of **2** (Figure 3a) were observed. To confirm that the reduced cellular uptake at 4 °C was due to reduced

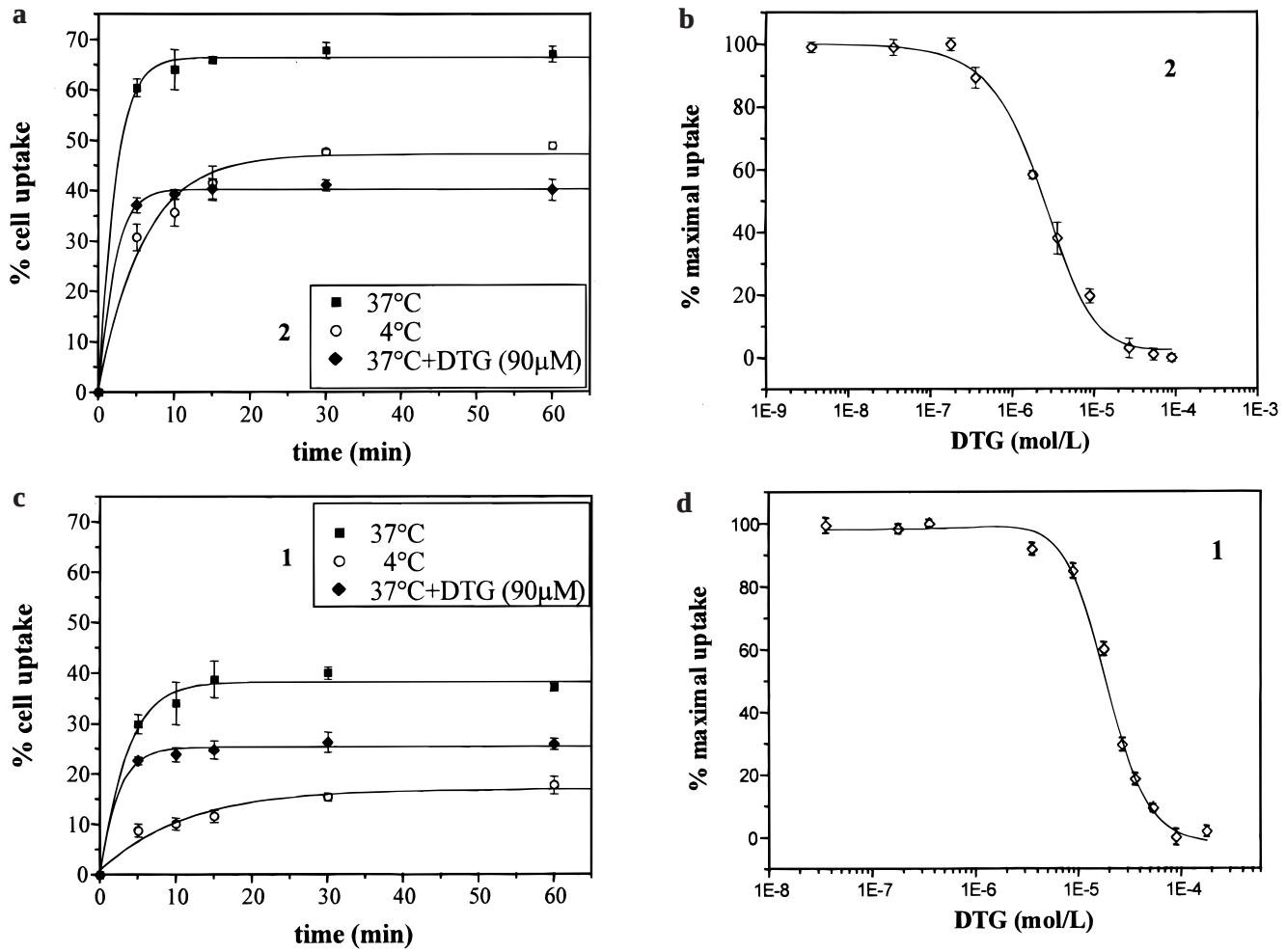


Figure 3. In vitro cell uptake in B16 murine melanoma (5×10^6 cells/mL) at 37, 4, and 37 °C with DTG (90 μM) of compounds **1** and **2**: (a) uptake kinetics of **2**, (b) dose-dependent uptake inhibition of **2** at 37 °C with DTG, (c) uptake kinetics of **1**, (d) dose-dependent uptake inhibition of **1** at 37 °C with DTG.

metabolism and not cell death, following a 4 °C (60 min) cell-uptake experiment, the cells were reincubated at 37 °C (60 min). This restored the uptake to the original values obtained at 37 °C (data not shown), thus indicating a significant active component of the total tumor-cell uptake. This active component is consistent with the lipophilicity measurements and indicates a greater nonspecific uptake component in **2** and, conversely, a greater active uptake component for the less lipophilic **1**. Similar cellular uptake kinetic studies for complexes **1** and **2** in rapidly dividing, non-melanoma murine C3H 10T1/2 fibroblasts at 37 and 4 °C displayed significantly less accumulation, with a maximum uptake of only 15−18% at 60 min (data not shown) compared with 68% (for **2**) and 37% (for **1**) in the B16 melanoma cells.

Although the exact nature and mechanism of uptake are not known for these complexes, the reduced uptake at 4 °C may be related to a decreased incorporation of these complexes in melanin synthesis as has been postulated for the parent iodinated benzamides.[17] Since structural analogues of the parent benzamides have been shown previously to possess σ-receptor affinity,[18,19] in vitro tumor-uptake studies of these technetium-99m complexes were performed in the presence of either *N*-(2-diethylaminoethyl)-4-iodobenzamide (BZA) or the structurally different 1,3-di-*o*-tolylguanidine (DTG), both known σ-ligands with differing σ-affinity ($K_i = 2.1$ and 28 nM, respectively).[18] A 30-min pretreatment of the tumor cells with either DTG or BZA (90 μM), followed by a 60-min incubation at 37 °C with the technetium-99m complexes, demonstrates a 43% and 44% reduction in the tumor-cell uptake of complexes **1** and **2**, respectively (Figure 3c,a). Consistent with the σ-receptor affinity of the inhibitors BZA and DTG, in vitro dose−response experiments at 37 °C also show a 50% inhibition of the specific cellular uptake of complex **1** at 18.50 μM DTG (Figure 3d) and complex **2** at 2.50 μM DTG (Figure 3b) in B16 cells. Likewise, BZA, a higher-affinity σ-receptor ligand of a different structural class, causes a 50% reduction of the cellular uptake of **1** at 1.57 μM and **2** at 1.32 μM (data not shown).

Inhibition experiments with either DTG or BZA performed at 4 °C indicate no dose-dependent decrease in the cell uptake of compounds **1** and **2**. Since a lower temperature should not decrease the binding of the technetium-99m complexes to the cell surface σ-receptor, given the lipophilic nature of these complexes, it is possible that specific σ-receptor binding may be a very small fraction of the total binding at 4 °C and, therefore, not observable in this whole-cell assay.

While binding to the σ-receptor by these technetium complexes may contribute to cellular accumulation and cannot be excluded, an alternative mechanism may involve the inhibitory effect of σ-ligands on cell growth

and proliferation[27,28] and thus indirectly affect the active uptake observed for these complexes.

Conclusions

'3+1' Mixed-ligand technetium-99m complexes with significant affinity for malignant melanoma were synthesized by incorporating into the monodentate portion of the complex *N*-(2-dialkylaminoalkyl) fragments that are known to contribute to the melanoma affinity of radioiodinated benzamides.[15] The effect of pK_a, log $D_{(pH\ 7.4)}$, and log *P* on the melanoma uptake of this series of complexes indicates that, although there is no correlation between uptake and lipophilicity (log *P*) of the '3+1' technetium-99m complexes, a pK_a of 9.9 and a log $D_{(pH\ 7.4)}$ of 1 may be optimum for in vivo melanoma uptake of these complexes. The high uptake of compound **2** compared with other small benzamide-containing, nonpeptidic technetium-99m complexes[20−22] indicates that such integrated complexes can lead to higher melanoma uptake and lends credence to the development of such SPECT imaging agents for the early diagnosis of malignant melanoma.

Experimental Section

All chemicals used were of analytical grade and were obtained from Sigma, Aldrich, or Fluka. Yields displayed are values obtained without attempts at optimization. Mass spectra were recorded on a MS device FINNIGAN MAT-95 using the "fast atom bombardment" (FAB) ionization technique. 400-MHz ^{1}H NMR measurements were carried out on a VARIO-400 device (Varian). Elemental analyses were performed on a LECO-CHNS-932 elemental analyzer. *N*-(2-Diethylamino)ethanethiol (**g**) and sodium methanethiolate (**h**), the monodentate ligands for compounds **1**, **5,** and **6,** were obtained from Fluka, and *N*-(2-diethylaminoethyl)-4-iodobenzamide was synthesized according to the literature.[18] Phosphate buffer (PB), 0.01 M, pH 7.4, contained Na_2HPO_4 and KH_2PO_4, while phosphate-buffered saline (PBS) contained 0.01 M PB (pH 7.4) with 0.12 M NaCl and 0.0027 M KCl.

All complexes were purified by HPLC using a Perkin-Elmer device consisting of a Turbo LC System with a quaternary pump (series 200 LC Pump), a programmable UV/VIS detector model 785A, and a homemade γ-detector (Bohrloch NaI(Tl) crystal) connected in series with the UV detector. All HPLC's were performed using a Hypersil-ODS column (250 × 8 mm, 10 μm; Knauer) and run under isocratic conditions with a 80:20 solvent mixture of methanol/phosphate buffer at a flow rate of 2 mL/min as the mobile phase. The column effluent was monitored simultaneously by both UV (254 nm) and γ-detection. The identity of the technetium-99m complexes was established by HPLC comparison with previously characterized analogous rhenium compounds (Table 1).[24]

Tris-Chelating Ligands. *N*-Methyl-3-azapentane-1,5-dithiol (a) (Complexes 1−3). *N*-Methyl-2,2′-aminodiethanol (12.000 g, 0.10 mol) was first converted to the hydrochloride salt by dissolving it in 30 mL of trichloromethane and treating it with HCl-saturated diethyl ether. The hydrochloride salt was then dissolved in 30 mL of trichloromethane, and thionyl chloride (17.800 g, 0.15 mol) was slowly added while stirring under nitrogen at room temperature. After the reaction mixture turned yellow and became homogeneous, an additional volume of thionyl chloride (17.800 g, 0.15 mol) was added and the reaction mixture was refluxed for 2 h. After cooling the mixture to room temperature and removing excess thionyl chloride by evaporation, the precipitated bis(2-chloroethyl)-methylamine hydrochloride was isolated via filtration and dried under vacuum: yield 19.260 g, 99%.

A mixture of bis(2-chloroethyl)methylamine hydrochloride (9.677 g, 50.40 mmol) and thiourea (8.252 g, 0.106 mol) was refluxed in 35 mL of ethanol for 5 h. After cooling the mixture to room temperature, the solution was allowed to stand at room temperature for 3−4 days whereupon the precipitated oil began to crystallize. Addition of methyl *tert*-butyl ether completed the crystallization and the isothiouronium salt was isolated by filtration of the precipitate followed by vacuum-drying over P_2O_5: yield 15.479 g, 88%.

The isothiouronium salt (6.810 g, 30.0 mmol) was dissolved in 20 mL of water, sodium hydroxide (3.600 g, 90.0 mmol) was added, and the reaction mixture was heated briefly at 100 °C for 3−5 min. The solution was quickly cooled to 20 °C in an ice bath and extracted with 3 × 10 mL of diethyl ether. The dried ether extract ($MgSO_4$) was filtered and reduced in volume to a pale yellow oil. The pure product was obtained as a colorless oil by subsequent distillation (Kugelrohr): yield 2.050 g, 13.6 mmol, 45%.

2-[(2-Mercaptoethyl)propylamino]ethanethiol (b), 2-[Benzyl(2-mercaptoethyl)amino]ethanethiol (c), and 2-[(2-Diethylaminoethyl)(2-mercaptoethyl)amino]ethanethiol (d) (Complexes 4−6). The appropriate primary amine (*n*-propylamine, benzylamine, *N*-(2-diethylamino)ethyleneamine) (41.0 mmol) was dissolved in 80 mL of toluene and saturated with dry argon in an autoclave bottle. To this solution was added ethylene sulfide (5.850 g, 80.0 mmol) dropwise while stirring under argon. The reaction mixture was tightly sealed and stirred for 2 h at room temperature followed by heating at 80 °C for 8 h. The slightly yellowish solution was filtered to remove a small amount of a solid white impurity. The filtrate was then concentrated to a pale yellow oil by evaporation and subsequently purified by vacuum distillation: yield (b) 6.444 g (88%), (c) 5,770 g (62%), (d) 5.737 g (66%).

Monodentate Ligands. *N*-(2-Dibutylamino)ethanethiol (e) and 2-Morpholin-4-ylethanethiol (f) (Complexes 2−4). The procedure described above was used with the following stoichiometric equivalents of the reactants: 16.9 mmol of secondary dibutylamine or morpholine and 16.50 mmol of ethylene sulfide: yield (e) 1.933 g (62%), (f) 2.183 g (90%).

'3+1' Rhenium Complexes. These were synthesized and characterized by procedures described in detail elsewhere.[24]

General Procedure for the Synthesis of '3+1' Technetium-99m Complexes (1−6). The complexes were prepared by mixing [^{99m}Tc]pertechnetate (13.5−27 mCi in 0.5−1.0 mL of saline), propylene glycol (400 μL), the appropriate monodentate ligand (**e**−**h**) (∼0.5 mg in 100 μL of methanol) and the appropriate tridentate ligand (**a**−**d**) (∼0.05 mg in 100 μL of methanol) with 20 μL of stannous chloride solution (1.0−2.0 mg $SnCl_2$ dissolved in 5 mL of 0.1 N HCl) and heating the reaction mixture at 45 °C for 20 min. After cooling to room temperature the radio-metalated complexes were purified by HPLC (vide supra). Yields generally ranged from 60% to 98% (Scheme 1).

Determination of Lipophilicity and pK_a Values. The lipophilicity and pK_a values of all complexes were determined using HPLC methods described earlier.[25,26] The log *P*, log $D_{(pH\ 7.4)}$ and pK_a values were determined on a Perkin-Elmer HPLC system 1020 using a PRP-1 (250 × 4.1 mm, 10 μm; Hamilton) reversed-phase column run under isocratic conditions with a flow rate of 1.5 mL/min at room temperature. The mobile phase was acetonitrile:phosphate buffer (0.01 M), 3:1, and pK_a's were determined between 3 and 11.[25,26]

The pK_{HPLC} values were obtained as the fitted points of inflection from the sigmoidal log D_{HPLC}/pH profiles. The aqueous ionization constants pK_a were calculated from the pK_{HPLC} values after correction with a predetermined correction factor obtained using standard amine compounds.[25,26] Log *P* values of the neutral complexes were estimated from the respective upper plateau of the sigmoidal log *D*/pH curve in the alkaline range.

Animal Studies. All animal experiments were performed in compliance with the *Principles of Laboratory Animal Care* (NIH Publication #85-23, revised 1985). Biodistribution studies and tumor-uptake measurements were performed in C57B16 mice (15−20 g) bearing the B16 murine melanoma on the hind limb.[14−17] The tumor cells (B16/F0), obtained from the German Cancer Research Centre (Heidelberg), were washed with PBS and transplanted subcutaneously on the left hind flank by an

inoculation of 0.5×10^6 cells (0.1 mL). Ten to 14 days later the animals developed palpable tumor nodules 3−5 mm in diameter. The biodistribution studies were carried out by tail-vein injection of 25−30 μCi (0.05−0.1 mL) of the ^{99m}Tc-labeled '3+1' derivative. At designated times postinjection, the animals were weighed and sacrificed. The harvested organs and tumors were blotted dry when appropriate, weighed, and counted in a γ-counter along with technetium-99m standards of the injected dose. The results are expressed as % ID/g tissue (Figure 2, Table 3).

In Vitro Cell Studies. Murine B16/F0 melanoma cells (ATCC) were grown in T-75 flasks in 14 mL of Dulbecco's modified Eagle medium (D-MEM; Gibco, Life Technology, Gaithersburg, MD) containing 4500 mg/L D-glucose, L-glutamine, and pyridoxine hydrochloride, 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), 0.2% gentamicin and 0.5% penicillin−streptomycin solution as the cell culture medium. C3H 10T1/2 fibroblast cells (ATCC) were cultivated in T-75 flasks in Basal medium Eagle (BME; Gibco) supplemented with 10% defined fetal bovine serum (Hyclone, cat. no. SH30070, matched lots AGM7413 and AFC5040) and 25 μg/mL gentamicin. All cells were harvested from cell culture flasks by trypsinization with 1 mL trypsin−EDTA solution (0.25% trypsin, 1 mM EDTA·4Na) (Gibco). After being washed with 12 mL of Dulbecco's phosphate-buffered saline (PBS) (Gibco), pH 7.2 (Ca^{2+}- and Mg^{2+}-free; g/L KCl, 0.20; KH_2PO_4, 0.20; NaCl, 8.00; Na_2HPO_4, 1.15), the cells were counted and resuspended in 8 mL of S-MEM (Gibco) (Ca^{2+}-free, with reduced Mg^{2+}content) and stored at 4 °C until use.

For in vitro tumor-cell-accumulation studies, 5×10^6 cells were incubated at 37 or 4 °C in polypropylene test tubes with intermittent agitation with 1−2 μCi (5 μL) technetium-99m complex (**1**−**6**) in a total volume of 350 μL of S-MEM. At appropriate time intervals the tubes were vortexed and 8-μL samples were layered on 350 μL of cold FBS in a 400-μL Eppendorf microcentrifuge tube. After centrifugation at 15000 rpm for 2 min, the tubes were frozen in a dry ice−acetone bath. While still frozen, the bottom tip of the microcentrifuge tube containing the cell pellet was cut and placed in a counting tube. The remaining portion of the tube with the supernatant was placed in a separate counting tube. Both fractions were counted for radioactivity in a γ-counter (Wallac, 1480 WIZARD 3″). The amount of supernatant in the cell pellet was determined to be <1% in separate experiments. The percentage cell uptake of the technetium-99m complex was calculated as:

$$\% \text{ uptake} = [\text{cpm (pellet)}]/[\text{cpm (pellet)} + \text{cpm (supernatant)}] \times 100$$

The effect of the inhibitors (DTG, BZA) on cell uptake of these complexes was studied by addition of the inhibitors at various concentrations to the cell suspension 30 min prior to addition of the ^{99m}Tc complexes. Fresh DTG stock solutions were made by dissolving DTG (3.0 mg, 12.5 μmol) in 0.38 mL of PBS and 0.12 mL of hydrochloric acid (0.1 N) and subjecting the mixture to ultrasound until a clear solution was obtained, followed by the addition of 0.50 mL of FBS to produce a neutral solution at pH 7.4. The stock solution of *N*-(2-diethylaminoethyl)-4-iodobenzamide hydrochloride (BZA) (4.8 mg, 12.5 μmol/mL) was made as above without the adddition of hydrochloric acid. The stock solutions were diluted by an appropriate amount of S-MEM, and aliquots between 5 and 25 μL were added to the cell suspension such that the final concentration of the inhibitors was between 0.02 and 120 μM in a total 350-μL cell suspension volume.

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