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ScienceDirect
Nuclear Medicine and Biology 37 (2010) 255–264
NUCLEAR MEDICINE — AND — BIOLOGY
www.elsevier.com/locate/nucmedbio

Preparation and biological evaluation of cyclopentadienyl-based ^{99m}Tc-complexes [(Cp-R)^{99m}Tc(CO)$_3$] mimicking benzamides for malignant melanoma targeting☆

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Received 26 October 2009; received in revised form 11 November 2009; accepted 25 November 2009

Abstract

The biological evaluation of half-sandwich ^{99m}Tc-complexes that surrogate iodobenzamide with a high affinity for melanin tumor tissue is described. We have synthesized via retro Diels–Alder reaction two models of ^{99m}Tc complexes which possess the piano stool [Cp^{99m}Tc(CO)$_3$] motif instead of a phenyl ring as in the original iodobenzamide ^{123}I-*N*-(*N*-benzylpiperidin-4-yl)-2-iodobenzamide (2-IBP) and *N*-(2-diethylaminoethyl)-4-iodobenzamide (BZA). Diels–Alder products **2a**–**b** (HCp-CONHR)$_2$ (**2a**, R=2-diethylaminoethyl; **2b**, R=benzylpiperidin-4-yl) were prepared and reacted with *fac*-[^{99m}Tc(H$_2$O)$_3$(CO)$_3$)]$^+$ **1** in water to produce the corresponding ^{99m}Tc complexes [(**2a**)^{99m}Tc(CO)$_3$)] **4a** and [(**2b**)^{99m}Tc(CO)$_3$)] **4b**. The structures of the ^{99m}Tc complexes on the no-carrier-added level have been confirmed by chromatographic comparison with the corresponding rhenium complexes **3a** and **3b**, macroscopically characterized by IR, NMR, ESI-MS and X-ray crystallography for **3a** [triclinic, P-1, *a*=7.3518(1) Å, *b*=8.0309(2) Å, *c*=17.5536(3) Å, α=99.1260(5)°, β=90.4215(14)°, γ=117.0187(11)°]. The radioconjugate **4b** showed good in vitro stability. In murine melanoma B16F1 cells, significant cellular uptake (43.9% of the total applied activity) was attained after 4 h at 37°C with about 50% of the cell-associated radioactivity being internalized in the cells (22% of the applied activity). Furthermore, in melanoma-bearing C57BL6 mice, tumor uptake values of 3.39±0.50 %ID g^{-1} and 3.21±0.26 %ID g^{-1} at 1 and 4 h postinjection, respectively, were observed indicating a good retention of **4b** in the tumor.

Keywords: Bioorganometallic Chemistry; Melanoma; Tumor uptake; Technetium; Radiopharmaceuticals; Labelling; Retro Diels–Alder

1. Introduction

Melanoma is a cutaneous neoplasm known for its high aggressiveness, its early dissemination of metastases and its poor prognosis once metastasized. Surgical treatment at early stages of melanoma is usually curative. However, patients with distant metastases have limited options since treatment would only be possible by accurate surgical removal of the primary tumor and all metastases [1]. Thus, early diagnosis and accurate follow-up decrease the mortality and provide the best chance for optimal clinical management. For this reason, the development of an effective radiopharmaceutical with melanoma affinity is of primary importance. A key feature of melanoma tumors is the extensive pigmentation (melanin) present in most melanoma tumor cells, thus making it a very attractive target for both diagnosis and treatment. Agents with melanin affinity such as monoclonal antibodies [2,3], nucleic acids [4,5] or small radioiodinated amino acids [6,7] have been evaluated with limited success.

A different class of radiopharmaceutical promising the scintigraphic detection of melanoma is radioiodinated benzamides. Among those, the iodobenzamides ^{123}I-*N*-(2-diethylaminoethyl)4-iodobenzamide (^{123}I-BZA) [8,9] and (*N*-benzylpiperidine-4-yl)-2-iodobenzamide (2-IBP) [10] displayed good tumor uptake. ^{123}I-BZA has been evaluated successfully in melanoma patients, resulting in excellent detection of melanoma and its metastases with high

☆ This work was supported by the Swiss Federal Secretariat for Research and Education under Contract No. SBF C06.0109 and by the University of Zurich.

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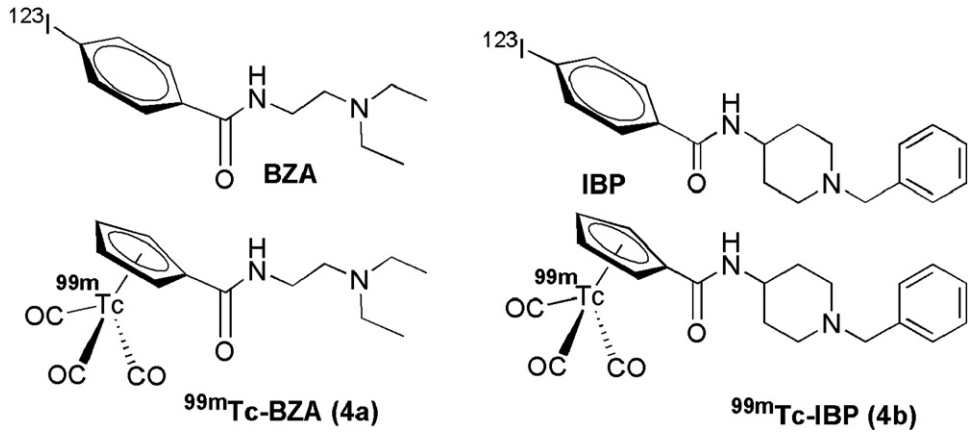
doi:10.1016/j.nucmedbio.2009.11.006



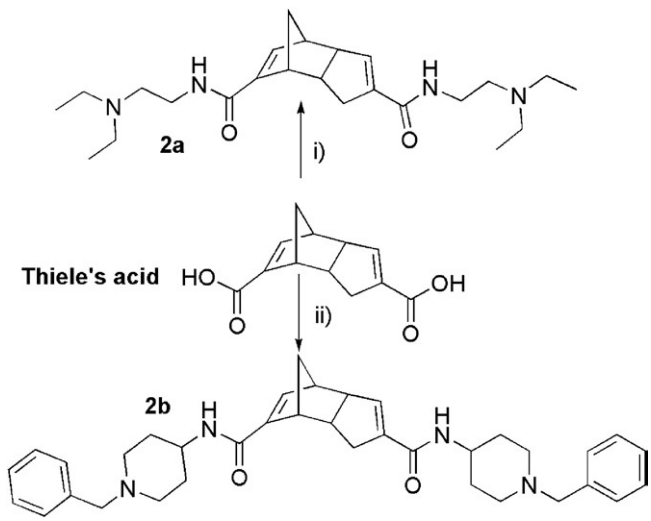
Scheme 1. Basic structures of the organic lead compounds BZA and IBP (upper row) and the corresponding [Cp^{99m}Tc(CO)$_3$] mimics (lower row).

sensitivity and selectivity [8]. The tumor uptake correlated with the high affinity to melanin, and BZA was found to localize in melanosomes, specific organelles of melanin synthesis and storage [11–13]. IBP was evaluated preclinically as well for melanoma detection. For IBP, tumor uptake was mediated by a σ receptor-based mechanism. In fact, benzamide ligands have been known to possess high affinity for the σ receptor which is expressed on various tumor cell types including melanoma [14,15].

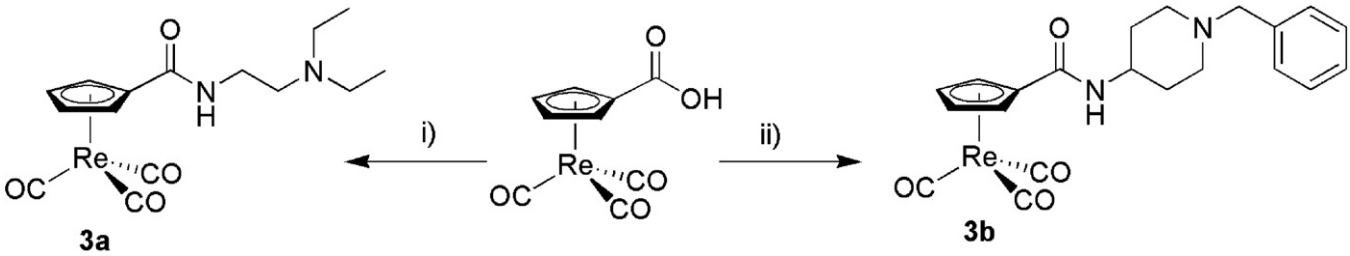
^{99m}Tc is the most widely used isotope in nuclear medicine [16–18], and the rising number of SPECT/CT γ-cameras and/or the advent of semiconductor cameras should attract even more interest. The most common approach for designing a target-specific ^{99m}Tc radiopharmaceutical is the conjugation of a complex to, e.g., carbohydrates [19], amino acids [20] or peptides [21–23]. The labeling of benzamide derivatives with ^{99m}Tc and their application to melanoma targeting are still limited to a few examples. These studies of Eisenhut et al. [24–26] applied the [^{99m}Tc=O]$^{3+}$ complexes with the N_2S_2 chelates conjugated to *N*-dialkylaminoalkyl groups and exhibited high tumor uptake [7.62 percent injected dose per gram (%ID g^{-1}) at 1 h postinjection (pi)] [24–26].

Clinical diagnostic agents have to be prepared in water which makes the use of sensitive organometallic ligand such as cyclopentadienyl (Cp) a challenging task. Despite these constraints, the Cp ligand is very attractive since it is small, has a low molecular weight and forms stable half-sandwich complexes [(Cp-R)M(CO)$_3$] (M=^{99m}Tc, Re). These inherent features are crucial advantages for minimizing sterical interference with the receptor binding part. In fact, previous studies in bioorganometallic chemistry showed that the piano-stool [(Cp-R)M(CO)$_3$] complex can often substitute a phenyl ring in biomolecules such as steroid hormones [27] and antibodies [28]. Whereas the Cp complexes of rhenium or iron can be prepared by classical synthetic routes, the ^{99m}Tc analogues must be synthesized under aqueous conditions. One approach used the double ligand transfer reaction and afforded [(Cp-R)^{99m}Tc(CO)$_3$] in organic solvents but in autoclaves [29–31]. Our group introduced a new synthetic concept from water [32,33]. Moreover, we recently established the direct reaction in water of [^{99m}Tc(OH$_2$)$_3$(CO)$_3$]$^+$ with Thiele's acid (HCp-COOH)$_2$ to prepare the half-sandwich [(Cp-COOH)^{99m}Tc(CO)$_3$] in very good yield [34]. This unexpected result makes the application of the Cp ligands for labeling convenient since stable Diels–Alder dimers can be directly involved in the reaction. We have exploited this concept towards a more general approach for the preparation of bioactive complexes of the [(Cp-R)^{99m}Tc(CO)$_3$] type wherein "R" is an amino acid or a benzamide group [35], and this procedure might lead to the application of half-sandwich complexes as mimics of phenyl rings in bioactive molecules.

In this report, we show that phenyl rings, as found in radioiodinated benzamides for melanoma imaging, can be replaced by [(Cp^{99m}Tc(CO)$_3$] under retention of tumor uptake and clearance from the body. These examples underline the possibility to design ^{99m}Tc radiopharmaceuticals based on the phenyl vs. [(Cp^{99m}Tc(CO)$_3$] analogy. As organic lead



Scheme 2. Synthesis of Thiele's acid based precursors. (i) NMM/i-BuO-Cl at RT for 15 min followed by 2-diethylaminoethylamine. (ii) NMM/i-BuO-Cl at RT for 3.5 h and addition of 1-benzyl-4-aminopiperidine dihydrochloride/NEt$_3$ and stirring for 22 h at RT.



Scheme 3. Synthesis of rhenium complexes **3a** and **3b**. (i) NMM/i-BuO-Cl at RT for 10 min and 2-diethylaminoethylamine/NEt_3 was added and reacted for 3 h at RT. (ii) NMM/i-BuO-Cl at RT for 10 min and 1-benzyl-4-aminopiperidine 2·HCl/NEt_3 were added and reacted for 3 h.

compounds, we selected ^{123}I-*N*-(2-diethylaminoethyl)4-iodobenzamide (BZA) and ^{123}I-*N*-(2-diethylaminoethyl)2-iodobenzamide (IBP) and replaced the iodo-phenyl ring with [Cp^{99m}Tc$(CO)_3$] to yield [(Cp-BZA)^{99m}Tc$(CO)_3$] (**4a**) and [(Cp-IBP)^{99m}Tc$(CO)_3$] (**4b**), respectively (Scheme 1). Both radiolabeled ^{99m}Tc complexes were biologically evaluated in melanoma cells and in tumor-bearing mice.

2. Results and discussion

2.1. Synthesis of ligands

The Diels–Alder dimer [$(HCp\text{-}COOH)_2$] can be regarded as protected cyclopentadiene (Cp) which is an evident source for HCp as demonstrated by Top et al. [36] for the preparation of [(Cp-COOH)Re$(CO)_3$] from $[ReO_4]^-$ and different isomers of Thiele's acid. The amide form of Thiele's acid [$(Cp\text{-}CONHR)_2$] has recently been used by us as a source for (Cp-CONHR) for the preparation of model ^{99m}Tc-benzamide [(Cp-CONH-Ph)^{99m}Tc$(CO)_3$] complexes [35]. Accordingly, we extended the approach towards "real" biomolecules. The preparation of ligands **2a** $(HCp\text{-}BZA)_2$ and **2b** $(HCp\text{-}IBP)_2$ was achieved by a classical amide formation procedure [37]. Thiele's acid was activated with butyl chloroformate/*N*-methyl morpholine in THF. The (HCp-CONHR) amides were obtained by the addition of the appropriate amines (**2a**, R=2-diethylamino-ethylamine; and **2b**, R=1-benzyl-4-aminopiperidine dihydrochloride) (Scheme 2). After chromatographic purification, **2a**–**b** were obtained in 41% and 29% yield, respectively, as off-white solids and characterized by NMR, ESI-MS and CHN analysis.

2.2. Synthesis of the rhenium complexes

The direct synthesis of piano stool-type complexes [(Cp-R)^{99m}Tc$(CO)_3$] in water from $[^{99m}TcO_4]^-$ or [^{99m}Tc$(CO)_3]^+$ is a convenient route for developing such complexes for imaging. The recent perspective of using [(Cp-R)^{99m}Tc$(CO)_3$] is encouraged by previous results where piano stool complexes of rhenium or iron (ferrocene) have been used as substitutes for phenyls in pharmaceuticals. To directly use stable Thiele's acid derivatives, $(HCp\text{-}COR)_2$ is a suitable strategy towards the corresponding ^{99m}Tc complexes [34,35].

For characterization of the ^{99m}Tc complexes, we have prepared the corresponding rhenium analogues (Scheme 3). The complexes [(Cp-BZA)Re$(CO)_3$] (**3a**) and [(Cp-IBP)Re$(CO)_3$] (**3b**) were directly accessible via amide coupling of the corresponding amines to [(Cp-COOH)Re$(CO)_3$] at 47% and 41% yield, respectively, and fully characterized by spectroscopic methods. Complex **3a** could be recrystallised by slow evaporation of a methanol solution to afford X-ray quality crystals. The structure could be elucidated and an ORTEP is given in Fig. 1, with relevant crystallographic data in Table 1. The compound crystallised as hydrochloride salt.

The rhenium central atom is η^5 coordinated to C_5H_4-CO-NH-C_2H_4-NH^+-Et_2. The geometry around the rhenium is pseudo octahedral, and the angles C-Re-C between the two adjacent CO are close to 90°. The amide group O4-C9-N1 has the usual geometrical parameters with C–N bond length of 1.337 Å. It is almost coplanar with the neighbouring cyclopentadienyl ring with a torsion angle of 5.11° between the Cp ring and the amide group.

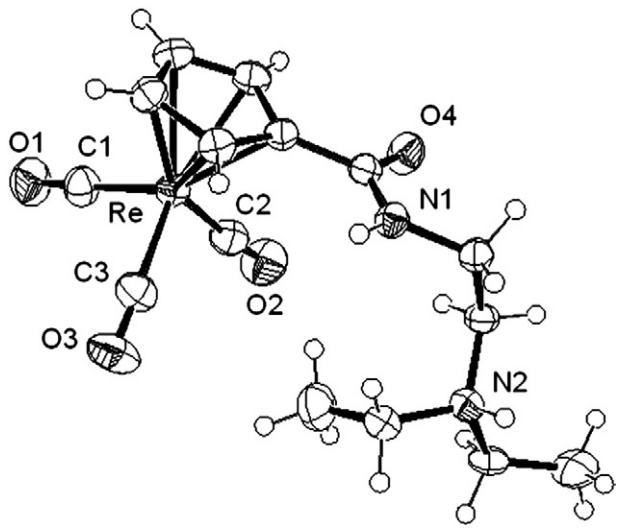


Fig. 1. ORTEP representation of [(Cp-BZA)Re$(CO)_3$] (**3a**). The chloride anion has been omitted for clarity. Important bond lengths (Å) and angles (°) are as follows: Re(1)-C(2), 1.912(6); Re(1)-C(3), 1.914(6); Re(1)-C(1), 1.928(6); Re(1)-C(4), 2.295(5); C(1)-O(1), 1.126(7); C(2)-O(2), 1.148(7); C(9)-N(1), 1.337(6); C(10)-N(1), 1.455(6); C(11)-N(2), 1.500(6); C(12)-N(2), 1.491(7); C(14)-N(2), 1.512(6); C(2)-Re(1)-C(3), 91.6(3); C(2)-Re(1)-C(1), 90.7(3); C(3)-Re(1)-C(1), 88.0(3); C(2)-Re(1)-C(8), 129.9(2); C(9)-N(1)-C(10), 119.7(4); C(12)-N(2)-C(11), 115.0(4).

Table 1
Crystallographic parameters for complex **3a**

Formula	$C_{15}H_{20}ClN_2O_4Re$
M (g mol^{-1})	513.98
Crystal system	triclinic
Space group	P-1
a (Å)	7.3518(1)
b (Å)	8.0309(2)
c (Å)	17.5536(3)
α (°)	99.1260(5)
β (°)	90.4215(14)
γ (°)	117.0187(11)
V (Å^3)	907.87(3)
Z	2
$D_{c\ calc}$ (g cm^{-3})	1.880
Linear absorption coefficient (mm^{-1})	6.858
Absorption correction	Semi-empirical
Relative $trans_{min}/trans_{max}$	0.56860/1.00000
Measured reflections	27908
Unique reflections/R_{int}	5556/0.0450
Refined parameters	210
$R_1(F)/wR_2$ (F^2) ($I>2\sigma(I)$)[a]	0.0348/0.0825
GooF	1.158

[a] $R_1=|F_o-F_c|/|F_o|$; $wR_2=[w(F_o^2-F_c^2)^2/(wF_o^2)]^{1/2}$.

The ^{1}H NMR spectrum of **3a** in DMSO showed Cp ring protons as two singulets at 6.10 and 5.92 ppm, and the NH amide proton and the methylene proton at 3.55 and 3.21–3.17 ppm as a triplet and a multiplet, respectively. The methyl protons are observed at 1.25 ppm as a triplet. The IR spectrum in KBr showed the two characteristic strong bands ν_{CO} of the *fac*-$[Re(CO)_3]^+$ at 2027 and 1934 cm^{-1}. ESI-MS measurements allowed to detect unambiguously the $[M+H]^+$ peak at 477 and 479 *m/z* as expected for the rhenium isotope pattern. Complex **3b** was characterized by ^{1}H NMR spectroscopy. The spectrum displayed the phenyl ring protons at 7.5 and the Cp proton at 6.21 (t) and 5.62 (t) ppm. The ESI-MS showed the cationic piperidinium $[M+H]^+$ at 551.1 and 553.1 *m/z*, and the *fac*-$[Re(CO)_3]^+$ is present with the two characteristic strong bands ν_{CO} at 2024 and 1918 cm^{-1}.

2.3. Preparation of the ^{99m}Tc complexes

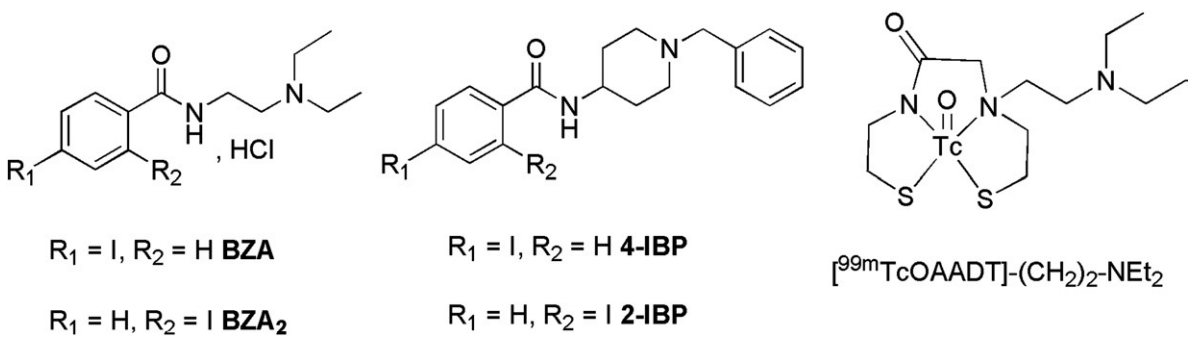
We have recently demonstrated that the metal-mediated retro Diels–Alder reaction is convenient to prepare cyclopentadienyl complexes $[(Cp\text{-}R)^{99m}Tc(CO)_3]$ in aqueous media in which R=amino acid or benzamide. The reaction may even be carried out with Thiele's acid derivative bound to polymeric beads to receive no-carrier-added ^{99m}Tc complexes [35]. To corroborate the principle of phenyl vs. $[(Cp\text{-}R)^{99m}Tc(CO)_3]$ analogy, we focused our investigations on the retro Diels–Alder synthesis of ^{99m}Tc analogues of ^{123}I-BZA and ^{123}I-IBP (Scheme 4).

The corresponding ^{99m}Tc complexes **4a** and **4b** were synthesized by metal-mediated retro Diels–Alder with **2a** and **2b** as shown in Scheme 5. Without optimizing the conditions, the reaction of **2b** with $[^{99m}Tc(OH_2)_3(CO)_3]^+$ at 90°C for 30 min gave **4b** in 28.4% radiochemical yield. After RP-HPLC purification, complex **4b** was obtained with high radiochemical purity (99%) and high specific activity which is essential for cellular internalization and tumor uptake studies. The retention times of the ^{99m}Tc and the Re analogue are well comparable, confirming the authenticity of **4b** (Fig. 2).

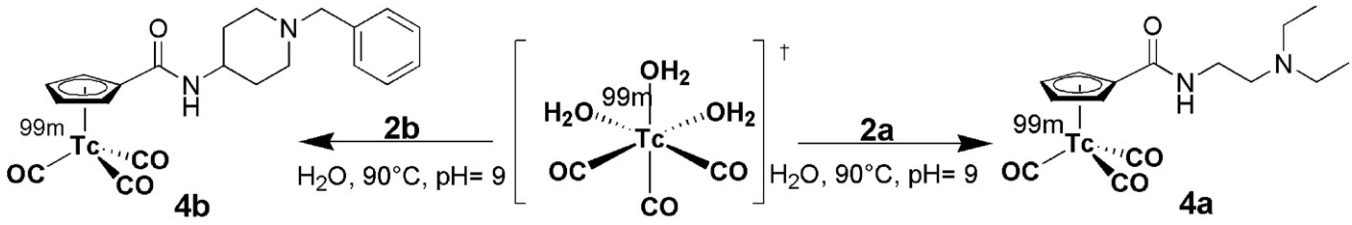
Applying the same procedure for the preparations of **4a** proved more difficult and was less efficient during 0.5–1 h at 90°C since side reactions were observed. The complex **4a** was formed in 9% yield together with the major product $[(Cp\text{-}COOH)^{99m}Tc(CO)_3]$ as a result of amide bond hydrolysis under our conditions. This amide bond cleavage might be due to the pH (≈9.5) and/or to the composition of the kit used for the preparation. However, after purification by preparative RP-HPLC, the ^{99m}Tc-labeled derivative was isolated with a purity >90%. Due to the low radiochemical yield and to the need of high activity for the biodistribution studies, **4a** was not biologically evaluated since its synthesis needs further optimization.

2.4. Biological studies

Iodo-benzamides with a piperidine moiety are "small molecule radiopharmaceuticals" and very attractive for melanoma imaging since they can specifically bind to σ receptors overexpressed in a variety of human tumors including malignant melanoma [15]. Particularly, benzylpiperidin-4-yl-iodobenzamides exhibit a high affinity for σ receptors and the pioneer (*N*-benzylpiperidine-4-yl)-4-



Scheme 4. Chemical structures of iodobenzamides based on diethylaminoethyl (BZA derivatives) and on benzylpiperidine moiety (IBP derivatives) and ^{99m}Tc (V)-related [26] diethylaminoethyl complex.



Scheme 5. Synthesis of ^{99m}Tc-benzamide complexes via metal-mediated retro Diels–Alder reaction.

iodobenzamide (4-IBP) derivative has been proposed as a targeting radiopharmaceutical for breast cancer imaging [38]. For obvious reasons, a ^{99m}Tc-benzylpiperidin would be favorable if biological properties were comparable or even superior to the radioiodinated reference compounds.

Since the hydrolytic and oxidative stability is crucial for biological studies, complex **4b** was incubated for 24 h at 37°C in PBS (pH=7.4). RP-HPLC did not reveal any decomposition products, underlining the very high stability of Cp complexes against hydrolysis, oxidation or trans-metallation.

Uptake and internalization studies performed for **4b** in murine B16F1 melanoma cells at 37°C revealed a time-dependent behavior as shown in Fig. 3. High levels of cellular uptake (activity on the membrane surface and inside the cell), and internalization (activity inside the cell) were achieved for this radioconjugate. For instance, at 4 h after incubation, while 43.9% of the radioactivity administered is associated with the cells, about 21% of the radioactivity was internalized. This assay is highly relevant since it is probably the best in vitro parameter to predict the tumor-targeting properties of the labeled ^{99m}Tc complexes.

The uptake mechanism of radioiodinated benzamides into melanoma cells has been extensively studied. In vitro studies showed that the uptake of aminoalkylbenzamide derivatives is related to the melanin content of cells with a selective accumulation of the signal in melanosomes and, thus, not σ-receptor mediated [11−13]. On the other hand, 4-piperidinyl-iodobenzamide derivatives are known to bind to σ receptors in a variety of tumor cells, including melanoma [10,14,15,38]. Although the structure of **4b** is more similar to that of IBP, the biological activity may be the result of different competing mechanisms, i.e., melanin uptake (cf. BZA_2) [39] rather than affinity to σ receptor (cf. IBP). To learn more about the binding and cellular localization of our radioconjugate, e.g., the importance of σ receptors vs. melanin, the first test is to evaluate the capacity of radioconjugate **4b** to bind synthetic melanin. This type of binding (saturable) strongly depends, in the case of BZA, on melanin concentration and incubation conditions such as the incubation time [40]. In this study, using the standard concentration of 0.5 mg of melanin/10 ml H_2O, and 1 h at 37°C, we observed that **4b** complex bound to the melanin with relatively high affinity (68%). BZA binds with melanin by mechanisms that probably involve an ionic interaction between the positively charge substance and the anionic carboxyl groups of melanin and other attractions such as van der Waals forces and/or a charge-transfer process involving the aromatic rings of the compound and the aromatic indole monomers of melanin [40]. The important binding of **4b** to melanin indicated this

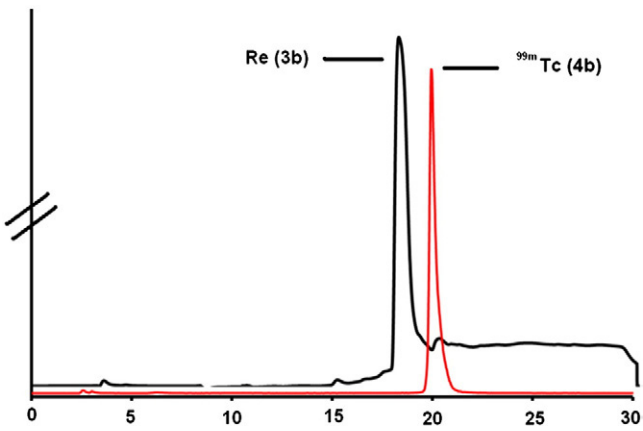


Fig. 2. HPLC superposition of rhenium complex **3b** and the corresponding ^{99m}Tc **4b**. Time delay of the radioactive peak due to separation of the detectors.

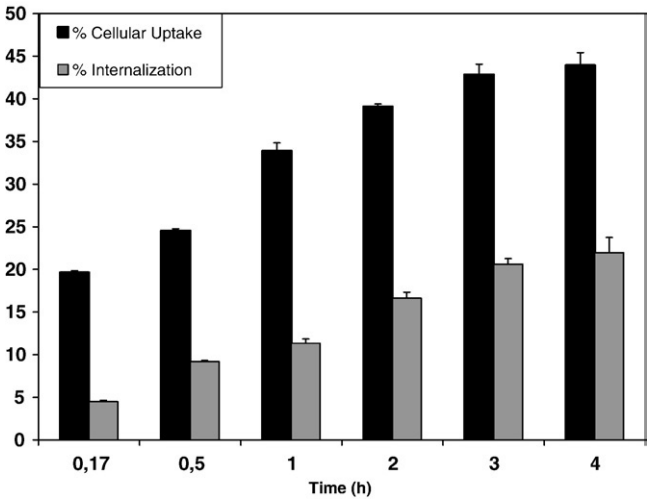


Fig. 3. Cellular uptake and cellular internalization of the radioconjugate **4b** in B16F1 melanoma cells at different time points (37°C). Cell associated and internalized activity expressed as a percentage of the total activity (mean±S.D., four replicates).

interaction to be partially or fully responsible for the high cellular uptake and internalization in melanotic B16F1 cells which may also favor cellular retention.

As the B16 melanoma cell line has also been reported to express σ receptors, further work will focus on the question of whether **4b** uptake and internalization mechanism are also σ-receptor mediated as referred for radioiodinated IBP. This would demonstrate that phenyl ring-[$Cp^{99m}Tc(CO_3)$] analogy (replacement) does not significantly affect the binding to σ receptors as compared to IBP. Whether binding to σ receptors or the melanin tropism is the major important mechanism in **4b** uptake will then be elucidated.

Due to the strong influence of physiological processes on drug kinetics, in vitro studies are not completely predictive of the in vivo suitability of tracers. Hence, **4b** was intravenously injected in B16F1 melanoma-bearing C57BL/6 mice to evaluate its potential as a tumor marker. Biodistribution data for 1 and 4 h pi are presented in Table 2. Fast clearance of **4b** from the blood stream was observed. At 4 h pi, 0.66±0.17 %ID g^{-1} was found in blood and 0.71±0.12 %ID g^{-1} in muscle. The total excretion of the radioactivity was, however, slow and only 33.8% was eliminated after 4 h. Elimination occurred via hepatobiliary excretion pathway. Liver activity was 14.7±3.9 %ID g^{-1} (1 h) and 11.50±2.92 %ID g^{-1} (4 h), and for the intestine 16.5±1.8 %ID g^{-1} (1 h) and 21.1±3.8 %ID g^{-1} (4 h). The kidney retained 16.2±2.4 %ID g^{-1} after 4 h which revealed a second path of excretion or eventually a specific receptor-mediated accumulation as the kidney is known to also express σ receptors.

A significant tumor uptake was observed at 1 h pi (3.39±0.50 %ID g^{-1}). The activity level remained about constant and was still 3.21±0.26 %ID g^{-1} at 4 h pi, indicating high tumor retention that may be in part attributed to the binding to melanin inside of the tumor cells. The tumor-to-blood ratios were 3.6 and 4.9 at 1 and 4 h pi, respectively. These relatively high ratios imply that the tumoral activities may not be related to intratumoral blood pool.

Table 2
Biodistribution of the radioconjugate **4b** in B16F1 melanoma-bearing C57BL/6 mice at 1 and 4 h pi (intravenously) (n=5)

Tissues (%ID g^{-1})	1 h	4 h
Tumor	3.39±0.50	3.21±0.26
Blood	0.93±0.26	0.66±0.17
Liver	14.71±3.89	11.50±2.92
Intestine	16.47±1.77	21.09±3.82
Spleen	6.03±1.20	3.49±0.65
Heart	3.26±0.45	1.62±0.35
Lung	8.60±2.42	1.86±0.66
Kidney	18.34±2.33	16.15±2.39
Muscle	1.23±0.27	0.71±0.12
Bone	1.76±0.27	1.36±0.79
Stomach	8.83±2.05	5.10±1.20
Pancreas	15.36±3.15	12.27±3.42
Eyes	6.95±1.10	5.54±0.75
Skin	2.41±0.60	2.06±0.61
Total excretion (33.8%)	5.4±1.4	33.8±3.9

Recently, two radioiodinated *N*-piperidin-4-yl analogues of IBP were presented as promising imaging agents for melanoma, exhibiting high and persistent tumor uptakes peaking at 12.5 and 11.7 %ID g^{-1} at 6 h and are partially related to σ_1-receptor affinity [41].

The extent of σ-receptor affinity of **4b** in tumors and other σ-expressing tissues such as liver, kidney and lung will be further evaluated by competition studies with haloperidol, a nonselective σ_1–σ_2 inhibitor.

Significant uptake of **4b** was also observed in the pigmented eyes of C57BL6 black mice (6.9 and 5.5 %ID g^{-1}, for 1 and 4 h pi, respectively), suggesting a melanin-related uptake and/or retention mechanism as described for both [^{123}I]-BZA [42] and 4-piperidinyl-[^{123}I]-iodobenzamide derivative [41]. The uptake in the pigmented structures in the eye is in agreement with the high in vitro binding value of **4b** to synthetic melanin (68%).

3. Conclusion

Metal-mediated retro Diels–Alder reaction enabled the preparations of stable [(Cp-R)^{99m}Tc(CO)$_3$]-type complexes, analogues of 2-[^{125}I]*N*-(*N*-Benzylpiperidin-4-yl)-2-iodo benzamide (IBP) for targeting melanoma. These ^{99m}Tc complexes are characterized by high cellular uptake and internalization. Despite slow excretion and high uptake in excretory organs, a significant tumor uptake and retention were observed in vivo. We demonstrated that metal-mediated retro Diels–Alder reaction is a suitable route for the aqueous synthesis of [(Cp-R)^{99m}Tc(CO)$_3$]-type complexes which can mimic phenyl rings in bioactive compounds. Further variations of the functionalities, mimicking purely organic melanoma-targeting agents bound to the Cp ring, will give structure–activity relationships to confirm the hypothesis that phenyl rings can be replaced by the [$Cp^{99m}Tc(CO)_3$] moiety.

4. Experimental part

4.1. General remarks

Reactions were carried out in oven-dried Schlenk glassware under an atmosphere of pure nitrogen when necessary. Solvents were dried over molecular sieves and degassed prior to use. All chemicals were obtained from commercial sources and used without further purification. The acid [(Cp-COOH)Re(CO)$_3$] was prepared according to a literature procedure [36]. NMR spectra were recorded on Bruker Advance 500 and 400 spectrometers. Chemical shifts δ in parts per million (ppm) relative to tetramethylsilane and coupling constants J are given in Hertz. Mass spectra were measured on a Bruker Esquire HCT (ESI) instrument; only characteristic fragments were given. The solvent flow rate

for ESI measurements was 5 μl min^{-1} with a nebulizer pressure of 15 psi and a dry gas flow rate of 5 L min^{-1} at a dry gas temperature of 300°C. IR spectra were recorded as KBr pellets on a Perkin-Elmer BX II IR spectrometer.

RP-HPLC was performed on a Perkin-Elmer system (LCPump, Series 200) coupled to a UV–Vis detector [LC 290 (Perkin-Elmer) or SDP-10AV (Shimadzu)] and a γ detector (LB 507 or LB 509; Berthold, Germany) for the ^{99m}Tc compounds. Analytical separations were performed on a Nucleosil C-18 column (100 Å, 5 μm, 250×3 mm). Semipreparative separations of the radioactive complexes were achieved on a Nucleosil C-18 column (100 Å, 7 μm, 250×8 mm). The columns were eluted with a flow rate of 1.0 ml min^{-1} (analytical) or 2.0 ml min^{-1} (semipreparative) using as eluents 0.1% TFA in H_2O (solvent A) and methanol (solvent B) with a variable gradient (0–3 min, 100% A; 3–3.1 min, 0 to 25% B; 3.1–9 min, 25% B; 9–9.1 min, 25% B to 34% B; 9.1–20 min, 34% B to 100% B; 20–25 min, 100% B; 25–25.1 min 100% B to 100% A; 25.1–30 min 100% A). The gradient was slightly modified to purify the radiolabeled complexes. Purification of complex **4a**: (0–3 min, 100% A; 3–3.1 min, 0 to 25% B; 3.1–9 min, 25% B; 9–9.1 min, 25% B to 34% B; 9.1–25 min, 34% B to 100% B; 25–35 min, 100% B; 35–35.1 min 100% B to 100% A; 35.1–40 min 100% A). Purification of complex **4b**: (0–3 min, 100% A; 3–3.1 min, 0 to 25% B; 3.1–9 min, 25% B; 9–9.1 min, 25% B to 34% B; 9.1–20 min, 34% B to 70% B; 20–30 min, 70% B; 30–35 min, 70% B to 100% B; 35–45 min 100% B; 45–50 min, 100% A).

4.2. Synthesis of ligands

4.2.1. Preparation of TA-BZA (**2a**)

Thiele's acid (660 mg; 3 mmol) was dissolved in 20 ml dry THF. *N*-Methylmorpholine (0.672 ml; 6 mmol) and isobutyl chloroformate (0.792 ml; 6 mmol) were added via syringe. A white precipitate formed. The reaction mixture was stirred during 15 min at ambient temperature under nitrogen.

2-Diethylaminoethylamine (0.6972 g; 6 mmol), dissolved in 5 ml THF, was added via syringe to the reaction mixture. The mixture was stirred during 18 h at ambient temperature under nitrogen. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography on silica gel (linear gradient from pure dichloromethane to dichloromethane/methanol/concentrated aqueous ammonia, 9:1:0.1), yielding 531 mg (42%) of a brownish solid. ESI-MS [*m*/*z*] (CH_3OH, pos. mode): 417.3 [M+H]; ^{1}H NMR (500 MHz, CD_3OD, δ 6.69): (m, 1H), 6.34 (m, 1H), 3.56–3.51 (m, 1H), 3.49–3.43 (m, 1H), 3.40–3.34 (m, 3H), 3.15 (m, 1H), 2.94–2.82 (m, 12 H), 2.50–2.42 (m, 1H), 2.08–2.01 (m, 1H), 1.66 (m, 1H), 1.46 (m, 1H), 1.17 (m, 12H). ^{13}C NMR (125 MHz, CD_3OD), δ in ppm: 168.7, 163.0, 144.3, 142.5, 141.5, 140.0, 55.8, 53.1, 52.8, 51.6, 50.0, 48.1, 48.0, 42.3, 36.6, 36.5, 34.4, 9.9, 9.4.

4.2.2. Preparation of TA-IBP (**2b**)

A total of 330 mg (1.5 mmol) of Thiele's acid was dissolved in 12 ml anhydrous THF. *N*-Methylmorpholine (0.42 ml; 3.75 mmol) and isobutyl chloroformate (0.495 ml; 3.75 mmol) were added. A white precipitate formed. The reaction mixture was stirred at RT under N_2 for 3.5 h. HPLC did not show free Thiele's acid after this time.

1-Benzyl-4-aminopiperidine dihydrochloride (1.579 g; 6 mmol) was suspended in 6 ml anhydrous DMF, and triethylamine (1.88 ml; 13.6 mmol) was added. The resulting turbid solution was added to the Thiele's acid mixture. The mixture was stirred at root temperature under N_2 for 22 h. A new product peak was found in HPLC. The solvent was removed by evaporation under reduced pressure. The product was purified by flash chromatography on silica gel (gradient from 100% CH_2Cl_2 to CH_2Cl_2/MeOH, 9:1), yielding 250 mg (29%) of a pale brownish solid. Calc. For M_r ($C_{36}H_{44}N_4O_2 \cdot 2HCl$) 637.68; ESI-MS [*m*/*z*] (CH_3OH, pos. mode): 565.3 $[M+H]^+$. Calc. C, 67.81; H, 7.27; N, 8.79; Found. C, 67.54; H, 7.75; N, 8.90. ^{1}H NMR (400 MHz, CD_3OD, δ in ppm): 7.32–7.24 (m, 10H), 6.66 (d, 1H, *J*=3.2 Hz), 6.31 (d, 1H, *J*=2.0 Hz), 3.77–3.60 (m, 2H), 3.49 (s, 4H), 3.36 (m, 1H), 3.11 (m, 1H), 3.0–2.8 (m, 5H), 2.5–2.4 (m, 1H), 2.2–2.0 (m, 5H), 1.85–1.65 (m, 4H), 1.65–1.35 (m, 6H). ^{13}C NMR (100 MHz, CD_3OD, δ in ppm): 167.2, 167.1, 142.7, 141.9, 139.8, 138.8, 138.7, 130.9, 129.5, 128.6, 64.1, 55.4, 53.7, 51.3, 48.6, 48.3, 48.0, 42.5, 34.1, 32.4.

4.3. Synthesis of rhenium complexes

4.3.1. Preparation of Re-BZA (**3a**)

To a stirred solution of cytectrene carboxylic acid (190 mg, 0.5 mmol) in THF (5 ml) was added *N*-methylmorpholine (0.056 ml, 51 mg, 0.5 mmol) followed by the addition of isobutyl chloroformate (0.066 ml, 68 mg, 0.5 mmol), resulting in the precipitation of a white solid. In a second flask, 2-diethylaminoethylamine (58 mg, 0.5 mmol) was added in THF (5 ml) containing NEt_3 (69 ml, 51 mg, 0.5 mmol). Both solutions were mixed and stirred for 1 h at room temperature (RT). After removal of the white precipitate by filtration, the solvent was removed under reduced pressure and the residual oil dissolved in $CHCl_3$ (10 ml). The solution was washed with water (5 ml) and the aqueous solution back-extracted with $CHCl_3$ (2× 5 ml). The combined organic solutions were extracted with 10% HCl solution. After evaporation of water, **3a** was obtained by RP-HPLC and crystallised by slow evaporation from MeOH (112 mg, 47%). Calc. For M_r ($C_{15}H_{19}N_2O_4Re$) 477.5; ESI-MS [*m*/*z*] (CH_3OH, pos. mode): 479.0 $[M+H]^+$; Calc. C, 35.05; H, 3.92; N, 5.45 Found. C, 35.14; H, 4.07; N, 5.50; ^{1}H NMR (500 MHz, DMSO, δ in ppm): 6.10 (s, 2H), 5.51 (s, 2H), 3.55 (t, 1H), 3.21–3.17 (m, 8H), 1.21–1.17 (t, 6H); ^{13}C NMR (100 MHz, DMSO, δ in ppm): 193.1 (CO), 93 (Cp), 87.55 (Cp), 86.01 (Cp), 35.26 (CH_2), 28.43 (CH_2), 18.47 (CH_3). IR *ν*(cm^{-1}, KBr) 3467, 2027, 1934, 1747, 1710.

*4.3.2. Preparation of Re-IBP (**3b**)*

Compound **3b** was obtained in an analogous procedure as 3**a** (112.9 mg, 41%). Calc. For M_r ($C_{21}H_{21}N_2O_4Re{\cdot}CF_3$-COOH) 551.61; ESI-MS [*m*/*z*] (CH_3OH, pos. mode): 551.1 and 553.1 $[M+H]^+$; Calc. C, 41.50; H, 3.33; N, 4.21; Found. C, 41.44; H, 3.69; N, 3.61; ^1H NMR (400 MHz, CD_3OD, δ in ppm): 7.5 (s, 5H; Ph), 6.21 (t, 2H, Cp), 5.62 (t, 2H, Cp), 4.35 (s, 2H, CH_2), 4.08 (m, 1H, CH), 3.59–3.56 (m, 2H, CH_2), 3.21–3.16 (m, 2H, CH_2), 2.21–2.19 (m, 2H, CH_2), 1.92–1.85 (m, 2H, CH_2); ^{13}C NMR (100 MHz, CD_3OD, δ in ppm): 192.78 (CO), 163.09 (CO), 131.0 (C, Ph), 130.00 (C, Ph); 129.05 (1C, Ph), 94.13 (Cp), 89.19 (Cp), 86.31 (Cp), 85.4 (Cp), 60.37 (CH_2), 51.42 (CH_2), 44.92 (CH), 28.48 (1C), 22.81 (C). IR ν(cm^{-1}, KBr) 3421, 2024, 1918, 1717.

*4.4. X-ray crystallographic data collection and refinement of the structures of technetium complex **4a***

Crystallographic data were collected at 183(2) K on an Oxford Diffraction Xcalibur system with a ruby detector using Mo Kα radiation (λ=0.7107 Å) that was graphite monochromated. Suitable crystals were covered with oil (Infineum V8512, formerly known as Paratone-N), mounted on top of a glass fibre and immediately transferred to the diffractometer. The program suite CrysAlis^{Pro} was used for data collection, semi-empirical absorption correction and data reduction [43]. The structure was solved with direct methods using SIR97 [44] and was refined by full-matrix least-squares methods on F^2 with SHELXL-97 [45]. The final model was checked for higher symmetry with the help of the program Platon [46]. Crystallographic data for the structural analysis has been deposited with the Cambridge Crystallographic Data Centre, CCDC 737441.

4.5. Radiolabeling

Na[$^{99m}TcO_4$] was eluted from a $^{99}Mo/^{99m}Tc$ generator, using 0.9% saline. With an Isolink kit (Mallinckrodt-Tyco, Inc.), the preparation of *fac*-[$^{99m}Tc(CO)_3(H_2O)_3]^+$ was performed according to a previously described procedure [47]. In a nitrogen-purged glass vial, 500 µl of a 10^{-3} M saline solution (or methanolic solution) of Thiele's acid-benzamide derivatives [HCp-COR)]$_2$ was added to 500 µl of a solution of *fac*-[$^{99m}Tc(CO)_3(H_2O)_3]^+$ (37–74 MBq) in 0.9% saline. The reaction mixture was incubated at 90°C for 30–60 min and then analyzed by RP-HPLC, using an analytical C-18 reversed-phase column. The radiolabeled compound was purified by semipreparative RP-HPLC. The activity corresponding to the $^{99m}Tc(CO)_3$ conjugate was collected in a 50-ml Falcon flask containing 200 µl of 0.9% saline for biodistribution and internalization studies, respectively. The solutions were concentrated to a final volume of 200 µl under nitrogen stream, and the product was controlled by analytical RP-HPLC to confirm its purity and stability after purification and evaporation.

4.6. In vitro stability

The in vitro stability of the radioconjugate was determined in PBS. The ^{99m}Tc-labeled complex (100 µl, ≈10 MBq) was incubated for 24 h at 37°C in PBS (pH 7.4, 1 ml) and then analyzed by HPLC.

4.7. Cell culture

B16F1 murine melanoma cells (ECACC, England, UK) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing GlutaMax I supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin antibiotic solution (all from Gibco-Invitrogen). Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C (Heraeus, Germany), with the medium changed every other day. The cells were adherent in monolayers and, when confluent, were harvested from the cell culture flasks with trypsin EDTA (Gibco-Invitrogen) and seeded farther apart.

4.8. Cellular uptake and internalization

Internalization assays of the radioconjugate **4b** were performed in B16F1 murine melanoma cells seeded at a density of 0.2 million/well in 24-well tissue culture plates and allowed to attach overnight. The cells were incubated at 37°C for a period of 5 min to 4 h, with about 200,000 cpm of the ^{99m}Tc-benzamide analogue in 0.5 ml of assay medium (DMEM with 25 mM HEPES and 0.2% BSA). Incubation was terminated by washing the cells with ice-cold assay medium. Cell surface-bound radioligand was removed by two steps of acid wash (50 mM glycine HCl/100 mM NaCl, pH 2.8) at RT for 5 min. pH was neutralized with cold PBS with 0.2% BSA, and, subsequently, the cells were lysed by a 10-min incubation with 1N NaOH at 37°C to determine the internalized radioligand. Internalization and cellular uptake (surface bound and internalized activity) results were based on four determinations for each time point and are expressed as the average value plus the standard deviation.

4.9. Melanin assay

An in vivo experiment was performed to evaluate the binding affinity of ^{99m}Tc-labeled complexes to melanin using synthetic tyrosine-melanin (Sigma) suspended in aqueous media. The general procedure used was as follows: ^{99m}Tc compound was added to melanin suspension (0.5 mg/10 ml). The reaction mixture was incubated for 1 h under stirring at 37°C. After incubation, the tubes were centrifuged at 35,000×*g* for 20 min to separate the melanin with bound ^{99m}Tc complex from the supernatant containing free ^{99m}Tc complex. The activity of an aliquot of supernatants was counted and expressed relative to that of a reference tube containing water and ^{99m}Tc complex. The percentages of free and bound ^{99m}Tc complex were then calculated.

4.10. Biodistribution

All animal experiments were performed in compliance with Portuguese regulations for animal treatment. The animals were housed in a temperature- and humidity-controlled room with a 12-h light/12-h dark schedule. Biodistribution of the ^{99m}Tc-benzamide complexes was estimated in melanoma-bearing C57BL/6 female mice (8–10 weeks old). Mice had been previously implanted subcutaneously with 1×10^6 B16F1 cells to generate primary skin melanoma. Ten to 12 days after the inoculation, tumors reached a weight of 0.2–1 g.

Animals were intravenously injected with the radiolabeled complex (0.7 and 3 MBq for 1 and 4 h biodistribution, respectively) diluted in 100 μl of saline solution into their retro-orbital sinus. Mice were sacrificed by cervical dislocation at 1 and 4 h pi. The administered dose and the radioactivity in the sacrificed animals were measured with a dose calibrator [Curiemeter IGC-3 (Aloka, Tokyo, Japan) or CRC-15W (Capintec, Ramsey, NJ, USA)]. The difference in radioactivity between the injected animal and the sacrificed animal was assumed to be due to excretion. Tumors and normal tissues of interest were dissected, rinsed to remove excess blood and weighed, and their radioactivity was measured using a γ counter (LB 2111; Berthold). The uptake in the tumor and healthy tissues of interest was calculated and expressed as a percentage of the injected radioactivity dose per gram of tissue (%ID g^{-1}). For blood, bone, muscle and skin, total activity was estimated by assuming that these organs constitute 6%, 10%, 40% and 15% of the total body weight, respectively. Urine was also collected and pooled together at the time of sacrifice.

Acknowledgment

We thank COST D39 for financial support.

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