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Novel ^{99m}Tc labelled complexes with 2-nitroimidazole isocyanide: design, synthesis and evaluation as potential tumor hypoxia imaging agents

Qing Ruan,^a Xuran Zhang,^a Xiao Lin,^{ab} Xiaojiang Duan^a and Junbo Zhang ^{*a}

Radiolabelled 2-nitroimidazoles have been used for imaging hypoxia. With the aim of developing novel ^{99m}Tc radiotracers for imaging hypoxia, four novel 2-nitroimidazole isocyanide derivatives (2a, 2b, 2c, and 2d) were synthesized and radiolabelling was carried out for preparing their corresponding ^{99m}Tc complexes. These ^{99m}Tc complexes were stable *in vitro* and could exhibit good hypoxic selectivity. The partition coefficient results indicated that they were hydrophilic, and an evaluation of biodistribution in mice bearing S180 tumors indicated that all of the complexes could accumulate in the tumors. Among them, ^{99m}Tc -2c exhibited the highest tumor uptake and tumor/blood and tumor/muscle ratios at 2 h post-injection. Further, single photon emission computed tomography (SPECT) imaging studies indicated clear accumulation in tumors, suggesting that ^{99m}Tc -2c was a promising candidate for hypoxia imaging.

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Introduction

Tumor hypoxia is one of the major causes of resistance to conventional radiotherapy and chemotherapy. Therefore, the evaluation of the oxygenation status of solid tumors plays an important role in tumor treatment planning.^{1–4} With the development of non-invasive imaging techniques, positron emission tomography (PET) and single photon emission computed tomography (SPECT) with hypoxia targeted radiopharmaceuticals are considered to be reasonable alternatives. In the development of a hypoxia imaging agent, nitroimidazole derivatives are enzymatically reduced and accumulate in hypoxic regions, therefore most labelled probes for tumor hypoxia have been based on nitroimidazole analogues.^{5,6} In particular, 2-nitroimidazole derivatives have been found to be more suitable for detection of tumor hypoxia, because 2-nitroimidazole has a more positive single electron reduction potential (SERP) value, which means that it can be efficiently reduced and retained in hypoxic cells.^{7–9} [^{18}F]Fluoromisonidazole ([^{18}F]FMISO), a 2-nitroimidazole radiotracer, is one of the most clinically studied radiotracers for hypoxia imaging.¹⁰ How-

ever, to some degree, the limited availability of the [^{18}F] isotope and limited PET scanners restricted its wide clinical use, especially in many developing countries. Although there have been a larger number of SPECT scanners in the world and advances being made in hardware and image reconstruction algorithms have optimized the spatial resolution of images of SPECT,¹¹ there is still a great need to develop effective SPECT tracers for hypoxia imaging. Since the discovery of ^{99m}Tc in the late 1930s and its introduction into nuclear medicine via the $^{99}\text{Mo}/^{99m}\text{Tc}$ generator in the 1950s, ^{99m}Tc has been the most widely used SPECT isotope. To date, many ^{99m}Tc labelled 2-nitroimidazole analogues have been reported.^{12–15} However, the properties of these ^{99m}Tc complexes are not yet ideal and ongoing efforts should be made to develop novel ^{99m}Tc labelled 2-nitroimidazole analogues for tumor hypoxia imaging.

Isocyanide (CN-R) is regarded as a monodentate ligand that strongly coordinates with the $^{99m}\text{Tc}(\text{I})$ core and [$^{99m}\text{Tc}(\text{I})(\text{CO})_3$]⁺ core to produce stable ^{99m}Tc complexes ([$^{99m}\text{Tc}(\text{CN-R})_6$]⁺ and [$^{99m}\text{Tc}(\text{CO})_3(\text{CN-R})_3$]⁺) with high yields.^{15–21} With the aim of developing novel ^{99m}Tc labelled 2-nitroimidazole analogues for tumor hypoxia imaging and exploring the influence of different lengths of the alkyl spacer between the 2-nitroimidazole moiety and the ^{99m}Tc core, in this study, by using 2-nitroimidazole as the targeting vector and isonitrile to coordinate with ^{99m}Tc , we synthesized four novel 2-nitroimidazole isocyanide derivatives and labelled them with ^{99m}Tc in an effort to develop novel tumor hypoxia imaging agents.

^a Key Laboratory of Radiopharmaceuticals (Beijing Normal University), Ministry of Education, College of Chemistry, Beijing Normal University, Beijing, 100875, P. R. China. E-mail: zhjunbo@bnu.edu.cn; Fax: +86 10 6220 5562;

Tel: +86 10 6220 8126

^b College of Water Sciences, Beijing Normal University, No 19, Xijiekouwai Street, Beijing 100875, People's Republic of China

Results and discussion

Chemistry

Synthesis of four novel 2-nitroimidazole isocyanide derivatives was achieved by reaction of the corresponding amino derivatives of 2-nitroimidazole (1) with different isocyanide-containing active esters in the presence of triethylamine, as shown in Scheme 1. The synthesis methods were very convenient for obtaining the desired products. The ligands (2a, 2b, 2c, and 2d) were characterized by ^1H NMR, ^{13}C NMR, IR and HR-MS, and the results obtained were in agreement with the proposed structures.

Radiolabelling and quality control

$^{99\text{m}}\text{Tc}$ -2a, $^{99\text{m}}\text{Tc}$ -2b, $^{99\text{m}}\text{Tc}$ -2c and $^{99\text{m}}\text{Tc}$ -2d complexes can be prepared by a direct labelling method with high radiochemical purities (RCP). The preparation of these complexes does not require heating and they can be easily formed at room temperature, while the preparation of $^{99\text{m}}\text{Tc}$ -MIBI (MIBI: 2-methoxy-2-isobutyl isonitrile) needs heating at 100 °C for 15 min. Just like $^{99\text{m}}\text{Tc}$ -MIBI, the proposed structure of these complexes would be a monovalent cation with a central technetium core that is surrounded by six similar ligands coordinated through the isonitrile carbon [Scheme 2].¹⁹

The RCP of the complexes were assessed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC). By TLC, in methanol, the $^{99\text{m}}\text{Tc}$ complexes migrated at the front ($R_f = 0.8$ –1.0), while $^{99\text{m}}\text{TcO}_4^-$ and $^{99\text{m}}\text{TcO}_2 \cdot 2\text{H}_2\text{O}$ remained at the origin ($R_f = 0$ –0.1). By HPLC, single radioactive peaks were observed with retention times (t_R) of 14.35 min, 14.63 min, 14.88 min and 15.38 min corresponding to $^{99\text{m}}\text{Tc}$ -2a, $^{99\text{m}}\text{Tc}$ -2b, $^{99\text{m}}\text{Tc}$ -2c and $^{99\text{m}}\text{Tc}$ -2d, respectively, while the retention time of $^{99\text{m}}\text{TcO}_4^-$ was 3.33 min. The mean radiochemical purities of the final products were all over 95%, suggesting that they can be used for *in vitro* and *in vivo* studies without further purification (Fig. 1).

As the structural characterization of $^{99\text{m}}\text{Tc}$ complexes is difficult to verify at the no-carrier-added level, the corresponding rhenium analogue can be synthesized at the macroscopic level.²² Tc and Re belong to the same group and they have similar chemistry. The structure of $^{99\text{m}}\text{Tc}$ complexes can be studied by using stable rhenium as a surrogate for $^{99\text{m}}\text{Tc}$. The co-injection HPLC patterns of $^{99\text{m}}\text{Tc}$ -2c and Re-2c are shown in Fig. 2. It could be noted that the radioactivity profile of $^{99\text{m}}\text{Tc}$ -2c ($t_R = 14.86$ min) nearly matched with the UV-

Scheme 2 Preparation procedures for $^{99\text{m}}\text{Tc}$ -2a, $^{99\text{m}}\text{Tc}$ -2b, $^{99\text{m}}\text{Tc}$ -2c and $^{99\text{m}}\text{Tc}$ -2d.

chromatogram of Re-2c ($t_R = 14.48$ min), suggesting that $^{99\text{m}}\text{Tc}$ -2c possessed the proposed structure.

In vitro stability study

The stability of the $^{99\text{m}}\text{Tc}$ complexes was assayed by measuring their radiochemical purity (RCP). The RCP of the complexes was more than 90% and all of the complexes did not show decomposition for 6 h in the labelling milieu at room temperature and in mouse serum at 37 °C, demonstrating their good *in vitro* stability.

Determination of the partition coefficient

The partition coefficient ($\log P$) values of $^{99\text{m}}\text{Tc}$ -2a, $^{99\text{m}}\text{Tc}$ -2b, $^{99\text{m}}\text{Tc}$ -2c and $^{99\text{m}}\text{Tc}$ -2d were -2.65 ± 0.14 , -1.95 ± 0.13 , -1.27 ± 0.04 and -0.45 ± 0.05 , respectively, suggesting that they were hydrophilic. Moreover, the $\log P$ values of the corresponding $^{99\text{m}}\text{Tc}$ complexes are in accordance with the structure of the ligands, since incorporation of more CH_2 groups increases the lipophilicity.

In vitro cellular uptake

The cellular uptake analysis of $^{99\text{m}}\text{Tc}$ -2a, $^{99\text{m}}\text{Tc}$ -2b, $^{99\text{m}}\text{Tc}$ -2c and $^{99\text{m}}\text{Tc}$ -2d was carried out using S180 cells under both

Scheme 1 Synthesis of 2a, 2b, 2c and 2d.

Fig. 1 HPLC patterns of $^{99\text{m}}\text{Tc}$ -2a (A), $^{99\text{m}}\text{Tc}$ -2b (B), $^{99\text{m}}\text{Tc}$ -2c (C) and $^{99\text{m}}\text{Tc}$ -2d (D).

Fig. 2 Co-injection analysis of ^{99m}Tc -2c and Re-2c.

hypoxic and aerobic conditions. As presented in Fig. 3, the uptake of the four ^{99m}Tc complexes in hypoxic cells was persistently higher than that in aerobic cells, suggesting that they had good hypoxia selectivity. Significant differences between each point under hypoxic and aerobic conditions were determined by Student's *t* test (independent, two-tailed). The criterion for significance was $p < 0.05$.

Biodistribution studies

Biodistribution studies were performed in Kunming mice bearing the S180 tumor, which is a representative hypoxia model of solid tumors.^{23–25} We conducted biodistribution studies at 0.5, 1 and 2 h after the ^{99m}Tc complexes were injected into mice. The results of ^{99m}Tc -2a, ^{99m}Tc -2b, ^{99m}Tc -2c and ^{99m}Tc -2d are shown in Tables 1–4, respectively. They all had a relatively high tumor uptake. The muscle uptake values were low, hence the tumor/muscle ratios were high. The initial accumulations in blood were high, but the clearance of activity from blood was faster than that of the tumor. For the four ^{99m}Tc complexes, excretion occurred through both the urinary and hepatobiliary tract. Low uptake in the thyroid and stomach suggested that no $^{99m}\text{TcO}_4^-$ uptake occurred.

Fig. 3 Cellular uptake of ^{99m}Tc -2a (A), ^{99m}Tc -2b (B), ^{99m}Tc -2c (C) and ^{99m}Tc -2d (D).

Table 1 Biodistribution of ^{99m}Tc -2a in mice bearing the S180 tumor ($n = 5$, T/B = tumor-to-blood ratio, T/N = tumor-to-muscle ratio)

% ID $g^{-1} \pm s$	0.5 h	1 h	2 h
Heart	1.04 ± 0.18	0.32 ± 0.09	0.28 ± 0.06
Liver	4.77 ± 0.55	1.86 ± 0.30	1.52 ± 0.34
Lung	2.26 ± 0.27	0.81 ± 0.04	0.61 ± 0.13
Kidney	8.80 ± 1.15	6.80 ± 2.01	5.28 ± 1.21
Spleen	0.77 ± 0.13	0.33 ± 0.07	0.24 ± 0.04
Stomach	0.33 ± 0.18	0.29 ± 0.17	0.26 ± 0.05
Bone	1.01 ± 0.15	0.49 ± 0.08	0.28 ± 0.07
Muscle	0.64 ± 0.22	0.19 ± 0.02	0.16 ± 0.02
Intestine	1.96 ± 0.53	0.75 ± 0.20	0.58 ± 0.26
Tumor	1.27 ± 0.37	0.83 ± 0.19	0.63 ± 0.09
Blood	2.87 ± 0.66	0.92 ± 0.08	0.70 ± 0.08
Thyroid (% ID)	0.03 ± 0.01	0.02 ± 0.00	0.01 ± 0.01
T/B	0.44	0.86	0.91
T/N	1.98	4.47	3.86

The comparison results of the biodistribution of the four ^{99m}Tc complexes in mice bearing the S180 tumor at 2 h post-injection are shown in Table 5. It was found that with the increase of log *P* of the complexes, the uptake in the liver was increased and the kidney uptake was decreased. By comparison, ^{99m}Tc -2c has lower muscle and blood uptake values and a higher tumor uptake, so the tumor/blood and tumor/muscle ratios of ^{99m}Tc -2c are much higher than those of the other three complexes.

By the analysis of previously reported data, we can find that the tumor uptake of ^{99m}Tc -2c and even the tumor/blood and tumor/muscle ratios were higher than those of some reported ^{99m}Tc -labelled 2-nitroimidazole derivatives. In 2017, Vats *et al.* reported a ^{99m}Tc -‘4 + 1’ mixed ligand complex of 2-nitroimidazole with isocyanide for hypoxia imaging. The tumor uptake of ^{99m}Tc -2c (0.93 ± 0.15) was more than two times better than that of $^{99m}\text{Tc}(\text{NS}_3)$ (2NimNC) (0.40 ± 0.07) at 1 h post-injection. As for the uptake of non-target organs and tissues, ^{99m}Tc -2c was much better than $^{99m}\text{Tc}(\text{NS}_3)$ (2NimNC). The liver uptake of ^{99m}Tc -2c (4.93 ± 0.37) was less than half of that of $^{99m}\text{Tc}(\text{NS}_3)$ (2NimNC) (12.42 ± 0.42), and the intestine uptake of ^{99m}Tc -2c (1.45 ± 0.40) was merely less

Table 2 Biodistribution of ^{99m}Tc -2b in mice bearing the S180 tumor ($n = 5$, T/B = tumor-to-blood ratio, T/N = tumor-to-muscle ratio)

% ID $g^{-1} \pm s$	0.5 h	1 h	2 h
Heart	0.47 ± 0.05	0.34 ± 0.02	0.26 ± 0.03
Liver	4.11 ± 0.49	3.20 ± 0.35	2.83 ± 0.26
Lung	0.99 ± 0.06	0.78 ± 0.02	0.64 ± 0.09
Kidney	7.72 ± 0.60	8.15 ± 0.42	8.22 ± 0.29
Spleen	0.53 ± 0.04	0.47 ± 0.07	0.39 ± 0.05
Stomach	0.10 ± 0.02	0.11 ± 0.04	0.09 ± 0.02
Bone	0.52 ± 0.10	0.47 ± 0.10	0.42 ± 0.12
Muscle	0.21 ± 0.03	0.14 ± 0.03	0.12 ± 0.02
Intestine	1.14 ± 0.49	0.51 ± 0.15	0.36 ± 0.09
Tumor	0.92 ± 0.18	0.63 ± 0.07	0.52 ± 0.08
Blood	1.17 ± 0.08	0.81 ± 0.09	0.53 ± 0.04
Thyroid (% ID)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
T/B	0.79	0.78	1.00
T/N	4.28	4.57	4.32

Table 3 Biodistribution of ^{99m}Tc -2c in mice bearing the S180 tumor ($n = 5$, T/B = tumor-to-blood ratio, T/N = tumor-to-muscle ratio)

% ID $\text{g}^{-1} \pm s$	0.5 h	1 h	2 h
Heart	0.64 ± 0.07	0.35 ± 0.03	0.29 ± 0.04
Liver	6.77 ± 0.81	4.93 ± 0.37	4.10 ± 0.55
Lung	2.31 ± 0.40	1.29 ± 0.08	1.01 ± 0.16
Kidney	5.70 ± 0.38	5.18 ± 0.39	4.91 ± 0.66
Spleen	1.19 ± 0.16	0.63 ± 0.06	0.69 ± 0.16
Stomach	0.35 ± 0.10	0.24 ± 0.08	0.53 ± 0.20
Bone	1.00 ± 0.04	0.67 ± 0.10	0.60 ± 0.08
Muscle	0.37 ± 0.04	0.21 ± 0.03	0.16 ± 0.04
Intestine	3.64 ± 0.53	1.45 ± 0.40	0.85 ± 0.21
Tumor	1.55 ± 0.19	0.93 ± 0.15	0.83 ± 0.14
Blood	1.31 ± 0.20	0.87 ± 0.01	0.63 ± 0.12
Thyroid (% ID)	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
T/B	1.18	1.06	1.32
T/N	4.15	4.39	5.05

Table 4 Biodistribution of ^{99m}Tc -2d in mice bearing the S180 tumor ($n = 5$, T/B = tumor-to-blood ratio, T/N = tumor-to-muscle ratio)

% ID $\text{g}^{-1} \pm s$	0.5 h	1 h	2 h
Heart	0.70 ± 0.17	0.55 ± 0.13	0.40 ± 0.08
Liver	15.67 ± 0.37	9.49 ± 0.69	6.96 ± 0.61
Lung	3.09 ± 0.92	3.76 ± 1.60	3.73 ± 0.45
Kidney	7.09 ± 1.42	3.36 ± 0.28	2.74 ± 0.41
Spleen	1.42 ± 0.12	0.94 ± 0.15	0.80 ± 0.12
Stomach	0.43 ± 0.02	0.31 ± 0.07	0.40 ± 0.14
Bone	1.06 ± 0.39	0.97 ± 0.24	0.60 ± 0.15
Muscle	0.42 ± 0.13	0.29 ± 0.08	0.22 ± 0.07
Intestine	3.70 ± 0.77	1.30 ± 0.16	1.47 ± 0.33
Tumor	1.30 ± 0.14	1.10 ± 0.23	0.78 ± 0.06
Blood	1.93 ± 0.21	1.47 ± 0.13	1.03 ± 0.13
Thyroid (% ID)	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.00
T/B	0.67	0.75	0.75
T/N	3.11	3.77	3.45

Table 5 Comparison of the biodistribution of the ^{99m}Tc complexes at 2 h post-injection

	^{99m}Tc -2a	^{99m}Tc -2b	^{99m}Tc -2c	^{99m}Tc -2d
Log P	-2.65	-1.99	-1.27	-0.44
Liver	1.52 ± 0.34	2.83 ± 0.26	4.10 ± 0.55	6.96 ± 0.61
Kidney	8.42 ± 1.18	8.22 ± 0.29	4.91 ± 0.66	2.74 ± 0.41
Tumor	0.63 ± 0.09	0.52 ± 0.08	0.83 ± 0.14	0.78 ± 0.06
T/B	0.91	1.00	1.32	0.75
T/N	3.86	4.32	5.05	3.45

than one thirteenth of that of $^{99m}\text{Tc}(\text{NS}_3)$ (2NimNC) (20.86 ± 0.62) at 1 h post-injection. As for the tumor/blood and tumor/muscle ratios, ^{99m}Tc -2c was superior to $^{99m}\text{Tc}(\text{NS}_3)$ (2NimNC) at 1 h post-injection.¹⁵

SPECT/CT imaging studies

As ^{99m}Tc -2c had a higher tumor uptake and tumor/blood and tumor/muscle ratios than the other three complexes, it was selected as a promising candidate for further SPECT/CT imaging studies. The SPECT/CT imaging results of ^{99m}Tc -2c

showed that the tumor uptake was observable (Fig. 4). However, the appreciable uptake of ^{99m}Tc -2c in the liver and kidneys is the defect of the complex. The value of the region-of-interest (ROI) ratio of the uptake for the tumor site to the corresponding non-tumor region was 5.39 ± 0.67 . The imaging data in mice were consistent with the biodistribution results in mice.

Experimental section

General

All chemicals were purchased from commercial sources and used without further purification. A $^{99}\text{Mo}/^{99m}\text{Tc}$ generator was obtained from the China Institute of Atomic Energy (CIAE). The murine sarcoma S180 cell line was obtained from Peking University Health Science Center (Beijing, China). The IR spectrum was obtained with an AVATAR 360 FT-IR spectrometer using KBr pellets. The NMR spectrum was recorded on a 400 MHz Bruker Avance spectrophotometer. The ESI-MS spectrum was recorded on a LC-MS Shimadzu 2010 series. High performance liquid chromatography (HPLC) was performed using a Waters 600 binary HPLC pump and a Waters 2487 UV absorbance dual λ detector (Milford, MA USA) with a reversed-phase column (Kromasil C18, 250×4.6 mm).

Chemistry

Compound 1 (2-(2-nitro-1H-imidazol-1-yl)ethan-1-amine) was prepared according to a literature method previously described.²⁶ The synthetic procedures and the spectral data for the final products (2a, 2b, 2c and 2d) were as follows.

Synthesis of 2a. 156 mg of compound 1 (1 mmol), 247 mg of compound a (2,3,5,6-tetrafluorophenyl 3-isocyanopropanoate) (1 mmol) and 0.21 mL triethylamine (1.5 mmol) were dissolved in 15 mL methanol. The mixture was stirred at room temperature for 6 h. The solvent was removed under reduced pressure and the residue was purified by chromatography ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$) to give 2a (3-isocyanato-N-(2-(2-nitro-1H-imidazol-1-yl)ethyl)propanamide) as a white solid (yield, 64%). IR (KBr)/ cm^{-1} : 3342.78 (νNH), 1487.18, 1359.87 (νNO_2), 1670.43 (νCO), 2152.65 (νNC); ^1H NMR (400 MHz, D_2O) δ (ppm): 7.29 (s, 1H), 7.03 (s, 1H), 4.49–4.46 (t, 2H), 3.60–3.58 (t, 2H), 3.53–1.3.51 (t, 2H), 2.54–2.51 (t, 2H); ^{13}C NMR (100 MHz, D_2O) δ (ppm): 172.62, 151.97, 144.32, 128.72, 127.27, 48.34, 38.67, 38.11, 34.55; HR-MS (ESI): found 238.0938, calcd 238.0934, $\text{C}_9\text{H}_{12}\text{N}_5\text{O}_3$ [$\text{M} + \text{H}^+$].

Fig. 4 SPECT/CT images of ^{99m}Tc -2c at 2 h post-injection, visual imaging (A), sagittal section (B), coronal section (C) and transverse section (D).

Synthesis of 2b. Following the same procedure used in the synthesis of 2a, 2b was isolated as a white solid (yield, 35%). IR (KBr)/cm⁻¹: 3254.05 (ν NH), 1489.11, 1327.08 (ν NO₂), 1633.78 (ν CO), 2150.72 (ν NC); ¹H NMR (400 MHz, D₂O) δ (ppm): 7.28 (s, 1H), 7.05 (s, 1H), 4.49–4.46 (t, 2H), 3.57–3.55 (t, 2H), 3.32–3.30 (t, 2H), 2.19–2.16 (m, 2H), 1.71–1.59 (t, 2H); ¹³C NMR (100 MHz, D₂O) δ (ppm): 175.55, 151.33, 143.70, 128.73, 127.72, 49.83, 40.56, 37.95, 32.43, 22.99; HR-MS (ESI): found 252.1098, calcd 252.1091, C₁₀H₁₄N₅O₃ [M + H⁺].

Synthesis of 2c. Following the same procedure used in the synthesis of 2a, 2c was isolated as a white solid (yield, 53%). IR (KBr)/cm⁻¹: 3366.99 (ν NH), 1491.04, 1363.73 (ν NO₂), 1655.00 (ν CO), 2146.86 (ν NC); ¹H NMR (400 MHz, D₂O) δ (ppm): 7.26 (s, 1H), 7.03 (s, 1H), 4.46–4.45 (t, 2H), 3.56–3.54 (t, 2H), 3.33–3.31 (t, 2H), 2.05–2.03 (m, 2H), 1.44–1.42 (m, 4H); ¹³C NMR (100 MHz, D₂O) δ (ppm): 176.87, 150.82, 144.331, 128.56, 127.58, 49.01, 40.86, 37.65, 34.21, 26.89, 22.83; HR-MS (ESI): found 266.1244, calcd 266.1247, C₁₁H₁₆N₅O₃ [M + H⁺].

Synthesis of 2d. Following the same procedure used in the synthesis of 2a, 2d was isolated as a white solid (yield, 69%). IR (KBr)/cm⁻¹: 3342.78 (ν NH), 1487.18, 1359.87 (ν NO₂), 1670.43 (ν CO), 2152.65 (ν NC); ¹H NMR (400 MHz, D₂O) δ (ppm): 7.27 (s, 1H), 7.05 (s, 1H), 4.48–4.46 (t, 2H), 3.56–3.54 (t, 2H), 3.35–3.32 (t, 2H), 2.04–2.02 (t, 2H), 1.52–1.50 (m, 2H), 1.38–1.31 (m, 2H), 1.20–1.15 (m, 2H); ¹³C NMR (100 MHz, D₂O) δ (ppm): 177.04, 150.33, 144.67, 128.53, 127.39, 49.52, 41.04, 37.92, 35.18, 27.80, 25.07, 24.32; HR-MS (ESI): found 280.1406, calcd 280.1404, C₁₂H₁₈N₅O₃ [M + H⁺].

Radiolabelling and quality control

The preparation procedures for ^{99m}Tc-2a, ^{99m}Tc-2b, ^{99m}Tc-2c and ^{99m}Tc-2d are as follows: 2 mg 2a (2b, 2c, 2d) was dissolved in 0.3 mL saline, then 0.2 mL sodium citrate buffer (0.1 mol L⁻¹, pH = 4.0) was added. After 0.03 mL SnCl₂·2H₂O (1 mg mL⁻¹) was added, 0.5 mL of saline containing ^{99m}TcO₄⁻ (37–74 MBq) was added right away. The mixture was kept at room temperature for 30 min. The radiochemical purity (RCP) of ^{99m}Tc-2a, ^{99m}Tc-2b, ^{99m}Tc-2c and ^{99m}Tc-2d was checked by TLC and HPLC. TLC was performed on a polyamide strip eluted with methanol. For HPLC, a water (A) and acetonitrile (B) mixture was adopted as the mobile phase at a flow rate of 1.0 mL min⁻¹, and the analytical conditions are described in Table 6.

Preparation of the rhenium analogue

In order to validate the proposed structure of ^{99m}Tc-2c, we synthesized the stable complex Re-2c. The preparation procedures for Re-2c were as follows: 100 mg of 2c was dissolved

in 0.3 mL saline, then 1 mL of sodium citrate buffer (0.1 mol L⁻¹, pH = 4.0) was added. After the addition of 1.5 mg SnCl₂·2H₂O, 2 mg KReO₄ was added right away. The mixture was kept at room temperature for 30 min. The desired product was purified by HPLC under the conditions described in Table 6. Then the solvent was removed and a brown residue was obtained. The product was analyzed by ¹H NMR and HR-MS. ¹H NMR (400 MHz, D₂O) δ (ppm): 7.30 (s, 1H), 7.08 (s, 1H), 4.52–4.48 (t, 2H), 3.60–3.57 (t, 2H), 3.39–3.36 (t, 2H), 2.10–2.06 (m, 2H), 1.54–1.47 (m, 4H); HR-MS(ESI): [(C₁₁H₁₅N₅O₃)₆ReNa]²⁺: *m/z* calcd. for C₆₆H₉₀N₃₀O₁₈ReNa, 900.3249; found, 900.3245.

In vitro stability study

The stability studies of ^{99m}Tc-2a, ^{99m}Tc-2b, ^{99m}Tc-2c and ^{99m}Tc-2d were performed by checking the RCP in the labelling milieu at room temperature for 6 h. *In vitro* serum stability studies in mouse serum were also assessed by a method reported earlier.²⁷ The RCP was checked by TLC and HPLC.

Determination of the partition coefficient (log *P*)

The lipophilicity of the four ^{99m}Tc complexes was evaluated in terms of the partition coefficients (log *P*) between 1-octanol and phosphate buffer (0.025 mol L⁻¹, pH 7.4) according to a previously reported method.²⁸ Briefly, the complex was mixed with an equal volume of the two phases. The mixture was vortexed for 3 min and then centrifuged at 10 000g for 5 min. From each phase, 0.1 mL of aliquot was pipetted and counted in a γ -counter. The partition coefficient, *P*, was calculated as the mean value of counts per minute per milliliter of the 1-octanol layer divided by that of the buffer. The final partition coefficient value was expressed as log *P* $\pm$ SD.

In vitro cellular uptake

In vitro uptake of the ^{99m}Tc complexes was performed using the murine sarcoma S180 cell line. The cells were incubated under hypoxic and aerobic conditions and evaluated by previously reported methods.²⁹ Briefly, S180 cells were suspended in fresh DMEM growth medium having 10% (v/v) fetal bovine serum at a cell concentration of 2 $\times$ 10⁶ cells per mL. Aliquots of 20 mL were put into glass vials and incubated at 37 °C under an atmosphere of 95% nitrogen plus 5% carbon dioxide (hypoxic conditions, oxygen concentration <10 ppm) or 95% air plus 5% carbon dioxide (aerobic conditions). After a 30 min equilibration period, 0.2 mL (3.7 MBq) of the ^{99m}Tc labelled complex was mixed with the suspension. 1 mL of cell suspension was pipetted and centrifuged at 3000g for 5 min at 0.5, 1, 2 and 4.0 h post-incubation. 0.9 mL of the supernatant was taken for counting (*C*_{out}) and the residual sample containing cells with 0.1 mL of the supernatant was also counted (*C*_{in}). The studies were conducted five times at each time point. The cell accumulation, *A*, was calculated as follows: *A* = (*C*_{in} - *C*_{out}/9)/(*C*_{in} + *C*_{out}). The final results were expressed as the average of the measurements $\pm$ standard deviation.

Table 6 The analytical conditions for HPLC

Time/min	0	5	15	20	22	32	35
A/%	90	90	30	30	10	10	90
B/%	10	10	70	70	90	90	10

Biodistribution studies

All biodistribution studies were performed according to the Regulations on Laboratory Animals of Beijing Municipality and all animal procedures were carried out in compliance with the Guidelines for Care and Use of Laboratory Animals of Beijing Normal University and approved by the Animal Ethics Committee of Beijing Normal University.

Experiments were performed using Kunming female mice (18–22 g) bearing S180 tumors.^{30,31} 0.1 mL of ^{99m}Tc complexes (7.4×10^5 Bq mL⁻¹) was injected *via* a tail vein. The mice were sacrificed in groups of five at 0.5, 1 and 2 h post-injection. The tumor, muscle, blood and other organs of interest were collected, weighed and counted using a gamma counter. The final results were expressed as the percent uptake of injected dose per gram of tissue $\pm$ standard deviation (% ID g⁻¹ $\pm$ SD).

SPECT/CT imaging studies

SPECT/CT images were acquired using a small animal Triumph SPECT/CT system (Trifoil imaging, USA). The SPECT data was reconstructed with HiSPECT software and analyzed with 40 vivoquant software. The Kunming female mice (18–20 g) bearing the S180 tumor were injected *via* a tail vein with ^{99m}Tc-2c (0.1 mL, 1.11×10^6 Bq). SPECT/CT images were acquired at 2 h after injection. The ROIs for the tumor site and the corresponding non-tumor region were selected and determined. The final results were expressed as mean $\pm$ standard deviation ($n = 3$).

Conclusion

In the present study, four novel 2-nitroimidazole isocyanide derivatives (2a, 2b, 2c, and 2d) were synthesized and their corresponding ^{99m}Tc complexes can be prepared by a direct labelling method without the need for heating. These ^{99m}Tc complexes exhibited hydrophilicity and *in vitro* stability as well as hypoxic selectivity. The preliminary biological evaluation in mice bearing the S180 tumor showed that they could accumulate in the tumor. Especially for ^{99m}Tc-2c, the biodistribution and SPECT imaging studies in mice bearing the S180 tumor showed that it had obvious tumor uptake and good target/no-target ratios, suggesting that it would be a potential tracer for hypoxia imaging in tumors.

Conflicts of interest

The author(s) confirm that this article content has no conflicts of interest.

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