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Synthesis and biological evaluation of an ^{123}I-labeled bicyclic nucleoside analogue (BCNA) as potential SPECT tracer for VZV-tk reporter gene imaging

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Abstract—An iodine-123 labeled bicyclic nucleoside analogue ([^{123}I]-**4**) has been synthesized and evaluated as a potential single photon emission tomography (SPECT) reporter probe for the non-invasive imaging of expression of the varicella zoster virus thymidine kinase (VZV-tk) reporter gene. In vitro enzymatic assays revealed that the non-radioactive mono-iodo derivative **4** has good affinity for VZV-TK (IC_{50}: 4.2 μM). Biodistribution of [^{123}I]-**4** was examined in normal mice. Evaluation of [^{123}I]-**4** in HEK-293T cells showed 1.74-fold higher accumulation in VZV-TK-expressing cells compared to control cells.

Bicyclic nucleoside analogues (BCNAs) are novel, lipophilic, highly potent, and selective inhibitors of varicella-zoster virus (VZV). VZV-thymidine kinase (VZV-TK) efficiently phosphorylates these BCNAs to the corresponding 5′-monophosphates and the thymidylate kinase activity of VZV-TK (Note: tk refers to the gene and TK refers to the protein) further converts BCNA-5′-monophosphates to BCNA-5′-diphosphates.[1,2] The phosphorylated metabolites are negatively charged and remain trapped in the cell. Therefore, radiolabeling of these BCNAs with a positron emission tomography (PET) or single photon emission computed tomography (SPECT) radioisotope may allow non-invasive imaging of VZV-tk activity, analogous to the widely studied herpes simplex virus type 1 thymidine kinase (HSV1-tk) reporter gene.[3,4]

We recently synthesized ^{11}C or ^{18}F labeled BCNAs (Fig. 1) as potential PET reporter probes for imaging expression of the VZV-tk gene.[5] In vitro evaluation using the human embryonic kidney cell line HEK-293T has revealed that these tracers selectively accumulate in VZV-TK-expressing cells,[5] in accordance to mentioned phosphorylation data. The short half-life of ^{11}C and ^{18}F ($t_{1/2}$ 20.4 and 110 min, respectively) allows for repeated or sequential imaging using PET. However, longer half-life radioisotopes would allow

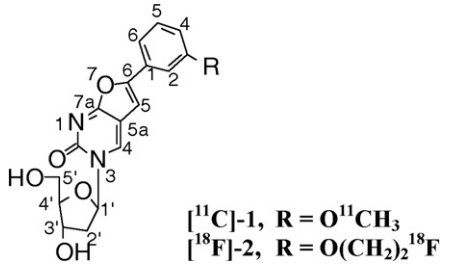

Figure 1. Chemical structures of the ^{11}C or ^{18}F labeled BCNAs for PET.

Keywords: Molecular imaging; BCNA; Iodine-123; SPECT; Radiopharmaceutical.
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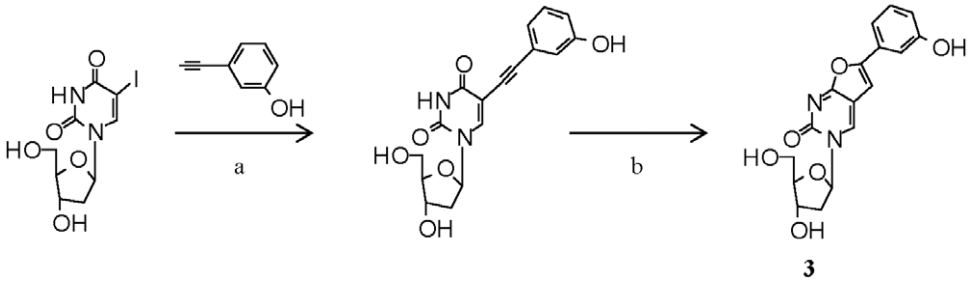
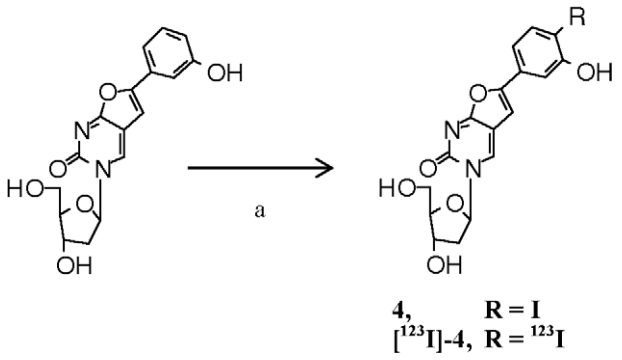


Scheme 1. Synthesis of radiolabeling precursor **3**. Reagents and conditions: (a) $Pd(PPh_3)_4$, iPr_2EtN, CuI, DMF, rt, 19 h; (b) Et_3N/MeOH, CuI, reflux, 4 h.

for imaging up to several hours after administration of the tracer and may allow to achieve images with a high signal/noise ratio because of physiologic wash-out of background radioactivity and retention of radioactivity in VZV-TK-expressing cells.[4] Iodine-123 ($t_{1/2}$ 13.2 h) is a SPECT radioisotope and can be conveniently introduced on an aromatic ring bearing a phenol group via an electrophilic substitution reaction. In this paper, we report the synthesis and preliminary biological evaluation of a mono-^{123}I-iodo labeled BCNA, namely 3-(2′-deoxy-β-D-ribofuranosyl)-6-(3-hydroxy, 4-[^{123}I]iodophenyl)-2,3-dihydrofuro[2,3-*d*]pyrimidin-2-one ([^{123}I]-**4**), and its evaluation as a reporter probe for the newly developed VZV-tk reporter gene.

Bicyclic nucleoside analogues are usually synthesized by Sonogashira coupling of aryl acetylenes to 5-iodo-2′-deoxyuridine under co-catalysis of Pd and cuprous ions, followed by copper(I)-promoted cyclization of the coupled product in situ or after isolation.[6,7] Following this reaction, the radiolabeling precursor **3** was synthesized by coupling 3-hydroxyphenyl acetylene and 5-iodo-2′-deoxyuridine in 38% yield as reported earlier (Scheme 1).[5,8] Compound **3** was used as starting material for the synthesis of non-radioactive mono-iodo derivative **4** through a standard electrophilic substitution reaction using sodium iodide (NaI) in the presence of peracetic acid as an oxidizing agent in acidic medium, in ethanol as solvent (Scheme 2).[9,10] It is evident from the structure of the phenol precursor **3** that iodination can occur at three possible positions (two *ortho*- and one *para*-position to the aromatic hydroxyl group). Interestingly, however, the iodination reaction resulted in one major mono-iodo compound **4** (as determined using mass spectrometry) that was isolated in 47% yield using RP–HPLC. Two-dimensional rotating frame Overhauser effect spectroscopy (^{1}H–^{1}H ROESY) experiments using HPLC purified **4** have revealed that the C-5 proton of the furopyrimidine ring interacts with C-2 and C-6 protons of the phenyl moiety, proving the absence of iodine atom on both these positions. This interaction of C-5 proton (furopyrimidine) with both C-2 and C-6 protons (phenyl ring) can be explained by the free rotation of the phenol group around the C-6(furopyrimidine)/C-1(phenyl) bond. Such interaction of C-5 proton (furopyrimidine) with C-5 (or C-4) proton of the phenyl ring is not very probable in view of the relative large distance between these protons. Further, the presence of iodine atom at C-5 position of the phenyl ring can also be excluded by the presence of a duplet at 7.76 ppm with a characteristic $^3J_{H-H}$ coupling of 8.2 Hz. These data strongly suggest that the location of the iodine atom in **4** is at C-4 of the phenyl ring.

The affinity of compounds **3** and **4** for purified recombinant VZV-TK enzyme was evaluated in vitro in competition with [CH_3-^{3}H]dThd (deoxythymidine) as the natural substrate for VZV-TK, and the results are presented in Table 1 as 50% inhibitory concentration (IC_{50}) values. The phenol precursor **3** displays good affinity for the enzyme with an IC_{50} value of 2.6 μM,[5] followed by the mono-iodo derivative **4** whose IC_{50} value is 4.2 μM. The affinity of the mono-iodo derivative **4** is comparable to that of previously reported [^{11}C]-**1** (IC_{50} of **1**: 4.8 μM), which proved to be a promising agent in cell uptake studies.[5] In contrast to their affinity for VZV-TK, BCNAs are not phosphorylated by other nucleoside kinase enzymes (e.g., HSV1-TK, cytosolic



Scheme 2. Synthesis of non-radioactive and radioactive **4**. Reagents and condition: (a) 0.5 M H_3PO_4, 0.2 M CH_3COOOH, [$^{127/123}$I]NaI, EtOH, rt, 30–60 min.

Table 1. Affinity (IC_{50}) of the synthesized non-radioactive compounds for the enzyme VZV-TK

Compound	R	IC_{50}[a] (μM)	Log *P*
3	H	2.6 ± 0.1	—
4	I	4.2 ± 0.6	—
[^{123}I]-**4**	^{123}I	—	0.83 ± 0.01

[a] 50% inhibitory concentration or compound concentration required to inhibit nucleoside kinase-catalyzed phosphorylation of 1 μM [CH_3-^{3}H]dThd by 50%.

TK-1) as shown before,[5] confirming the basis for the specificity of these compounds for VZV-TK.

Similar to the synthesis of non-radioactive **4**, radiosynthesis of ^{123}I-labeled **4** was achieved by incubating compound **3** with no-carrier-added (nca) [^{123}I]NaI as labeling agent in the presence of peracetic acid at room temperature (rt) for 30 min.[11] The reaction mixture was purified using HPLC on an XTerra™ RP C_{18} column (5 μm, 4.6 × 250 mm; Waters, Milford, USA), employing 35% EtOH in 0.05 M NaOAc buffer (pH 5.5) as a mobile phase at a flow rate of 0.9 mL/min. The radiolabeled product [^{123}I]-**4** was isolated at 21 min. The radiochemical yield (RCY) of [^{123}I]-**4** was rather low (12 ± 1%; $n = 2$) but sufficient for the preliminary biological evaluation of the tracer. However, this low RCY is assumed to be partly due to the formation of isomers in the radioactive synthesis. At least two other peaks with similar relative intensity as [^{123}I]-**4** were observed in the HPLC-chromatogram of the reaction product after radioactive synthesis. Although the formation of one such mono-iodo isomer (apart from compound **4**) was also observed in the non-radioactive synthesis, its relative amount was low and hence it was not isolated and characterized. However, the formation of other isomers was more pronounced during radiosynthesis resulting in a relative low radiochemical yield of [^{123}I]-**4**. Therefore, use of a *para*-hydroxy compound as starting material may help to achieve a higher RCY, in which case the iodination can occur only at the *meta*-position of the phenyl ring, resulting in a single product. The identity of the purified [^{123}I]-**4** was confirmed by co-elution with authentic non-radioactive compound **4** after co-injection on the above-described analytical HPLC system (Fig. 2). The collected fraction containing [^{123}I]-**4** was concentrated using a flow of nitrogen at 40 °C. An aliquot was reinjected on the same HPLC system to check the stability of the tracer, and the analysis showed that the product did not decompose upon heating at 40 °C for about 20 min.

The lipophilicity of the RP–HPLC purified radiolabeled product [^{123}I]-**4** was determined by partitioning between 1-octanol and 0.025 M phosphate buffer, pH 7.4 ($n = 6$). The log partition coefficient (log P) value of [^{123}I]-**4** was 0.83 ± 0.01.

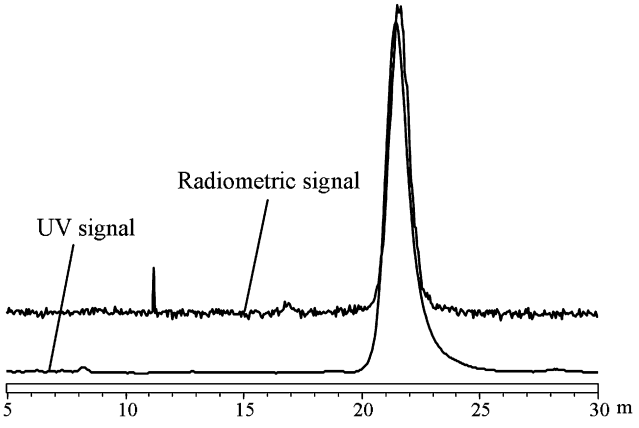


Figure 2. RP–HPLC chromatogram of purified [^{123}I]-**4** co-injected with its non-radioactive analogue (rt = 21.3 min).

Table 2. Biodistribution of [^{123}I]-**4** in normal mice at 2 and 60 min post-injection

Organ	%ID[a]		SUV[b]	
	2 min	60 min	2 min	60 min
Urine	4.9 ± 3.0	23.3 ± 9.3	—	—
Kidneys	12.3 ± 0.9	3.3 ± 1.7	9.0 ± 0.7	2.4 ± 1.1
Liver	26.1 ± 0.7	20.3 ± 5.0	4.9 ± 0.2	4.0 ± 1.0
Spleen + pancreas	1.2 ± 0.1	0.4 ± 0.0	1.7 ± 0.1	0.5 ± 0.0
Lungs	2.2 ± 0.8	0.2 ± 0.1	3.7 ± 0.3	0.7 ± 0.1
Heart	0.5 ± 0.1	0.1 ± 0.0	1.6 ± 0.2	0.4 ± 0.2
Stomach	1.5 ± 0.1	4.1 ± 3.0	—	—
Intestines	11.1 ± 1.6	30.1 ± 12.3	—	—
Brain	0.1 ± 0.0	0.0 ± 0.0	0.4 ± 0.1	0.0 ± 0.0
Blood	25.5 ± 5.5	4.4 ± 1.8	3.6 ± 0.8	0.6 ± 0.3
Carcass	29.5 ± 0.9	16.5 ± 3.6	—	—

Data are expressed as means ± SD; $n = 3$ per time point.

[a] Percentage of injected dose calculated as cpm in organ/total cpm recovered.

[b] Standard uptake values calculated as (radioactivity in cpm in organ/weight of the organ in g)/(total counts recovered/body weight in g).

In vivo distribution of the tracer [^{123}I]-**4** was studied in male NMRI mice and the results are presented in Table 2 as percentage of the injected dose (% ID), or, where possible, as standard uptake values (SUVs). SUVs were calculated as (radioactivity in cpm in organ/weight of the organ)/(total counts recovered/body weight). The mice were injected via a tail vein with 37 kBq of [^{123}I]-**4** and sacrificed by decapitation at 2 min or 60 min after injection ($n = 3$ per time point). Blood and organs were recovered and weighed, followed by counting of the radioactivity in an automated γ-counter. A high fraction of the injected radioactivity was present in blood at 2 min post-injection (pi) (25.5% ID), but this cleared rapidly with only 4.4% ID remaining at 60 min pi. This indicates a good clearance of the tracer from blood and its possible usefulness for in vivo imaging starting from the early hours after injection of the tracer. The compound was cleared mainly by the hepatobiliary system (50.4% of ID in liver + intestines at 60 min pi) and to a lesser extent to the urine (23.3% of ID at 60 min pi). The excretion of [^{123}I]-**4** is comparable to that of [^{11}C]-**1** and [^{18}F]-**2**, which showed 65–67% of ID in the hepatobiliary system and 28–30% ID in urine at 60 min pi. The activity in other organs (spleen, pancreas, lungs, heart, and brain) was negligible at 60 min pi (⩽0.4% ID). In addition, no brain uptake of the tracer was observed at 2 min or 60 min pi. At 2 min pi, the highest concentration of radioactivity was found in the kidneys with a SUV of 9.0, followed by liver (SUV = 4.9) and lungs (SUV = 3.7).

The accumulation of [^{123}I]-**4** was evaluated in VZV-TK- or beta-galactosidase-(control) expressing HEK-293T cells. HEK-293T cell line is preferred because they can be readily transduced to high copy numbers, allow for good selection using puromycin, and because they are

sufficiently adherent to tolerate the multiple wash-steps during the uptake studies. The cells were transduced with a lentiviral vector (LV)[12] encoding the VZV-tk gene or the beta-galactosidase gene and a puromycin resistance gene (*pac*, puromycin-*N*-acetyl-transferase) linked by an internal ribosome entry sequence (IRES). Cells were incubated in triplicate with about 18 kBq of [^{123}I]-**4** at 37 °C for 30 min. The incubation was followed by removal of the medium, triple washing of the cells with ice-cold phosphate-buffered saline, cell lysis using Cell Culture Lysis Reagent (Promega Corporation, Madison, USA), and subsequent counting of the radioactivity in combined wash fractions as well as in cell lysate (γ-counter). The accumulation of the tracer was normalized for total protein content in the cell fraction for each well and expressed as percent of total radioactivity per mg protein (% tracer/mg protein). The uptake of [^{123}I]-**4** was 1.74-fold higher in VZV-TK-expressing cells than in beta-galactosidase-expressing cells (1.23% vs 0.7% of tracer/mg protein in control cells; $n = 3$; $p < 0.05$, unpaired bidirectional Student's *t*-test). However, this uptake ratio (VZV-TK+ cells vs control) is rather low considering the good affinity of the non-radioactive compound **4** against VZV-TK (IC_{50}: 4.2 μM). The previously reported PET tracer [^{11}C]-**1** (IC_{50} of **1**: 4.8 μM) showed an uptake ratio of 53 (VZV-TK+ cells vs control) in the same cell line. However, the log *P* of [^{11}C]-**1** is 1.27 compared to 0.83 for [^{123}I]-**4**. Most nucleosides and their analogues are hydrophilic and are transported across the cell membrane by specialized membrane nucleoside transporter (NT) proteins belonging to the family of either concentrative or equilibrative NTs (CNTs or ENTs, respectively). In contrast, BCNAs that are pyrimidine nucleoside analogues cross the cell membrane presumably by passive diffusion due to the lipophilicity of the tracer, which is a crucial factor for its accumulation in VZV-tk transduced cells. The log *P* value should ideally be between 1 and 2.5 for passive diffusion of neutral compounds through cell and tissue membranes.[13] Hence, the low log *P* value of [^{123}I]-**4** may explain its lower uptake in VZV-TK-expressing cells. However, detailed transport studies are required to establish the exact mode of transportation of BCNAs across cell and tissue membranes.

In conclusion, further in vivo evaluation of [^{123}I]-**4** as a SPECT tracer for the VZV-tk reporter gene is warranted in view of its favorable biodistribution, good affinity for the VZV-TK, and its significant uptake in VZV-TK-expressing cells.

Acknowledgments

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8. Compound **3**: To a stirred solution of 5-iodo-2′-deoxyuridine (2.9 g, 8.19 mmol) in DMF (30 mL) under N_2 at rt were successively added DIEA (3.0 mL, 16.95 mmol), $Pd(PPh_3)_4$ (0.96 g, 0.828 mmol), CuI (0.31 g, 1.65 mmol), and a solution of 3-hydroxyphenylacetylene (2.9 g in 5 mL DMF, 24.57 mmol). The resulting mixture was stirred for 19 h after which CuI (0.29 g), MeOH (73 mL), and TEA (55 mL) were added and the reaction mixture was heated under reflux for 4 h. The solvents were evaporated under reduced pressure and the resulting residue was stirred with Amberlite IRA-400 (HCO_3^-) in CH_3OH/CH_2Cl_2 (1:1) (20 mL) for 1 h. The resin was filtered, washed with methanol, and the combined filtrates were evaporated to dryness. The crude product was purified using column chromatography on silica gel eluted with gradient mixtures of CH_2Cl_2 and CH_3OH (up to 10%) to obtain a yellow substance, which was treated with hot CH_3CN. The pure product was obtained as yellow crystals (1.1 g, 38%). δ_H (DMSO-d_6) 9.8 (1H, Ph-OH), 8.85 (1H, s, 4-H), 7.34–7.16 (3H, 3× Ph-H), 7.20 (1H, s, 5-H), 6.83 (1H, m, Ph-H), 6.18 (1H, dd, $^3J = 6.1$ Hz, 1′-H), 5.32 (1H, d, $^3J = 4.4$ Hz, 3′-OH), 5.20 (1H, t, $^3J = 5.0$ Hz, 5′-OH), 4.24 (1H, m, 3′-H), 3.93 (1H, m, 4′-H), 3.66 (2H, m, 5′-CH_2), 2.40 (1H, m, 2′-H), 2.09 (1H, m, 2′-H); MS (ES)$^+$ Accurate mass: $[C_{17}H_{16}N_2O_6+Na]^+$ theor. 367.0906; found 367.0904.
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10. Compound **4**: To a stirred solution of compound **3** (100 mg, 0.29 mmol) in EtOH (200 mL) were sequentially added 0.5 M H_3PO_4 (33.3 mL), 0.2 M peracetic acid (66.6 mL), EtOH (70 mL), and dropwise a solution of NaI in 0.01 M NaOH (2 mg/mL; 21.7 mL). The reaction mixture was stirred at rt for 1 h, followed by evaporation of the solvents under reduced pressure. The crude product was purified using HPLC that consisted of a Waters 600 pump (Waters) and a C-18 semi-preparative column (Econosphere, 10 × 250 mm; Alltech, Deerfield, USA) eluted with 25% CH_3CN in 0.01 M NH_4OAc solution at a flow rate of 3.0 mL/min (rt = 18.7 min). Detection was carried out using a UV-spectrometer (Waters 2487 Dual λ absorbance detector) set at 254 nm. After evaporation of the solvent from the combined HPLC fractions, the

product was obtained as a white solid (64 mg, 47%). δ_H (DMSO-d_6) 8.86 (1H, s, 4-H), 7.76 (1H, d, ^3J = 8.2 Hz, Ph-5-H), 7.30 (1H, d, ^4J = 1.6 Hz, Ph-2-H), 7.21 (1H, s, 5-H), 7.05 (1H, dd, ^3J = 8.2 Hz, ^4J = 1.6 Hz, Ph-6-H), 6.18 (1H, t, ^3J = 6.1 Hz, 1′-H), 5.32 (1H, d, ^3J = 4.4 Hz, 3′-OH), 5.20 (1H, t, ^3J = 5.0 Hz, 5′-OH), 4.25 (1H, m, 3′-H), 3.93 (1H, m, 4′-H), 3.67 (2H, m, 5′-CH_2), 2.41 (1H, m, 2′-H), 2.10 (1H, m, 2′-H); MS $(ES)^+$ Accurate mass: $[C_{17}H_{15}IN_2O_6+Na]^+$ theor. 492.9872; found 492.9825.

11. Synthesis of [^{123}I]-**4**: To a labeling reaction vial containing 37 MBq of nca [^{123}I]NaI in 2 μL of 0.05 M NaOH were sequentially added a solution of **3** in EtOH (0.5 mg/mL; 150 μL), 0.5 M H_3PO_4 (25 μL), 0.2 M peracetic acid solution (50 μL), and EtOH (50 μL). The reaction mixture was incubated at rt for 30 min, followed by purification using RP–HPLC on an XTerra™ RP C_{18} column (5 μm, 4.6 × 250 mm; Waters), eluted with 35% EtOH in 0.05 M NH_4OAc buffer (pH 5.5) at a flow rate of 0.9 mL/min (rt = 21.3 min). Detection of the compounds was carried out using a UV-detector set at 254 nm, and a 3-inch NaI(Tl) scintillation detector connected to a single channel analyzer. [^{123}I]-**4** was obtained in 12 ± 1% yield (relative to starting ^{123}I activity, n = 2).
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