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4-*N*-Alkanoyl and 4-*N*-Alkyl Gemcitabine Analogues with NOTA Chelators for 68-Gallium Labelling

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Abstract

The conjugation of 4-*N*-(3-aminopropyl)-2'-deoxy-2',2'-difluorocytidine with 2-(*p*-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (SCN-Bn-NOTA) ligand in 0.1 M Na₂CO₃ buffer (pH 11) at ambient temperature provided 4-*N*-alkylgemcitabine-NOTA chelator. Incubation of latter with excess of gallium(III) chloride (GaCl₃) (0.6 N AcONa/H₂O, pH = 9.3) over 15 min gave gallium 4-*N*-alkylgemcitabine-NOTA complex which was characterized by HRMS. Analogous [⁶⁸Ga]-complexation of 4-*N*-alkylgemcitabine-NOTA conjugate proceeded with high labeling efficiency (94% to 96%) with the radioligand almost exclusively found in the aqueous layer (~95%). The high polarity of the gallium 4-*N*-alkylgemcitabine-NOTA complex resulted in rapid renal clearance of the ⁶⁸Ga-labelled radioligand in BALB/c mice.

GRAPHICAL ABSTRACT

Keywords

Nucleosides; Gemcitabine; Prodrugs; NOTA; PET imaging; 68-Gallium

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Conflict of Interest

The authors have declared no conflict of interest.

1. Introduction

Gemcitabine (2'-deoxy-2',2'-difluorocytidine, dFdC) is a potent chemotherapeutic drug used in the treatment of cancers and solid tumors.^{1–2} Various prodrug strategies have been developed to improve uptake and to diminish intracellular deamination of gemcitabine into toxic 2'-deoxy-2',2'-difluorouridine (dFdU) by deoxycytidine deaminase (dCDA).³ Most of these prodrugs possess lipophilic acyl modifications on the exocyclic 4-*N*-amine or 5'-hydroxyl groups.^{4–9} Gemcitabine analogues have also been designed as theranostic anticancer prodrugs with cellular specificity and imaging capabilities. These include H-gemcitabine¹⁰ and gemcitabine-coumarin-biotin conjugates,¹¹ among others.^{12–13}

Recently, 1-(2-deoxy-2-[¹⁸F]fluoro-β-D-arabinofuranosyl)cytosine [¹⁸F-FAC] was developed as a noninvasive predictor probe of tumor response to gemcitabine.^{14–17} Although FAC generally follows the known biodistribution of gemcitabine, it is missing a *geminal* difluoromethylene unit at C2', that is critical for anticancer properties of dFdC, its inhibitory activity of ribonucleotide reductases,¹⁸ and its physicochemical properties.¹⁹ Practical syntheses of gemcitabine are based on incorporation of a *geminal* difluoro unit into the ribose moiety in the early synthetic stages.^{20–21} Preparation of 2'-[¹⁸F]dFdC following these synthetic approaches would result in an impractical multistep radiosynthesis, given the short half-life of the ¹⁸F isotope (110 mins) and the lack of availability of the proper synthetic precursors.²² To address these challenges, we have explored developing gemcitabine radiotracers in which ¹⁸F labeling is incorporated at a different position than at sugar C2' and in the later stages of their synthesis (Figure 1).

We have developed 4-*N*-acyl gemcitabine analogues with modifications suitable for incorporation of fluorine radiotracers (e.g. **A**, Figure 1). However, 4-*N*-acyl analogues **A** showed cleavage of the amide linkage under typical radiosynthetic protocols for ¹⁸F labeling (KF/K₂CO₃/K_{2.2.2}/CH₃CN/110°C). We found, however, that 4-*N*-alkyl gemcitabine derivatives²⁴ are suitable for incorporation of ¹⁸F radiolabels to 4-*N*-alkyl chain (e.g., **B** → **C**), yet showed ¹⁸F *in vivo* defluorination, which resulted in marked bone uptake as Na¹⁸F during mice studies.²⁵ Subsequently, we explored lipophilic 4-*N*-alkyl gemcitabine analogues bearing terminal β-ketosulfonate (e.g. **D**) in which the lack of hydrogen at carbon β was expected to prevent loss of fluorine. However, fluorination and deprotection under conditions required for ¹⁸F labeling afforded fluoro product **E** in low yields.²⁶ We recently also reported 4-*N*-acyl and 4-*N*-alkyl gemcitabine analogues with silicon-fluoride moieties that have shown to be good substrates for ¹⁸F-labeling (e.g., **F** → **G**). Preliminary positron-emission tomography (PET) imaging in mice showed the biodistribution of [¹⁸F]-**G** to have initial concentration in the liver, kidneys and GI tract followed by increasing signal in the bone.²⁷

Hydrolysis of the 4-*N*-amide linkage under radiosynthetic protocols employed in the preparation of ¹⁸F radiotracers and observed early loss of ¹⁸F through the elimination processes during *in vivo* mice studies prompted us to search for other gemcitabine conjugates suitable for PET imaging. Recent advances in aza-crown ethers bifunctional chelators with the ability to bind gallium generated significant interest in ⁶⁸Ga-based radiopharmaceuticals^{28–32} and radiotracers.^{33–34} As a metal salt, gallium has been reported

to possess anti-proliferative properties in various cancer cells mainly attributed with its ability to mimic Fe^{3+} .^{35–37} Among bifunctional chelators, NODA-SA³⁸ (1,4,7-triazacyclononane-1-succinic acid-4,7-diacetic acid) and NODA-GA³⁹ (1,4,7-triazacyclononane-1-glutamic acid-4,7-diacetic acid), which possess a NOTA (1,4,7-triazacyclononane-1,4,7-triacetic acid) complexation site, have a coordination cavity highly accommodating to Ga(III) (ionic radius, 0.65 Å). Also, anti-TF (Tissue Factor) antibody (ALT-836) conjugated to 4-isothiocyanatobenzyl-NOTA (SCN-Bn-NOTA) labelled with ⁶⁴Cu showed uptake in pancreatic cancer cells overexpressing TF and has been advanced to Phase I clinical trials as a combination therapy with gemcitabine.⁴⁰ These results encouraged us to investigate 4-*N*-alkanoyl/alkyl gemcitabine analogues conjugated with aza-crown ether chelators for ⁶⁸Ga-labelling.

2. Results and Discussion

2.1. 4-*N*-Alkanoyl gemcitabine-NOTA conjugates

Initially, we attempted direct condensation of gemcitabine **1** with commercially available NODAGA(tBu)₃ using conditions previously employed for coupling of the weakly nucleophilic 4-amino group of gemcitabine with carboxylic acids.²³ Thus, condensation of **1** with NODA-GA(tBu)₃ in the presence of *N*-methylmorpholine (NMM), 1-hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) gave protected NODA - 4-*N*-alkanoylgemcitabine conjugate **2** in only 10% yield after RP-HPLC purification (Scheme 1). Nevertheless **2** was found to be unstable and upon standing slowly decomposed back to **1**. Deprotection of **2** with TFA afforded NODA-GA-4-*N*-alkanoylgemcitabine conjugate **3** (37%) but also promoted further decomposition to **1**. Moreover, coupling of **1** with 2-(*p*-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (SCN-Bn-NOTA) in 0.1 M sodium carbonate buffer (pH = 9.5)³³ failed to produce the corresponding thiourea conjugate.

In an effort to overcome the poor nucleophilicity associated with cytosine's exocyclic amine, we also explored gemcitabine analogues having a more nucleophilic primary amine at the 4-*N*-alkanoyl linker for subsequent conjugation with NOTA chelators. Thus, condensation of **1** with commercially available *N*-Boc-β-alanine (NMM/HOBt/EDC) provided **4a** in 65% yield (Scheme 2). Attempted removal of the Boc protection at 4-*N*-(3-aminopropanoyl) linker in **4a** with TFA was problematic to control and led mainly to the cleavage of the amide linkage yielding gemcitabine **1** in addition to desired **4b** in very low yields.⁴¹ Besides, condensation of the crude **4b** with SCN-Bn-NOTA in 0.1 M Na₂CO₃ (pH = 11) over 14 h was unsuccessful to provide a stable gemcitabine-NOTA ligand **5**.

2.2. 4-*N*-Alkyl gemcitabine-NOTA conjugates

Since 4-*N*-alkylgemcitabine derivatives are resistant to chemical hydrolysis as well as enzymatic deamination by cytidine deaminase,²³ we next undertook an effort to prepare gemcitabine-NOTA ligand conjugated via 4-*N*-alkyl linker. The 4-*N*-tosylgemcitabine **6a** (prepared from gemcitabine in 96% yield²³) and 3',5'-di-*O*-Boc protected 4-*N*-tosylgemcitabine **6b**²³ served as convenient substrates. Displacement of the *p*-toluenesulfonamido group from **6b** with commercially available *N*-Boc-1,3-propanediamine

in 1,4-dioxane afforded as main product the partially deprotected 4-*N*-(3-*N'*-Boc-3-aminopropyl)gemcitabine **7** (59%, Scheme 3) in addition to 3',5'-di-*O*-Boc protected product (31%). Subsequent deprotection of **7** with TFA yielded the 4-*N*-(3-aminopropyl) derivative **8** (93%), with a primary amine group suitable for coupling with ligands. Amine **8** was also prepared efficiently in a “one-pot” synthesis from **6a** (94% overall yield) without isolation of **7**.

Condensation of **8** with commercially available SCN-Bn-NOTA in 0.1 M Na₂CO₃ buffer (pH = 11) at ambient temperature for 48–72 h afforded the 4-*N*-(3-SCN-Bn-NOTA-propyl)gemcitabine conjugate **9** identified by HRMS (ESI⁺) *m/z* 771.2879. The 4-*N*-alkylgemcitabine-NOTA conjugate **9** was found to be stable as a solid, however some minor deterioration back to **8** (TLC/HPLC) was observed after some time (few weeks) even when stored at –20°C. This showed again that gemcitabine analogues with 4-*N*-alkyl chains have better stability than the corresponding 4-*N*-acyl counterparts (e.g., **2** or **5**).

2.3. [⁶⁸Ga]-Labeling and evaluation of NOTA-4-*N*-alkylgemcitabine radioligand.

Incubation of **9** with excess of gallium(III) chloride (GaCl₃) mimicking radiosynthetic conditions (0.6 N AcONa/H₂O, pH = 9.3) over 15 min gave the gallium NOTA-4-*N*-alkylgemcitabine complex **10**, characterized by HRMS (ESI⁺) *m/z* 837.1977 (Scheme 4). The reaction products were isolated by HPLC on a Phenomenex Gemini semi-preparative RP-C18 column [0% → 100% ethanol gradient in 0.01% TFA/H₂O from 0 to 30 min at a flow rate = 3 mL/min] giving **9** (*t_R* = 12.8 min) and **10** (*t_R* = 11.5 min), respectively. The gallium complexation met criteria for working with [⁶⁸Ga]³⁺ (half-life 68 min) and was applied towards the radiosynthesis of the [⁶⁸Ga]-**10** radioligand.

The ⁶⁸Ga was eluted from an IGG-100 ⁶⁸Ge/⁶⁸Ga generator. Purified [⁶⁸Ga]³⁺ (10 mCi in 1 mL of ≈0.1 N HCl) for the labeling was obtained following previously elaborated conditions.⁴² The [⁶⁸Ga]-labeling of **9** was completed within 15 min and analyzed by developing TLC plates eluted with 0.1 M citric acid on a phosphoscreen (Figure 2). Phosphoscreen analysis (Table 1) indicated a high labeling efficiency (94% to 96%) for the complexation of the NOTA-4-*N*-alkylgemcitabine **9** with [⁶⁸Ga]³⁺ to yield [⁶⁸Ga]-**10**.

The distribution coefficient of [⁶⁸Ga]-radioligand **10** was evaluated in octanol/H₂O and EtOAc/H₂O. The radioligand **10** was almost exclusively found in the aqueous layer, with less <5% of the observed counts occurring in the organic phase (data not shown) of either system. The hydrophilic character of [⁶⁸Ga]-radioligand **10** suggests that the radioligand will be rapidly excreted.

Labeling of **9** with ⁶⁸Ga was repeated to test for distribution of **10** in BALB/c mice. Chelation of ⁶⁸Ga to NOTA moiety in **9** was quantitative after 5 minutes incubation. However, a shoulder and some minor impurities peaks were observed. The purity did not change after Sep-Pak purification, showing a shoulder (RT: 3.27 min) and a minor peak (RT: 4.55 min) that are still visible and quantifiable (Figure 3). However, these impurities were not expected to have a large effect on imaging studies. Only minimal hepatobiliary excretion during *in vivo* mice studies. The rapid renal clearance of [⁶⁸Ga]-**10** is most likely due to the increased hydrophilicity caused by the addition of the NOTA chelator as compared to ¹⁸F-

FAC¹⁴ or more hydrophobic ¹⁸F radiolabeled 4-*N*-alkyl gemcitabine analogues **C**²⁵ and **G**²⁷ (see Figure 1). Although not within the scope of the present work, this compound could result in higher contrast images due to higher lesion to background ratio, which could produce significant clinical impact. The one hour post-injection images are presented in Figure 4.

In conclusion, we have developed the synthesis of 4-*N*-alkyl gemcitabine–NOTA conjugates and their efficient complexation with gallium that provides the basis for development of the 4-*N*-modified gemcitabine derivatives, bearing an elongated alkyl chains with more lipophilic character, as potential 68-gallium PET imaging radioligands.

3. Experimental

The ¹H (400 MHz), ¹³C (100 MHz), or ¹⁹F (376 MHz) NMR spectra were recorded at ambient temperature in MeOH-*d*₄, unless otherwise noted. The reactions involving non-radioactive materials were followed by TLC with Merck Kieselgel 60-F₂₅₄ sheets and products were detected with a 254 nm UV lamps. Column chromatography was performed using Merck Kieselgel 60 (230–400 mesh). Perkin Elmer high-performance liquid chromatography (HPLC) was used for purifying both the non-complexed and complexed compounds on RP-C18 semi-preparative Phenomenex Gemini or preparative XTerra columns with mobile phase and flow rate as indicated. High-resolution mass spectra (HRMS) were obtained with a Solarix instrument (FT-MS). The SCN-Bn-NOTA and NODA-GA(tBu)₃ bifunctional chelators were purchased from Macrocyclics Inc, TX, USA and CheMatech, Dijon, France, respectively. Gemcitabine hydrochloride was purchased from LC Laboratories, MA, USA. All other reagent grade chemicals were purchased from Sigma Aldrich.

4-*N*-[4-(4,7-Bis(2-*tert*-butoxy-2-oxoethyl)-1,4,7-triazonan-1-yl)-5-*tert*-butoxy-5-oxopentanoyl]-2'-deoxy-2',2'-difluorocytidine (**2**).

Gemcitabine **1** (12.6 mg, 0.042 mmol) was treated with 4-(4,7-bis(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7-triazacyclononan-1-yl)-5-(*tert*-butoxy)-5-oxopentanoic acid [NODA-GA(tBu)₃; 25 mg, 0.046 mmol] according to coupling conditions as described for **4a**. After 20 h, reaction mixture was treated with H₂O and EtOH, the volatiles evaporated under reduced pressure and the resulting residue (75 mg) diluted with a solution of 95% H₂O/CH₃CN to a total volume of 1.5 mL, passed through a 0.2 μm PTFE syringe filter and then injected into a preparative HPLC column (XTerra Prep RP-C18 column; 5μ, 19 × 150 mm) *via* 2 mL loop. The HPLC column was eluted with a gradient program (isocratic mobile phase mixture 95% H₂O/CH₃CN for 60 min, gradient 95 → 0% H₂O/CH₃CN over 30 min) at a flow rate = 2.5 mL/min, to give **2** (3.4 mg, 10%; *t*_R = 100–125 min) as a clear oil: ¹H NMR δ 1.44–1.53 (m, 27H, 9 × CH₃), 1.58–1.67 (m, 2H), 1.90–2.03 (m, 2H), 2.05–2.12 (m, 2H), 2.68–2.75 (m, 2H), 2.76–3.05 (m, 13H), 3.80–3.88 (m, 1H, H5'), 3.97–4.03 (m, 2H, H4', H5''), 4.26–4.36 (m, 1H, H3'), 6.28 ("t", *J* = 7.1 Hz, 1H, H1'), 7.52 (d, *J* = 7.7 Hz, 1H, H5), 8.22 (d, *J* = 7.6 Hz, 1H, H6); ¹⁹F NMR δ –119.03 (d of m, *J* = 246.1 Hz, 1F), –120.09 (d of m, *J* = 247.4 Hz, 1F); MS (ESI) *m/z* 789 (100, [M+H]⁺).

4-*N*-[4-(4,7-Bis(carboxymethyl)-1,4,7-triazonan-1-yl)-4-carboxybutanoyl]-2'-deoxy-2',2'-difluorocytidine (3).

Compound **2** (3.4 mg, 0.004 mmol) was dissolved in TFA (1.0 mL) and the mixture was stirred at ambient temperature overnight. After 15 h, the reaction mixture was diluted with toluene, the volatiles were evaporated, and the residue co-evaporated with a fresh portion of toluene. The resulting residue was then dissolved with a solution of 95% H₂O/CH₃CN to a total volume of 1.5 mL, passed through a 0.2 µm PTFE syringe filter and then injected into a preparative HPLC column (XTerra Prep RP-C18 column; 5µ, 19 × 150 mm) via 2 mL loop. The HPLC column was eluted with a gradient program (isocratic mobile phase mixture 95% H₂O/CH₃CN for 60 min, gradient 95 → 0% H₂O/CH₃CN over 30 min) at a flow rate = 2.5 mL/min, to give **3** (1.0 mg, 37%; *t*_R = 63–70 min) as a clear oil: ¹H NMR (D₂O) δ 1.78–1.95 (m, 2H, CH₂), 2.33–2.38 (m, 2H, CH₂), 2.97–3.02 (m, 5H), 3.04–3.09 (m, 6H), 3.11–3.16 (m, 5H), 3.37–3.43 (m, 1H), 3.60–3.67 (m, 1H, H5'), 3.75–3.82 (m, 1H, H5''), 3.84–3.88 (m, 1H, H4'), 4.08–4.16 (m, 1H, H3'), 5.96 (d, *J* = 7.9 Hz, 1H, H5), 5.99 ("t", *J* = 7.6 Hz, 1H, H1'), 7.67 (d, *J* = 7.8 Hz, 1H, H6); ¹⁹F NMR (D₂O) δ -117.39 (d of m, *J* = 230.5 Hz, 1F), -117.71 (d of m, *J* = 236.1 Hz, 1F); MS (ESI) *m/z* 621 (100, [M+H]⁺).

4-*N*-[3-*N*-(*tert*-Butoxycarbonyl)-3-aminopropanoyl]-2'-deoxy-2',2'-difluorocytidine (4a).

N-Methylmorpholine (NMM; 15.6 mg, 0.154 mmol), 1-hydroxybenzotriazole (HOBt; 20.8 mg, 0.169 mmol), commercially available *N*-Boc-β-alanine (29.2 mg, 0.154 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 28.3 mg, 0.182 mmol) were sequentially added to a stirred solution of gemcitabine hydrochloride (**1**; 42 mg, 0.140 mmol) in DMF/DMSO (3:1, 2 mL) at ambient temperature under Argon. The reaction mixture was then gradually heated to 65°C (oil-bath) and kept stirring overnight. After the reaction was completed (TLC), the reaction mixture was cooled to 15°C and partitioned between a small amount of brine and EtOAc. The organic phase was separated, and the aqueous layer extracted with fresh portions of EtOAc (2 × 10 mL). The combined organic layers were then washed with NaHCO₃/H₂O, dried over Na₂SO₄ and evaporated. The crude product was column chromatographed (5% MeOH/EtOAc) to give **4a** (40.1 mg, 65%) as a white solid: ¹H NMR δ 1.42 (s, 9H, 3 × CH₃), 2.63 (t, *J* = 6.5 Hz, 2H, CH₂), 3.37 (t, *J* = 6.5 Hz, 2H, CH₂), 3.81 (dd, *J* = 3.2, 12.8 Hz, 1H, H5'), 3.95–3.99 (m, 2H, H4', H5''), 4.24–4.34 (m, 1H, H3'), 6.26 (t, *J* = 7.3 Hz, 1H, H1'), 7.49 (d, *J* = 7.6 Hz, 1H, H5), 8.33 (d, *J* = 7.6 Hz, 1H, H6); ¹⁹F NMR δ -121.01 (br. d, *J* = 237.0 Hz, 1F), -119.16 (dd, *J* = 243.4, 12.1 Hz, 1F); HRMS (ESI⁺) *m/z* calcd for C₁₇H₂₅F₂N₄O₇ [M+H]⁺, 435.1686, found 435.1699.

4-*N*-[3-*N*-(*tert*-Butoxycarbonyl)-3-aminopropanyl]-2'-deoxy-2',2'-difluorocytidine (7).

In a tightly sealed vessel, a mixture of **6b**²³ (11.5 mg, 0.018 mmol) and commercially available *N*-Boc-1,3-propanediamine (150 µL, 150 mg, 0.86 mmol) in 1,4-dioxane was stirred at 65°C. After 48 h, the volatiles were evaporated the resulting residue was column chromatographed (30% EtOAc/hexane → 5% MeOH/EtOAc) to give fully protected 3',5'-di-*O*-Boc-4-*N*-(3-*N'*-Boc-3-aminopropanyl)gemcitabine [3.6 mg, 31%; MS (ESI⁺) *m/z* 621 (100, [M+H]⁺)] followed by **7** (3.5 mg, 59%) as colorless oil: ¹H NMR δ 1.43 (s, 9H, 3 × CH₃), 1.74 ("quint", *J* = 6.8 Hz, 2H, CH₂), 3.10 (t, *J* = 6.7 Hz, 2H, CH₂), 3.41 (t, *J* = 6.9 Hz, 2H, CH₂), 3.77 (dd, *J* = 3.2 Hz, 12.6 Hz, 1H, H5'), 3.87 (dt, *J* = 2.8, 8.3 Hz, 1H, H4'), 3.93

("d," $J = 2.1, 12.6$ Hz, 1H, H5"), 4.24 (dt, $J = 8.4, 12.1$ Hz, 1H, H3'), 5.85 (d, $J = 7.6$ Hz, 1H, H5), 6.21 ("t," $J = 8.0$ Hz, 1H, H1'), 7.75 (d, $J = 7.6$ Hz, 1H, H6); ^{19}F NMR δ -119.94 (br. d, $J = 240.6$ Hz, 1F), -118.83 (dd, $J = 10.3, 238.3$ Hz, 1F); MS (ESI) m/z 421 (100, $[\text{M} + \text{H}]^+$).

4-*N*-(3-Aminopropanyl)-2'-deoxy-2',2'-difluorocytidine (8).

Method A.—Compound **7** (3.4 mg, 0.008 mmol) was dissolved in TFA (1.0 mL) and the mixture was stirred at ambient temperature overnight. After 15 h, the reaction mixture was diluted with toluene, the volatiles were evaporated, and the residue co-evaporated with a fresh portion of toluene. The resulting residue was then column chromatographed [EtOAc $\rightarrow$ 50% EtOAc/SSE (EtOAc/*i*-PrOH/H₂O, 4:2:1; upper layer)] to give **8** (2.4 mg, 93%) as a white solid: ^1H NMR δ 1.95 ("quint", $J = 6.8$ Hz, 2H, CH₂), 3.19 (t, $J = 6.8$ Hz, 2H, CH₂), 3.51–3.57 (m, 2H, CH₂), 3.81 (dd, $J = 3.1, 12.0$ Hz, 1H, H5'), 3.91–3.99 (m, 2H, H4', H5'), 4.28 (dt, $J = 8.2, 12.1$ Hz, 1H, H3'), 5.92 (d, $J = 7.5$ Hz, 1H, H5), 6.25 ("t," $J = 8.1$ Hz, 1H, H1'), 7.88 (d, $J = 7.1$ Hz, 1H, H6); ^{13}C NMR δ 34.7, 38.6, 39.1, 60.5 (C5'), 70.1 (dd, $J = 21.0, 24.2$ Hz, C3'), 82.8 (dd, $J = 4.1, 5.1$ Hz, C4'), 86.2 (C1), 97.8 (C5), 125.0 (t, $J = 256.8$ Hz, C2'), 140.1 (C6), 158.9 (C2), 165.9 (C4). ^{19}F NMR δ -118.92 (br. d, $J = 241.2$ Hz, 1F), -120.11 (dd, $J = 10.4, 237.8$ Hz, 1F); HRMS (ESI⁺) m/z calcd for C₁₂H₁₉F₂N₄O₄ $[\text{M} + \text{H}]^+$, 321.1369, found 321.1346.

Method B.—In a tightly sealed container, a solution of **6a**²³ (19.6 mg, 0.048 mmol), commercially available *N*-Boc-1,3-propanediamine (100 μL , 100 mg, 0.58 mmol) and TEA (3 mL) in 1,4-dioxane was stirred at 75°C. After 96 h, the volatiles were evaporated and the resulting residue was treated with TFA (1.5 mL) and the mixture stirred at ambient temperature overnight. After 15 h, the reaction mixture was diluted with toluene, the volatiles were evaporated, and the residue co-evaporated with a fresh portion of toluene. The resulting residue was then column chromatographed (10% MeOH/EtOAc) to give **8** (15.6 mg, 94%) with data as reported above.

4-*N*-[3-*N*-(SCN-Bn-NOTA)aminopropanyl]-2'-deoxy-2',2'-difluorocytidine (9).

A mixture of SCN-Bn-NOTA (10 mg, 0.031 mmol) and **8** (10 mg, 0.018 mmol) in 3 mL of 0.1 M Na₂CO₃ buffer (pH 11) was stirred at ambient temperature for 60 h. The reaction mixture was injected into a Phenomenex Gemini semi-preparative RP-C18 column (5 μ , 25 cm $\times$ 1 cm) via 2 mL loop and eluted with 0% $\rightarrow$ 100% ethanol gradient in 0.01% TFA/H₂O from 0 to 30 min at a flow rate = 3 mL/min. Product **9** (12.5 mg, 54% yield) eluted with $t_r = 12.8$ min. ^1H NMR δ 1.90 ("quint", $J = 6.5$ Hz, 2H, CH₂), 2.68–2.75 (m, 2H), 2.87–2.95 (m, 4H), 3.06–3.15 (m, 3H), 3.17–3.29 (m, 5H), 3.41–3.48 (m, 4H), 3.50–3.62 (m, 1H), 3.65–3.72 (m, 4H), 3.79 (dd, $J = 3.2$ Hz, 12.5 Hz, 1H, H5'), 3.88–3.98 (m, 2H, H4, H5'), 4.20–4.29 (m, 1H, H3'), 5.89 (d, $J = 7.6$ Hz, 1H, H5), 6.26 ("t", $J = 8.0$ Hz, H1'), 7.22–7.28 (m, 2H, Ar), 7.31–7.37 (m, 2H, Ar), 7.79 (d, $J = 7.6$ Hz, 1H, H6). ^{19}F NMR δ -118.80 (d of m, $J = 242.1$ Hz, 1F), -119.45 (d of m, $J = 244.1$ Hz, 1F). MS (ESI⁺) m/z 771 (100, $[\text{M} + \text{H}]^+$); HRMS (ESI⁺) m/z calcd for C₃₂H₄₅F₂N₈O₁₀S $[\text{M} + \text{H}]^+$ 771.2942; found 771.2879.

Gallium 4-*N*-[3-*N*-(SCN-Bn-NOTA)aminopropanyl]-2'-deoxy-2',2'-difluorocytidine (10).

Gallium(III) chloride (5.1 mg, 0.029 mmol) was added to a stirred solution of **9** (1.0 mg, 0.001 mmol) in 0.6 N NaCH₃CO₂/H₂O (1 mL, pH = 9.3) at ambient temperature. After 30 min, the reaction was diluted to a total volume of 1.5 mL with H₂O and injected into a Phenomenex Gemini semi-preparative RP-C18 column (5μ, 25 cm × 1 cm) via 2 mL loop and eluted with 0 → 100% ethanol gradient in 0.01% TFA/H₂O from 0 to 30 min at a flow rate = 3 mL/min. Product **10** (0.5 mg, 46% yield) eluted with *t_R* = 11.5 min. HRMS (ESI⁺) *m/z* calcd for C₃₂H₄₂F₂GaN₈NaO₁₀S [M+H]⁺ 837.1963; found 837.1977.

Generation of ⁶⁸Ga radiotracer of 10.

⁶⁸Ge/⁶⁸Ga Radioisotopic Generator: Two different ⁶⁸Ge/⁶⁸Ga generators were used in this study, a 50 mCi IGG-100 (Eckert & Ziegler, Germany), based on the TiO₂ resin technology; and a 50 mCi ITG generator (ItM, Germany), based on a modified silica resin.

Labeling with IGG-100 ⁶⁸Ge/⁶⁸Ga Generator: The generator was eluted with 5 mL of 0.1 M ultrapure HCl (Sigma-Aldrich, USA) solution. Since the elution contains metal contaminants, a combination of chromatographic exchange resins were used for pre-purification of the eluent as described previously.⁴² Briefly, two luer-fitting column beds were prepared. First, 40 mg of AG-50Wx8 cation exchange (Eichrom, USA) column was connected to a three-way stopcock. Next, 15 mg of UTEVA anion exchange (Eichrom, USA) resin in a column was positioned. Finally, another three-way stopcock was located at the end. The system was designed to be used in four simple steps: elution (from the generator, 5mL of 0.1 M HCl); cleaning (1 mL of 0.1 M HCl); purification (1 mL of 5 M HCl); extraction (1 mL of Millipore Water).

The purified ⁶⁸Ga elution (1 mL, pH = 0.6–1; activity = 10–20 mCi) obtained from the purification system was collected in a 15 mL centrifuge tube. A solution containing **9** (10 μg of a 1 mg/mL solution in DMSO) was later added and immediately buffered using 0.3 mL of 3 N ultrapure sodium acetate (Sigma-Aldrich, USA). Labeling was performed using a thermomixer stirring at 750 rpm for 15 minutes at room temperature (Eppendorf, Germany), taking samples at 5, 10 and 15 minutes of incubation. No further purification was done for initial testing. Final quality control was performed in Silicagel 60 TLC plates with 0.1 M citric acid as the mobile phase. Plates were quantified in OptiQuant software (Perkin Elmer, USA) by exposing a phosphor screen for 5 min and reading it in a Packard Cyclone Phosphorimager (Perkin Elmer, USA). This labeled product was not used for imaging.

Labeling with the ITG ⁶⁸Ge/⁶⁸Ga Generator: This generator was eluted using 4 mL of 0.05M HCl and collected in a 15 mL sterile centrifuge tube. The obtained solution containing up to 1850 MBq (50 mCi) of ⁶⁸Ga was directly used for labeling by adding 25 μL of a 1 mg/mL compound **9** stock solution in DMSO, followed by 80 μL of a 3N NaOAc solution for buffering (pH = 4–5). The mixture was then placed in a thermomixer (FisherSci, USA) at 95°C and 750 rpm shaking for 15 minutes. A sample of the reaction was taken and analyzed by radio HPLC to confirm the labeling. Immediately after labeling confirmation, a purification/reformulation was performed by trapping the labeled product in a C-18 Sep-Pak Plus Lite (Waters, USA) followed by a wash with 5 mL of saline solution. The product was

then eluted with 200 µl of a 50 % EtOH/saline solution, followed by 0.9 ml of saline to produce a final injectable solution containing 8–9% EtOH in 1 ml saline. A final QC HPLC was performed to assess purity.

Quality Control: Radiochemical and chemical purity were determined on a dual pump Varian Dynamax HPLC (Agilent Technologies, USA) fitted with an Agilent ProStar 325 Dual Wavelength UV-Vis Detector and a NaI(Tl) flow count detector (Bioscan, USA). UV absorption was monitored at 220 nm and 280 nm. Solvent A was 0.01 % trifluoroacetic acid (TFA) in H₂O and solvent B was 0.01 % TFA in 90:10 v/v MeCN:H₂O. Analytical (radio)HPLC was performed a Symmetry[®] C18, 4.6×50 mm column (Waters, USA), a flow rate of 2 mL/min and a gradient of 0% B 0–1 min., 0–100% B 1–8 min and 100–0% B 8–10 min. Compound identity was confirmed by co-injection with known standards.

Imaging: Animals (BALB/c) were injected with 100 µl of the final solution and placed immediately in the camera under 2% isoflurane anesthesia. Imaging was performed in a Siemens Inveon µPET/CT for 30 min from 45 to 75 minutes post injection. Final Maximum Intensity Projection (MIP/µPET CT) images are presented for 4 animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The 4-*N*-acyl and 4-*N*-alkyl gemcitabine analogues suitable for ¹⁸F-radiolabelling.

Figure 2.

TLC for complexation of NOTA-4-*N*-alkylgemcitabine 9 with ^{68}Ga . Free $[^{68}\text{Ga}]^{3+}$ (lane A) and $[^{68}\text{Ga}]$ -complexed-10 (lane B).

Figure 3.

HPLC purification of [^{68}Ga]-**10** with minor impurities after Sep-Pak purification.

Interestingly, in contrast with previously published studies with ^{18}F -FAC,¹⁴ compound [^{68}Ga]-**10** showed

Figure 4. BALB/c maximum intensity projection (MIP) μ PET/CT mice images at one hour post injection showing rapid renal clearance.

Scheme 1.

Synthesis of the 4-*N*-alkanoyl gemcitabine-NOTA conjugate.

Scheme 2.

Attempted synthesis of gemcitabine-NOTA conjugate with 4-*N*-alkanoyl linker

Scheme 3. Preparation of the gemcitabine-NOTA conjugate with 4-*N*-alkyl linker.

Scheme 4.

Labeling of 4-*N*-alkylgemcitabine-NOTA conjugate with gallium or ^{68}Ga .

Table 1.

Labeling values for 4-*N*-alkylgemcitabine-NOTA ligand 9 with ^{68}Ga .

	Trial 1		Trial 2		Trial 3	
	Control (Free ^{68}Ga)	Cmpd [^{68}Ga]-10	Control (Free ^{68}Ga)	Cmpd [^{68}Ga]-10	Control (Free ^{68}Ga)	Cmpd [^{68}Ga]-10
Counts in upper	363069018	25232330	240306449	10064331	219716549	6458287
region Counts in lower	7970885	374787348	4208914	256461215	3319270	166566169
Region Labeling Yield	≈ 2% Appl. Point	94%	≈ 2% Appl. Point	96%	≈ 1% Appl. Point	96%