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N-Succinimidyl guanidinomethyl iodobenzoate protein radiohalogenation agents: Influence of isomeric substitution on radiolabeling and target cell residualization

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ABSTRACT

Introduction: *N*-succinimidyl 4-guanidinomethyl-3-[*I]iodobenzoate ([*I]SGMIB) has shown promise for the radioiodination of monoclonal antibodies (mAbs) and other proteins that undergo extensive internalization after receptor binding, enhancing tumor targeting compared to direct electrophilic radioiodination. However, radiochemical yields for [^{131}I]SGMIB synthesis are low, which we hypothesize is due to steric hindrance from the Boc-protected guanidinomethyl group ortho to the tin moiety. To overcome this, we developed the isomeric compound, *N*-succinimidyl 3-guanidinomethyl-5-[^{131}I]iodobenzoate (*iso*-[^{131}I]SGMIB) wherein this bulky group was moved from ortho to meta position.

Methods: Boc$_2$-*iso*-SGMIB standard and its tin precursor, *N*-succinimidyl 3-((1,2-bis(*tert*-butoxycarbonyl) guanidino)methyl)-5-(trimethylstannyl)benzoate (Boc$_2$-*iso*-SGMTB), were synthesized using two disparate routes, and *iso*-[*I]SGMIB synthesized from the tin precursor. Two HER2-targeted vectors — trastuzumab (Tras) and a nanobody 5 F7 (Nb) — were labeled using *iso*-[*I]SGMIB and [*I]SGMIB. Paired-label internalization assays in vitro with both proteins, and biodistribution in vivo with trastuzumab, labeled using the two isomeric prosthetic agents were performed.

Results: When the reactions were performed under identical conditions, radioiodination yields for the synthesis of Boc$_2$-*iso*-[^{131}I]SGMIB were significantly higher than those for Boc$_2$-[^{131}I]SGMIB (70.7 ± 2.0% vs 56.5 ± 5.5%). With both Nb and trastuzumab, conjugation efficiency also was higher with *iso*-[^{131}I]SGMIB than with [^{131}I]SGMIB (Nb, 33.1 ± 7.1% vs 28.9 ± 13.0%; Tras, 45.1 ± 4.5% vs 34.8 ± 10.3%); however, the differences were not statistically significant. Internalization assays performed on BT474 cells with 5 F7 Nb indicated similar residualizing capacity over 6 h; however, at 24 h, radioactivity retained intracellularly for *iso*-[^{131}I]SGMIB-Nb was lower than for [^{125}I]SGMIB-Nb (46.4 ± 1.3% vs 56.5 ± 2.5%); similar results were obtained using Tras. Likewise, a paired-label biodistribution of Tras labeled using *iso*-[^{125}I]SGMIB and [^{131}I] SGMIB indicated an up to 22% tumor uptake advantage at later time points for [^{131}I]SGMIB-Tras.

Conclusion: Given the higher labeling efficiency obtained with *iso*-SGMIB, this residualizing agent might be of value for use with shorter half-life radiohalogens.

1. Introduction

Targeted radiotherapy using monoclonal antibodies (mAbs) specifically reactive with internalizing receptors that are either uniquely or over expressed on cancer cells has been widely investigated [1]. To circumvent the loss of radioactivity from tumor cells after intracellular degradation of the radioiodinated mAbs [2,3], a variety of residualizing labeling approaches have been developed including the use of iodotyramine-cellobiose conjugates [4–6], and D-amino acid peptide-DTPA conjugates [7], and peptides containing charged D-amino acids [8,9].

Our group has been pursuing the development of an alternative approach for labeling internalizing mAbs that seeks to enhance retention of radiohalogens in cancer cells while attempting to avoid concomitantly increased normal tissue retention. The design hypothesis has been to utilize acylation agents that will result in the generation of labeled catabolites that would be charged at lysosomal pH and therefore will not be able to cross the lysosomal and cell membranes [10–14]. The most successful residualizing agent developed to date based on this premise is the guanidine-moiety-containing

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acylation agent, *N*-succinimidyl 4-guanidinomethyl-3-[^{131}I]iodobenzoate ([^{131}I]SGMIB) [15,16], which likely reflects the fact that guanidine has a pKa of ~13, and should remain exclusively positively charged at lysosomal pH. Indeed, when the anti-epidermal growth factor receptor variant III-reactive mAb L8A4 was labeled with [^{131}I]SGMIB, significantly higher intracellularly retained radioactivity in tumor cells in vitro, compared to that from the directly labeled mAb was seen even at 24 h [15]. Moreover, an up to fivefold tumor targeting advantage was demonstrated for radioiodination of this and other internalizing vectors with SGMIB prosthetic group [17–19].

When we designed the SGMIB reagent, we opted to use the 1,3,4- over 1,3,5-isomer simply due to the ready availability of the starting material for the synthesis of both its standard and tin precursor. However, the yields for the synthesis of Boc_2-[^{131}I]SGMIB from its tin precursor were only 60–65%, significantly lower than that obtained with similar compounds [10,20]. Hypothesizing that the lower radiochemical yield might be due to steric hindrance imparted by the bulky Boc_2-guanidinomethyl group present at the ortho position of the tin moiety in the precursor, in the current study, we have developed an isomeric agent, *N*-succinimidyl 3-guanidinomethyl-5-[^{131}I]iodobenzoate (*iso*-[^{131}I]SGMIB) wherein the guanidinomethyl moiety has been moved to the 3-position to give a 1,3,5-substitution. Direct comparisons were made between Boc_2-*iso*-[^{131}I]SGMIB and Boc_2-[^{131}I]SGMIB with regard to radiochemical yield and the efficiency of conjugating *iso*-[^{131}I]SGMIB and [^{131}I]SGMIB to two HER2-targeting proteins — trastuzumab (Tras) and 5 F7 nanobody. Cellular retention of radioactivity after internalization of two biomolecules, each labeled using the two isomeric prosthetic agents, was evaluated in vitro on HER2-expressing BT474 breast carcinoma cells. Finally, a paired-label biodistribution of trastuzumab radioiodinated using *iso*-[^{125}I]SGMIB and [^{131}I]SGMIB was performed in mice with subcutaneous BT474M1 breast carcinoma xenografts.

2. Materials and methods

2.1. General

Chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless noted otherwise. Sodium [^{125}I]iodide and sodium [^{131}I]iodide in 0.1 N NaOH with specific activities of 2200 Ci/mmol and 1200 Ci/mmol respectively, were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). The Boc-protected tin precursor of 1,3,4-SGMIB and radioiodinated SGMIB were synthesized as reported previously [15,16]. Aluminum-backed sheets (Silica gel 60 F254) used for analytical TLC and silica gel 60 for normal-phase column chromatography were obtained from EM Science (Gibbstown, NJ). In some cases, chromatography was also performed with the Biotage Isolera chromatography system (Charlotte, NC) using their pre-packed columns. Preparative thick layer chromatography was used for small-scale purification with plates obtained from Whatman (Clifton, NJ) or EM Science. High pressure liquid chromatography (HPLC) was performed using a Beckman Gold HPLC system equipped with a Model 126 programmable solvent module, a Model 166 NM variable wavelength detector, a Model 170 radioisotope detector and a Beckman System Gold remote interface module SS420X; data were acquired using the 32 Karat® software (Beckman Coulter, Inc., Brea, CA). Recently, the gamma detector in this system was replaced with a ScanRam RadioTLC scanner/HPLC detector combination (LabLogic; Brandon, Fl), and later radio HPLC analyses were performed with that detector. A 4.6 × 250 mm Partisil silica column (10 μm; Alltech, Deerfield, IL) was used for normal phase HPLC. PD-10 desalting columns for gel filtration were purchased from GE Healthcare (Piscataway, NJ). Instant thin layer chromatography (ITLC) was performed using silica gel impregnated glass fiber sheets (Pall Corporation, East Hills, NY) eluted with PBS, pH 7.4. Developed sheets were analyzed for radioactivity either using the TLC scanner described above or cutting the sheet into small strips and counting them in an automated gamma counter (LKB 1282, Wallac, Finland or Perkin Elmer Wizard II, Shelton, CT). Proton NMR spectra were obtained on a Varian 400 MHz NMR spectrometer (Palo Alto, CA); chemical shifts are reported in δ units using the residual solvent peak as reference. Mass spectra were recorded using an Agilent LC/MSD Trap for electrospray ionization (ESI) LC/MS or an Agilent LCMS-TOF with DART, a high resolution mass spectrometer used for ESI, DART and LC-MS.

2.2. Nb, mAb, cells, and culture conditions

Trastuzumab (Tras; Herceptin®) was purchased from Genentech (San Francisco, CA), and the anti-HER2 Nb [17,18], 5 F7 was a gift from Ablynx (Ghent, Belgium). All reagents used for cell studies were obtained from Invitrogen (Grand Island, NY). BT474 human breast carcinoma cells were cultured in DMEM/F12 medium containing 10% fetal calf serum (FCS), streptomycin (100 μg/mL), and penicillin (100 IU/mL) (Sigma Aldrich, MO). Cells were cultured at 37 °C in a humidified incubator under 5% CO_2 with media changed every two days. When about 80% confluent, cells were sub-cultured by trypsinization (0.05 % Trypsin–EDTA).

2.3. Synthesis of Boc-protected iodo standard and tin precursor-method 1

2.3.1. 3-Amino-5-(methoxycarbonyl)benzoic acid (**1**)

A suspension of 10% Pd/C (0.35 g, 3.33 mmol) in ethanol (2 mL) was carefully added to a solution of 3-(methoxycarbonyl)-5-nitrobenzoic acid (2.5 g, 11.1 mmol) in ethanol (5 mL) in an argon-purged flask. Argon was displaced by hydrogen by repeated evacuation and hydrogen flushes, and the mixture was stirred at 20 °C for 5 h under a hydrogen atmosphere (balloon). After reduction reaction was completed, the reaction mixture was carefully filtered through a bed of Celite in a fritted funnel. Ethanol was evaporated from the filtrate, and the crude product chromatographed (40:60 ethyl acetate:hexanes) to yield 2.03 g (94%) of white solid: ^{1}H NMR (CD_3OD): δ 3.88 (s, 3H), 7.51 (d, 2H), 7.89 (s, 1H). MS (ESI), *m/z*: (positive mode) 196.1 $(M + H)^+$; (negative mode) 194.1 $(M\text{-}H)^-$.

2.3.2. 3-Iodo-5-(methoxycarbonyl)benzoic acid (**2**)

Aqueous hydriodic acid (57 wt %, 0.85 mL) was added to a solution of 3-amino-5-(methoxycarbonyl)benzoic acid (**1**; 1.96 g, 10.10 mmol) in THF (3 mL) at 0 °C, and the mixture stirred for 5 min. A solution of sodium nitrite in water (2.08 g, 30.14 mmol; 3 mL) was added drop wise to the above mixture, and the stirring continued for another 40 min at 0 °C. A solution of potassium iodide (5.00 g, 30.12 mmol) in 3 mL of water was added to the resultant diazonium intermediate, and the mixture stirred for 2 h at 20 °C. The reaction mixture was diluted with 300 mL of water and extracted with EtOAc (3 × 150 mL). The combined organic extract was washed with brine, dried with Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by chromatography (1:1 ethyl acetate:hexanes) to yield 0.2 g (26%) of white solid: ^{1}H NMR ($CDCl_3$): δ 3.86 (s, 3H), 7.84 (s, 1H), 7.88 (s, 1H), 8.18 (s, 1H). MS (ESI), *m/z*: (positive mode) 329 $(M + Na)^+$; (negative mode) 305 $(M\text{-}H)^-$.

2.3.3. Methyl 3-(hydroxymethyl)-5-iodobenzoate (**3**)

Borane dimethyl sulfide complex (0.20 g; 2.63 mmol) was added drop wise to a solution of 3-iodo-5-(methoxycarbonyl)benzoic acid (**2**; 0.70 g, 2.29 mmol) in anhydrous chloroform (10 mL), and the mixture refluxed for 3 h. The mixture was partitioned between 0.1 M potassium carbonate and ethyl acetate, and the pooled ethyl acetate extract was washed with brine, and dried with Na_2SO_4. The crude product was purified by chromatography using 3:7 ethyl acetate:hexanes to yield 0.44 g (78%) of an oil: ^{1}H NMR ($CDCl_3$): δ 3.87 (s, 3H), 4.65 (s, 2H), 7.86 (s, 1H), 7.90 (s, 1H), 8.21(s, 1H). GCMS *m/z*: 292 $(M + H)^+$.