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Radioiodinated O^6 -Benzylguanine Derivatives Containing an Azido Function

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Abstract

Introduction—Drug resistance to alkylator chemotherapy has been primarily attributed to the DNA repair protein alkylguanine-DNA alkyltransferase (AGT); thus, personalizing chemotherapy could be facilitated if tumor AGT content could be quantified prior to administering chemotherapy. We have been investigating the use of radiolabeled O^6 -benzylguanine (BG) analogues to label and quantify AGT in vivo. BG derivatives containing an azido function were sought to potentially enhance the targeting of these analogues to AGT, which is primarily present in the cell nucleus, either by conjugating them to nuclear localization sequence (NLS) peptides or by pretargeting via bioorthogonal approaches.

Methods—Two O^6 -(3-iodobenzyl)guanine (IBG) derivatives containing an azido moiety— O^6 -(4-azidohexyloxymethyl-3-iodobenzyl)guanine (AHOMIBG) and O^6 -(4-azido-3-iodobenzyl)guanine (AIBG)—and their tin precursors were synthesized in multiple steps and the tin precursors were converted to radioiodinated AHOMIBG and AIBG, respectively. Both unlabeled and radioiodinated AHOMIBG analogues were conjugated to alkyne-derivatized NLS peptide heptynoyl-PK₃RKV. The ability of these radioiodinated compounds to bind to AGT was determined by a trichloroacetic acid precipitation assays and gel electrophoresis/phosphor imaging. Labeling of an AGT-AIBG conjugate via Staudinger ligation using the ¹³¹I-labeled phosphine ligand, 2-(diphenylphosphino)phenyl 4-¹³¹Iiodobenzoate, also was investigated.

Results—¹³¹I]AHOMIBG was synthesized in two steps from its tin precursor in $52.2 \pm 7.5\%$ (n = 5) radiochemical yield and conjugated to the NLS peptide via click reaction in $50.7 \pm 4.9\%$ (n = 6) yield. The protected tin precursor of AIBG was radioiodinated in an average radiochemical yield of $69.6 \pm 4.5\%$ (n = 7); deprotection of the intermediate gave ¹³¹I]AIBG in $17.8 \pm 4.2\%$ (n = 9) yield. While both ¹³¹I]AHOMIBG and its NLS conjugate bound to AGT pure protein, their potency as a substrate for AGT was substantially lower than that of ¹²⁵I]IBG. Uptake of ¹³¹I]AHOMIBG-NLS conjugate in DAOY medulloblastoma cells was up to 8-fold higher than that of ¹²⁵I]IBG; however, the uptake was not changed when the cellular AGT content was first depleted with BG treatment. ¹³¹I]AIBG was almost equipotent as ¹²⁵I]IBG with respect to binding to pure AGT; however, attempts to radiolabel AGT by treatment with unlabeled AIBG followed by Staudinger ligation using the radiolabeled phosphine ligand, 2-(diphenylphosphino)phenyl 4-¹³¹Iiodobenzoate were not successful.

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Conclusion—Although AHOMIBG, and AIBG were synthesized successfully in both unlabeled and radioiodinated forms, the radioiodinated compounds failed to label AGT either after NLS peptide conjugation or via Staudinger ligation. Currently, other bioorthogonal approaches are being evaluated for labeling AGT by pretargeting.

Keywords

Alkylguanine-DNA alkyltransferase (AGT); *O*⁶-benzylguanine; Nuclear localization sequence peptide; Bioorthogonal conjugation; click chemistry; Staudinger ligation

1. Introduction

The drug resistance seen in alkylator chemotherapy has been primarily attributed to the repair of alkyl lesions from the *O*⁶-position of guanine residues by the DNA repair protein alkylguanine-DNA alkyl transferase (AGT; a.k.a. MGMT); indeed, an inverse correlation between tumor AGT levels and therapeutic outcome has been demonstrated [1,2]. Thus, a prior knowledge of tumor AGT status will certainly help in personalizing alkylator chemotherapy. For this reason, we have been interested in developing a non invasive, radionuclide-based imaging method to quantify AGT in vivo on a real time basis.

*O*⁶-benzylguanine (BG), the prototypical AGT substrate has been used clinically to modulate AGT thereby increasing the susceptibility of tumors to alkylators [3–5]. Because AGT is inactivated by BG via the initial transfer of benzyl group to a cysteine in AGT, we hypothesized that a BG analogue containing an imageable radionuclide on its benzyl moiety could be a useful agent for the quantification of AGT in vivo by SPECT or PET. We have developed radioiodinated and ¹⁸F-labeled BG analogues and demonstrated their ability to label AGT protein in pure form, and to a degree, when present in tumor cells in vitro [6–8]. However, differentiation of tumor with and without AGT in vivo was not possible with these radiolabeled molecules [9]. We speculate that difficulties in achieving adequate delivery of the intact tracer to cell nucleus where AGT is predominantly present was the primary reason for this lackluster performance.

Nuclear localizing sequence (NLS) peptides have been utilized for the efficient delivery of DNA to the cell nucleus [10–14]. NLS peptides also have been used for the targeted delivery of antibodies [15,16] and peptides as well as other vectors into the cell nucleus [17]. Thus we are interested in developing BG analogues conjugated to one or more NLS peptides as a means to facilitate their nuclear importation. In addition to our intended goal of using them for AGT quantification by radionuclide imaging, NLS conjugates also might improve their potency for AGT depletion prior to chemotherapy compared to BG itself.

The fact that systemically administered BG does modulate AGT suggests that at least a fraction of the dose is reaching the cell nucleus. Thus an alternative means for radiolabeling AGT in vivo will be to employ an Activity-Based Protein Profiling (ABPP)/bioorthogonal approach [18–22]. In this case, the strategy would involve the administration of an unlabeled BG derivative containing a chemical reporter followed by a radiolabeled reactive partner/probe molecule that a) has a bioorthogonal moiety and b) can be delivered to the cell nucleus (Figure 1). Several bioorthogonal reactions have been reported. These include copper-catalyzed and copper-free Huisgen 1,3-dipolar cycloaddition (click and strain-promoted click reaction/chemistry) between an azide and alkyne (terminal alkyne or cyclooctyne) [23–26], Staudinger and traceless Staudinger ligation (azide and triarylphosphine) [27,28], Staudinger-phosphite reaction [29], tetrazine/*trans* cyclooctene cycloaddition [30,31], condensation between a terminal cysteine and 2-cyanobenzothiazole [32], and a recently reported alkyne-nitrone cycloaddition [33].

In the most commonly utilized bioorthogonal conjugations such as click reactions and the Staudinger ligation, the azide function is one of the bioorthogonal moieties. Although one can envisage BG derivatives with either of the binding bioorthogonal moieties, we chose to develop BG analogues containing an azido function in order to be able to explore the feasibility of using them both to conjugate to NLS peptides and to pre-target AGT with them. In our preliminary work reported herein, the synthesis of two radioiodinated azido-BG derivatives and some results of their biological evaluations are described.

2. Materials and Methods

2.1. General

All chemicals were purchased from Sigma-Aldrich unless otherwise specified. Sodium [^{125}I]iodide (2200 Ci/mmol) and sodium [^{131}I]iodide (1200 Ci/mmol), as solutions in 0.1N NaOH, were procured from Perkin Elmer Life and Analytical Sciences (Boston, MA). Radioiodinated O^6 -(3-iodo)benzylguanine (IBG) was synthesized as reported before [8]. Purified human AGT with a (His)₆ tag [34] was generously provided by Dr. Anthony E. Pegg of Hershey Medical Center, Pennsylvania State University, or obtained from Sigma-Aldrich. *Para*-(tri-*n*-butylstannyl)benzoic acid [35] was synthesized by the palladium-catalyzed stannylation of 4-iodobenzoic acid with hexabutylditin in 92% yields following a literature procedure for the synthesis of the trimethylstannyl analogue [36]. It was converted to its *N*-succinimidyl ester using HATU and the latter radioiodinated following the work of Wilbur et al. [35].

Aluminum-backed sheets (Silica gel 60 F254) were used for analytical TLC, and normal-phase column chromatography was performed using silica gel 60 (both obtained from EM Science, Gibbstown, NJ). Preparative thick-layer chromatography was performed using 20 × 20 cm, 1000:μ plates (Whatman, Clifton, NJ). High-pressure liquid chromatography (HPLC) was performed using the following systems: 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, using 32 Karat[®] software; and b) Waters Model Delta 600 semi-preparative system with a Model 600 controller and a Model 2487 dual wavelength absorbance detector; data were acquired using Millenium software. Reversed-phase HPLC was performed using a 4.6 × 250-mm XTerra RP18 (5:μ) column and a 19 × 150-mm XTerra RP18 (7:μ) column for analytical and semi-preparative runs, respectively. Radio-TLC was analyzed initially using a System 200 Imaging Scanner (BioScan, Washington, DC), and then the sheets were cut into strips and counted using an automated gamma counter (LKB 1282, Wallac, Finland). Unless specified otherwise, proton NMR spectra of samples were obtained using CDCl₃ as the solvent on a Varian Mercury 400 MHz spectrometer. Chemical shifts are reported in δ units using the residual CHCl₃ peak as a reference. Low resolution mass spectra were obtained on a JEOL SX-102 high-resolution mass spectrometer (FAB, and EI), Applied Biosystems Voyager DE-Pro (MALDI) or on an Agilent 1100 LC/MSD Trap SL (electrospray). High-resolution mass spectra were obtained using the JEOL SX-102 instrument or by DART-MS using an Agilent TOF-MS model 6224 mass spectrometer. Phosphor-imaging of electrophoresed gels was performed on a Cyclone Phosphor Scanner (Packard Bioscience Company) using Optiquant[™] 4.00 software.

2.2. Methyl 4-(hydroxymethyl-2-iodobenzoate (1)

The title compound was synthesized in two steps from 1-methyl-2-aminoterephthalate (1.95 g, 10 mmol) by initially converting it to 1-methyl-2-iodoterephthalate via the diazonium salt [37] in 81% (2.49 g) yield. Borane dimethyl sulfide (1.11 ml of 10 M; 11.1 mmol) was added dropwise to a refluxing solution of 1-methyl-2-iodoterephthalate (2.4 g; 7.8 mmol) in

20 ml of dry THF and the mixture stirred at reflux for 30 min. The reaction mixture was quenched by careful addition of methanol and the solvents were evaporated under reduced pressure. The crude mixture was subjected to silica gel chromatography using a gradient of 25% to 75% ethyl acetate in hexanes to yield **1** (44%) of **1** [38]. ¹H-NMR (CDCl₃-400MHz): 2.60 (br, 1 H), 3.86 (s, 3H), 4.61 (s, 2H), 7.29 (d, 1H), 7.70 (d, 1H), 7.91 (s, 1H).

2.3. Methyl 4-((tert-butyldimethylsilyloxy)methyl)-2-iodobenzoate (**2**)

A solution of **1** (3.2 g; 10.96 mmol), imidazole (821 mg; 12.10 mmol), and TBDMS-Cl (485 mg; 12.10 mmol) in dry DMF (6 ml) was stirred under argon at 20 °C for 24 h. The reaction mixture was partitioned between ethyl acetate and water and the aqueous layer extracted twice with ethyl acetate. The pooled ethyl acetate layer was washed with brine, dried with anhydrous sodium sulfate and the ethyl acetate was evaporated. The crude product was purified by silica gel chromatography using 25% ethyl acetate in hexanes to obtain **2** in almost quantitative yield. ¹H-NMR (CDCl₃-400MHz): 0.10 (s, 6H), 0.94 (s, 9H), 3.92 (s, 3H), 4.71 (s, 2H), 7.35 (d, 1H), 7.79 (d, 1H), 7.95 (s, 1H). MS (FAB⁺) *m/z*: 406 (M⁺), 349. HRMS (FAB⁺) Calcd for C₁₅H₂₃IO₃Si (M⁺): 406.0461 Found: 406.0453 ± 0.0008 (n = 4).

2.4. (4-((Tert-butyldimethylsilyloxy)methyl)-2-iodophenyl)methanol (**3**)

Borane dimethylsulfide (3.2 ml of 10 M; 32.00 mmol) was added dropwise to a refluxing solution of **2** (4.35 g; 10.71 mmol) in 20 ml of dry THF and the mixture was let refluxing for 2–3 h. Methanol (10 ml) was added dropwise and the solvents were evaporated. The crude residue was subjected to silica gel chromatography using a gradient of 10–30% ethyl acetate in hexanes to yield 3.61 g (89%) of a clear oil. ¹H-NMR (CDCl₃-400MHz): 0.10 (s, 6H), 0.94 (s, 9H), 1.96 (t, 1H), 4.66 (d, 2H), 4.67 (s, 2H), 7.31 (d, 1H), 7.39 (d, 1H), 7.79 (s, 1H). MS (EI⁺) *m/z*: 378 (M⁺), 363, 321, 291. HRMS (EI⁺) Calcd for C₁₄H₂₃IO₂Si (M⁺): 378.0512. Found: 378.0510 ± 0.0012 (n = 4).

2.4. Tert-butyl(3-iodo-4-(iodomethyl)benzyloxy)dimethylsilane (**4**)

Imidazole (1.30 g; 19.11 mmol) and triphenylphosphine (3.74 g; 14.26 mmol) were dissolved in 95 ml of 3:1 diethyl ether:acetonitrile. Iodine (3.62 g; 14.25 mmol) was added to the above mixture followed by a solution of **3** (3.60 g; 9.52 mmol) in 14 ml of 3:1 diethyl ether:acetonitrile dropwise. The mixture was sonicated to break up clumpy materials and stirred at 20 °C for 2 h. Solvents were evaporated and the residue was chromatographed using 3% ethyl acetate in hexanes to yield 2.54 g (55%) of a yellow solid. ¹H-NMR (CDCl₃-400MHz): 0.10 (s, 6H), 0.94 (s, 9H), 4.55 (s, 2H), 4.65 (s, 2H), 7.24 (d, 1H), 7.42 (d, 1H), 7.76 (s, 1H). MS (EI⁺) *m/z*: 473 (M-CH₃)⁺, 432, 361. HRMS (EI⁺) Calcd for C₁₄H₂₂I₂O₂Si (M⁺): 487.9529. Found: 487.9515 ± 0.0005 (n = 3).

2.5. (4-((6-Azidohexyloxy)methyl)-3-iodobenzyloxy)(tert-butyl)dimethylsilane (**5**)

T-Azidohexanol [39] (1.74 g; 8.41 mmol), synthesized by the treatment of 6-bromohexanol with sodium azide in DMSO, was dissolved in THF (50 ml) and treated with potassium *tert*-butoxide (8.41 ml of 1 M solution in THF) at room temperature for 30 min. A solution of **4** (2.04g mg; 4.18 mmol) in THF (7.5 ml) was added dropwise to the above and the mixture stirred at 20 °C for 20 h. Acetic acid (0.8 ml) was added and the solvents were evaporated and the crude product was chromatographed using silica gel and a gradient of 0–20% ethyl acetate in hexanes to yield 2.11 g (100%) of **5**. ¹H-NMR (CDCl₃-400MHz): 0.10 (s, 6H), 0.94 (s, 9H), 1.43 (m, 4H), 1.64 (m, 4H), 3.26 (t, 2H), 3.54 (t, 2H), 4.46 (s, 2H), 4.68 (s, 2H), 7.30 (d, 1H), 7.37 (d, 1H), 7.79 (s, 1H). MS (FAB⁺) *m/z*: 503 (M⁺), 502, 475, 446. HRMS (FAB⁺) Calcd for C₂₀H₃₃IN₃O₂Si (M-H)⁺: 502.1386. Found: 502.1384 ± 0.0002 (n = 4).

2.6. (4-((6-Azidohexyloxy)methyl)-3-iodophenyl)methanol (6)

A solution of TBAF in THF (1 M; 4.6 ml) was added to **5** (2.11 g; 4.2 mmol) and the mixture stirred at 20 °C for 1 h. THF was evaporated and the residue partitioned between water and ethyl acetate. The aqueous layer was extracted twice with ethyl acetate and the combined ethyl acetate solution was washed with brine, dried with anhydrous sodium sulfate and the ethyl acetate evaporated. The crude product was subjected to silica gel chromatography using a gradient of 25–50% ethyl acetate in hexanes to yield 1.4 g (86%) of **6**. ¹H-NMR (CDCl₃-400MHz): 1.42 (m, 4H), 1.63 (m, 4H), 1.80 (br, 1H), 3.26 (t, 2H), 3.54 (t, 2H), 4.46 (s, 2H), 4.64 (s, 2H), 7.32 (d, 1H), 7.39 (d, 1H), 7.83 (s, 1H). MS (FAB⁺) *m/z*: 390 (M+H)⁺, 362. HRMS (FAB⁺) Calcd for C₁₄H₂₁IN₃O₂ (M+H)⁺: 390.0678. Found: 390.0685 ± 0.0010 (n = 4).

2.7. 6-(4-((6-Azidohexyloxy)methyl)-3-iodobenzyloxy)-9-((2-(trimethylsilyl)ethoxy)methyl)-9H-purin-2-amine (7)

First, 6-chloro-9-((2-(trimethylsilyl)ethoxy)methyl)-9H-purin-2-amine (SEM-ACP) [40] was synthesized starting with 6-chloro-9H-purin-2-amine (2-amino-6-chloropurine; ACP). For this, sodium hydride (60%; 0.71 g, 29.5 mmol) was added to anhydrous DMF (25 ml) under argon and the slurry cooled to 0–5 °C. After stirring for 5 min, 6-chloro-9H-purin-2-amine (2.0 g, 11.79 mmol) was added to the above over a period of 10 min while maintaining the temperature at 10–15 °C. To this stirred mixture was then added (2-(chloromethoxy)ethyl)trimethylsilane (2.36 g, 14.15 mmol) over 5 min after which it was allowed to warm to room temperature and the stirring continued overnight. The reaction mixture was poured over ice and the precipitated crude product was extracted with ethyl acetate and then purified by silica gel chromatography using 7:3 hexanes:ethyl acetate to yield 2.1 g (59.4%) of a white solid: ¹H-NMR (CDCl₃-400MHz): 0.01 (s, 9H), 0.98 (m, 2H), 3.60 (m, 2H), 5.16 (br, 2H), 5.46 (s, 2H), 7.90 (s, 1H). MS (CI) *m/z*: 300 (M+H)⁺. HRMS (FAB⁺) Calcd for C₁₁H₁₉ClN₅OSi (MH⁺) 300.1047. Found: 300.1046 ± 0.0005 (n = 4). A mixture of SEM-ACP (216 mg; 0.72 mmol), **6** (1.40 g; 3.60 mmol), DABCO (48 mg; 0.43 mmol), and potassium carbonate (199 mg; 1.44 mmol) in anhydrous DMSO (7.2 ml) was heated at 60 °C for 2 h. The reaction mixture was cooled and carefully treated with HCl (1M, 75 ml) and the product was extracted into ethyl acetate. The aqueous layer was further extracted twice with ethyl acetate and the combined ethyl acetate solution was washed with brine and dried with anhydrous sodium sulfate. Ethyl acetate was evaporated and the crude mixture was chromatographed over silica gel using a gradient of 25–75% ethyl acetate in hexanes to yield 349 mg (75%) of **7**. ¹H-NMR (CDCl₃-400MHz): -0.03 (s, 9H), 0.92 (m, 2H), 1.41 (m, 4H), 1.63 (m, 4H), 3.25 (t, 2H), 3.52 (t, 2H), 3.57 (m, 2H), 4.45 (s, 2H), 4.92 (br, 2H), 5.43 (s, 2H), 5.49 (s, 2H), 7.39 (d, 1H), 7.48 (d, 1H), 7.70 (s, 1H), 7.96 (s, 1H). MS (FAB⁺) *m/z*: 653 (M+H)⁺. HRMS (FAB⁺) Calcd for C₂₅H₃₈IN₈O₃Si (M+H)⁺: 653.1881. Found: 653.1885 ± 0.0012 (n = 4).

2.8. 6-(4-((6-Azidohexyloxy)methyl)-3-(trimethylstannyl)benzyloxy)-9-((2-(trimethylsilyl)ethoxy)methyl)-9H-purin-2-amine (8)

A mixture of **7** (35.6 mg; 0.05 mmol), hexamethylditin (178 mg; 0.55 mmol), (Ph₃P)₂PdCl₂ (19 mg; 0.03 mmol) in dry dioxane (4.5 ml) was heated at 80 °C for 90 min. The reaction mixture was cooled, and filtered over a bed of Celite. The dioxane from the filtrate was evaporated and the residue was loaded onto a silica gel bed that was then eluted with copious amounts of hexanes to remove non polar byproducts. The silica bed was then eluted with ethyl acetate to extract the product along with other more polar impurities. The ethyl acetate was evaporated from the eluent and the residue subjected to preparative TLC using 50:50 ethyl acetate:hexanes to yield 3.6 mg of **8** (10%). ¹H-NMR (CDCl₃-400MHz): -0.07 (s, 9H), 0.25 (s, 9H, [¹¹⁹Sn-H d]), 0.89 (m, 2H), 1.34 (m, 4H), 1.55 (m, 4H), 3.22 (t, 2H), 3.41 (t, 2H), 3.54 (m, 2H), 4.42 (s, 2H), 4.85 (br, 2H), 5.40 (s, 2H), 5.51 (s, 2H), 7.25 (d,

1H), 7.44 (d, 1H), 7.58 (s, 1H), 7.68 (s, 1H). MS (FAB⁺) *m/z*: cluster peaks at 691 (M+H)⁺, 665. HRMS (FAB⁺) Calcd for C₂₈H₄₇N₈O₃Si¹²⁰Sn (M+H)⁺: 691.2562. Found: 691.2560 ± 0.0010 (n = 4).

2.9. 6-(4-((6-Azidohexyloxy)methyl)-3-iodobenzyloxy)-9H-purin-2-amine (9)

TBAF (1 M in THF; 2.65 ml) was added to **7** (138 mg; 0.21 mmol) and the mixture heated at 60 °C for 1 h. Silica gel (10g) was added to the cooled reaction mixture, which was diluted with methanol and solvents were evaporated. The silica gel adsorbed with the mixture was eluted with 5% methanol in ethyl acetate. Solvents were evaporated from the eluent, and the residue subjected to preparative TLC developed sequentially with 5% and 10% methanol:ethyl acetate to yield 14 mg (13%) of **9**. ¹H-NMR (CD₃CN-400MHz): 1.38 (m, 4H), 1.57 (m, 4H), 3.26 (t, 2H), 3.51 (t, 2H), 4.43 (s, 2H), 5.22 (br, 2H), 5.45 (s, 2H), 7.41 (d, 1H), 7.51 (d, 1H), 7.58 (s, 1H), 7.99 (s, 1H), 10.40 (br, 1H). MS (FAB⁺) *m/z*: 523 (M+H)⁺. HRMS (FAB⁺) Calcd for C₁₉H₂₄IN₈O₂ (M+H)⁺: 523.1067. Found: 523.1068 ± 0.0003 (n = 4).

2.10. Heptynoyl-PK₃RKV

N[∇]-Fmoc-protected valine, lysine (N[∇]-Boc), arginine (Pbf), 3 lysines (N[∇]-Boc), and proline as well as heptynoic acid (each 1 mmol) were sequentially attached to Fmoc-amide resin (Applied Biosystems, Foster City, CA; 0.25 mmol) on an automated peptide synthesizer (433A, Applied Biosystems, Foster City, CA) using their Fastmoc chemistry to obtain 807 mg (84%) of the loaded resin. The peptide was cleaved from the loaded resin (572 mg) by treatment with 4 ml of 95:95:5:5 (v/v/v/v) DCM:TFA:water:triisopropylsilane at 20 °C for 1 h. The resin was filtered, the filtrate concentrated and the peptide precipitated with the addition of a large excess of ether to obtain 55 mg (37%) of the peptide. MS (FAB⁺) *m/z*: Calcd for C₄₇H₈₇N₁₅O₈ (M⁺) 989.7. Found: 990.9 (M+H)⁺. MS (MALDI) *m/z*: 991.2 (M+H)⁺, 1013.2 (M+Na)⁺.

2.11. AHOMIBG-Peptide Conjugate (10)

Heptynoyl-PK₃RKV peptide (4.3 mg; 4.3:μmol) and **9** (2.7 mg; 5.2:μmol) were dissolved in 200:1 of DMSO. Copper sulfate (0.4 M; 50:1) and sodium ascorbate (0.6 M; 50:1) were added to the above and the mixture was stirred at 20 °C for 3–4 h. The progress of the reaction was monitored by reversed-phase HPLC. For this the analytical column was eluted with a gradient consisting of 0.1% TFA in both water (solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml/min. The proportion of solvent B was maintained at 5% for 5 min and then linearly increased to 100% over 30 min. The *t_R* of the starting peptide, compound **9**, and the product peptide (**10**) were 19.1, 19.9 and 26.2 min, respectively. The reaction mixture was diluted with water (1 ml) and subjected to preparative HPLC. For this, the semi-preparative column was eluted at 7 ml/min with a gradient consisting of 0.1% TFA in both water (solvent A) and acetonitrile (solvent B); the proportion of solvent B was linearly increased from 10% to 100% in 30 min. Yield 4.9 mg (75%). (MS (MALDI) *m/z*: Calcd for C₆₆H₁₁₀IN₂₃O₁₀ 1512.6. Found: 1512.3 (M⁺), 1534.3 (M+Na)⁺).

2.12. (4-Amino-3-iodophenyl)methanol (12)

This compound was prepared from methyl 4-amino-3-iodobenzoate following a protocol similar to that reported for its synthesis from ethyl 4-amino-3-iodobenzoate [41]. DIBAL-H (32.5 ml, 32.5 mmol) was added over a period of 30 min to a solution of methyl 4-amino-3-iodobenzoate (3.0 g, 10.83 mmol) in anhydrous THF (50 ml) cooled to 0–5 °C while maintaining a temperature below 15 °C. The reaction mixture was allowed to warm gradually to room temperature and stirred further for 16 h. It was then added portion wise to a saturated solution of citric acid and the product from the aqueous mixture was extracted

with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate, and after filtering the drying agent, concentrated to give a crude orange solid. The crude product was purified by silica gel chromatography using 4:1 hexanes:ethyl acetate to obtain 1.7 g (63.0%) of a solid: $^1\text{H-NMR}$ (CDCl_3 -400MHz): 4.05 (bs, 2H), 4.50 (s, 2H), 6.70 (d, 1H), 7.11 (d, 1H), 7.63 (s, 1H).

2.13. (4-Azido-3-iodophenyl)methanol (13)

Sodium nitrite (0.57 g, 8.19 mmol) dissolved in 15 ml of cold water was added to a slurry of **12** (1.7 g, 6.83 mmol) in 1 N HCl (50 ml) maintained at 0–5 °C. After the addition was complete the mixture was stirred at 0–5 °C for an additional 20 min. A solution of sodium azide (1.78 g, 27.3 mmol) in 25 ml of water was then added while maintaining the temperature below 10 °C. The reaction mixture was gradually warmed to room temperature and the stirring continued for 2 h. The mixture was poured over ice and the pH adjusted to ~8.0 by the careful addition of solid sodium bicarbonate. The crude product was extracted with ethyl acetate and chromatographed over silica gel using 4:1 hexanes:ethyl acetate to yield 0.73 g (39.0%) of a solid [42,43]. $^1\text{H-NMR}$ (CDCl_3 -400MHz): 1.65 (t, 1H), 4.58 (d, 2H), 7.05 (d, 1H), 7.32 (d, 1H), 7.73 (s, 1H).

2.14. 6-(4-Azido-3-iodobenzyloxy)-9-((2-(trimethylsilyl)ethoxy)methyl)-9H-purin-2-amine (14)

A mixture of SEM-ACP (100 mg, 0.334 mmol), **13** (275 mg, 1.0 mmol), potassium carbonate (115 mg, 0.834 mmol), DABCO (0.037 ml, 0.334 mmol), in anhydrous DMSO (15 ml) was stirred at 65 °C for 3 h. The reaction mixture was cooled to room temperature and partitioned between water and ethyl acetate. The crude product obtained by concentrating the ethyl acetate solution was purified by silica gel chromatography using 1:1 hexanes:ethyl acetate to yield 111 mg (61.8%) of an off-white solid. $^1\text{H-NMR}$ (CDCl_3 -400MHz): 0.01 (s, 9H), 0.95 (t, 2H), 3.61 (t, 2H), 4.91 (bs, 2H), 5.47 (s, 2H), 5.51 (s, 2H), 7.14 (d, 1H), 7.57 (dd, 1H), 7.76 (s, 1H), 7.97 (s, 1H). LCMS m/z : 539.2 ($\text{M}+\text{H}^+$). HRMS (DART-TOF) Calcd for $\text{C}_{18}\text{H}_{24}\text{I}\text{N}_8\text{O}_2\text{Si}$ ($\text{M}+\text{H}^+$): 539.0836. Found: 539.0840 $\pm$ 0.0002 ($n=4$).

2.15. 6-(4-Azido-3-(trimethylstannyl)benzyloxy)-9-((2-(trimethylsilyl)ethoxy)methyl)-9H-purin-2-amine (15)

A mixture of **14** (100 mg, 0.186 mmol), and hexamethylditin (0.385 ml, 1.857 mmol) in anhydrous 1,4-dioxane (10 ml) was heated to 100 °C. Bis(triphenylphosphine)palladium(II) dichloride (32.6 mg, 0.046 mmol) was added to the above and the resultant golden solution was stirred at 100 °C for 1 h. The ensued black slurry was cooled to 20 °C and the precipitated metallic palladium was filtered over a bed of Celite. The filtrate was concentrated to dryness and the resultant oil was subjected to preparative TLC by application to three silica gel plates followed by elution with 1:1 hexanes:ethyl acetate to afford 47 mg (44.0 %) of a solid. $^1\text{H-NMR}$ (CDCl_3 -400MHz): 0.01(s, 9H), 0.34(s, 9H, [$^{119}\text{Sn-H}$ d]), 0.95 (t, 2H), 3.61 (t, 2H), 4.98 (bs, 2H), 5.45 (s, 2H), 5.55 (s, 2H), 7.17 (d, 1H), 7.56 (m, 2H), 7.80 (s, 1H). LCMS m/z : 580 (cluster peaks, ($\text{M}+\text{H}^+$)), 565.2 (cluster peaks, ($\text{M}-\text{CH}_3$) $^+$) HRMS (DART-TOF) $\text{C}_{18}\text{H}_{24}\text{I}\text{N}_8\text{O}_2\text{Si}$ ($\text{M}+\text{H}^+$): 577.1518. Found: 577.1531 $\pm$ 0.0001 ($n=4$).

2.16. 6-(4-Azido-3-iodobenzyloxy)-9H-purin-2-amine (16)

Acetyl chloride (27:1) was added to a solution of **14** (15 mg; 0.03 mmol) in 1.4 ml dry methanol and the homogeneous solution was stirred at 20 °C for 1–2 h. Triethylamine (28:1) was added and the solvents were removed. The residue was triturated with ether, dissolved in methanol and purified by preparative TLC using ethyl acetate to obtain 2.4 mg (21%) of a white solid. HPLC: The analytical column was eluted at 1 ml/min with a gradient consisting

of 0.1% TFA in both water (solvent A) and acetonitrile (solvent B); 5% B to 100% B in 30 min; t_R = 18.2 min. $^1\text{H-NMR}$ ($\text{DMSO-}d_6$ -400MHz): 5.40 (s, 2H), 6.27 (br, 2H), 7.33 (d, 1H), 7.58 (d, 1H), 7.78 (s, 1H), 7.95 (s, 1H), 12.42 (br, 1H). MS (LCMS) m/z : 409 ($\text{M}+\text{H}$)⁺, 381 ($\text{M}-\text{N}_2$). HRMS (FAB⁺) Calcd for $\text{C}_{12}\text{H}_{10}\text{IN}_8\text{O}$ ($\text{M}+\text{H}$)⁺: 409.0022. Found: 409.0020 $\pm$ 0.0014 (n = 4).

2.17. 2-(Diphenylphosphino)phenyl 4-iodobenzoate (20)

A mixture of 4-iodobenzoic acid (53.5 mg, 0.216 mmol), HATU (82 mg, 0.216 mmol), and triethylamine (0.038 ml, 0.270 mmol) in dry acetonitrile (10 ml) was stirred at 20 °C for 1 h. *Ortho*-(diphenylphosphino)phenol [27,44] (50 mg, 0.180 mmol) was added to the above and the mixture stirred at 20 °C overnight. This reaction mixture was partitioned between aqueous bicarbonate and ethyl acetate. The pooled ethyl acetate layers were dried over anhydrous Na_2SO_4 , filtered, and the ethyl acetate from the filtrate was evaporated. The crude product was subjected to silica gel column chromatography by eluting the column with hexanes:EtOAc (95:5 to 80:20) to give 36 mg (39.4 %) of a solid. $^1\text{H-NMR}$ (CDCl_3 -300MHz): 6.78 (m, 2H), 7.11 (m, 1H), 7.25 (m, 10H), 7.35 (m, 1H), 7.44 (d, 2H), 7.65 (d, 2H).

2.18. 2-(Diphenylphosphino)phenyl 4-(tributylstannyl)benzoate (21)

A mixture of 4-(tri-*n*-butylstannyl)benzoic acid (177 mg, 0.431 mmol), HATU (115 mg, 0.302 mmol), and triethylamine (0.076 ml, 0.539 mmol) in DMF (3 ml) was stirred at 20°C for 1 h and then 2-(diphenylphosphino)phenol (100 mg, 0.359 mmol) was added and the stirring continued overnight. The reaction mixture was worked up as in the synthesis of **20** and subjected to silica gel chromatography by eluting the column with hexanes:EtOAc (95:5 to 80:20) to yield 83 mg (34.4 %) of an oil. $^1\text{H-NMR}$ (CDCl_3 -400MHz): 0.89 (t, 9H), 1.06 (m, 6H), 1.31 (m, 6H), 1.52 (m, 6H), 6.84 (m, 2H), 7.15 (m, 1H), 7.32 (m, 10H), 7.40 (m, 1H), 7.44 (d, 2H), 7.73 (d, 2H). HRMS (DART-TOF) $\text{C}_{37}\text{H}_{46}\text{O}_2\text{PSn}$ ($\text{M}+\text{H}$)⁺: 673.2257. Found: 673.2257 $\pm$ 0.0004 (n =4).

2.19. Radioiodinated AHOMIBG-Peptide conjugate (^{131}I 10)

Radioiodine (1–5 mCi) in 0.1N NaOH was added to a ½ dram vial containing 50:g of **8**, and the water was dried with a stream of argon. A 3:1 (v/v) mixture of glacial acetic acid and 30% hydrogen peroxide (10:1) was added to the above and after capping the vial the mixture was sonicated for 30 seconds. The solvents were evaporated by purging with argon and 100:1 TFA was added to the dried activity. The deprotection reaction was allowed to proceed for 5 minutes at 20°C and then TFA was removed with a gentle stream of argon and co-evaporation with 2 H 25:1 of ethyl acetate. The residue was taken in 25:1 of methanol and injected into the HPLC analytical column eluted at flow rate of 1 ml/min with a gradient consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B); the proportion of solvent B was kept at 10% for 5 min and then linearly increased to 100% over 30 min. The HPLC fractions containing [^{131}I]AHOMIBG (t_R = 24.5 min) were purged with argon, diluted with water and then passed through a reversed-phase solid-phase cartridge (Waters C18 sep pak) that was activated with 2 ml each of methanol and water in succession. The cartridge was washed with water and then the radioactivity was eluted with 0.25 ml aliquots of methanol. The methanol fractions containing [^{131}I]AHOMIBG were concentrated, transferred to a ½ dram vial and dried. For the click reaction, a solution of heptynoyl-PK₃RKV (0.25mg/25:1 DMSO) was added to the above dried radioactivity followed by 3:1 of 0.4M copper sulfate and 3:1 of 0.6M sodium ascorbate and the mixture was stirred at 20 °C for 30 min. The reaction mixture was diluted with 10 ml water and then passed through a C18 Sep-Pak cartridge that was activated by sequential elution with 2 ml each methanol, water, and 5 mM hexane sulfonic acid. After passing the diluted reaction mixture, the cartridge was washed with 2 H 5 ml H_2O , 0.25 ml 5mM hexane sulfonic acid,

and the radioactivity was eluted with 0.25 ml aliquots of methanol. The methanol fractions containing the radioactivity were combined, evaporated to dryness and reconstituted in PBS, pH 7.14. Radiochemical purity was determined by HPLC using the above conditions (t_R = 17.7 min).

2.20. 6-(4-Azido-3-[^{131}I]iodobenzoyloxy)-9H-purin-2-amine ([^{131}I]16)

Iodine-131 (0.1–2 mCi) in 1–5:1 was added to **15** (5:g) in a ½-dram vial, and the water was evaporated with a gentle stream of argon. A 3:1 (v/v) mixture of glacial acetic acid and 30% hydrogen peroxide (10:1) was added to the above and the reaction mixture sonicated for 30 sec. The solvents were evaporated with a gentle stream of argon. Ethyl acetate 2 H 25:1 was added evaporating it each time and finally the radioactivity was reconstituted in 25:1 acetonitrile and injected into the HPLC analytical column. The column was eluted at a flow rate of 1 ml/min with a gradient consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The proportion of B was maintained at 10% for 5 min and then linearly increased to 100% over 30 min. The HPLC fractions corresponding to [^{131}I]14 (t_R = 26.8 min) were combined, diluted with water and the radioactivity was extracted into ethyl acetate (2 H 2 ml). The combined ethyl acetate solution was washed with 2 ml brine, dried with anhydrous sodium sulfate and the ethyl acetate was evaporated. A freshly prepared solution of acetyl chloride in methanol (6.8:1 of acetyl chloride in 171:1 of methanol; 25:1) was added to the above radioactivity in a ½-dram vial. The reaction mixture was vortexed and incubated at room temperature for 15 min and then injected into the analytical column. The column was eluted at a flow rate of 1 ml/min with a gradient consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The proportion of B was linearly increased from 25% to 60% in 30 min and then to 100% in the next 10 min. The HPLC fractions containing [^{131}I]16 (t_R = 10.1 min) were diluted with 6 – 8 ml of water and passed through a C18 solid-phase cartridge that had been activated with 2 ml each of ethanol and water in that order. The cartridge was washed with 2 H 5 ml water and 0.25 ml of 5 mM hexane sulfonic acid and the radioactivity was then eluted with 0.25 ml portions of ethanol. The ethanol from the eluents was evaporated and the residual radioactivity was reconstituted in PBS, pH 7.14.

2.20. 2-(Diphenylphosphino)phenyl 4-[^{131}I]iodobenzoate([^{131}I]20)

N-hydroxysuccinimidyl 4-[^{131}I]iodobenzoate ([^{131}I]SIB) was synthesized as reported [35] and purified by normal-phase HPLC. A solution of 2-(diphenylphosphino)phenol (3 mg; 0.01 mmol) in 25:1 dry THF was mixed with 10:1 of 1 M potassium *tert*-butoxide in THF and 8:1 of diisopropylethylamine and this mixture was added to 125:1 of dried [^{131}I]SIB. The entire reaction mixture was injected onto the analytical HPLC column and eluted at a flow rate of 1 ml/min with a gradient consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The proportion of B was linearly increased from 5% to 100% in 30 min and held at 100% for 5 min. HPLC fractions containing [^{131}I]20 (t_R = 27.3 min) were extracted with ethyl acetate, the combined ethyl acetate layers were dried with anhydrous sodium sulfate, and the ethyl acetate evaporated from the supernatant.

2.21. Paired-label binding of [^{131}I]AHOMIBG-NLS ([^{131}I]10) and [^{125}I]IBG to pure AGT

About 100 nCi of each tracer was incubated with AGT (5:g) in 0.1 ml of 50 mM Tris-Cl, pH 7.5, 5 mM DTT, and 0.1 mM EDTA in the presence of 10:g of calf thymus DNA. After incubation for 30 min at 37°C, the protein was precipitated by the addition of 200:g carrier BSA and 1 ml cold 12% TCA. The precipitated proteins were collected on GF-C (Whatman) filters which were washed extensively with 5% TCA. As controls, two other assays were performed in parallel: in the first one, 10:M BG was added 30 min before adding the tracers and in the second AGT was omitted. The assays were performed in duplicate and the results are expressed as the percentage of input activity retained on the filter.

2.22. Paired-label uptake and retention of [¹³¹I]AHOMIBG-NLS and [¹²⁵I]IBG by DAOY cells as a function of time

DAOY cells [45] were plated at a density of 5 × 10⁵ cells per well in 6-well plates and incubated overnight in Zinc Option medium containing 2.2 g/l sodium bicarbonate, 8 mM HEPES, and 10% fetal bovine serum (FBS). The medium was removed and replenished with fresh medium with or without 10:1 BG and the cells were incubated at 37 °C for 4 h. Medium was removed and fresh medium containing 100 nCi of [¹³¹I]AHOMIBG and [¹²⁵I]IBG was added to both sets of wells and the cells were incubated at 37 °C for 30 min, 1 h, 2 h, 3 h, and 4 h. At the end of each incubation period, the medium was aspirated and the cells were washed to remove any unbound radioactivity. The cells were solubilized by incubation with 0.5N sodium hydroxide for 30 min and cell-associated radioactivity was determined using an automated gamma counter.

2.23. Paired-label binding of [¹³¹I]AHOMIBG and [¹²⁵I]IBG to pure AGT

This experiment was performed essentially as described for the NLS conjugate in Section 2.21. In addition, the assay was also repeated with and without pre-incubation with BG but in a single label format, and aliquots of the incubation mixture in each case were subjected to gel electrophoresis and subsequent phosphor imaging.

2.23. Paired-label binding of [¹³¹I]AIBG and [¹²⁵I]IBG to pure AGT

This was done as described for [¹³¹I]AHOMIBG (section 2.23) and its NLS-conjugate (Section 2.21).

2.24. Bioorthogonal approach to label pure AGT using Staudinger ligation

Pure AGT protein (2: g) was incubated in duplicate with 4: M of AIBG (**16**) for 30 min at 37 °C in a 0.1 ml of 50 mM Tris-Cl, pH 7.5, 5 mM DTT, 0.1 mM EDTA, 10 ug CT-DNA. For negative controls, a parallel assay was performed by omitting AIBG. Buffer was exchanged by passing these samples through corresponding spin gel filtration columns (GE Healthcare, Buckinghamshire, UK) each of which was pre-eluted with PBS. About 1: Ci of 2-(diphenylphosphino)phenyl 4-[¹³¹I]iodobenzoate ([¹³¹I]**21**) in 5:1 DMSO was added to each of the above samples in PBS and the mixture incubated for 2 h at 37 °C. Carrier BSA protein (200: g) was added followed by 1 ml of cold 12% TCA. The precipitate was pelleted, washed with 5% TCA a couple of times, and the pellets and the combined supernatants were counted for radioactivity.

3. Results

3.1. Chemistry and Radiochemistry

3.1.1. 6-(4-((6-Azidohexyloxy)methyl)-3-iodobenzyloxy)-9H-purin-2-amine and precursor—The benzyl alcohol derivative **6** was synthesized starting from 1-methyl-2-aminoterephthalate in seven steps (Figure 2) in an overall yield of 15%. Coupling of **6** to SEM-ACP rendered the protected target compound **7** (75% yield), which was then converted to 6-(4-((6-Azidohexyloxy)methyl)-3-iodobenzyloxy)-9H-purin-2-amine (**9**) by treatment with TBAF in 13% yield. The protected tin precursor **8** was synthesized from **7** via palladium-catalyzed stannylation in 10% yield.

3.1.2. AHOMIBG-NLS peptide conjugate—The NLS peptide appended with an alkyne function (heptynoyl PK₃RKV) was obtained by solid-phase peptide synthesis in 37% yield. This was conjugated to AHOMIBG using the click reaction to provide the conjugate in 75% yield (Figure 3).

3.1.3. 6-(4-Azido-3-iodobenzoyloxy)-9H-purin-2-amine and precursor—The key intermediate, 4-azido-3-iodobenzyl alcohol (**13**) [42,43] was synthesized from commercially available methyl 4-amino-3-iodobenzoate (**11**) in two steps in an overall yield of 25% (Figure 4). It was conjugated to SEM-ACP to provide **14** in 62% yield. The tin precursor **15** was synthesized from **14** in 44% yield. The target compound **16** was obtained from **13** in 21% yield by its treatment with methanolic HCl [46] (Figure 5).

3.1.4. 2-(Diphenylphosphino)phenyl 4-iodobenzoate and precursor—HATU-mediated esterification of 4-iodobenzoic acid with 2-(diphenylphosphino)phenol, which was synthesized following a literature procedure [27,44], yielded a standard of the phosphine phenol ester **20** [44] in 39% yield (Figure 6). The tin precursor **21** was similarly derived using 4-(tri-*n*-butylstannyl)benzoic acid in 34% yield.

3.1.5. 6-(4-((6-Azidohexyloxy)methyl)-3-[¹³¹I]iodobenzoyloxy)-9H-purin-2-amine-NLS conjugate—The tin precursor **8** was radioiodinated and the resultant [¹³¹I]**7** deprotected in situ to [¹³¹I]**9** (Figure 3). An average of $52.2 \pm 7.5\%$ ($n = 5$) of the total radioactivity injected into the HPLC eluted in the peak corresponding to that of [¹³¹I]**9**. Click reaction of [¹³¹I]**9** with the NLS peptide proceeded with an average radiochemical yield of $50.7 \pm 4.9\%$ ($n = 6$). The radiochemical purity of [¹³¹I]**9** was almost 100% and that of [¹³¹I]**10** was 85–90%. No peaks were seen on the UV profile other than the occasional presence of **9** or **10** presumed to reflect the presence of carrier iodide in ¹³¹I preparation.

3.1.6. 6-(4-Azido-3-[¹³¹I]iodobenzoyloxy)-9H-purin-2-amine ([¹³¹I]16**)**—Radioiodination of **15** gave [¹³¹I]**14** in an average radiochemical yield of $69.6 \pm 4.5\%$ ($n = 7$). Deprotection of [¹³¹I]**14** with methanolic HCl gave the target compound [¹³¹I]**16** in $17.8 \pm 4.2\%$ yield ($n = 9$) (Figure 5). The radiochemical purity of [¹³¹I]**14** was greater than 95%. With the HPLC conditions utilized, the byproduct alcohol [¹³¹I]**13** had a t_R (12.5 min), distinctly different from that of [¹³¹I]**16** (10.2 min). Nonetheless, it was difficult to isolate [¹³¹I]**16** devoid of [¹³¹I]**13** and as a result, the radiochemical purity of the final [¹³¹I]AIBG was only about 60–70%. Except for the occasional presence of unlabeled congener, produced as a result of the presence of the carrier iodide in ¹³¹I, no other UV peaks were seen in either case.

3.1.7 2-(Diphenylphosphino)phenyl 4-[¹³¹I]iodobenzoate([¹³¹I]20**)**—Attempts to synthesize [¹³¹I]**20** by radioiododestannylation of tin precursor **21** were not successful (Figure 6) nor was it possible to conjugate the phosphine phenol **17** to the pre-labeled 4-[¹³¹I]iodobenzoic acid or its *N*-hydroxysuccinimide ester **23** under the variety of conditions that were investigated. Eventually [¹³¹I]**20** was synthesized by the treatment of [¹³¹I]**23** with the potassium salt of **17** in an average radiochemical yield of $68.2 \pm 11.6\%$ ($n = 2$).

3.2. Suitability of AHOMIBG-NLS as a substrate for AGT

The suitability of the AHOMIBG-NLS conjugate as a substrate for the pure AGT protein was assessed using a standard paired-label assay performed with the pure protein. As depicted in Figure 7, in the absence of a blocking dose of BG, the percent of input activity that was TCA precipitable was similar for both [¹³¹I]AHOMIBG-NLS ($81.2 \pm 3.9\%$) and the positive control [¹²⁵I]IBG ($73.7 \pm 6.6\%$). However, the fraction of ¹³¹I activity that was TCA-precipitable was reduced only marginally either in the presence of 10:M BG ($56.0 \pm 12.2\%$) or in the absence of AGT ($53.7 \pm 4.1\%$). In contrast, a substantial reduction in TCA-precipitable ¹²⁵I activity was seen, with $9.8 \pm 6.2\%$ and $8.8 \pm 3.4\%$, respectively of the ¹²⁵I activity in the precipitate under the latter two conditions of nonspecific binding.

3.3. Uptake of [^{131}I]AHOMIBG-NLS and [^{125}I] IBG in DAOY cells

The uptake of [^{125}I]IBG and the ^{131}I -labeled NLS conjugate in AGT-expressing DAOY cells in vitro as a function of time was determined. The uptake of [^{131}I]AHOMIBG-NLS by DAOY cells increased with time and was substantially higher ($p < 0.05$) than that seen for the co-incubated [^{125}I]IBG at all time points with an 8-fold higher uptake observed at 4 h for the peptide conjugate (Figure 8). As seen before [6], the uptake of [^{125}I]IBG was not changed when the cells were pre-treated with increasing concentrations of BG and removing the BG-containing medium before incubating with the tracers. A similar trend was seen in the case of [^{131}I]AHOMIBG as well.

3.4. Suitability of AHOMIBG as a substrate for AGT

Because the AHOMIBG-NLS conjugate was demonstrated to exhibit a high degree of nonspecific binding to AGT, the suitability of AHOMIBG itself as a substrate for AGT was evaluated in a paired-label assay as described for its NLS conjugate. As shown in Figure 9, only $8.3 \pm 0.8\%$ of the input ^{131}I dose was precipitable compared to $45.0 \pm 0.6\%$ for ^{125}I . On the other hand, its binding to AGT was demonstrated to be specific as it was reduced by about 75% from the control values determined when unlabeled BG was added to the incubation mixture or AGT was omitted. The ability of [^{131}I]AHOMIBG to bind to AGT was confirmed by gel electrophoresis of the incubation mixture (single label format) and visualization of the bands with phosphor imager (Figure 9, right panel). As seen with [^{125}I]IBG, the major band was seen at a molecular weight corresponding to the AGT protein in the absence but not the presence of an excess of BG.

3.5. AIBG is a substrate for AGT

A paired-label binding assay of [^{131}I]AIBG and [^{125}I]IBG was performed and indicated that $49.6 \pm 6.4\%$ and $83.3 \pm 12.2\%$, respectively of the input radioiodine activity was TCA-precipitable (Figure 10). The nonspecific binding was similar for both compounds. Because the radiochemical purity of [^{131}I]AIBG was only 60–70%, one possibility is that AIBG is an equally good substrate for AGT as IBG.

3.6. Labeling AGT in a pretargeted manner via Staudinger Ligation

About 82% of the added [^{131}I]20 activity was insoluble in TCA; however, a similar result was obtained when unlabeled AIBG was omitted from the initial incubation mixture. HPLC of a DMSO solution of [^{131}I]20 used for the assay that was left at 37°C for about 2 h showed no peak corresponding to [^{131}I]20 ($t_R = 27.3$ min). Instead, a single peak with a t_R of 23.8 min was seen.

Discussion

A means to noninvasively quantify tumor AGT content in real time could have a significant impact on the care of cancer patients. Towards this end, our efforts have been directed at the development of methods to radiolabel endogenous AGT in vivo and quantify its presence by SPECT or PET. We have successfully developed radiolabeled analogues of BG, the prototypical AGT modulating agent, and have shown their ability to radiolabel AGT in vitro. However, they could not differentiate between tumors that expressed AGT and those from which AGT was depleted in vivo. This has been attributed to both metabolic decomposition [47] and poor delivery of these agents after systemic administration to the nuclear compartment of tumor cells in which AGT primarily resides. We have considered two approaches to surmount these problems especially the delivery issue. One is to conjugate the labeled BG analogue to an NLS peptide in order to enhance its delivery to the cell nucleus; the other is a pretargeting approach wherein an unlabeled BG analogue containing a reporter

group is administered first followed by a radiolabeled chaser decorated with a complementary moiety and that also is capable of nuclear localization. It was envisaged that BG analogues containing an azido function might be appropriate for the execution of both of these approaches (Figure 1). Azido-functionalized BG analogues can be conjugated to an NLS peptide containing the complementary alkyne function via the click reaction and are also amenable to bioorthogonal conjugation via click reaction or Staudinger ligation.

The compound initially designed for this purpose was AHOMIBG (**9**), which includes an 8-atom spacer in order to minimize steric hindrance in its interaction or that of its NLS conjugate with AGT. An iodine substituent was included that could be replaced with an appropriate radioiodine nuclide. The key intermediate benzyl alcohol was synthesized in multiple steps and conjugated to the purine derivative SEM-ACP; the SEM protecting group was utilized to facilitate both synthesis and chromatographic purification. TBAF-mediated deprotection of SEM-AHOMIBG (**7**) yielded the target compound while its stannylation rendered the protected tin precursor (**8**). The tin precursor **8** could be radioiodinated to [¹³¹I]**7** in excellent yield. Initially, deprotection of HPLC purified [¹³¹I]**7** was attempted using TBAF at 60–70 °C as was done with the unlabeled compound. While formation of [¹³¹I]**9** was seen by HPLC, considerable deiodination also was observed. In addition, this protocol involved an extra HPLC purification. Subsequently deprotection of [¹³¹I]**7** was achieved in situ using TFA. The potential formation of alcohol [¹³¹I]**6** under these conditions was monitored by both HPLC and TLC. The alcohol [¹³¹I]**6** could be well-separated from [¹³¹I]**9** by HPLC (*t_R* = 27.3 min vs 24.6 min for [¹³¹I]**7**), and little if any formation of [¹³¹I]**6** was observed. Finally, the NLS conjugate [¹³¹I]**10** was synthesized by the click reaction of [¹³¹I]**7** with the alkyne-modified NLS peptide.

BG derivatives can be appended with moieties as large as oligonucleotides and nanoparticles without losing their ability to be recognized as substrates by AGT [48,49]. Encouraged by this fact, we embarked on our strategy of conjugating a NLS peptide to BG in order to enhance uptake in the cell nucleus. The ability of this conjugate ([¹³¹I]**10**) to serve as a substrate for AGT was evaluated by measuring its binding to pure AGT protein in a TCA precipitation assay. About 80% of activity was TCA precipitable suggesting that the NLS conjugate may be a substrate for AGT. However, up to 56% of the ¹³¹I activity could still be precipitated in control experiments in which the incubation medium either contained excess BG or lacked AGT. With regard to possible explanations for this behavior, it seems unlikely that the NLS peptide conjugate itself is insoluble in 12% TCA because poly-cationic nature of the peptide would enhance solubility in aqueous solutions. On the other hand, it could associate with the carrier protein used in the assay in some noncovalent fashion. Another possibility is that the lipophilicity of the spacer present in the conjugate enhanced hydrophobicity, decreasing solubility.

We next determined the ability of the NLS conjugate [¹³¹I]**10** to bind to a tumor cell line known to express high levels of AGT. Although up to 8-fold higher uptake of [¹³¹I]**10** was seen in DAOY cells compared to that for [¹²⁵I]IBG, cellular uptake remained essentially unchanged when the AGT in these cells was depleted by prior treatment with excess BG, again suggesting a high degree of binding of [¹³¹I]**10** not specific to AGT expression. Given these results, we investigated whether AHOMIBG itself (i.e. without spacer and NLS peptide) was a specific substrate for AGT. While the results of both the TCA precipitation assay and gel electrophoresis analysis indicated that [¹³¹I]AHOMIBG could act as a substrate for AGT, it did not label AGT as efficiently as [¹²⁵I]IBG. Although the properties of the radiolabeled NLS-conjugate were not found to be useful for its use as an agent for quantification of AGT by molecular imaging, it is possible that its higher cellular uptake might be exploited for better AGT modulation; however, this remains to be investigated.

As mentioned above, we designed AHOMIBG to include an 8-atom spacer to minimize potential steric interference of the NLS moiety with the interaction of the conjugate with AGT. To investigate whether this spacer was detrimental to specific AGT recognition, we wanted to develop an analogue either lacking or containing a shorter spacer between the azido function and the ring. Initially, we set out to synthesize an azidomethyl derivative using 4-azidomethyl-3-iodobenzyl alcohol following a similar synthetic pathway as used for AHOMIBG. While we were successful in the syntheses of both the iodo standard and the tin precursor in the *N*-9-SEM protected form, attempts to deprotect the iodo derivative with TBAF resulted in the formation of an unexpected product. NMR spectroscopy indicated that although the SEM group remained intact, the azidomethyl group was converted to an aldehyde function (details not given). Attempts to deprotect to the radioiodinated SEM derivative with TFA, as done for the AHOMIBG, were also not successful. Thus we turned our attention to a derivative in which the azido function is directly attached to the benzene ring of IBG. The synthesis of the protected form of both the iodo standard and the tin precursor was achieved as in the case of AHOMIBG via the conjugation of 4-azido-3-iodobenzyl alcohol with SEM-ACP and the stannylation of the resultant product. However, deprotection of the SEM group from the iodo derivative proved to be challenging. The TBAF-mediated reaction was extremely slow and resulted in multiple side products, and TFA deprotection resulted in the decomposition of the guanine and concomitant formation of the alcohol **13**. A reasonable yield was obtained, albeit with the formation of a considerable amount of the above alcohol when methanolic HCl, produced in situ with acetyl chloride [46], was used for this purpose. Likewise, it was possible to deprotect the radioiodinated analogue under these conditions in modest yield; however, formation of the alcohol [¹³¹I]**13** and several other unidentified byproducts was observed. Due to the close proximity of the product **16** peak to that of the alcohol **13** under the HPLC conditions used for [¹³¹I]**16** isolation, it was not possible to exclude [¹³¹I]**13** as a contaminant as of yet.

The TCA precipitation assay indicated that **16** exhibited good binding to AGT and assuming that all of the radioactivity from the [¹³¹I]**13** impurity did not bind, it nearly performed as well as [¹²⁵I]IBG. On that basis we investigated whether unlabeled **16** could be used to pre-target AGT for subsequent labeling via a bioorthogonal approach. Because click reaction involves the use of copper, which can be toxic, we resorted to traceless Staudinger ligation [27], notwithstanding its slower rate of reaction, as a means to label AGT. Towards this end, we developed a traceless phosphine reagent containing an iodobenzoyl moiety (**20**). While our work was in progress, Mamat et al. reported the synthesis of this compound but using slightly different methods [44]. Considering the susceptibility of phosphines to oxidation, we initially strayed away from developing a tin precursor lest the phosphine may get oxidized in the presence of the oxidant employed in the radioiodination protocol. However, it was not possible to conjugate the phenol **17** to pre-radiolabeled 4-[¹³¹I]iodobenzoic acid or its *N*-hydroxysuccinimide ester under various sets of conditions. Next, we synthesized the tin precursor (**21**) hoping that it could be radioiodinated with the oxidized radioiodine extracted into an organic solvent and thus free of any oxidant; however, these attempts were also futile. Eventually, we were successful in the synthesis of [¹³¹I]**20** by the treatment of the pre-formed potassium salt of the phosphine phenol **17** with the known ester **23**.

To evaluate proof of principle, the labeling of pure AGT treated with AIBG (**16**) via Staudinger ligation was attempted using the above radioiodinated phosphine ligand. The results were the same whether AGT was treated with AIBG or not. One of the contributing factors for this observation could be the oxidation of the phosphine before it had a chance to react with the azido group attached to AGT. It should be noted that a small amount of DMSO, a mild oxidant, was used to dissolve [¹³¹I]**20** and incubation of this solution at 37 °C for about 2 h indeed resulted in its complete conversion to a more polar product, presumably the phosphine oxide, as evidenced by HPLC analysis. It is not clear however

how more than 80% of the radioactivity was TCA precipitable even without AIBG in the pre-incubation mixture. It should be pointed out that in a recent work, the feasibility of labeling a pretargeted antibody with radiolabeled phosphine ligands has been demonstrated without considerable oxidation of these ligands [50,51]. It may be possible to exchange the bioorthogonal moieties — that is to place the phosphine moiety on the BG molecule and use a chaser molecule such as radiolabeled AZT. In future work we may consider such an approach.

In summary, we have synthesized two IBG analogues containing an azido function in both unlabeled and radioiodinated form. The derivative with the azido moiety attached directly to the ring was a reasonable analogue of IBG. On the other hand, the one with a spacer between the azido function and the ring had a sub-par performance as an AGT substrate. The NLS-conjugate of the latter radiolabeled with ^{131}I had a significantly higher uptake in tumor cells compared to [^{125}I]IBG; however, it was not able to differentiate between tumor cells with AGT and those from which AGT has been depleted. Although we were able to develop a radioiodinated traceless phosphine ligand, AGT labeling with this via Staudinger ligation was not feasible. Experiments to label AGT using other bioorthogonal approaches are under way.

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Figure 1. Schematic representation showing the attachment of NLS peptide to radiolabeled BG derivative containing an azido function (upper) and the bioorthogonal approach for labeling AGT (lower).

Figure 2.
Scheme for the synthesis of N^9 -SEM protected tin precursor of AHOMIBG.

Figure 3.
Synthesis of unlabeled and labeled AHOMIBG and its NLS conjugate

a) DIBAL; b) HNO_2 , NaN_3 ; c) SEM-ACP, DABCO, K_2CO_3 ;
d) $(\text{Me}_3\text{Sn})_2$, $(\text{Ph}_3\text{P})_2\text{PdCl}_2$.

Figure 4.
Scheme for the synthesis of protected tin precursor for AIBG

Figure 5.
Scheme for the synthesis of labeled and unlabeled AIBG.

Figure 6.
Scheme for the synthesis of radioiodinated triaryl phosphine for traceless Staudinger ligation.

Figure 7. Paired-label binding of $[^{131}\text{I}]\text{AHOMIBG-NLS}$ and $[^{125}\text{I}]\text{IBG}$ to pure AGT in presence of calf-thymus DNA. After incubation, carrier BSA was added and the proteins were precipitated by TCA (black bars). The experiment was also done in presence of excess BG (white bar) or excluding AGT (hashed bar).

Figure 8.

Paired-label uptake of $[^{131}\text{I}]\text{AHOMIBG-NLS}$ (circle) and $[^{125}\text{I}]\text{IBG}$ (triangle) by BG-treated (open) and untreated (filled) DAOY cells as a function of time. Plated cells were treated with 10:M BG for 4 h; just medium was substituted for controls. After removing medium, all cells were incubated with both tracers for various periods of time and the cell-associated radioactivity was determined at each time points.

Figure 9.

Left: Paired-label binding of $[^{131}\text{I}]\text{AHOMIBG}$ (white bars), and for comparison $[^{125}\text{I}]\text{IBG}$ (black bars) to pure AGT. Right: SDS PAGE/Phosphor imaging of AGT incubated individually with the two tracers in the presence and absence of excess BG.

Figure 10. Paired-label binding of $[^{131}\text{I}]\text{AIBG}$ and $[^{125}\text{I}]\text{IBG}$ to pure AGT in the presence (white bars) and absence (black bars) of 10:M BG.