Nuclear Medicine and Biology 43 (2016) xxx–xxx

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

$^{67/68}$Galmydar: A metalloprobe for monitoring breast cancer resistance protein (BCRP)-mediated functional transport activity

Jothilingam Sivapackiam [a], Scott E. Harpstrite [a], Julie L. Prior [a], Stephen Mattingly [a,b], Vijay Sharma [a,b,c,d,*]

[a] ICCE Institute, Molecular Imaging Center, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, MO 63110, USA
[b] Students and Teachers As Research Scientists (STARS), Washington University School of Medicine, St. Louis, MO 63110, USA
[c] Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110, USA
[d] Department of Biomedical Engineering, School of Engineering & Applied Science Washington University School of Medicine, St. Louis, MO 63105, USA

ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form 23 November 2015
Accepted 3 December 2015
Available online xxxx

Keywords:
BCRP
Metalloprobe
Gallium

ABSTRACT

Introduction: For stratification of chemotherapeutic choices, radiopharmaceuticals capable of imaging breast cancer resistance protein (BCRP/ABCG2)-mediated functional transport are desired. To accomplish this objective, Galmydar, a fluorescent and moderately hydrophobic Ga(III) cationic complex and its $^{67/68}$Ga-radiolabeled counterparts were interrogated in HEK293 cells stably transfected with BCRP and their WT counterparts transfected with empty vector. Additionally, the sensitivity and specificity of ^{68}Ga-Galmydar to evaluate functional expression of BCRP at the blood–brain barrier (BBB) was investigated in gene-knockout *mdr1a/1b*$^{(-/-)}$ (double knockout, dKO) and *mdr1a/1b*$^{(-/-)}$*ABCG2*$^{(-/-)}$ (triple knockout, tKO) mouse models.
Methods: For radiotracer uptake assays and live cell fluorescence imaging, either ^{67}Ga-Galmydar or its unlabeled counterpart was incubated in HEK293 cells transfected with BCRP (HEK293/BCRP) and their WT counterparts at 37 °C under a continuous flux of CO_2 (5%) in the presence or absence of Ko143, a potent BCRP antagonist, and cellular uptake was measured to assess the sensitivity of Galmydar to probe BCRP-mediated functional transport activity *in cellulo*. For assessing the potential of Galmydar to enable diagnostic imaging of targeted tissues *in vivo*, the ^{67}Ga-radiolabeled counterpart was incubated in either human serum albumin or human serum at 37 °C and the percentage of unbound ^{67}Ga-Galmydar was determined. To evaluate the sensitivity of ^{68}Ga-Galmydar for molecular imaging of BCRP-mediated efflux activity *in vivo*, microPET/CT brain imaging was performed in dKO and tKO mice and their age-matched WT counterparts, 60 min post-intravenous injection.
Results: ^{67}Ga-Galmydar shows uptake profiles in HEK293 cells inversely proportional to BCRP expression, and antagonist (Ko143) induced accumulation in HEK293/BCRP cells, thus indicating target sensitivity and specificity. Furthermore, employing the fluorescent characteristics of Galmydar, optical imaging in HEK293/BCRP cells shows an excellent correlation with the radiotracer cellular accumulation data. ^{67}Ga-Galmydar shows > 85% unbound fraction and presence of parental compound in human serum. Finally, microPET/CT imaging shows higher retention of ^{68}Ga-Galmydar in brains of dKO and tKO mice compared to their age-matched WT counterparts, 60 min post-intravenous tail-vein injection.
Conclusions: Combined data indicate that Galmydar could provide a template scaffold for development of a PET tracer for imaging BCRP-mediated functional transport activity *in vivo*.

1. Introduction

Breast cancer resistance protein (BCRP, ABCG2, 72 kDa, a member of the adenosine binding cassette (ABC) family of transporter proteins) [1] is known to be expressed on the plasma membrane of tumor stem cells, and therefore postulated to be involved in stem cell differentiation [2,3], mediating protection against xenobiotics [4], and aiding tumor cell survival under hypoxia conditions [5]. Physiologically within the human body, BCRP is known to be localized on the luminal surface of brain endothelial cells (blood–brain barrier, BBB) [6], human placenta, bile canaliculi, the colon, and small bowel [7], thus effectively facilitating normal excretory functions. Literature precedents also indicate that BCRP shares significant substrate homology with *MDR1* P-glycoprotein (Pgp, ABCB1) [8,9], restricting delivery of tyrosine kinase inhibitors, such as imatinib, nilotinib, gefitinib, erlotinib, dasatinib, sorafenib, and lapatinib [10,11], and chemotherapeutics, such as topotecan, irinotecan, epirubicin, doxorubicin, daunorubicin, and mitoxantrone [12,13], into targeted sites. Furthermore, while BCRP is postulated to be downregulated in primary CNS lymphomas [14], in neuroepithelial tumors, such as ependymomas [15] and glioma [16], tumor stem-like cells show upregulation of BCRP expression, thus complicating CNS delivery of drugs. Importantly,

* Corresponding author at: Mallinckrodt Institute of Radiology, Washington University School of Medicine, Box 8225, 510 S. Kingshighway Blvd., St. Louis, MO 63110, USA. Tel.: +1 314 362 9358; fax: +1 314 362 0152.
E-mail address: sharmav@mir.wustl.edu (V. Sharma).

http://dx.doi.org/10.1016/j.nucmedbio.2015.12.001
0969-8051/

quantitative LCMS analysis of BCRP expression indicates that it is 1.3–1.5-fold higher and 3.2–4.6-fold lower than Pgp in human [17,18] and murine [19,20] BBB, respectively. Although LCMS data measure total protein content of both monomer and dimer forms of BCRP, the dimer represents its functional form [21]. Therefore, for better understanding of relationships between chemotherapeutic delivery and BCRP-mediated transport activity, assessment of protein expression at the functional level will provide more biochemically relevant information than the overall quantification of the protein. Thus, imaging agents capable of interrogating BCRP-mediated functional transport activity could enable stratification of patients likely to benefit from a given therapeutic choice.

To accomplish this objective, tariquidar [22], elacridar [22], gefitinib [23], and verapamil [24], previously characterized either as Pgp/BCRP substrates or inhibitors, have been incorporated with cyclotron-produced radionuclides (^{11}C and ^{18}F) for PET imaging of BCRP-mediated functional transport activity *in vivo*. While promising, some of these agents, such as verapamil, suffer from rapid metabolism, thus complicating the region-specific analysis *in vivo* [25,26]. To further enhance general access to biomedical imaging resources, the incorporation of an on-site generator-produced radionuclide (^{68}Ga) into an organic scaffold could enable deployment of a cost-efficient methodology for site-specific production of PET radiopharmaceuticals. Previously, we have reported moderately hydrophobic and cationic Ga(III) metalloprobes capable of interrogating Pgp-mediated functional transport *in cellulo* and *in vivo* [27–29]. Herein, we investigate potential of Galmydar (Scheme, Fig. 1) to monitor BCRP-mediated transport activity *in cellulo* and *in vivo*. Additionally, exploiting its modest fluorescent characteristics, we demonstrate the ability of Galmydar to monitor BCRP-driven functional transport in live cells using optical imaging, and correlate information from fluorescence data with that of radiotracer cellular accumulation.

2. Materials and methods

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. ^{1}H NMR and proton-decoupled ^{13}C NMR spectra were recorded on either 300 MHz or 400 MHz spectrometer (Varian); chemical shifts are reported in δ (ppm) with reference to TMS. Mass spectra were obtained from the Washington University Resource for Biomedical and Bioorganic Mass Spectrometry using samples diluted in 50/50 methanol/water containing 0.1% formic acid and analyzed via HRESI. Elemental analyses were performed by Galbraith Laboratories, Knoxville, TN. HPLC analysis was performed with a Waters System 600 equipped with dual λ-detector 2487 (280 and 214 nm) and a γ-detector (Bioscan) for identification of radiopeaks. Galmydar and its $^{67/68}$Ga-radiolabeled counterparts were assessed for purity on a C-18 reversed-phase column (Vydac TP, 10 μm, 300 Å) using an eluent gradient of ethanol and saline (20% ethanol in saline from 0–5 min, 20–90% ethanol in saline from 5–25 min, 90% ethanol in saline from 25–30 min, 100% ethanol from 30–35 min, and 100% saline from 35–40 min; at a flow of 2 mL/min). Radiochemical purity was also determined on C-18 plates employing a mobile eluent mixture of 90/10 ethanol/saline, using a radio-TLC (Bioscan System 200 Image Scanner).

2.1. Chemistry

The linear tetramine, 1,2-ethylenediamino-bis{(1-amino-2,2-dimethyl)propane}, 2-hydroxy-3-isopropoxy-benzaldehyde (**2**), and precursor ligand (**3**; 3,3′-(2-hydroxy-3-isopropoxyphenylimidazolidine-1,3-diyl)bis[1-{(2-hydroxy-3-isopropoxyphenyl)methyleneamino-2,2-dimethyl}propane]) were synthesized as described earlier [29–31].

2.1.1. Synthesis of Galmydar

Galmydar was synthesized by treating the precursor ligand dissolved in ethanol with a dropwise addition of gallium(III) acetylacetonate dissolved in ethanol involving the ligand exchange reaction described earlier [31]. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 0.79 (s, 6H), 0.96 (s, 6H), 1.30–1.33 (dd, 12H), 2.63 (d, 2H), 2.79 (d, 4H), 2.94 (br, s, 2H), 3.61–3.75 (m, 4H), 4.63 (sept, 2H), 4.79 (br, s, 2H), 6.62 (t, 2H), 6.87 (d, 2H), 7.04 (d, 2H), 8.18 (s, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 22.0, 22.1, 22.2, 26.2, 35.6, 47.7, 59.2, 68.9, 69.5, 115.7, 119.2, 119.5, 125.8, 148.7, 158.1, and 170.3; MS (HRESI) Calcd for $[C_{32}H_{48}N_4O_4Ga]^+$: 621.2926, found: m/z = 621.2930; and Calcd for $[^{13}C_{32}H_{48}N_4O_4Ga]^+$: 622.2959, found: m/z = 622.2967. Elemental analysis calculated for $C_{32}H_{48}N_4O_4Ga + CH_4O$: C 50.72; H 6.71; N 7.17; Ga 8.92%. Found: C 50.51; H 6.68; N 7.08; Ga 9.05%.

2.2. Radiochemistry

2.2.1. Preparation of ^{67}Ga-Galmydar

Radiolabeled ^{67}Ga-metalloprobe was synthesized by following a procedure described earlier [29–31] with slight modifications. Briefly, ^{67}Ga was obtained as a commercial citrate salt in water (Triad Isotopes), converted into chloride using HCl (6 N), extracted in ether (2 × 2 mL), and evaporated, and the residue was converted into ^{67}Ga(acetylacetonate)$_3$ by reacting with acetylacetone using standard procedures. The radiolabeled ^{67}Ga-metalloprobe was obtained through a ligand exchange reaction involving ^{67}Ga(acetylacetonate)$_3$ and heptadentate Schiff-base precursor ligand **3** dissolved in ethanol heated at 100 °C for 40 min. The reaction was followed using thin-layer chromatography plates (C-18) employing a radiometric scanner (Bioscan), using an eluent mixture of 90/10 ethanol/saline (R_f: 0.80). Finally, ^{67}Ga-Galmydar was purified by radio-HPLC on a C-18 reversed-phase column (Vydac TP, 10 μm, 300 Å), using the gradient eluent mixture of ethanol and saline described above. The fraction eluting at a retention time of 27.0 min was collected, concentrated, and employed for bioassays.

2.2.2. Preparation of ^{68}Ga-Galmydar

Radiolabeled ^{68}Ga-metalloprobe was synthesized by following a procedure described previously [32,33] with slight modifications. Briefly, ^{68}Ga was eluted from a generator (Eckert & Ziegler Eurotope) using 0.1 M HCl, which was passed through cation exchange column (Phenomenex; Strata-X-C 33 μm Polymeric Strong Cation; 30 mg/mL) to remove metal impurities and then pure ^{68}Ga was eluted with 400 μL of 0.02 M HCl in 98/2 acetone/water [32]. Thereafter, HEPES buffer (pH 5.45, 400 μL) was added to the eluent mixture, the pH was

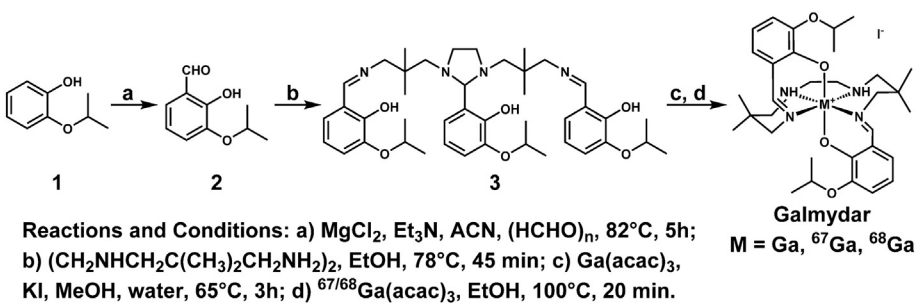

Fig. 1. Scheme for chemical synthesis of Galmydar [(ENB-3-isopropoxy-PIDMP)Ga]$^+$.

Please cite this article as: Sivapackiam J., et al, $^{67/68}$Galmydar: A metalloprobe for monitoring breast cancer resistance protein (BCRP)-mediated functional transport activity..., Nucl Med Biol (2016), http://dx.doi.org/10.1016/j.nucmedbio.2015.12.001