Radiolabeling and Preclinical Evaluation of a New S-Alkylated Cysteine Derivative Conjugated to C-Substituted Macrocyle for Positron Emission Tomography

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Supporting Information

ABSTRACT: A new S-alkylated cysteine-derivatized tumor targeting agent, 2,2′-(12-(2-(2-acetamido-2-carboxyethyl)thio)-acetamido)-11,13-dioxo-1,4,7,10-tetraazacyclotetradecane-4,7-diyl)-diacidic acid was developed for positron emission tomography (PET) imaging. N-Acetyl cysteine (NAC) was conjugated to ATRIDAT as a specific targeting agent toward L-type and ASC amino acid transporter systems in the oncogenic cells. NAC was attached via S-alkylation to prevent its incorporation at undesired recognition sites affecting the signal-to-noise ratio. NAC-ATRIDAT was subjected to gallium-68 complexation with >75% radiolabeling yield. The radiocomplex was purified through the tc18 cartridge to obtain 99.89% radiochemical yield. IC-50 of the NAC-ATRIDAT conjugate was 0.8 mM in A549 cells as evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide assay. Binding affinity experiments on A549 cells showed noteworthy binding with $K_m$ in the nanomolar range. A time course study showed a $K_m$ value of 0.19 $\mu$M and $V_{max}$ value of 0.49 pmol/µg protein/min showing reasonable tumor kinetics. Efflux studies showed that the synthesized radioligand is transported majorly by LAT followed by the ASC system. Clearance was found to be renal with 7.67 ± 1.48% ID/g uptake at 30 min which substantially declined to 0.52 ± 0.6% ID/g at 4 h. A significant uptake of 10.06 ± 1.056% ID/g was observed at the tumor site in mice at 1 h. $\mu$PET images revealed a high contrast with a tumor-to-kidney ratio of 4.8 and a tumor-to-liver ratio of 35.85 at 1 h after injection. These preclinical in vitro and in vivo evaluation supports its potential on the way of becoming a successful $^{68}$Ga-radiolabeled amino acid-based PET imaging agent.

INTRODUCTION

Amino acids and their derivatives labeled with radioactive isotopes are being extensively used in clinics and research labs as positron emission tomography (PET)/single-photon emission computed tomography imaging agents. Enhanced accretion of amino acids in tumor cells as a consequence of increased amino acid transport and protein synthesis is primarily responsible for their role in cancer imaging.1−5 The increase in the number of amino acid transporters in oncogenic proliferating cells has been documented by scientists in their reports. Among the transporters, the L-type transport system is primarily responsible for their role in cancer imaging.1−5

Many $^{18}$F-radiolabeled amino acids have been advanced for clinical PET applications. 4-[$^{18}$F]fluoro-L-phenylalanine ([$^{18}$F]FPhe) and 2-[$^{18}$F]fluoro-L-tyrosine ([$^{18}$F]FET) have shown promising results, yet their complicated radiolabeling procedures and low radiochemical yields are a limitation.6−8 O-(2-[$^{18}$F]fluoroethyl)-L-tyrosine ([$^{18}$F]FET), 2-[$^{18}$F]fluoro-R-methyl-L-tyrosine ([$^{18}$F]FMT), and [$^{18}$F]FDOPA are other $^{18}$F-labeled PET tracers, but their use focusses on brain-related abnormalities mainly.14−19 Outside the brain, their uptake is moderate in tumor tissues as compared to the surrounding healthy tissues, giving a poor to average target-to-nontarget ratio. Some recently synthesized compounds, 3-[[$^{18}$F]fluoro-2-methyl-2-(methylamino)propanoic acid ([[$^{18}$F]NMMeFAMP), 2-amino-3-[[$^{18}$F]fluoro-2-methylpropanoic acid ([[$^{18}$F]FAMP),1-amino-3-[[$^{18}$F]fluorocyclobutyl-1-carboxylic acid ([[$^{18}$F]FACBC), and 1-amino-3-[[$^{18}$F]fluoromethyl-cyclobutane-1-carboxylic acid ([[$^{18}$F]FMACBC) have shown good localization at the brain and tumor site.20−23

$^{11}$C-Methyl-L-methionine ($^{11}$C-MET) is an extensively used PET tumor imaging agent based on an amino acid. However, owing to its incorporation in the protein segments via the amino-acyl-transfer RNA pathway along with its degradation products, $^{11}$C-MET produces a significant amount...
of radioactive metabolites which leads to a decrease in its specificity and selectivity in vivo. Also, MET gets assimilated into nonprotein matter like phospholipids and RNA through the transmethylation process (transfer of its radiolabeled methyl group) via S-adenosyl-l-methionine. Both these side reactions create substantial nonspecific background signal, making quantitative imaging very difficult at the tumor target site.

All these amino acid derivatives have been radiolabeled with $^{11}$C or $^{18}$F, restricting their use in PET centers having an in-house cyclotron facility or nearby cyclotron center. Bourdier et al. reported that an S-alkylated amino acid derivative PET imaging agent with a chelator, shown to possess high stability with metal ions in the $M^{2+}$ and $M^{3+}$ form, especially Ga(III), was proposed. ATRIDAT, already reported by our group to form thermodynamically stable and strong complexes with Ga(III) was used for conjugating the cysteine derivative after slight modification in the pendant arm, keeping the macrocyclic framework intact. PET is a highly sensitive technique which provides a method to visualize and quantify the uptake at the target site. PET aids in early diagnosis, as it detects the changes in the biochemical pathways, allowing detection of disease before the appearance of anatomical changes. Being a generator-produced radioisotope along with an ideal half-life, $^{68}$Ga with its optimum isotope energy makes an attractive radioisotope for PET imaging. The comparatively shorter half-life of $^{68}$Ga and hydrophilic nature of the complex allows rapid renal clearance which ultimately reduces the radiation burden to the patient. $^{68}$Ga-PET is extensively used

Scheme 1. Synthesis of 2,2′-(12-(2-((2-Acetamido-2-carboxyethyl)thio)acetamido)-11,13-dioxo-1,4,7,10-tetraazacyclotridecane-4,7-diyl)diacetic Acid$^{a}$

$^{a}$Conditions: (i) EAA/HCl, r.t.; (ii) ClCH$_2$COCl, K$_2$CO$_3$, CHCl$_3$/H$_2$O, 0 °C; (iii) N-acetyl l-cysteine, NaOMe, anhydrous MeOH, r.t.; (iv) TFA, dichloromethane (DCM), 0 °C.
in the diagnosis and management of tumors owing to defined chemistry of Ga(III) with triaza- and tetraaza macrocyclic molecules.31,32

## RESULTS AND DISCUSSION

**Synthesis.** Synthetic strategy utilizes S-alkylation of N-acetyl cysteine (NAC) with a chloroacetylated derivative of a 13-membered protected macrocycle, di-tert-butyl 2,2′-(12-((tert-botoxycarboxy)amino)-11,13-dioxo-1,4,7,10-tetraazaatri-decane-4,7-diyli)diacetate (1)30 (Scheme 1). The Boc group of this macrocycle (1) was selectively cleaved using HCl gas dissolved in ethyl acetate, exposing the amine group for further reaction to give compound 2. This reaction occurs in 2–3 h, and the product formed precipitates as a pale yellow solid in the reaction solvent which can be used as such without further purification. The free amine group was then chloroacetylated (3) to alkylate NAC (bio vector) from the thiol side to synthesize compound 4. Conjugation of the biovector was confirmed by the presence of an additional peak for 3 protons at 2 ppm (for methyl protons of the acetyl group) in the 1H NMR spectra and the peak at 23 ppm for the carbon of this methyl group in the 13C spectra. A peak at 176 ppm appears in the 13C spectra, for the carboxyl group of the cysteine. In mass spectra, the conjugation is confirmed by the [M + H]+ peak at 661.5 m/z. This intermediate on trifluoroacetic acid (TFA) treatment gave the final compound (5) confirmed by the [M − H]+ peak at m/z 547.3 in the mass spectra. All synthesized compounds were characterized through spectroscopic techniques such as NMR and mass spectrometry (see the Supporting Information). The synthesized molecule targets the enhanced amino acid uptake and the elevated AA transporters in the tumor cells and S-alkylated to prevent its incorporation in the nondesired protein/nonprotein segments for high tumor-to-background ratio.

**Radiolabeling, Serum Stability and log P.** The labeling of ATRIDAT-NAC was performed with 68Ga using middle fraction of the 68Ge/68Ga generator elute. 68Ga-radiolabeling of ATRIDAT-NAC was accomplished at the ambient temperature of 90 °C for 10 min at pH 4.5. Temperature less than 90 °C or reduced time of heating significantly decreased the radiolabeling yield. A decent radiochemical yield of 78% with 98.9% radiochemical purity and 5 MBq/nmol of specific activity was obtained after C-18 cartridge purification. The radiocomplex was confirmed through EZ-thin-layer chromatography (TLC), with the dominant peak showing the 68Ga-ATRIDAT-NAC moiety. The radiolabeled compound exhibited more than 97% stability in human serum up to 60 min which reduced to 75% after 4 h incubation when traced with instant TLC (ITLC) (see the Supporting Information for the HPLC profile, EZ-TLC, radiolabeling optimization and serum stability graphs). The intact percentage of the radiotracer is reasonable to perform the biological studies with 3–4 h duration. The distribution of the radiotracer in octanol and phosphate buffered saline (PBS) gave a log P value of −2.63 ± 0.04 showing its greater affinity toward the hydrophilic system.

**Cell Viability Assay.** The in vitro cytotoxicity of the conjugate was assessed by incubating A549 cells with different concentrations of ATRIDAT-NAC at 24, 48, and 72 h time intervals. At lower concentrations of the ligand, no significant toxicity was observed. At 1 mM concentration of the compound, 70.76 ± 1.64% viability was obtained at 24 h which decreased to 67.52 ± 0.54% at 48 h and 56.37 ± 1.73% at 72 h of incubation (Figure 1). IC50 in A549 cells was observed to be 0.8 mM, indicating its safe and nontoxic behavior. Toxicity of cells was observed to be dose-dependent at all time points.

![Figure 1. Cytotoxicity assay curve of ATRIDAT-NAC in A549 cells performed for 24, 48, and 72 h duration from 1 pM to 10 mM concentration range.](Image)

**Time Course and Kinetics Study.** Time progression analysis showed that the transport of 68Ga-ATRIDAT-NAC into A549 cells was fast and linear up to 30 min, then reaching saturation at 60 min of incubation. Linear correlation was seen as a result of a saturable system because of L followed by the ASC transporter system in Eadie–Hofstee transformation (Figure 2). A kinetic study revealed a Michaelis constant (Km) value of 0.19 μM and Vmax (maximal transport rate) with a value of 0.49 pmol/μg protein/min.

![Figure 2. Time course of 68Ga-ATRIDAT-NAC. Uptake was seen from 0.5 to 60 min. The inset depicts an Eadie–Hofstee graph of saturable transport. The experiment was performed in triplicates, and values are mean SD of 3 measurements.](Image)

**Cell Binding Studies.** Cell binding assay of 68Ga-ATRIDAT-NAC was done on A549 cells to assess the binding of the radioconjugate to the transporters on the surface of tumor cells. Nonspecific binding was evaluated by the addition of 100 times excess of unlabeled cold NAC. The binding curve revealed appreciable binding of ATRIDAT-NAC on the A549 tumor cell line surface (Figure 3). The Scatchard plot analysis showed significant affinity of the synthesized compound on the
tumor cells. The dissociation constant \((K_D)\) was observed to be 5.5 nM.

**Na\(^+\)**-Dependent/-Independent Efflux Study.** Amino acid efflux studies were conducted with and without the presence of Na\(^+\) to assess the specificity of \(^{68}\)Ga-ATRIDAT-NAC for its transporter associated with uptake by A549 cells. This experiment was performed with the control set where no amino acid was added in the buffer solutions. It is worthwhile to note that the transport kinetics was similar to that of cysteine. The addition of Na\(^+\) has shown a pattern of efflux in the presence of other amino acids including alanine, DOPA, phenyl alanine, and glutamine. The replacement of Na\(^+\) by choline showed slightly decreased the uptake of \(^{68}\)Ga-ATRIDAT-NAC in cysteine-containing buffer solution. There was 90% efflux of \(^{68}\)Ga-ATRIDAT-NAC as compared to control in the case of Na\(^+\)-independent conditions (Figure 4). The results suggested that \(^{68}\)Ga-ATRIDAT-NAC is primarily transported and mediated mainly by L type with minor transport system ASC.

**Incorporation of \(^{68}\)Ga-ATRIDAT-NAC into Proteins.** This experiment was done to check the influence of S-alkylation on the amino acid-based radioligand incorporation in the protein segments. Less than 2% radioactivity was found in samples precipitated with trichloroacetic acid (TCA) in the acid insoluble fraction after 30 min incubation with \(^{68}\)Ga-ATRIDAT-NAC. Almost all the activity was retained in the acidic fraction. Incorporation of \(^{68}\)Ga-ATRIDAT-NAC into protein was not observed.

**Blood Kinetics, \(\mu\)PET Imaging, and Biodistribution.** The blood clearance study was performed in rats till 4 h. It showed rapid clearance of the radioligand, \(^{68}\)Ga-ATRIDAT-NAC from blood with 31.99% remaining after 30 min. After 30 min, the compound showed gradual clearance from the blood with 19.8% remaining at 2 h and 16.3% remaining at 4 h (see the Supporting Information, Figure S13).

\(\mu\)PET scanning was carried out to evaluate the potential of \(^{68}\)Ga-ATRIDAT-NAC to target A549 tumor cells xenograft in the forelimb of athymic mice for diagnostic applications. An increased uptake of the radiotracer at the tumor site was seen which improved with time as compared to other organs. In vivo \(\mu\)PET scans of A549 xenograft in nude mice showed high localization of \(^{68}\)Ga-ATRIDAT-NAC at the desired site, while there was minimal activity accumulation in the blood pool organs (Figure 5). A slight uptake at the kidney and liver sites was seen at 1 h which decreased further when seen in the 2 h delayed images. Low retention in liver and kidneys was observed because of the hydrophilic nature of the radioligand conjugate. The regions of interest (ROIs) as calculated semi-quantitatively have shown 95.23 kBq/mm\(^3\) of the dose in tumor, whereas in the kidney and liver, the doses were found to be 19.72 and 2.66 kBq/mm\(^3\) respectively (Table 1). The tumor-to-kidney ratio of 4.8 and tumor-to-liver ratio of 35.85 were achieved.

Biodistribution studies were carried out at different time intervals, namely, 0.5, 1, 2, and 4 h. The path of excretion was observed to be kidneys with 7.67 ± 1.48% ID/g accumulation

| organ  | ROI’s (kBq/mm\(^3\)) |
|--------|-----------------|
| tumor  | 95.23           |
| kidney | 19.72           |
| liver  | 2.66            |

![Figure 3](image1.png)

**Figure 3.** Displacement of \(^{68}\)Ga-ATRIDAT-NAC using excess unlabeled cold NAC. Scatchard plot showing the specific binding of the radiolabeled compound to the ratio of bound to free for A549 cells.

![Figure 4](image2.png)

**Figure 4.** Efflux study of \(^{68}\)Ga-ATRIDAT-NAC by different amino acids in sodium-containing and sodium-absent media in A549 cells at \(t = 60\) min at 25 °C. The experiment was performed in duplicates in 3 sets with control, \(n = 3\), values are mean ± SD.

![Figure 5](image3.png)

**Figure 5.** \(\mu\)PET/computed tomography (CT) scintigraphic scan of \(^{68}\)Ga-ATRIDAT-NAC injected in nude mice with A549 tumor cells xenograft in the forelimb at 1 h after injection. 3D-rendered reconstructed \(\mu\)PET/CT image using AMIRA 4.1.1.

![Table 1](table1.png)

**Table 1.** Table Representing ROIs of Different Organs
at 30 min after which the radioactivity in the kidneys declined considerably at 1 h with 2.08 ± 0.66 and 0.52 ± 0.08% ID/g retaining at 4 h. The brain showed less than 0.1% at all time intervals. The uptake in the bone was also negligible at all time points with 0.19 ± 0.07% ID/g at 30 min, the highest uptake value. The radioconjugate showed accretion of 4.15 ± 0.16% ID/g at 0.5 h in the liver which decreased to 0.38 ± 0.09 and 0.16 ± 0.04% ID/g at 1 and 4 h, respectively. The log P value plays a pivotal role in deciding the fate of excretion and formation of cationic species. The log P value of −2.89 was obtained for [68Ga-ATRIDAT-NAC] because of which it followed the renal route of excretion followed by excretion through the liver. The tumor/kidney and tumor/liver ratios at different time intervals are shown in Table 2. Less than 0.5% uptake was observed in the stomach. In tumor, a considerable uptake of 2.47 ± 0.16% ID/g at 30 min was seen and rapid accumulation with a value of 10.06 ± 1.05% ID/g at 1 h. Further, a decrease of activity was seen at 2 h with 6.80 ± 0.57% ID/g value which reduced to 4.52 ± 0.61% ID/g at 4 h (Figure 6). The biodistribution statistics shows speedy clearance of the radiolabeled probe from the blood pool and other vital tissues. Apart from hydrophilic nature of the compound, the alkylated thiol group helps in reducing accumulation of activity in the background regions. This results in amplification of the signal at the tumor site.

### MATERIALS AND METHODS

Chemicals and reagents consumed in the syntheses were obtained commercially and utilized without further purification unless notified. Sodium methoxide in methanol, N,N-dimethyl formamide (anhydrous), N-acetyl-i-cysteine, ethyl acetate, pet ether, chloroacetyl chloride, I-octanol, sodium sulphate anhydrous, potassium carbonate, TFA, acetonitrile, methanol, DCM, chloroform, and water were purchased from E. Merck Ltd. (India). Among these, solvents were purchased of HPLC grade. Dulbecco’s modified Eagle’s medium (DMEM), penicillin, streptomycin sulphate, nystatin, and trypsin–ethylenediaminetetraacetic acid (EDTA) (10×) were bought from Sigma, USA. Foetal bovine serum was obtained from Gibco.

The reactions demanding anhydrous conditions were carried under an inert N2 atmosphere. Anhydrous methanol was prepared by using 4 Å molecular sieves. The reaction temperatures mentioned denote the temperature of the reaction vessel’s water bath and not the reaction vessel itself. Aluminium-coated silica gel sheets (silica gel 60 F, Merck, Germany) were used to run TLC and seen by potassium permanganate developing solution. ITLC was done to assess radiocomplexation yield and percentage of radiochemical purity.

**Instrumentation Details.** The Bruker AVANCE II 400 MHz NMR system (ultra-shielded) was used for recording 1H and 13C NMR spectra. The Agilent 6310 system ion trap mass spectrometer was used for electrospray ionization mass spectroscopy (ESI-MS in positive and negative ion mode). High-resolution mass spectrometry—liquid chromatography mass spectrometry (HRMS–LCMS) coupled with the quadrupole time-of-flight mechanism was utilized to obtain accurate mass of the compounds at University of Delhi. The scintigraphic scan was taken using the GE FLEX Triumph LABPET4. μPET module () having 2’2’10 mm3 LYSO/LGSO scintillators in an 8-pixel with avalanche photodiode detector module. Image analyses and reconstruction was accomplished using Amira 4.1.1 and AMIDE software. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldisubstituted benzene (MTT) assay plates were recorded at 570 nm with a reference wavelength of 630 nm on a Synergy Hybrid H4 (BioTek Instruments, Winooski, VT, USA).

**HPLC Details.** The Agilent 1260 (infinity analytical scale purification) system was used to analyze the final compounds on the Atlantis T3 C18 reverse phase column (5 μm, 4.6 mm × 250 mm). The mobile phase used was 0.05% TFA in water (solvent A, 60%) and acetonitrile (solvent B, 40%) in the isocratic mode with a flow rate of 0.8 mL/min for 30 min and 10 μL of injection volume. RT (retention time) of 4.114 min was obtained for the final compound (see the Supporting Information).
Cell Culture Details. DMEM supplemented media (10% foetal bovine serum, 50 µg/mL streptomycin sulfate, 2 µg/mL nystatin, and 50 U/mL penicillin) was used to maintain the monolayer cultures of A549 cells. Cells were regularly cultured two times in a week using trypsin−EDTA (Sigma, USA) and kept at 37 °C in a CO2 incubator maintained at 5% CO2 and 95% air.

Animal Models. Animal procedures were permitted and executed according to the recommendations of INMAS’s Animal Ethics Committee (reg. no. */GO/RBI/S/99/ CPCSEA). Animals were kept under sterile and controlled temperature of 22 ± 2 °C and water and diet ad libitum. Xenografted athymic nude mice were used for scintigraphic and biodistribution studies. Cell suspension (100 L) of A549 cells was used for hot labeling. Wistar rat was used for blood kinetics.

Statistical Analysis. The Scatchard plot was analyzed through nonlinear regression using GraphPad Prism 5.0. Biodistribution data are represented as a graph depicting mean ± SD.

EXPERIMENTAL SECTION

Synthesis of Di-tert-butyl-2,2′-(12-amino-11,13-dioxo-1,4,7,10-tetraazacyclotridecane-4,7-diyl)-diacetate (2). To 5 mL 1 M dry solution of HCl, 5 mL in ethyl acetate (containing 500 mol % of HCl), compound 1 (200 mg, 0.36 mmol) was added and stirred for 4–5 h in a round-bottom flask at room temperature. The product starts to precipitate out in ethyl acetate as the reaction progresses. Completion of reaction was checked through TLC for precipitates out in ethyl acetate (containing 500 mol % of HCl), compound diacetate (2).

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To a solution of NaOMe in methanol (15 mg, 0.28 mmol) was added equimolar NAC (30 mg, 0.19 mmol) and compound 3 (100 mg, 0.19 mmol) in anhydrous MeOH (3 mL) at room temperature under inert conditions. After completion of reaction (checked through TLC), the reaction was quenched with a small amount of water, and the solvent was evaporated using a rotary evaporator to give a sticky yellow compound. The crude compound was then subjected to column chromatography purification using neutral alumina and 20% methanol in chloroform as a mobile phase to give 4 as a pale yellow-colored compound. (92.41 mg, 0.14 mmol, 74.8%): 1H NMR (400 MHz, 25 °C, CDCl3): 1.4 (s, 18H, CH3), 2.0 (s, 1H, NH), 2.6–2.7 (m, 8H, CH2), 3.2–3.3 (m, 8H, CH2), 4.1 (s, 1H, CH) 7.8 (br, 2H, NH), 7.9 (br, 1H, NH). 13C NMR (100 MHz, 25 °C, CDCl3): 21.6, 33.3, 34.3, 51.8, 53.0, 53.7, 58.7, 162.3. HRMS (positive): m/z calc for C28H48N6O10S [M+H]+, 661.32; found [M + H]+, 661.5.

Synthesis of 2,2′-(12-(2-(Acetamido-2-carboxyethyl)thio)acetamido)-11,13-dioxo-1,4,7,10-tetraazacyclotridecane-4,7-diyl)diacetic Acid (5). To a solution of 68Ga in DCM (5 mL) was added compound 4 (100 mg, 0.15 mmol) under ice-cold conditions. The reaction was left overnight to stir at room temperature. The resulting solution was applied to a rotatory evaporator for complete removal of TFA. This gave compound 5 as an yellow solid. 68Ga (half-life = 68 min and positron emission of 89%) was fractionated from 68Ge to 68Ga generator. In the generator, 68Ga-1/2 value of 68 min and positron emission of 89% was injected into the generator to elute 68Ga(III) from the matrix column. The middle fraction was used for radiolabeling to minimize 68Ga breakthrough. HCl (12 N, 1.25 mL) was added to the eluate to obtain a 5 N solution of [GaCl4]−. Dowex-1 of 200–400 mesh size (50 mg) anion exchange resin was set for trapping Ga in the gallium chloride [GaCl4]− form. Thereafter, 68Ga− elution was accomplished with water. This carrier-free solution was used for hot labeling. CH3COONa was used to make the pH ≈ 4. To it, 20 µmol of ATRIDAT-NAC was added, and the resulting mixture was heated at 90 °C for a

4. Radiolabeling of the Compound. Radiolabeling was performed using the previously published method. 68Ge (T1/2 value of 68 min and positron emission of 89%) was fractionated from 68Ge to 68Ga generator. In the generator, 68Ge (half-life = 270 days) is adsorbed on the titanium dioxide-based inorganic matrix. A total of 5 mL of 0.1 N HCl was injected into the generator to elute 68Ga(III) from the matrix column. The middle fraction was used for radiolabeling to minimize 68Ga breakthrough. HCl (12 N, 1.25 mL) was added to the eluate to obtain a 5 N solution of [GaCl4]−. Dowex-1 of a 200–400 mesh size (50 mg) anion exchange resin was set for trapping Ga in the gallium chloride [GaCl4]− form. Thereafter, 68Ga− elution was accomplished with water. This carrier-free solution was used for hot labeling. CH3COONa was added to make the pH ≈ 4. To it, 20 µmol of ATRIDAT-NAC was added, and the resulting mixture was heated at 90 °C for a
duration of 10 min. It was then cooled to room temperature and subjected to a C-18 cartridge purification (preconditioned with ethanol and water and air-dried). $^{68}$Ga(III) was washed away with water, and the radiolabeled compound was retained on the cartridge. The $^{68}$Ga(III)-labeled ATRIDAT-NAC was eluted with 60% ethanol. ITLC-SG was done to estimate the radiochemical yield in 15% ammonium acetate/methanol in the ratio of 1:4 as the mobile phase. The ITLC was scanned and analyzed with the OmniScan EZ-TLC scanner and PeakSimple 3.0, respectively.

**Cytotoxicity of NAC-ATRIDAT.** Cytotoxicity was assessed in the A549 cells using the MTT assay. Cells growing exponentially at a uniform cell density of 4000–5000 cells/well were plated in a 96-well microtiter plate. Cells were treated with increasing concentrations of the test compound (pM to mM range) at intervals of 24, 48 and 72 h. At the end of treatment, negative control and treated cells were incubated with MTT (0.05 mg/mL) for 2 h at 37 °C, and the medium was removed. Triplicate wells were lysed, and 150 μL of DMSO was added to dissolve the formazan crystals. Optical density of 150 μL extracts was measured at 570 nm (reference filter: 630 nm). Surviving fraction for the concentration range was plotted against the concentration for ATRIDAT-NAC.

**Human Serum Stability Evaluation.** Blood was taken from healthy persons (volunteers) and allowed to clot for 1 h was plotted against the concentration for ATRIDAT-NAC.

**Human Serum Stability Evaluation.** Blood was taken from healthy persons (volunteers) and allowed to clot for 1 h at 37 °C in a humidified incubator maintained at 5% CO₂ balanced with 95% air. After clotting, the blood was centrifuged at 400 g. The serum was filtered through a 0.22 μm syringe filter. To 900 μL of human serum, 100 μL of the radiolabeled compound was added and placed in a CO₂ chamber incubated at 37 °C. ITLC was done for dissociation of the complex in the ratio of 1:4 of the 15% ammonium acetate/MeOH solvent system.

**log P.** In a presaturated solution of 500 μL each of 1-octanol and PBS, a solution of 20 μL of $^{68}$Ga-labeled-ATRIDAT-NAC (50 μM) was added. The presaturated solution containing the $^{68}$Ga-labeled-ATRIDAT-NAC was vortexed and centrifuged at 3000 rpm for 10 min. The two phases were separated, and the radioactivity in a volume of 100 μL from each layer was measured in the radioactivity associated with the cells was determined on a gamma counter. Specific binding was calculated by subtracting nonbinding fraction from the total binding.

**Blood Kinetics.** The blood kinetics study was performed in female Wistar rats (∼230 g) to analyze the kinetics of radioconjugate clearance from the blood after circulation. $^{68}$Ga-ATRIDAT-NAC (1 MBq) was injected in the blood stream through the tail vein of the female Wistar rat. The blood samples (200 μL) were collected from the ocular vein through capillary at different time intervals (10–240 min), and counts were measured on a gamma counter. Calculations were done assuming the 64 mL/kg blood volume of the rat. Data are given as percentage injected dose (% ID) at different time frames.

**Cell uptake Studies.** For cell uptake studies, 0.3 × 10⁶ cells were seeded in DMEM supplemented medium in 24-well culture plates which were incubated overnight. On the next day, the cells/well were counted. The growth medium was replaced by HBSS prior to the experiment. The cells were incubated with the 0.37 MBq of the radiolabeled compound (500 μL) and incubated at 37 °C for 60 min. To terminate the uptake, the tracer solution was removed, and cells were washed with ice-cold PBS. The cells were lysed with 0.2 N NaOH (500 μL), and the radioactivity associated with the cells was counted using the γ-counter. The results were presented as percentage dose per 10⁵ cells (all values were decay corrected).

**Cell Binding Studies.** Monolayer cultures of the A549 cell line were grown in normal DMEM, supplemented with 10% FBS. The monolayers were washed with HBSS and left in HBSS for 2 h at 37 °C prior to the conduct of the experiment. Saturation and displacement experiments were carried out at 37 °C. Cells were incubated for 1 h with (1 pM to 10 μM) concentration of $^{68}$Ga-ATRIDAT-NAC with and without the presence of the unlabeled NAC (100 folds excess) to calculate the total and nonspecific binding, respectively. The monolayers were washed with PBS four times after the incubation time. The radioactivity associated with the cells was determined on a gamma counter. Specific binding was calculated by subtracting nonbinding fraction from the total binding.

**Time Course and Transport Kinetics of $^{68}$Ga-ATRIDAT-NAC.** The time course of the $^{68}$Ga-ATRIDAT-NAC uptake was measured by treating the cells with 10 μM of the radiolabeled compound in HEPES-buffered Krebs solution (0.5 mL; 37 °C) for different time intervals (30 s to 60 min). Transport of the radiolabeled compound was stopped by removing the radioactive and washing three times with PBS. The total activity in cells was measured as stated above in the cell uptake study. A549 cells were incubated for 10 min in HEPES-buffered Krebs solution (50 μL; 37 °C) with concentrations (0.005–0.1 μM) of $^{68}$Ga-ATRIDAT-NAC for kinetic experiments. The data are plotted using the Michaelis–Menten equation.

**Selectivity and Sodium Ion Dependency of Transport.** The inhibition of $^{68}$Ga-ATRIDAT-NAC transport by amino acids in competition was studied in two different conditions. In the first, cells were incubated with Krebs solution and 10 μM solution of $^{68}$Ga-ATRIDAT-NAC. In second, inhibition studies were carried with Na⁺-free Krebs solution by substituting sodium salts (NaCl, NaHCO₃, and NaH₂PO₄) with its choline and potassium analogues (choline chloride, choline bicarbonate, and KH₂PO₄). A549 cells were treated with $^{68}$Ga-ATRIDAT-NAC for 1 h at 37 °C in standard Krebs buffer. After incubation, cells were rinsed and treated with Krebs buffer (standard and modified) consisting of 1 mM of either L-alanine, L-cysteine, DOPA, L-phenyl alanine, or L-glutamine in the group of 4 sets. After 1 h, cells were washed with PBS thrice, followed by the addition of lysis buffer and counts were taken of all the fractions. $^{68}$Ga-ATRIDAT-NAC efflux was stated as percent of radioactivity effluxed from the cells.

**Incorporation of $^{68}$Ga-ATRIDAT-NAC into Proteins.** The incorporation of $^{68}$Ga-ATRIDAT-NAC into protein was done in concordance to the previously published protocol with slight modifications. For radioligand incorporation in proteins, the samples were treated with 1 MBq (500 μL) of $^{68}$Ga-ATRIDAT-NAC at 37 °C for 30 min. At the completion of treatment, the radioactive media were removed, followed by cell washing with 1 mL ice-cold PBS thrice. The cells were dislodged using 0.5 mL EDTA (1%), and 0.5 mL of 20% TCA was added to the cells. The samples were kept for 10 min on ice prior to centrifugation at 10 000 rpm for 5 min. The pellet obtained was washed thrice with ice cold PBS. The radioactivity associated with the pellet and the supernatant was quantified by a gamma counter. $^{68}$Ga-ATRIDAT-NAC integration in the proteins was calculated as the percentage of radioactivity in the precipitated fraction after the TCA addition.
Biodistribution Studies in Tumor Xenograft Mice Models. Biodistribution studies were done on athymic mice after 10 days of tumor inoculation. The biodistribution of the radiolabeled compound was assessed in A549 tumor xenograft mice after intravenous injection of 100 µL of saline holding 1 MBq of 68Ga-ATRIDAT-NAC. After 30, 60, 120, and 240 min injection of the 68Ga-ATRIDAT-NAC, mice (n = 3) for each time interval were sacrificed by cervical dislocation following CO2 administration. Organs were harvested and washed with saline and dried on blotting sheets. Organs were then weighed, and the radioactivity in the whole organ was measured by a gamma counter. Percent injected dose per gram of tissues was calculated and plotted.

PET Imaging Studies in Tumor Xenograft Mice Models. The PET imaging study was performed on anesthetized animals by breathing of 2% isoflurane dissolved in oxygen (2 L/min). Each mouse was given 68Ga-ATRIDAT-NAC (30 MBq) intravenously in 100 µL of saline intravenously. CT scan was taken of the respective animals for the anatomical frame prior to PET acquisitions at 1, 2, and 4 h venously. CT scan was taken of the whole organ was measured by an AMIRA, and semiquantitative analysis was done using AMIDE. Data are stated as activity in kBq per mm3 of the tissue.

■ ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b00059.

[pp. 15–13S] NMR and mass spectra of compounds, ITLC, analytical HPLC, blood clearance, and serum stability graph (PDF)

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■ ABBREVIATIONS

NAC, N-acetyl l-cysteine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide; PET, positron emission tomography; ROI, region of interest; MLEM, maximum likelihood expectation maximization

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