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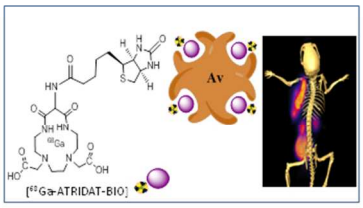

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Biotinidase resistant ^{68}Gallium-radioligand based on Biotin/Avidin interaction for pretargeting: Synthesis and Preclinical evaluation

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ABSTRACT: A new macrocyclic system 2,2'-(12-amino-11,13-dioxo-1,4,7,10-tetraazacyclotridecane-4,7-diyl)diacetic acid (ATRIDAT) was designed for coordinating metals in +2 and +3 oxidation states particularly ^{68}Ga(III), for PET imaging. ATRIDAT was conjugated to biotin for pretargeting via biotin-avidin interaction. This model provides high tumor targeting efficiency, stability to biotinidase activity leading to modest signal amplification at the tumor site. Cyclization of triethylenetetramine with protected amino malonate resulted in the formation of 13 membered diamide ring. (D)-biotin was then anchored on the pendant amine rendering alpha methyne carbon to the biotinamide bond which blocks the biotinidase enzyme activity. Biotinidase stability assay showed remarkable stability towards the action of biotinidase with ~95% remaining intact after treatment following 4 h. Binding affinity experiments such as HABA assay, competitive displacement studies with (D)-biotin and CD showed high binding affinity of the molecule with avidin in nanomolar range. Biotin conjugate was successfully radiolabeled with ^{68}Ga with radiolabeling efficiency of ~ 70% and then purified to get 99.9% radiochemical yield. IC_{50} of the compound was found to be 2.36 mM in HEK cell line and 0.82 mM in A549 as assessed in MTT assay. In biodistribution studies, major route of excretion was found to be renal. Significant uptake of 4.15 ± 0.35 % was observed in tumor in the avidin pretreated mouse at 1 h. μPET images also showed a high tumor to muscle ratio of 26.8 and tumor to kidney ratio of 1.74 at 1 h post injection after avidin treatment.

INTRODUCTION

Selective delivery of the radiolabeled compound to the target cells to avoid possible uptake/damage to the surrounding healthy cells is an important aspect of tumor imaging and therapy. One of the most encouraging line consists in functionalization of molecules with biotin analogues due to the latter's extremely strong affinity for the proteins avidin (Av) and streptavidin (SAv).[1-4] Strong hydrogen bonding interactions within the binding site of the biotin due to the presence of ureido group and its polarization through charged aspartate results in remarkable affinity/avidity in femtomolar range.[5-7] This high selectivity of biotin for avidin and fast blood clearance gives the advantage of better tumor to non-tumor ratio over conventionally used radiolabeled monoclonal antibodies and long peptides which have slow blood clearance and moderate tumor to muscle ratio.[8] This makes avidin-biotin system an ideal method for delivering diagnostic or therapeutic radionuclides to cancer cells.

Pretargeting was developed to circumvent the prolonged circulation of macromolecules thereby reducing the radiation burden by using non-radioactive macromolecule.[9,10] This approach involves sequential administration of first a macromolecule (Avidin/ antibody based targeting molecule) and second a low molecular weight non-immunogenic radiolabeled effector molecule (vitamin biotin). The radioactive species was injected after a lag period for allowing the targeted macromolecule to accumulate at the tumor site and any residual to be cleared from the blood circulation. These two component interaction must be strategically modified to ensure strong interaction at the target site. The superior pharmacokinetics of the effector molecule results in the improvement of imaging contrast at earlier time point and decrease localization in the non-target organs.[11]

Avidin is a highly glycosylated positively charged protein having terminal N-acetyl glucosamines and mannose residues that bind to lectins. Lectins are expressed in tumor cells at varying levels. Tumor cells with high metastatic propensity express substantial level of surface lectins.[12] A study reported by Konishi et.al, demonstrated that the carbohydrate moiety on the avidin surface is responsible for binding to the lectins which are overexpressed in a variety of rodent and human sarcomas, hepatomas, lung carcinomas, squamous cell carcinomas, ovarian, mammary carcinomas, hepatomas etc.[13, 14] Lectins on the tumor cell surface aid in metastasis by increasing their ability to form aggregates with each other or by helping in adhering to the host cells or the extracellular matrix. Binding of avidin to lectin overexpressing tumor cells have been used by Hama et al. for optical imaging of cancer cells.[15, 16]

Biotin is preferred because of its various favourable properties, for e.g. its comparatively simple chemical structure, easy to work with functional groups and high affinity for Av/ SAv making it a highly tumor specific biomarker. The binding of avidin-biotin is a highly adaptable tool and has been used for wide applications like biological imaging, sensing and target delivery.[17]All these benefits make biotin a

desirable candidate for target specific diagnostic agents. The application of biotin derivatives is not just limited to tumor imaging; its utility has been explored in the area of infection localization, biochemical assays and antibacterial properties also.[18-22]

Positron Emission Tomography (PET) is performed to map physiological and biological processes in organisms to detect disease-related biochemical abnormalities prior to the appearance of anatomical changes.[2368]^{68}Ga was chosen as the PET radioisotope owing to its ideal decay characteristics (89% positron emission) and sufficiently long half-life (68 min). Being a generator produced radioisotope, ^{68}Ga has a practical advantage of being utilized in places without cyclotron facility.[24, 25]

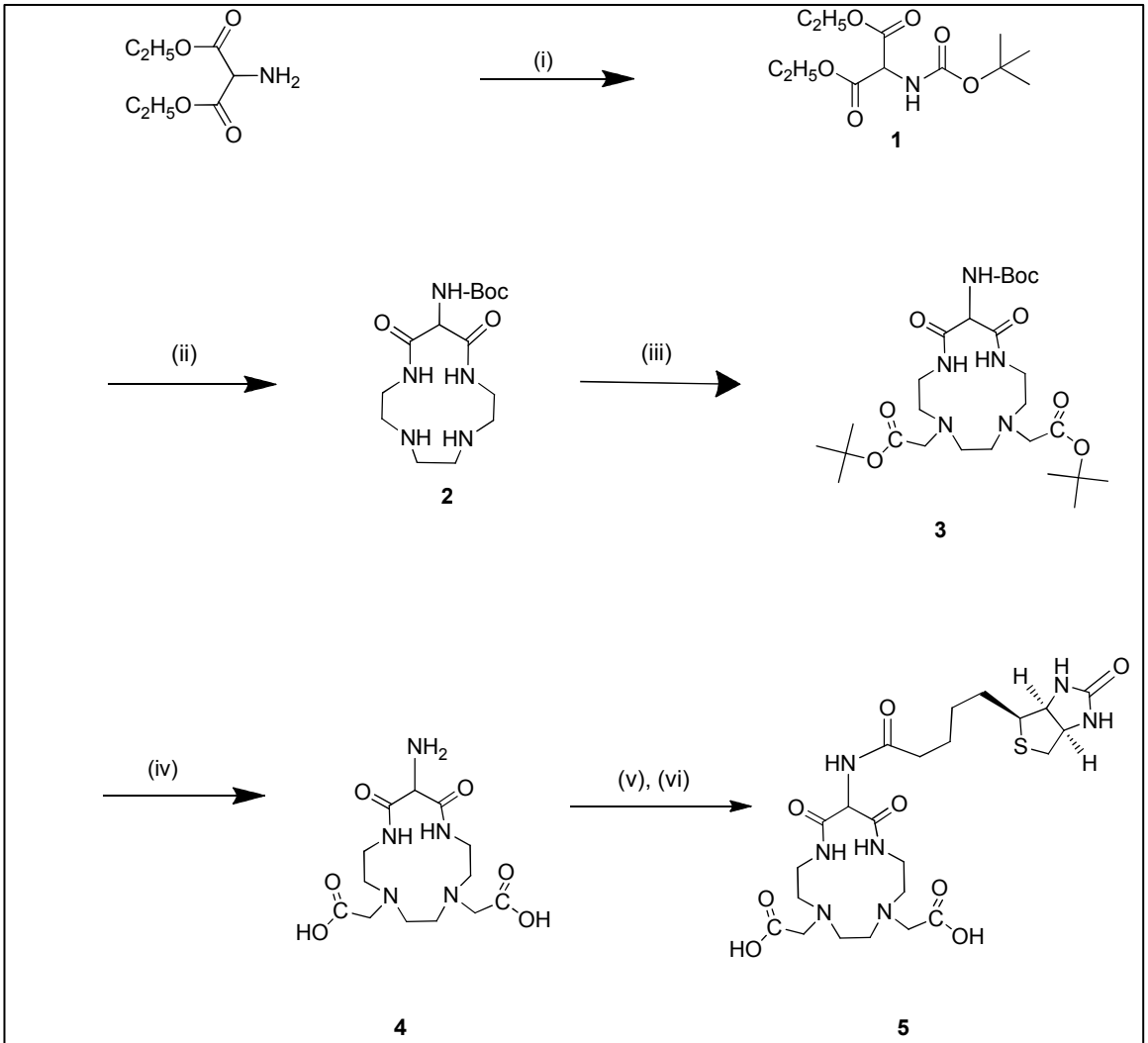
For complexation to metal ions, macrocyclic polyamines, particularly tetraazacycloalkanes have gained lot of importance due to their ligating and biological properties.[26-28] Their strong affinity and selective binding with certain metals make them very useful in the field of radioactive diagnosis. An alternative to the conventional *N*-substituted macrocyclic frameworks is the functionalization of the carbon skeleton of the macrocycle which restores the reactivity of the secondary amines making them available for reaction with other coordinating groups.[29]

In this pursuit we came up with a new chelating agent 2, 2'-(12-amino-11, 13-dioxo-1, 4, 7, 10-tetraazacyclotridecane-4, 7-diyl)diacetate (ATRIDAT) which can be complexed with various metal ions in +2 and +3 states with high stability. The uniqueness of this molecule lies in the presence of two amide bonds and two carboxylic groups which provide a framework for metal chelation along with amine group on the carbon skeleton of the macrocycle which can be readily conjugated with the biological probe. The amide bonds can be further reduced, giving a flexibility to increase the denticity of the compound. In the present study, we have introduced two carboxylates for binding to ^{68}Ga.

Biotin derivatives generally synthesized by reaction of its side arm carboxylic group with amine group of the chelator linker are an easy target for the serum biotinidase enzyme which is known to cleave biotinyl peptide bonds.[30-32] Previous approaches to synthesize biotinidase resistant compounds had limitations in the extent of avidin binding.[33-35]So before synthesizing a 2-step tumor targeting agent containing avidin-biotin system for accumulation at the tumor site, these two factors were given utmost importance. Thus with this aim, the synthesized macrocyclic system was conjugated to biotin to create a tumor targeting agent which is stable to biotinidase as well as its property to bind with avidin is not compromised. It gives high tumor to noise ratio which may also provide useful information with appreciably high sensitivity and specificity in diagnostic/prognostic value for determining tumor lesions in clinical applications.

RESULTS AND DISCUSSION

Design and Synthesis of ATRIDAT macrocycle. A new macrocyclic chelating agent(ATRIDAT) was designed which forms stable metal complexes with +2 and +3 metal ions. ATRIDAT (2, 2'-(12-amino-11, 13-dioxo-1, 4, 7, 10-tetraazacyclotridecane-4, 7-diyl)diacetic acid) was synthesized with two carboxylates and functionalized with amine group for further conjugation. Synthesis was started from boc-protection of diethylaminomalonate as shown in scheme 1. The introduction of amine unit on the macrocycle was accomplished by diethylaminomalonate. This boc-protected diethylaminomalonate (**1**) underwent aminolysis or macrocyclization in presence of very slow addition of triethylenetetramine in high dilution conditions in anhydrous methanol to give **2**. High dilution conditions were maintained to promote the



[a]Conditions: (i) Boc-anhydride, EtOH, 0 °C; (ii)Triethylene tetramine, anhydrous MeOH, Reflux; (iii) tert-butylbromoacetate, $CHCl_3/H_2O$, 0 °C; (iv) TFA,DCM, 0 °C; (v) Biotin, DCC, NHS, DMF; (vi) Biotin-NHS, **4**, TEA, DMF

Scheme 1. Synthesis of (2, 2'-(12-amino-11, 13-dioxo-1, 4, 7, 10-tetraazacyclotridecane-4, 7-diyl)diacetic acid) ATRIDAT conjugated Biotin[a]

the macrocycle formation in higher concentrations. Lower solvent conditions enhance the formation of mono-substituted and bis-substituted side products. When the macrocyclization was carried out in lower dilution conditions, the yield of product was under 10% compared to 25% in case of high dilution conditions. Completion of reaction was checked through elimination of reactants in TLC. Compound **2** was purified through alumina column ($CHCl_3$/ MeOH system). Peaks at 155 and 167 ppm in the ^{13}C spectra of compound **2** shows the presence of two different –NH groups of the carbamate and amide functionality. White colored compound (**2**) thus obtained after purification was reacted with tert-butyl bromoacetate in biphasic system ($CHCl_3/H_2O$). Compound **3** thus obtained had boc-protected amine group and two tert-butyl arms. To expose the amine group towards next set of reactions, compound **3** was subjected to 20 % TFA in DCM which gave compound **4** with a free amine group and two acetate arms. All intermediates and final macrocycle were characterized by NMR and Mass spectroscopy. (See Supporting Information).

Conjugation of ATRIDAT to Biotin. After synthesizing the bifunctional chelator**4**, the carboxyl group of (D)-biotin was activated with the help of NHS and DCC in DMF. It was then reacted in situ with **4** to give ATRIDAT-BIO as a white solid with 75% yield. The formation of product was indicated by presence of m/z peak at 572.2501 in HRMS. The macrocycle was directly conjugated with biotin for the synthesis of a two-step tumor pretargeting agent using increased uptake of avidin in tumor cells mediated by lectins. This avidin-biotin based chelator works as a model for enhancement of signal at the tumor site and rapid clearance from the blood pool organs.

Radiochemistry. The biotin conjugate was labeled with ^{68}Ga(III) cation using a solution in 0.05 M hydrochloric acid of the radiometal eluted from the $^{68}Ge/^{68}Ga$ generator system. The labeling reaction was performed at pH 4.7 at 90 °C. As seen in the species distribution curve pH > 5 leads to the formation of Gallium hydroxide species [$Ga(OH)_3$, $Ga(OH)_2L$] preventing complexation with the macrocylic moiety as assessed from species distribution curve (See supporting information). Therefore the optimum pH was found to be in between 4.5-5.0. Any lower temperature conditions significantly lowered the radiolabeling yield. The radiochemical yield was found to be 76 % and after cartridge purification, 99.9 % purity with 10 MBq/nmol of specific activity was obtained. EZ-TLC scan confirmed the formation of [^{68}Ga-ATRIDAT-BIO] with major peak showing the radiolabeled product. The stability of the radiolabeled complex was checked with ITLC for 4 h and it was observed that the radiolabeled conjugate was intact with less than 0.12% dissociation. The ^{68}Ga half-life of 68 min in combination with the short synthesis time may allow the use of the resulted radiotracer for biological studies during 3-4 h. Facile and single step radiosynthesis is a key to wider acceptance of the radiotracer at clinical level. Thus the procedure afforded translatable radiochemical synthesis for the preparation of ^{68}Ga radiopharmaceutical.

Log $P_{\text{octanol/ PBS}}$. Log *P* is the logarithm of the ratio of distribution of the labeled compound in the two solvents namely octanol and PBS. It helps in deducing the extent of hydrophilic or lipophilic character after complexation. Log $P_{\text{octanol/PBS}}$ value of -2.79 ±0.06 was obtained for ^{68}Ga ATRIDAT-BIO which shows its hydrophilic nature.

Protonation Constant. Potentiometric titrations were performed to check the stability of the ligand with metal ions. The metal-ligand bond should be stable and restrain dissociation under physiological conditions, to avoid toxicity experienced due to the free metal ion as well as no background signals. This is also an important parameter to see if the chelate undergoes transmetallation with the endogenous metals.After potentiometric titration, two pKa values were obtained for the ligand. (Table 1) The pKa1 and pKa2 values of the ligand are lower than the pKa values of DOTA and DTPA.[36] This shows that the ligand, ATRIDAT-BIO will ionise faster and liberate its protons in the solution.

Table 1. pKa values of ligand DOTA, DTPA and ATRIDAT-BIO in NMe_4Cl (0.1 M) at 298 K

pKa	DOTA	DTPA	ATRIDAT-BIO (after HP's correction)
pKa1	11.14	10.49	7.23
pKa2	9.69	8.37	4.91
pKa3	4.85	4.09	

Stability Constant. The stability constants of the ligand were evaluated with Ga(III), Tb(II), Cu(II), Zn(II) and Pb(II). The ligand forms fairly stable metal complexes with a range of metal ions with its highest selectivity for Ga(III).(Table 2)

Table 2. Stability constant of metal complexes of ATRIDAT-BIO with Ga^{3+}, Tb^{2+}, Cu^{2+}, Zn^{2+} and Pb^{2+} (298 K, μ = 0.1 M, NMe_4Cl)

	Ga-ATRIDAT-BIO	Tb-ATRIDAT-BIO	Zn-ATRIDAT-BIO	Cu-ATRIDAT-BIO	Pb-ATRIDAT Biotin
log β	20.7	19.8	16.5	15.9	16.2

Binding affinity of ATRIDAT-BIO to Avidin (HABA Assay, Competitve binding and CD experiment). 3-hydroxyaza benzoic acid (HABA) is a dye which undergoes various spectral changes in presence of avidin. Free HABA gives a peak at around 348 nm while when it is bound to Avidin i.e. in form of HABA/Av complex it gives a strong absorption peak at 500 nm. This assay is based on the difference in affinity of avidin for HABA and biotin. The binding between HABA and Avidin is relatively weak (K_d = 5.8 x10^{-6} M) as compared to biotin with Avidin (K_d= 1x10^{-15} M) wherein biotin can easily replace HABA from HABA/Av complex. This assay testifies the difference in binding of biotin derivative to Avidin compared with biotin as a standard.

It is worthwhile to note that ATRIDAT-BIO was able to inhibit the HABA/Av complex in a dose dependent fashion. At a concentration of 440 μM of biotin, 100 % HABA/ Av complex was inhibited while at the same concentration of ATRIDAT-BIO, almost 80% HABA/Av complex was inhibited (Figure1). It can be inferred from the binding affinity that the synthesized compound is comparable to biotin with regard to its binding affinity for Avidin.

To further evaluate its binding potential, displacement assay was done by immobilising Avidin and treatment with various concentration of (D)-biotin and biotin derivative with method already reported in the literature.[21] K_d value of 7.64 nM with B_{max} value of 65.23 nM was obtained through competitive binding of [^{68}Ga-ATRIDAT-BIO] to avidin. K_d value was found to be comparable to the earlier synthesized compounds.[37] CD experiment also showed that during melting experiments, ATRIDAT-BIO stabilizes Avidin even at elevated temperatures. (For K_d and CD graphs, See supporting information). These data demonstrated high binding affinity of ATRIDAT-BIO to avidin. It is imperative that the newly synthesized compound does not alter its ability to bind with Avidin.

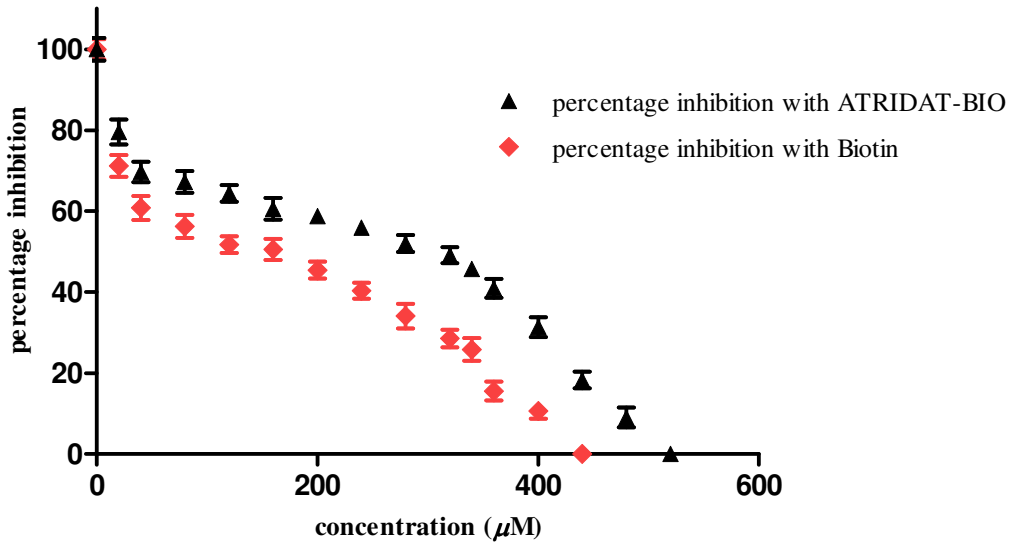


Figure 1. Inhibition of HABA/avidin complex with biotin and ATRIDAT-BIO. Varying concentrations (10-600 μM) of competitors [(D)-biotin and ATRIDAT-BIO derivative were added to the HABA/Av complex, obtained by mixing avidin and HABA to a final concentration of 100 μM and 5 μM, respectively. Experiment was done in triplicates.

Biotinidase Resistance Assay: Biotinidase, a naturally occurring hydrolytic enzyme present in humans and mouse family, is involved in assimilation of biotin from diet through hydrolysis of biocytin and its analogues. This assay was performed in freshly collected human serum of healthy volunteers with their

consent. The principle underlying this assay is that cleavage of biotinamide bond results in separation of biotin moiety (bound to Avidin) from the radioactivity. The enzymatic activity of biotinidase enzyme can be quenched with the help of p-hydroxymercuribenzoic acid (pHMB) since the active site of enzyme contains sulfhydryl group. So, a comparison of extent of Avidin binding in presence and absence of pHMB is a way to confirm that the free radioactivity is a consequence of enzymatic activity.

The radiolabeled biotin derivative did not get hydrolyse by enzyme remained with avidin at the top of Amicon 30 kDa filters while the cleaved fragment passed through the membrane filter. In the absence of inhibitor, pHMB it was found that 94.40 ± 0.956 % [^{68}Ga-ATRIDAT-BIO] remained on the filter after 4 h of experiment. It was evident that [^{68}Ga-ATRIDAT-BIO] was inert to the effect of biotinidase in absence of pHMB. In the presence of pHMB, taken as control, not much difference was found in the percentage of intact radiolabeled compound with 97.3 ± 0.908% left on the filter membrane after 4 h (Figure 2).

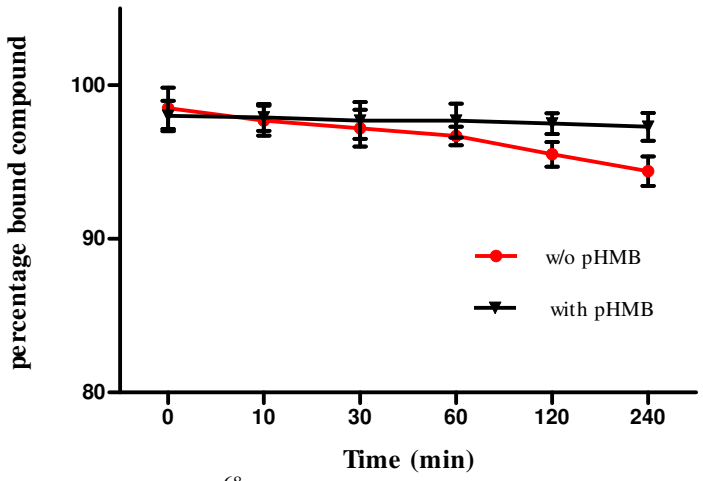


Figure 2. Biotinidase stability assay on [^{68}Ga-ATRIDAT-BIO] performed with and without the presence of p-hydroxymercuribenzoic acid incubated in fresh human serum at 37 ºC for 2 h. Graph is plotted as Percentage of bound radioligand vs. time. All values represent the percentage radioactivity retained on Amicon Ultra-30 KDa in presence of Avidin. The average of triplicate assays ± SD is presented.

Blom et al. synthesized series of DOTA-biotin analogues (with alkyl and ether linkers) showing only 20-30 % intact tracers with one analogue having -$(CH_2)_2$-O-$(CH_2)_2$-O-$(CH_2)_2$ as linker, remaining 80 % intact post 120 min.[38] Eriksson et al. synthesized ^{68}Ga-DOTA-$(PEG)_2$-biotin with 85 % stability towards enzyme cleavage till 30 min (higher time points not shown).[39] Sabatino et al. synthesized reduced

biotinamidohexylamine-DOTA conjugate which was enzyme resistant but its avidin binding ability got compromised.[34] Hainsworth et al. synthesized DOTA-lysine-biotin conjugate with substituted alpha position (to biotinamide bond) blocking the enzyme as well shown high binding affinity for SAv.[40]Another approach was used by Germeroth et al. by incorporating triazole linker between biotin and DOTA. It showed encouraging results but rely on the use of Cu catalysed click reactions which may have certain limitations with the metal binding ability of the macrocycle.[41]

The inertness of radiolabeled ATRIDAT-BIO towards biotinidase could be due to the presence of two amidic groups on methyne carbon alpha to the biotinamide bond which hindered the action of enzyme. This is also previously reported by Wilbur and group that substituents in the methylene carbon alpha to the biotinamide bond block the biotinidase enzyme.[42]

Cell viability Assay and *In vitro* reactivity. Effect of the compound on cells viability was analysed with respect to the concentration of compound as well as duration of treatment. The MTT assay was performed on HEK and A549 cells at different concentration range (pM to mM) at 24, 48 and 72 h. Insignificant toxicity was observed in either cell lines at lower concentration range. At higher concentrations, toxicity was observed. Percentage viable cells in HEK cell line at 2 mM concentration was found to be 47.5 ± 0.4 % at 24 h which increased to 74.62 ± 0.521 % at 72 (Figure3a). The IC_{50} of compound for HEK cells was found to be 2.36 mM. IC_{50} value was found to be 0.82 mM for A549 cells, percentage viability at 1 mM was found to be 72.4 ± 0.341 % at 24 h and 58.10 ± 0.217 % at 72 h (Figure 3b). Dose dependent toxicity of cells was observed in both cell lines. More than 99 % of [^{68}Ga-ATRIDAT-BIO] showed substantially higher binding to the A549 tumor cells where Avidin treatment was given whereas negative control showed less than 5 % binding.

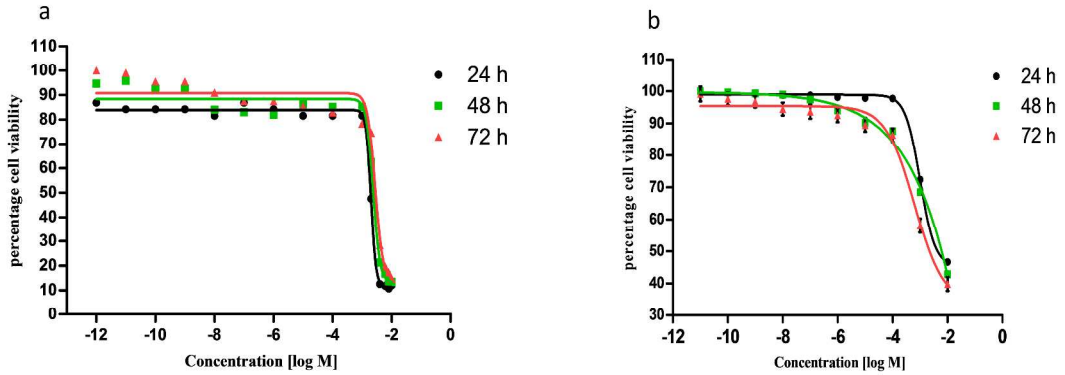


Figure 3. MTT Assay of ATRIDAT-BIO Concentration (1 pM–10 mM) and time dependent (24 h, 48 h, and 72 h) cytotoxicity of ATRIDAT-BIO in (a) HEK cell line (b) A549 cell line

Blood Kinetics and *μ*PET Imaging. Blood kinetics showed rapid clearance of the labeled compound from the blood with only 16 % remaining in blood after 30 min and only 10 % after 60 min post injection (see supporting information). For evaluating the efficacy of pretargeting for diagnostic applications, ^{68}Ga-ATRIDAT-BIO was acquired after 4 h avidin pretreatment. The tumor uptake in comparison with the rest of body increased with time indicating it as a potential two-step targeting agent. Delayed image showed clearance from the kidneys at 4 h thereby increasing the tumor to kidney ratio. *In vivo* *μ*PET A549 tumor bearing athymic mice showed high uptake of ATRIDAT-BIO at the pretargeted tumor site (Figure 4). In the absence of pretreatment of avidin at 1 h, very low uptake was observed. Comparative images are shown in supporting information. The ROI values of tumor were 35.62 KBq/mm^3 whereas in the kidney, it was 20.38 KBq/mm^3. The tumor to muscle ratio reached 26.8 and tumor to kidney ratio was observed to be 1.74 at 1 h.

Biodistribution Study. Biodistribution studies were performed at 30 min, 1 h and 2 h. Major route of excretion was found to be through kidneys with 9.56 ± 2.6 % ID/g at 30 min in treated mice whereas 3.25 ± 0.28 % ID/g uptake was found in the control mice. The activity in the kidneys decreased significantly at 1 h in both treated and control mice with 2.09 ± 0.85 % ID/g and 1.81± 0.6 % ID/g respective uptake in the organ. There was no uptake in brain with less than 0.1 % in both sets. Radiolabeled compound showed an uptake of 1.55 ± 0.07 % ID/g and 1.75 ± 0.26 % ID/g at 30 min in liver of treated and control mice which reduced to 0.29 ± 0.04 % ID/g and 0.41 ± 0.03 % ID/g at 120 min. In spite of the reported accumulation of avidin in liver in the same time frame, low uptake and early washout from the liver was seen which could be attributed due to the hydrophilic nature of the radiolabeled compound. Log *P* values play a major role in deciding the route of excretion. [^{68}Ga-ATRIDAT-BIO] with log *P* value of -2.79 follows renal route of excretion as seen with other compounds in the same log *P* range.[43] In tumor, an appreciable uptake of 2.08 ± 0.18 % ID/g at 30 min was found in the treated mice whereas in control 0.6 ± 0.07% uptake was seen in the same time frame. The activity escalated to 4.15 ± 0.35% ID/g and 1.61 ± 0.04 % ID/g in treated mice at 60 min and 120 min respectively whereas in control mice, activity

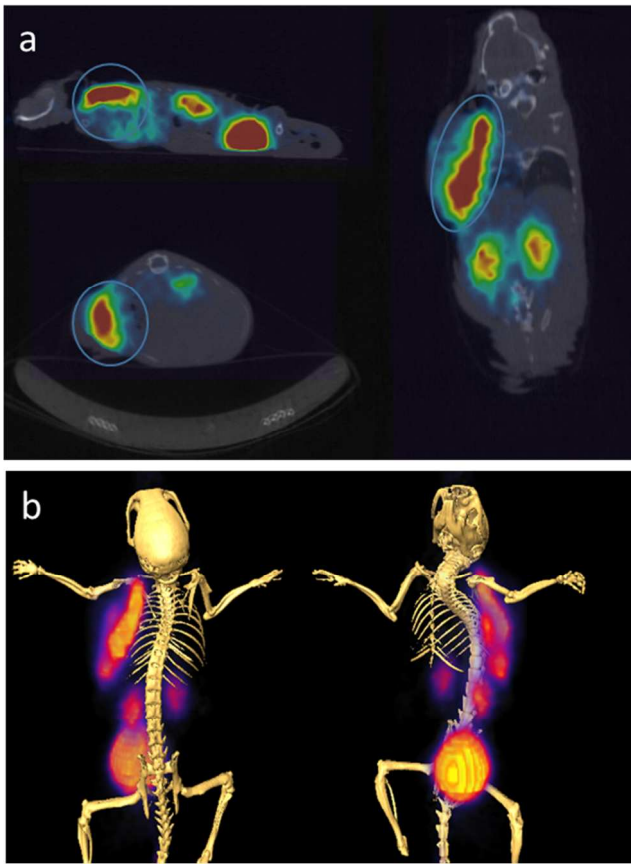


Figure 4. μPET/ CT images of ^{68}Ga-ATRIDAT-BIO (30 MBq) injected in Avidin treated nude mice xenografted with A549 tumor cells a) 2D reconstructed µPET/ CT image b) 3D reconstructed µPET/ CT @ AMIRA 4.1.1

decreased to 0.46 ± 0.06 % ID/g and 0.12 ± 0.02 % ID/g at 60 min and120 min respectively (Figure 5). Tumor to blood ratio showed ten times increased uptake background in treated mice at 60 min interval compared to the control mice (Figure 5f). The biodistribution data shows the early washout of the labeled compound from blood pool organs as well as other organs except tumor (Figure 6). Moreover, in control group in absence of Avidin, rapid clearance from the tumor was also observed (Figure 7). It shows avidin mediation is required for tracer binding as corroborated by the previous reports.[39] Presence of avidin is enhanced at the tumor site due to increased lectins expression which aids in signal amplification through radiolabeled ATRIDAT-BIO. Rapid kinetics helps to clear background levels and hence decrease noise signals during imaging.

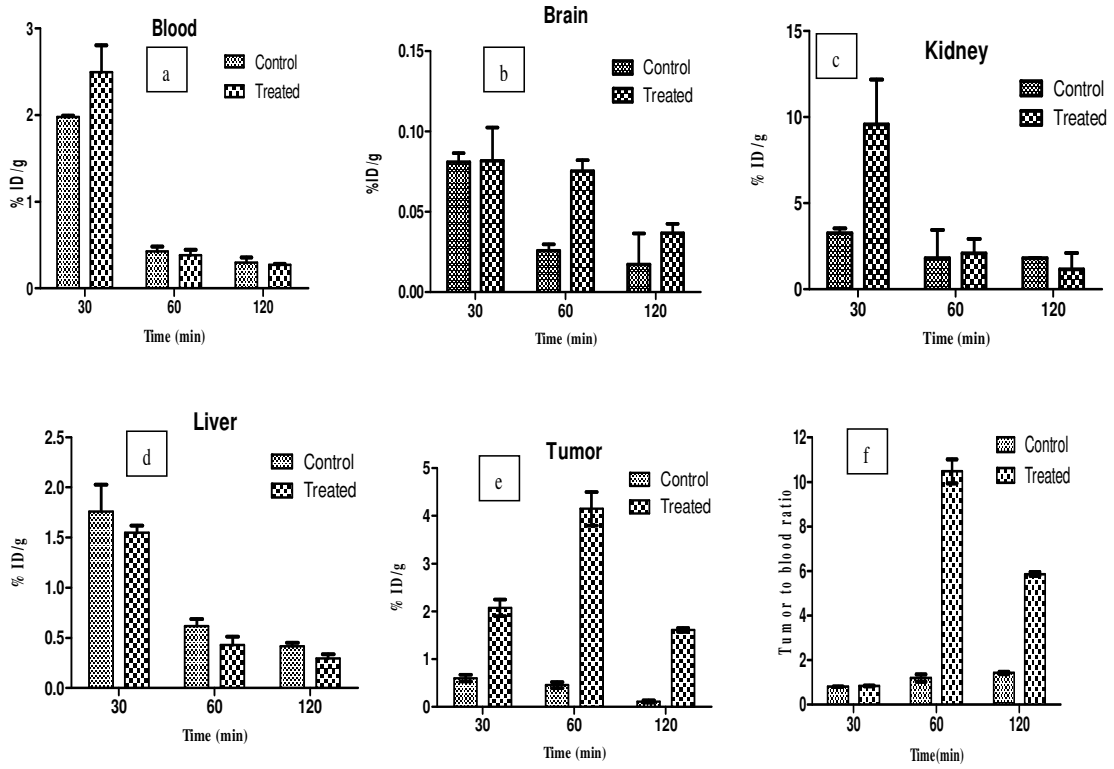


Figure 5. (a-e) Comparative biodistribution of [^{68}Ga-ATRIDAT-BIO] in Avidin injected (treated) and without avidin preinjection (control) in athymic A549 tumor grafted mice (f) Tumor to blood ratio at 30, 60 and 120 min time intervals in treated (avidin pretreated) and control (without avidin preinjection) mice

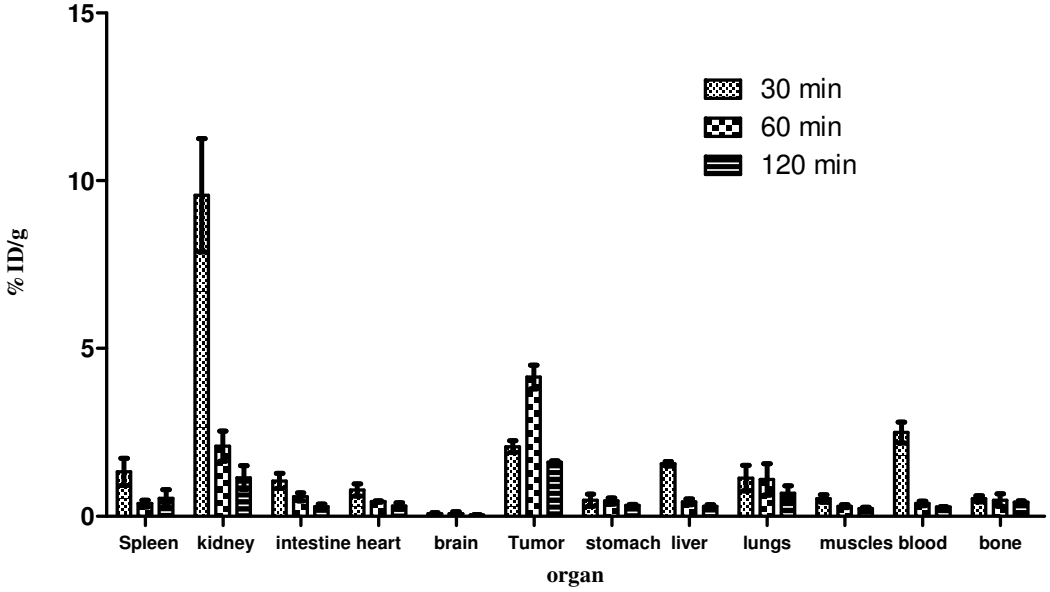


Figure 6. Biodistribution of [^{68}Ga-ATRIDAT-BIO] in athymic mice bearing A549 Tumor following avidin intravenous (i.v.) injection (treated)

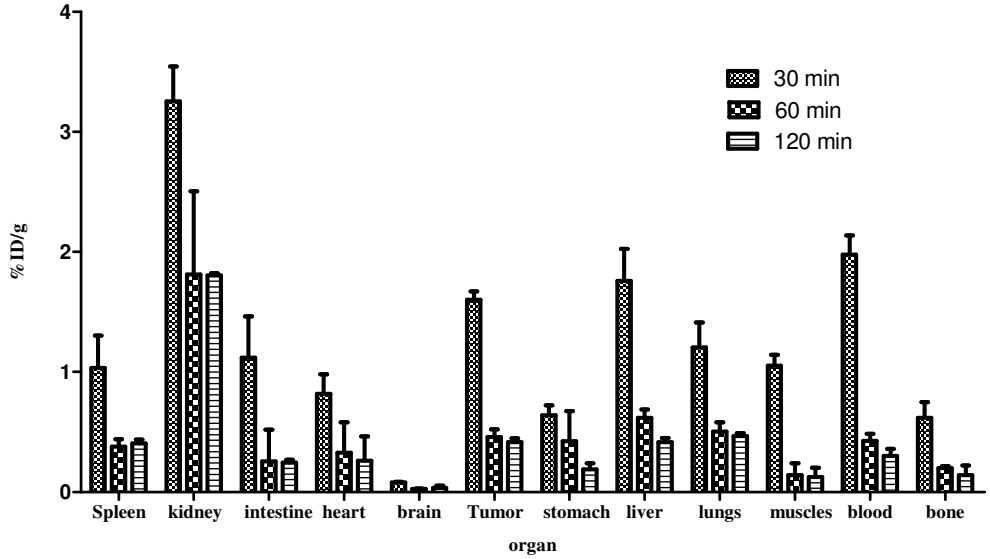


Figure 7. Biodistribution of[68Ga-ATRIDAT-BIO] inathymic mice bearing A549 tumor injected without avidin pre-treatment (control)

CONCLUSIONS

ATRIDAT-BIO as a new probe for ^{68}Ga-radiocomplexation has been designed, synthesized and characterized. ATRIDAT-BIO was found to be stable in serum containing biotinidase as well as binds with high affinity to avidin. This showed that the synthesized biotin derivative is an attractive system where neither affinity to bind with avidin is compromised nor it is susceptible to the enzymatic cleavage by biotinidase. This was our major focus in designing and synthesizing a biotin based PET imaging agent for pretargeting. Previous methodologies to synthesize different DOTA-biotin constructs were based on incorporation of a linker between biotin and the chelating agent, mainly to provide immunity from biotinidase. ATRIDAT has been made in a way that it fulfills the purpose of chelator as well as the linker. ATRIDAT-BIO gives all the desirable qualities of a pretargeting probe without any additional synthetic steps for the introduction of a linker. Moreover, ATRIDAT-BIO being C-substituted preserves the reactivity of the N-atoms of the ring resulting in the formation of kinetically stable complex with ^{68}Ga with more than 70 % radiolabeling efficiency. A high uptake [^{68}Ga-ATRIDAT-BIO] at the tumor site in athymic mice pre-injected with avidin gave diagnostically useful target to non-target ratio that reinstates

its probable use in the area of PET imaging using two-step targeting and gives a prospective lead for the development of further diagnostic/therapeutic probes.

MATERIALS AND METHODS

Chemicals. Triethylene tetramine, diethylamino2-malonate, di-tert-butyl dicarbonate, tert-butyl bromoacetate, potassium carbonate, sodium sulphate anhydrous, trifluoroacetic acid, 1-octanol, N-hydroxy succinimide, dicyclohexylcarbodimide, d-biotin, 4-hydroxymercurybenzoic acid (pHMB) and 4´-hydroxyazabenzene-2-carboxylic acid (HABA) were purchased from Sigma- Aldrich. Acetonitrile (HPLC grade), N'N'-dimethylformamide (HPLC grade), triethylamine, ethanol, methanol, Dichloromethane (HPLC grade), chloroform and Water (HPLC grade) and were obtained from E. Merck Ltd., India. Amicon Ultra, 30KDa filters were purchased from Merck. The reactions demanding anhydrous conditions or involving moisture sensitive reactants were carried out under an atmosphere of dry nitrogen using oven dried (80°C) glassware. All reaction temperatures reported indicate the temperature of the bath in contact with the reaction vessel.TLC was run on the silica gel coated aluminum sheets (Silica gel 60 F_{254}, Merck, Germany) and visualized in iodine impregnated silica and potassium permanganate developing solution. Instant thin layer chromatography was used to detect radio-complexation and radiochemical purity.

Instrumentation. 1H and 13C NMR spectra were recorded on a Bruker Avance II 400 MHz system (Ultra shield). Mass spectra (ESI-MS in positive and negative ion mode) were performed on in-house Agilent 6310 system ion trap. High resolution mass spectroscopy (HRMS LC/MS) was done using accurate mass Quadrupole time-of-flight (Q-TOF) at Delhi University.

The acquisitions were performed using a GE FLEX Triumph micro-PET/SPECT/CT scanner. This scanner consists of a micro-PET module (LabPET4) with 2'2'10 mm^3 LYSO/LGSO scintillators in an 8-pixel, quad-APD detector module arrangement. Amira 4.1.1 is used for image reconstruction and Amide software was used for the semi quantitative analysis.

Radiolabeling studies and potentiometric measurements were carried out using a γ-scintillation counter GRS230, ECIL (India) and an automatic titration system consisting of Metrohm 713 pH meter equipped with a Metrohm A.60262.100 electrode and 800 Dosino autoburet, respectively. CD spectra were measured on a JASCO 815 spectrophotometer in Delhi University, and the MTT assay absorbance was read at 570 nm using 630 nm as a reference wavelength on an ELISA reader (BioTek instruments,

Vinooski, VT, USA). PET imaging was performed using a Discovery STE 16 (GE) system. UV−vis and fluorescence studies were performed in a Biotek synergy H4 hybrid multiplate reader.

HPLC Details. Analytical HPLC was performed on Atlantis T3 C-18 reverse-phase column (5 μm, 4.6 mm x 250 mm) using Agilent1260 Infinity Analytical-Scale Purification System. The mobile phase was 0.05 % TFA in water (solvent A) and acetonitrile (solvent B) with a flow rate of 0.8 mL/min. Isocratic 80% A and 20% solvent B for 20 min. UV detection was performed at 230 nm using a UV-Vis detector. Injection volume was 10 μL. Retention time was found to be 5.7 min (See supporting Information).

Cell Culture. Monolayer cultures of normal embryonic kidney cells, HEK, in DMEM (Sigma, USA) supplemented with 10% fetal bovine serum (GIBCO), 50 U/mL penicillin, 50 μg/mL streptomycin sulfate, and 2 μg/mL nystatin were maintained at 37 °C in a humidified CO_2 incubator (5% CO_2, 95% air). Cells were routinely subcultured using 0.05% Trypsin (Sigma, USA) in 0.02% EDTA in humidified atmosphere of 95% air and 5% CO_2 at 37^{o}C twice a week.

Animal Models: Animal Models. All animal experiments were conducted in accordance with the guidelines of INMAS animal ethics committee. Blood clearance, imaging, and biodistribution were carried on athymic mice and Wistar rats. Rats and mice were housed under conditions of controlled temperature of 22 ± 2°C and normal diet.

Statistical Analysis. The competition curve of the receptor binding experiments was analyzed by nonlinear regression using algorithms in Graph Pad PRISM 5.0 (San Diego, CA). Biodistribution Data is reported as mean ± Standard Deviation (S.D.).

Synthetic Approach.

Synthesis of diethyl 2-(tert-butoxycarbonylamino)malonate (1) : Solution of diethyl 2-aminomalonate hydrochloride (1000 mg, 4.72 mmol) in ethanol (5 mL) was cooled to 0°C and kept under inert conditions. To this boc anhydride (1546.8 mg, 7.08 mmol) dissolved in ethanol (10 mL) was added dropwise for a period of 30 min. This was followed by addition of triethylamine (1969.9 μL, 14.61 mmol), temperature maintained at 0-5°C for 3 h after which it was allowed to stir at room temperature overnight. The resulting solution was filtered and filtrate evaporated under reduced pressure. The concentrate was then dissolved in water and extracted with dichloroform (3 x 30 mL). The organic layer was collected, dried with anhydrous sodium sulphate and evaporated to give **1** as a colorless viscous liquid (1227.9 mg, 4.4 mmol, 94.6 %): ^{1}H NMR (400 MHz, 25 °C, $CDCl_3$) δ (ppm): 1.3 (t, 6H, CH_3, J = 7.2 Hz), 1.4(s, 9H, CH_3), 4.3 (q, 4H, CH_2, J= 3.2 Hz), 4.9 (d, 1H, CH, J=7.6 Hz), 5.6 (br,1H,NH) ^{13}C NMR(100 MHz, 25°C, $CDCl_3$), δ(ppm): 13.9, 28.1, 57.4, 62.4, 80.5, 154.7, 166.6; ESI-MS (positive ions): m/z calcd for $C_{12}H_{21}NO_6$ $[M + Na]^+$ 298.1; found 298.1 $[M + Na]^+$.

Synthesis of tert-butyl (11, 13-dioxo-1, 4, 7, 10-tetraazacyclotridecan-12-yl)carbamate (2): Triethylenetetramine (270 μL, 1.8 mmol) was dissolved in anhydrous methanol (150 mL) and potassium carbonate (49.6 mg, 0.36 mmol) was added to it in catalytic amount. The solution was heated to 60°C and solution of **1**(500 mg, 1.8 mmol) in anhydrous methanol (150 mL) was added dropwise at a very slow rate for a period of 1 h. The resulting solution was then refluxed for 15 days. After this the solution was cooled to room temperature, filtered and evaporated under reduced pressure. The yellow crude product obtained was purified through column chromatography (neutral alumina, 5% methanol in chloroform) to yield compound **2** as a white product**.** (148 mg, 0.45 mmol, 25%): ^{1}H NMR (400 MHz, 25 °C, $CDCl_3$) δ (ppm): 1.4 (s, 9H, CH_3), 2.6-2.8 (m, 8H, CH_2), 3.3 (m, 2H, CH), 3.5 (m, 2H, CH), 4.6 (s, 1H, CH) ^{13}C NMR (100 MHz, 25°C, $CDCl_3$), δ (ppm):28.2, 39.5, 46.7, 48.0, 60.0 (), 80.5 (4°C), 155, 167.5; ESI-MS (positive ions): m/z calcd for $C_{14}H_{27}N_5O_4$ $[M + H]^+$ 330.2; found 330.1 $[M + H]^+$.HRMS (positive ions): m/z calcd. for $C_{14}H_{27}N_5O_4$ $[M + H]^+$ 330.2141; found 330.2132 $[M + H]^+$.

Synthesis of di-tert-butyl 2, 2'-(12-((tert-butoxycarbonyl)amino)-11, 13-dioxo-1, 4, 7, 10-tetraazacyclotridecane-4, 7-diyl)diacetate (3) : Solution of **2** (200 mg, 0.61 mmol) in $CHCl_3$ (5 mL) was kept at 0-5°C and solution of potassium carbonate (251.6 mg, 1.82 mmol) in water (5 mL) and tert-butyl bromoacetate (269.2 μL, 1.82 mmol) in $CHCl_3$(5mL) were added simultaneously to it. After addition, ice cold conditions were maintained for 4 h after which it was allowed to come at r.t. and stirred overnight. Then the solution was transferred to a separating funnel and extracted with $CHCl_3$ (3 x 50mL). The organic layer was collected, dried with anhydrous sodium sulphate and evaporated to give **3** as a white solid. (275.2 mg, 0.49 mmol, 81%): ^{1}H NMR (400 MHz, 25 °C, $CDCl_3$)1.4 (s, 27H, CH_3), 2.4 -2.8 (m, 8H, CH_2), 3.1 (s, 2H, CH_2), 3.3 (m, 4H, CH_3), 3.6 (s, 2H, CH_2), 4.7 (s, 1H, CH), 5.9 (br, 1H, NH) 7.5 (br, 2H, NH)^{13}C NMR (100 MHz, 25°C, $CDCl_3$), δ (ppm):28.2, 37.5, 52.2, 54.5, 58.9, 79.9 (4°C), 81.8 (4°C), 155.3, 166.2, 171.5; ESI-MS (positive ions): m/z calcd for $C_{26}H_{47}N_5O_8$ $[M + H]^+$ 558.3; found 558.1 $[M + H]^+$. HRMS (positive ions): m/z calcd. for $C_{26}H_{47}N_5O_8$ $[M + H]^+$ 558.3503; found 558.3518 $[M + H]^+$.

Synthesis of 2, 2'-(12-amino-11, 13-dioxo-1, 4, 7, 10-tetraazacyclotridecane-4, 7-diyl)diacetic acid (4): Compound 3(250 mg, 0.45mmol) was dissolved in 20% TFA in DCM (5mL) under ice cold conditions. The reaction was then stirred overnight at room temp. Then the resulting solution was evaporated under reduced pressure till complete removal of TFA to yield **4** as a white solid (148 mg, 0.43 mmol, 96%): ^{1}H NMR (400 MHz, 25 °C, D_2O) 2.8-3.1 (m, 10H, CH_2), 3.6 – 3.8 (s, 6H, CH_2), 4.7 (s, 1H, CH)^{13}C NMR (100 MHz, 25°C, D_2O), δ (ppm): 36.7, 52.7, 53.9 , 55.7, 164.2, 172.6; ESI-MS (positive ions): m/z calcd for $C_{13}H_{23}N_5O_6$ $[M + H]^+$ 346.2; found 346.4 $[M + H]^+$. HRMS (positive ions): m/z calcd. for $C_{13}H_{23}N_5O_6$ $[M + H]^+$ 346.1727; found 346.1717$[M + H]^+$.

Synthesis of 2, 2'-(11, 13-dioxo-12-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3, 4-d]imidazol-4-yl)pentanamido)-1, 4, 7, 10-tetraazacyclotridecane-4, 7-diyl)diacetic acid (5): Biotin (100 mg, 0.41 mmol) was dissolved in dry DMF (5mL) under inert conditions. To this N-hydroxy succinimide (70 mg, 0.62 mmol) and dicyclohexylcarbodimide (127 mg, 0.62 mmol) were added portionwise at room temperature and stirred overnight. Then the solution was filtered and to the filtrate, solution of **4** (120 mg, 0.35 mmol) in dry DMF (2 mL) was added dropwise under inert conditions at 40-50°C. Triethylamine (242 μL, 1.7 mmol) was added dropwise after complete addition of **4**. Temperature was maintained at 40-50°C for 5 h, after this reaction was stirred for 48 h at room temperature. The resulting solution was then filtered and concentrated under reduced pressure. The slight yellowish compound was then triturated with cold methanol to give **6** as a white solid compound. (171 mg, 0.29mmol, 86%): ^{1}H NMR (400 MHz, 25 °C, D_2O)1.1 (m, 1H, CH), 1.3 (m, 2H, CH_2),1.5 (m, 4H, CH_2), 2.2 (t, 2H, CH_2), 2.6 (m, 1H, CH), 2.8 (m, 1H, CH), 3.0-3.3(m, 10H, CH_2), 3.5-3.6(m, 6H, CH_2), 4.2 (m, 1H, CH) 4.4 (m, 1H, CH), 4.8 (s, 1H, CH)^{13}C NMR (100 MHz, 25°C, D_2O), δ (ppm):24.8, 27.6, 34.8, 35.5, 39.9, 51.8,52.9, 54.0, 55.4, 58.8,60.2,62.0,165.4, 167.9, 172.6, 176.8; ESI-MS (negative ions): m/z calcd for $C_{23}H_{37}N_7O_8S$[M - H] $^+$ 570.2; found 570.3 [M + H] $^+$.HR-MS: m/z calcd for $C_{23}H_{37}N_7O_8S$ [M + H] $^+$ 572.2429 found 572.2501 [M + H] $^+$.

Radiolabeling of ATRIDAT-BIO. ^{68}Ga ($T_{1/2}$ = 68 min, β^+ = 89%, and EC= 11%) was obtained from a ^{68}Ge/^{68}Ga generator in which 68Ge ($T_{1/2}$ = 270 d) was attached to a column of an inorganic matrix based on titanium dioxide. 5 mL of 0.1N HCl was used to elute ^{68}Ga from the column. Fractionation was done to keep the middle fraction of the elute having minimum ^{68}Ge breakthrough. To this, 1.25 mL of 12 N HCl was added to obtain a $GaCl_3$ solution of 5 N. A mini-column using Dowex-1 (50 mg, 200–400 mesh) anion exchange resin was prepared. Gallium in $[GaCl_4]^-$ form, pH <1 retains fully on the anion exchange column while the metal cations are passed through the column. ^{68}Ga (III) was eluted with water and this solution was used for radiolabeling purpose. Sodium acetate was added to maintain 4.5 to 5 pH of the solution and the solution slightly warmed. Then, 20 μmol of compound, ATRIDAT-BIO was added and the resulting solution was heated at 90 °C for 10 min. After this, the reaction mixture was cooled to room temperature and applied to a C-18 SPE-column which was preconditioned with ethanol, water and air dried. Free ^{68}Ga is not trapped on the cartridge and is washed with water. The ^{68}Ga labeled compound is then eluted with ethanol, then ITLC-SG was performed to determine the labeling efficiency in solvent system 1: 4 of 15% Ammonium acetate: MeOH as mobile phase. The ITLC strip was scanned using Omniscan EZ-TLC scanner.

Octanol–Water Partition Coefficient (log $P_{octanol/water}$). To determinethe log P (pH = 7.4) value, a solution of the ^{68}Ga-labeled-ATRIDAT-BIO (20 μL, 50 μM) was added to a solution of 1-octanol and

phosphate buffered saline (PBS) (presaturated with each other), containing 500 μL of each, in a 2 mL mili centrifuge tube. The obtained solutions were vortexed and centrifuged at 3000 rpm for 10 min. A volume of 100 μL was removed from the two phases separately and the radioactivity was measured in a γ-counter. The experiments was performed in triplicate. The method was published previously.[44]

Potentiometric Titrations. Potentiometric measurements were carried out with an automatic titration system consisting of Metrohm 713 pH meter equipped with Metrohem A.60262.100 electrode, 800 Dosino autoburet. The electrode of the pH meter was pre caliberated using standard buffer solutions. Protonation constants were determined by titrating 1 mM of ligand with 10 mM tetrabutyl-ammoniumhydroxide (TBAOH) in the pH range of 2–12. Titrations were performed at 25^{o}C withtetrabutylammoniumhydroxide (TBAOH), I = 0.1M to maintaining constant ionic strength.The stability constants with different metals were deduced using similar conditions to the protonation constants (1 mM metal salt and 1 mM ligand solution).The metal solutions were prepared by dissolving their corresponding salts in deionized water. A solution was prepared containing ligand and metal in a 1: 1 ratio of ligand/metal and complexes were titrated with TBAOH (0.1 M). The experiments were performed in triplicate. The protonation and stability constants of the ligand were calculated from the titration data using the program Tiamo 2.0.

CD spectroscopic Measurements. Each spectrum (195-260 nm) was obtained from averaging three scans and subtracting the contribution from the buffer solution. Other experimental settings were as follows: 20 nm x min scan speed, 2.0 nm bandwidth, 0.2 nm resolution, 50 mdeg sensitivity and 4 s response. Melting curves of wild type avidin, with or without 4 equivalents of D-biotin and its ATRIDAT-BIO derivative, were recorded by following the decrease of dichroic signal at 225 nm, keeping the temperature in the range 25-95 °C. Instrument settings were as follows: bandwidth 1 nm, response 0.5 s, data pitch 1 °C, temperature slope 10 °C/min. At every temperature increment of 5 °C, a far-UV CD spectrum was collected in the experimental conditions reported above.

HABA Assay. Preliminary experiments were performed by varying the concentration of HABA (from 2-100 μM) and avidin (10, 50and 100 μM) to calculate their optimal concentrations in the competitive binding studies. In the final experiments, carried out in 1 mL sample cuvettes, various concentrations of competitors [(D)-biotin and ATRIDAT-BIO derivatives] were added to the HABA/Av complex, obtained by mixing avidin and HABA to a final concentration of 100μM and 5μM, respectively. Changes in absorbance were measured at 500 nm. All experiments were in triplicate, and the % inhibition was calculated as {[(absorbance with competitor) - (absorbance without HABA)/(Av complex)]/[(absorbance without competitor) - (absorbance without HABA)/(Av complex)]}x100.

Biotinidase Resistance Assay. Previously published experiment has been slightly modifiedto conductthe following experiment.[28] Radiolabeled ^{68}GaATRIDAT-BIO was diluted to obtain approximately 8000 cpm per 10 μL in a well counter. To a 0.5 mL aliquot of diluted serum (1:10 with 20 mM phosphate buffer, pH 6.8) was added 10 μL of the tc-18 cartridge purified radiolabeled biotin derivative in water in 6 sets depending on the time points. Each tube was slightly vortexed and placed in a 37 °C heating block/ water bath. After the desired time (e.g.,0,15,30,1h, 2 h,4 h), the vials were removed, and 40 μL of 2 mM 4-(hydroxymercuri) benzoic acid (pHMB), sodium salt in H_2O, was added. To this solution was added an excess of avidin (40 μL; 0.24 mg), and incubated at 37 °C for another 30 min. The solution was split into two 200 μL aliquots, and the samples were transferred to two Amicon 30 kDa filters and centrifuged at 3000*g* for 10 min. The substrate remaining on the filter was washed with 4 x 100 μL of phosphate buffer, concentrating by centrifugation each time. The top and bottom (containing filtrate and washes) of the Amicon 30 were counted in a well counter. The ratio of bound counts (top) to the total counts (top and bottom) was calculated to determine the percent of the biotin derivative that had not been cleaved. As a control, this procedure was repeated as above except that the 4(hydroxymercuri)-benzoic acid was added prior to the radiolabeled biotin derivative to inhibit biotinidase activity.

MTT Assay. To test the cytotoxic effect of ATRIDAT-BIO exposure on cells, MTT assays were conducted.Exponentially growing cells were plated in a 96 well plate at a cell density of 4000 cells/well 24 h before treatment. Cells were treated with the varying concentrations of the drug at 24 h, 48 h and 72 h time intervals and MTT assays were done. At the end of treatment, both the treated cells and negative control were incubated with MTT at a final concentration of 0.05 mg/mL for 2 h at 37 ^{o}C and followed by removal of the medium. Triplicate wells from each treatment were lysed and the formazan crystals were dissolved using 150 µL of DMSO. Optical density of 150 μL extracts at 570 nm was measured (reference filter: 630 nm). Surviving fraction for 0.001-10 mM concentration range was plotted against concentration for ATRIDAT-BIO.

***In Vitro* reactivity**. ^{68}Ga-ATRIDAT-BIO binding was assessed on immobilized human cancer cells A549 derived from tumors with nude mice xenograft. The cell suspension was homogenized and treated with Avidin (50 μg) with increasing no of cancer cells suspended in 100μL of PBS in micro centrifuge tubes for 1 h at 4 °C. ^{68}Ga-ATRIDAT-BIO was added and incubated for another 30 min. The supernatant was aspirated from the pellet and the counts in pellet were recorded. The bound radioactivity in %age was determined by an auto well gamma system. Negative control was taken as cells without the treatment of avidin.

Animal Model. All animal experiments were performed in accordance with guidelines of Institute's Animal Ethics Committee (Reg. No. 8/GO/a/99/CPCSEA). Athymic nude mice were used for PET

imaging and ex vivo biodistribution studies. Animals were housed under conditions of controlled temperature of 22 ± 2 °C and normal diet. Mice were inoculated subcutaneously with 0.1 mL of cell suspension of A549 (3×10^6 cells) in the right front flank under sterilized condition. When the tumor volume reached ~400 mm^3 (after 1-2 weeks of inoculation), the A549 tumor bearing mice were used for biodistribution and imaging studies.

Blood Kinetics Study. The blood kinetics was carried out in female Wistar rat weighing approximately 264 g to study the clearance of the radiolabeled complex from the blood circulation. 1 MBq ^{68}Ga radiolabeled compound was injected intravenously through the tail vein of the rat and blood samples (0.2 mL) were taken from the occular vein at different time interval starting from 10 min until 240 min and counts were monitored on a gamma counter. Whole body blood was considered to an amount equal to 7% of the body weight of the rat. Data were expressed as percent administered dose at different time intervals.

Biodistribution Studies. All nude mice (n=30, weighing 18-20 g) were inoculated with 3x 10^6 human tumor lung cancer cells (A549 cells). The mice were divided into two groups, the first group was treated with Avidin (1µg/ µL) and 100 µL was injected intravenously to each mice. In the experiment, after 4 h of Avidin treated mice were divided into three groups for different time intervals with 4-6 mice in each group. The biodistribution was examined after the injection of Avidin for pretargeting at 30 min, 1 h and 2 h. The biodistribution (3 groups, n= 15) of ^{68}Ga-ATRIDAT-BIO was also performed in mice without avidin pretreatment. Intravenous injection of ^{68}Ga-ATRIDAT-BIO (in saline, pH 7) was injected to each mouse (100 μL, 1 MBq/ mouse, 0.1-0.2 nmol) through its tail vein. Treated and control mice were dissected at 30 min, 1h and 4h post injection. The organs and tumors were harvested, made free of adhering tissue, rinsed with chilled saline, blotted to remove excess liquid, weighed, and radioactivity measured in each organ in a gamma counter calibrated for ^{68}Ga energy. Uptake of the radiotracer in each tissue was evaluated and expressed as percentage injected dose per gram of the tissue (% ID/g). Animal protocols have been approved by Institutional Animal Ethics Committee and in accordance with the guidelines of INMAS animal ethics committee (CPCSEA Regn No.8/GO/a/99).

***µ*PET Acquisition.** Animals were anesthetized by inhalation of 2 % isoflurane in 2 L/min oxygen in the prone position. Animals were injected with 5 MBq ^{68}Ga-ATRIDAT-BIOimmediately prior to their μPET scan on the camera bed at the start of an acquisition.μPET static images were acquired for 10 min at 1, 2 and 4 h after injection. The resulting PET was reconstructed using 50 iterations of the Maximum Likelihood Expectation Maximization algorithm. ROI semi-quantitative analysis was done using amide. ROIs were drawn over tumors and the average signal levels in the ROI were measured. Data is expressed as KBq/mm^3 of the tissue.

ASSOCIATED CONTENT

Supporting Information available: [**pp. 1S-11 S** NMR and mass spectra (ESI and HRMS) of all synthesized compound, radio-tlc, species distribution curve, HPLC profile, K_D graph and experimental method, CD, Blood Clearance graph, μPET Images control and Avidin treated mice. This material is available free of charge via the internet at http:// pubs.acs.org

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Notes

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ABBREVIATIONS

DOTA, 2,2',2'',2'''-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetic acid; DTPA, Diethylenetriaminepentaacetic acid ; ITLC, instant thin layer chromatography ; ROI, Region of Interest; MTT,3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide ; PET positron emission tomography

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