Original Article

Preparation and preclinical evaluation of ^{68}Ga-DOTA-amlodipine for L-type calcium channel imaging

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ABSTRACT

Aim: In order to develop a possible tracer for L-type calcium channel imaging, we here report the development of a Ga-68 amlodipine derivative for possible PET imaging. **Materials and Methods:** Amlodipine DOTA conjugate was synthesized, characterized and went through calcium channel blockade, toxicity, apoptosis/necrosis tests. [^{68}Ga] DOTA AMLO was prepared at optimized conditions followed by stability tests, partition coefficient determination and biodistribution studies using tissue counting and co incidence imaging up to 2 h. **Results:** [^{68}Ga] DOTA AMLO was prepared at pH 4–5 in 7–10 min at 95°C in high radiochemical purity (>99%, radio thin layer chromatography; specific activity: 1.9–2.1 GBq/mmol) and was stable up to 4 h with a log *P* of −0.94. Calcium channel rich tissues including myocardium, and tissues with smooth muscle cells such as colon, intestine, and lungs demonstrated significant uptake. Co incidence images supported the biodistribution data up to 2 h. **Conclusions:** The complex can be a candidate for further positron emission tomography imaging for L type calcium channels.

Keywords: Amlodipine, apoptosis/necrosis assay, biodistribution, calcium channel blockade activity, Ga-68

INTRODUCTION

The existence of voltage-dependent calcium channels was demonstrated more than 20 years ago in voltage clamp experiments on multicellular cardiac preparation. Among them, L-type calcium channels intercede depolarization-dependent entry of calcium ions through the plasma membrane and control excitation-contraction coupling in muscular tissues.[1] Pharmaceuticals referred to as "calcium channel antagonists" such as nitrendipine, verapamil, and *d-cis*-diltiazem inhibit L-type calcium channels and have been extensively used for the treatment of human cardiovascular disorders.[2]

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Furthermore, several pathologies, such as congestive heart failure, myocardial hypertrophy, irritable bowel syndrome, and colon cancer are related to change in L-type calcium channels abundance and function.[3-7] The noninvasive imaging and quantification of L-type calcium channels in living tissues required the synthesis of the radiolabeled compound with high affinity to bind to calcium channel. Several [^{11}C] radiolabeled 1,4-dihydropyridine antagonist have been suggested as promising agents for molecular imaging such as, S11568 that appeared to be suitable for imaging myocardial L-type Ca^{2+} channels and was suggested to provide new insights in the study of myocardial hypertrophy and congestive heart failure.[3] A lipophilic nonionized derivative of S12968, ^{11}C-labelled N-Boc-S12968, was synthesized to facilitate its penetration into the brain. However, its uptake was not sufficient to be used for imaging brain calcium channels.[8]

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In one study, a single direct Tc-99m complex of amlodipine has been reported without structure determination, radiochromatographic data and biological studies and was used in drug delivery studies using nanoemulsions.[9] Recently, Ga-67 and In-111 DTPA-amlodipine complexes have been reported and were evaluated as possible L-type calcium channel tracers.[10,11] However, DOTA-conjugates usually offer more radiolabeling opportunities with other diagnostic radionuclides such as Y-86, In-111, Cu-64, and Cu-62, whereas DTPA is mainly applied to In-111 complexes and sometimes lanthanides.

In recent years, several pharmaceuticals have been conjugated with different chelating agents for labeling with either gamma-emitting or positron-emitting radionuclides as diagnostic agents in molecular imaging. Covalent attachment of DOTA (as a strong chelating agent), with biologically active ligands, leads to developing therapeutic/diagnostic (theranostic) agents while radiolabeled with appropriate radionuclides.

Due to suitable characteristics of Ga-68 for nuclear medicine applications (half-life: 68.3 min, positron decay, gamma: 0.511 MeV [176%], 0.8 MeV [0.4%], 1.078 MeV [3.5%]. 1.24 MeV [0.14%], 1.87 MeV [0.15%]), development of a possible Ga-68 tracer seemed interesting.

As a part of our efforts to synthesize targeting radiopharmaceuticals for L-type calcium channels, we here report the synthesis, characterization, and radiochemistry of ([^{68}Ga]-3-O-ethyl 5-O-methyl 2-[2-N-(carbonyl-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) aminoethoxymethyl]-4- (2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate ([^{68}Ga]-DOTA-AMLO) [Figure 1]. Biological evaluation and imaging study were performed in normal rats.

PROCEDURE

The ^{68}Ge/^{68}Ga generator (30 mCi/day activity) was obtained from Pars Isotope Co., Karaj, Iran. Chemicals were purchased from the Aldrich Chemical Co., (Germany).

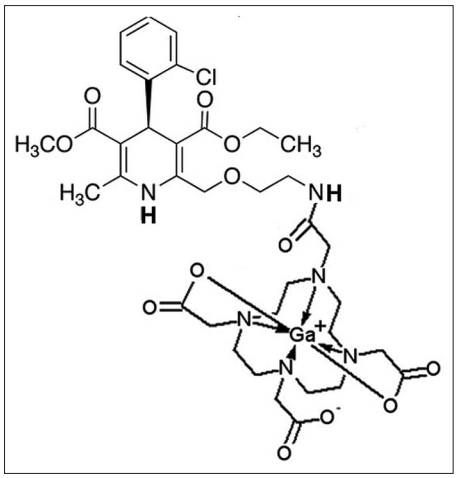

Figure 1: Possible chemical structure of ^{68}Ga-DOTA-amlodipine

Thin layer chromatography (TLC) for DOTA-AMLO quality control was performed on polymer-backed silica gel, F 1500/LS 254, 20 cm × 20 cm, TLC Ready Foil, Schleicher and Schuell®, (Germany). Normal saline and sodium acetate used for radiolabeling were of high purity and had been filtered through 0.22 μm Cativex filters. Instant TLC was performed by counting Whatman No. 2 papers using a TLC scanner, Bioscan AR2000, Bioscan Europe Ltd., (Paris, France). Biodistribution data were acquired by counting normal saline washed tissues after weighting on a Canberra™ high purity germanium (HPGe) detector (model GC1020-7500SL). Radionuclidic purity was checked with the same detector. For activity measurement of the samples, a CRC Capintec Radiometer (NJ, USA) was used. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edition. Images were taken in coincidence mode of a Dual-Head SPECT system (SMV, France, Sopha DST-XL).

Synthesis of DOTA-conjugated amlodipine

DOTA-AMLO was synthesized by conjugation of the DOTA-N-hydroxy succinimide (NHS) ligand to an amlodipine free base according to similar DOTA-NHS conjugation reactions with amine groups.[12] The amlodipine free base was prepared based on McDaid and Deasy.[13] Briefly, amlodipine besylate (500 mg) was dissolved in 50 mL of water at 50–55°C. 1 mL of a NaOH solution (1 M) was added. The mixture put at 3–5°C and stirred at this temperature for 1 h. The solid was filtered off and washed with water and dried in the vacuum oven. The infrared (IR) spectrum of white solid was in accordance with the literature, and its melting point was 140–143°C. The DOTA-NHS conjugation to amlodipine free base was performed based on reported similar methods with slight modifications. To a magnetically stirred solution of DOTA-NHS (10 mg, 0.013 mmol) in dimethylformamide (DMF) (2 mL) was added triethylamine (as a proton scavenger) (0.01 mL, 0.075 mmol) under nitrogen. After 5 min, a solution of amlodipine free base (10.6 mg, 0.026 mmol) in DMF (1 mL) was added to the reaction mixture, and it was stirred for 24 h at room temperature. After 24 h, the progress of the reaction was confirmed by TLC. The reaction mixture was evaporated to dryness under vacuum, and the residue was dispersed in ethanol and filtered to remove amlodipine residue. The filtered mass was washed several times with ether, methanol, and ethanol. Yield: 40% (5 mg). Mp: 150–155°C FT-IR (KBr): 3280, 1731, 1646 cm^{-1}. Liquid chromatography-mass spectrometry- (LC-MS): 794 $[M + H]^+$, 817 $[M + Na]^+$. ^{1}H NMR (300.13 MHz, D_2O): δ_H = 1.04 (t, ^3J = 7 Hz, 3 H, OCH_2CH_3), 2.18 (s, 3 H, CH_3), 2.96–3.77 (CH_2 of DOTA, NH-CH_2-CH_2-O, OCH_3, 31 H), 3.92–3.97 (m, 2 H, OCH_2CH_3), 4.51 (dd, ^2J = 14.5 Hz, 2 H, $CH_2OCH_2CH_2NH$), 5.20 (s, 1 H, CH of 1,4-dihydropyridine), 7.04 (t, ^3J = 6.9 Hz, 1 H, 1 CH of Ar), 7.14 (t, ^3J = 7.2 Hz, 1 H, 1 CH of Ar), 7.22 (d, ^3J = 7.7 Hz, 1 H, CH of Ar), 7.33 (t, ^3J = 7.6 Hz, 1 H, 1 CH of Ar).

Cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Cell viability test performed according to the common techniques

presented by Mosmann.[14] HT-29 cells were seeded in clear 96-well plates at a density of 4000 cells per well for 24 h to reach in suitable confluent monolayer. After 24 h, the cells were subsequently incubated with 1 mg/mL, 500 μg/mL, 50 μg/mL, 5 μg/mL, 500 ng/mL and 50 ng/mL of DOTA-AMLO and the cells were kept in the incubator for 24 h. After incubation for time intervals at 37°C, the cells were incubated for 4 h with a 0.5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (10 μL), which was replaced with equal parts of DMSO to dissolve formazan crystals produced by mitochondrial dehydrogenase activity of viable cells and absorbance was measured at 570 nm. Data shown are based on five different experiments, and the results were expressed as a mean ± standard deviation.

Apoptosis/necrosis assay of DOTA-amlodipine conjugate

The assay was performed according to Annexin-V-FLUOS Staining Kit procedure from Roche Co.[15] To evaluate the presence of apoptotic cells, the HT-29 cell line (1×10^5 cells/well) were incubated with amlodipine and amlodipine-DOTA in a 24-well microplate for 24 h. Data were confirmed by an Annexin V-propidium iodide (PI) staining kit following the manufacturer's recommendations to discriminate the apoptotic cells from necrotic cells. The subsets of cells that were Annexin V-positive, PI-negative and Annexin V-positive (apoptotic) and PI-positive (necrotic and/or cells in advanced apoptosis) were determined. Each concentration was tested in duplicate.

Calcium channel blockade activity evaluation

The *in vitro* calcium channel antagonist activity (IC50) of DOTA-amlodipine conjugate was determined as the molar concentration of the test compounds required to produce 50% inhibition of the high K^+ concentration of guinea pig ileal longitudinal smooth muscle (GPILSM). Briefly, male guinea pigs, weighing 300–400 g, were killed by a blow on the head. The nonterminal part of the ileum was removed and cut into segments of 10–15 mm length. Each ileal segment was suspended in an organ bath and connected to an isometric transducer (K30, Hugo Sachs Electronic, Germany). Contractions of the ileal segments were recorded, using an amplifier (Plugsys, Hugo Sachs Elektronik, Germany) and a recorder (Graphtec, model WR3320). From concentration-response curves, the pIC50 value of the compound was calculated and compared with that of amlodipine as a reference compound.[16]

Preparation of ^{68}Ga-DOTA-amlodipine

A prototype 30 mCi ^{68}Ge/^{68}Ga generator developed at Pars Isotope Co. Iran, was used in this study. The eluted activity was analyzed carefully for radiolabeling according to a recent report.[17] In a typical run, the acidic solution (0.61 mL) of [^{68}Ga] $GaCl_3$ (197.95 MBq, 5.35 mCi) was transferred to a 5 mL-borosilicate vial and heated to dryness using a flow of nitrogen gas at 50–60°C. After adjusting the pH by the addition of 400 μL of 1 M HEPES buffer (pH. 4), 20 μL of DOTA-AMLO dissolved in ethanol (4 mg/mL ≈ 5.04 μmoles) was added to the gallium-containing vial and vortexed at 90°C. The active solution was checked for radiochemical purity by radio-TLC (RTLC) method at 5, 10, 20 and 30 min after labeling. The final solution with acceptable radiochemical purity was sterile filtered using a 0.22 μm membrane, and the pH was adjusted to 5.5–7. A 5-μL sample of the final solution was spotted on silica gel paper, and a 10% ammonium acetate:methanol (1:1) mixture was used as the mobile phase and ascending of the desired compound on the paper was compared with RTLC of [^{68}Ga] $GaCl_3$ and of [^{68}Ga]-DOTA.

Determination of the partition coefficient

The partition coefficient (logP) of ^{68}Ga-DOTA-AMLO was calculated followed by the determination of *P* (P = the ratio of activities of the organic and aqueous phases). A mixture of 1 mL of 1-octanol and 1 mL of isotonic acetate buffered saline (pH = 7) containing approximately 3.7 MBq of the radiolabeled gallium complex at 37°C was vortexed 1 min. Following centrifugation at >1200 g for 5 min, the octanol and aqueous phases were sampled and counted in an automatic well-type counter.

Biodistribution in wild-type rats

The distribution of the radiolabeled complex, as well as free Ga-68 cation among tissues, was determined in rats. The total amount of radioactivity injected into each rat was measured by counting the 1-mL syringe before and after injection in a dose calibrator with fixed geometry. The animals were sacrificed by CO_2 asphyxiation at 15, 30, 45, 60, 90, and 120 min after injection ($n = 5$ for each time interval), the tissues (blood, heart, lung, brain, intestine, skin, stomach, kidneys, liver, muscle, and bone) and stool, were weighed and rinsed with normal saline and their activities were determined with a HPGe detector equipped with a sample holder device as percent of injected dose per gram of tissues.

Imaging of [^{68}Ga]-DOTA-amlodipine in normal rats

Volumes of the final [^{68}Ga]-DOTA-AMLO solution (0.1 mL; 4.4 MBq activity) were injected into the dorsal tail vein of healthy rats. The total amount of radioactive material injected into each rat was measured by counting the 1-mL syringe before and after injection in a dose calibrator with fixed geometry. The animals were relaxed by halothane and fixed in a suitable probe. Images were taken 30, 60, and 120 min after administration of the radiopharmaceutical in coincidence mode. The useful field of view was 540 mm × 400 mm. The spatial resolution in the coincidence mode was 10 mm full width at half maximum at the center of the field of view, and sensitivity was 20 Kcps/μCi/cc.

RESULTS AND DISCUSSION

Production of the precursor

Various reaction conditions were tried for amlodipine-DOTA conjugation. The conjugation was performed in DMF, DMSO as well as aqueous mixtures as the best reaction solvent showed

to be DMF since the reaction workup seemed feasible due to the precipitation of the final compound followed by solid washing using various solvents.

For better yields the amlodipine free base (1) was added to the DOTA-NHS. (2) Nitrogen atmosphere was also mandatory for the conjugation reaction since the presence of water can reduce the conjugation yields. The reaction scheme is shown in Figure 2.

In the IR spectrum, the formation of peaks at 1646 and 1731 demonstrate the amide group formation among the DOTA moiety and amlodipine. The peak at 3280 shows the existence of COOH groups in the molecule. In LC-MS data, the molecular weight of the parent molecules was not observed; however, due to the presence of Na in the sample and carboxylates in the molecule the M + Na species is reported. In ^{1}HNMR studies, the existence of all protons from amlodipine molecule as well as NH-CH_2-CH_2-O moiety from the DOTA part are observed with appropriate ratios area under the curve for protons.

Pharmacology

The *in vitro* calcium channel antagonist activities (IC50) of DOTA-AMLO conjugate and amlodipine reference drug were determined as the molar concentration of the test compounds required to produce 50% inhibition of the high K^+ concentration of GPILSM and are presented in Table 1. These results indicate that DOTA-AMLO exhibits less potent calcium channel antagonist activity than reference drug amlodipine (IC50 = 0.5 ± 0.2 × 10^{-8} M), yet it is active at the micromolar range. These data showed that production of radiolabeled DOTA-AMLO can result in a possible imaging tracer for L-type calcium channels.

Table 1: IC_{50} values for DOTA-AMLO synthesized in this study

Compound	MeanIC_{50}±se	MeanIC_{30}±se	*n*
DOTA-AMLO	3.11±1.68 (×10^{-5})	1.07±0.65 (×10^{-6})	4
Amlodipine	2.13±1.85 (×10^{-6})	3.68±2.46 (×10^{-7})	4

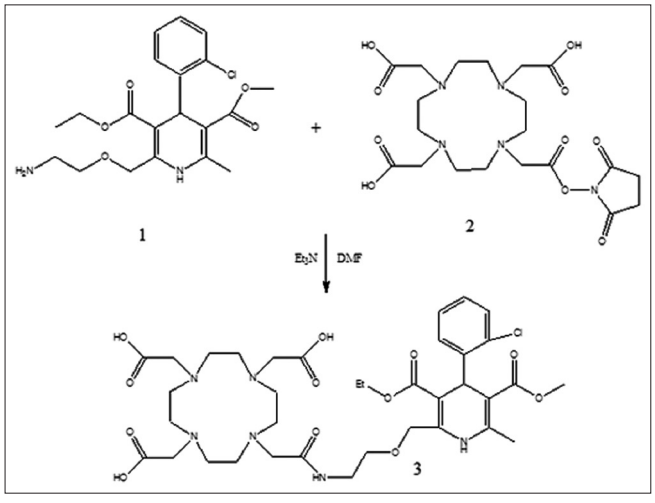

Figure 2: Reaction steps for preparation of DOTA-amlodipine conjugate

Cell viability assay

The toxicity results of the three species including amlodipine itself as the standard are demonstrated in Figure 3. At almost 1 mg/mL the degree of toxicity is very close for amlodipine and DTPA-AMLO, used in previous studies[10,11] about 45% cell survival is recorded. However in the case of DOTA-AMLO, at least 35% cell viability is observed showing more toxic effects compared to the other species. The maximum used the amount of tracer in this study is a 4.4 MBq for each animal (equivalent to 2.2 μg DOTA-AMLO).

Apoptosis/necrosis assay of DOTA-amlodipine conjugate

Necrosis and apoptosis are two ways that a cell can die. Necrosis occurs when a cell is damaged by an external force. On the other hand, apoptosis is the process of programmed cell death that occurs in multicellular organisms and under normal physiological conditions. In these experiments, no statistically significant difference was observed in the effect of DOTA-AMLO on apoptotic cells compared with amlodipine. The same confirming evidence was observed using an MTT cellular assay. Figure 4 demonstrates the flow cytometry results in HT-29 cells for DOTA-AMLO conjugate as well as amlodipine for better comparison. The apoptosis data does not comply with the toxicity tests and just was performed to discover the mechanism of cell death.

Radiolabeling

Many considerations must be taken into account for radiolabeling, some influence the radiochemical purity of the DOTA-complex and some would change the quality of the formulation for future applications.[18] The elution pattern of the generator are important since the narrower the activity/volume peak, a sample with higher specific activity is obtained for radiolabeling, however, using 1 M or higher concentration of HCl solutions although usually yield a better radioactivity peak/elution, however the formation of other gallium species not entering the radiolabeling occurred at these concentrations. By the choice of a suitable concentration (0.6–0.7 M), most of the daily generator eluted activity was obtained and also the peak of activity ranges for 0.5–1.5 mL of the first elution.

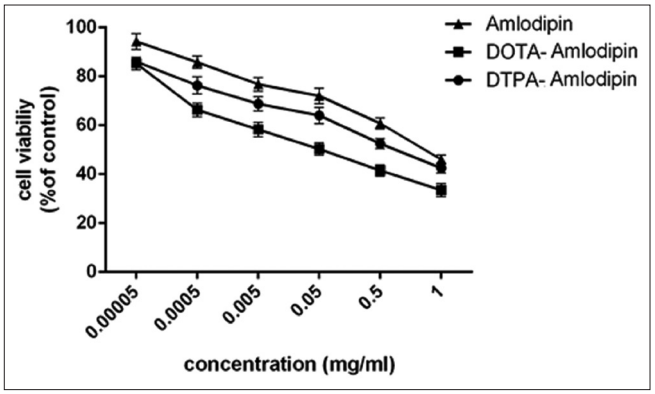

Figure 3: Toxicity effects of DOTA-amlodipine, DTPA-amlodipine[10] and amlodipine on HT29 cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

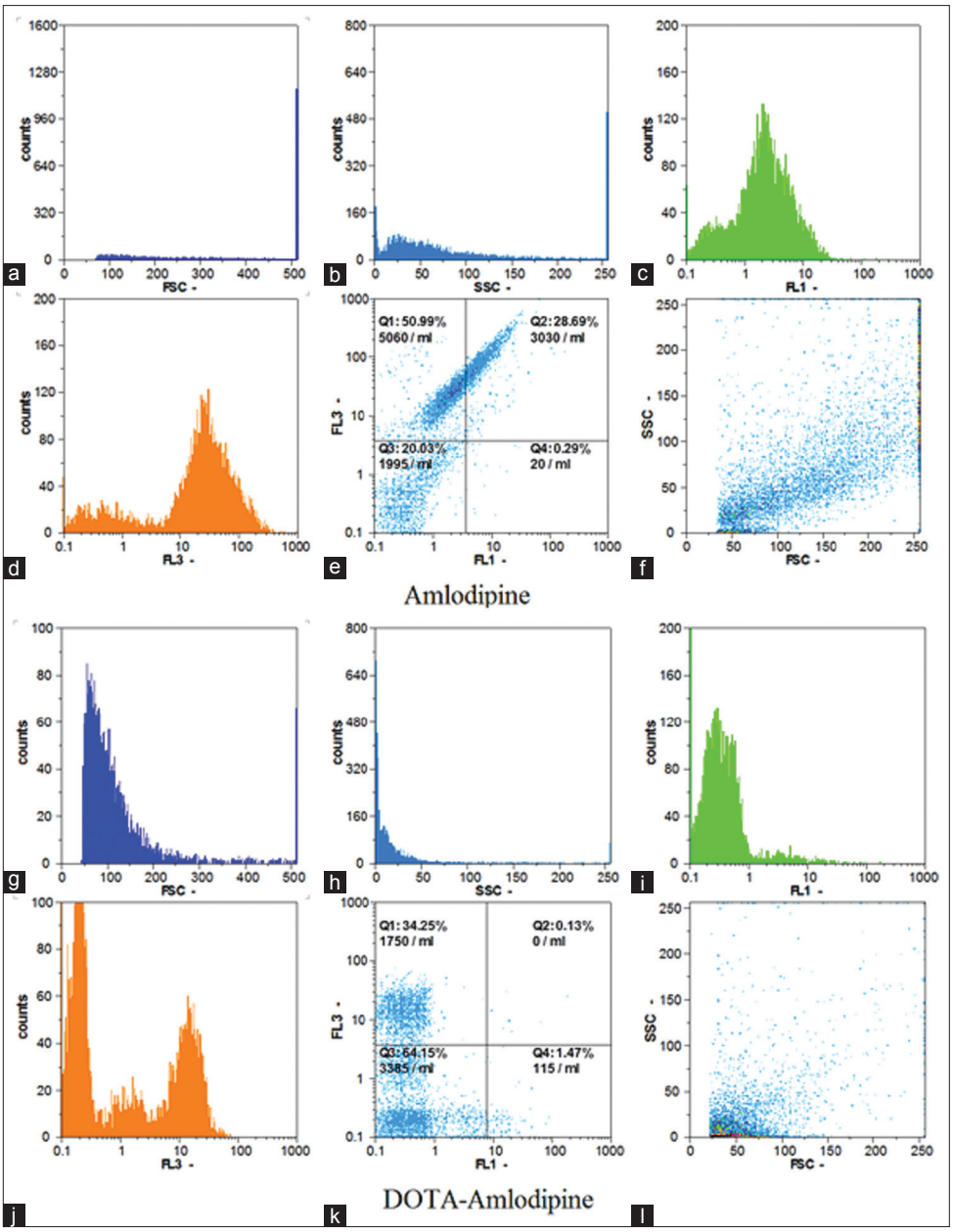

Figure 4: Percentage of cellular apoptosis/necrosis obtained by flow cytometry in HT-29 cell lines. The HT-29 cell lines were treated with: 1 mg, 500 μg, 50 μg, 5 μg, 500 ng, 50 ng amlodipine (a-f) and DOTA-amlodipine (g-l)

DOTA-AMLO is an efficient biological compound, effective at 10^{-7}. The existence of unlabeled compound in formulations may block the calcium channels competing the application of radiotracer at the molecular level. A series of reactions performed to reach the minimal ligand amount needed leading to the application of 4–5 μM of ligand used for a typical labeling. Figure 5 demonstrates the chromatograms of [^{68}Ga]-DOTA-AMLO complex as well as the free Ga-68 at the optimized conditions.

The presence of metal impurities affects complexation reaction due to the presence of impurities at ppm levels compared to the existence of Ga-68 cation at ppb or even less amounts. Usually, the radiolabeling yield decreases with the competition of other cations especially ferric cation in the radiolabeling reaction, leading not only to lower specific activity but also competition at the receptor level. This is why the amount of cation release from a generator should be checked so often not only at the beginning of the generator application in any center but also routinely. Long-term radiolysis reaction occurring in the matrix of the generator is a major concern. Furthermore, the interaction of Sn, Zn with DOTA moiety is reported leading to the unwanted reaction with the conjugated peptide. The routine daily or twice a week elution of the generator even if not used for the radiolabeling, would help the removal of unwanted cold cation presence in the radiolabeling eluent.

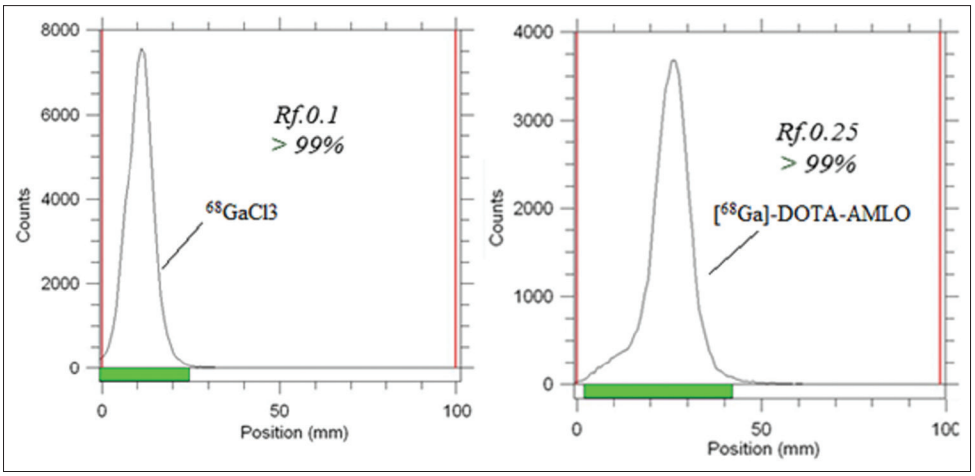

Figure 5: Radio-thin-layer-chromatography of [^{68}Ga]-$GaCl_3$ (left) and [^{68}Ga]-DOTA-amlodipine (right) on silicagel paper developed in 10% ammonium acetate: methanol (1:1) mixture as the mobile phase at the optimized conditions

Partition coefficient

As expected from the chemical behaviors, the lipophilicity of [^{68}Ga]-DOTA-AMLO was low due to the presence of the three carboxylic groups at the DOTA moiety. The measured octanol/water partition coefficient, P, for the complex was found to depend on the pH of the solution. At the pH 7, the logP for the complex was logP: −0.940.

Stability

The chemical stability of [^{68}Ga]-DOTA-AMLO was high enough to perform further studies. Incubation of the complex in freshly prepared human serum for 2 h at 37°C showed no loss of ^{68}Ga from the complex. The radiochemical purity of complex remained at starting radiochemical purity for 2 h under physiologic conditions.

Biodistribution

Biodistribution studies were performed for ^{68}Ga-DOTA-AMLO and free Ga^{3+}. As reported previously, ^{68}Ga is excreted majorly from the gastrointestinal tract with high blood content due to transferrin binding at early time intervals, as well as significant colon, bone and stomach activity content is observed, the kidney is not a significant accumulation site (not shown). The radiolabeled complex is rapidly washed out from the blood circulation into receptor-rich organs with very low uptake in the liver.

Specific uptakes were observed in the myocardium as a striatal tissue containing calcium channels. Other smooth muscle-containing tissues such as veins, intestines, colon, lung, and stomach expressing higher levels of L-type calcium channels at the cell surface showed higher uptakes. As shown in Figure 6, in early time intervals (0–30 min) postinjection, the best specific uptake is observed in the stomach with >2% in 15 min. Lung shows the longest stable uptake up to 1.8–1.9% at 15–45 min postinjection. Intestine reached the maximum uptake of 2.5% in 30 min postinjection however then diminished.

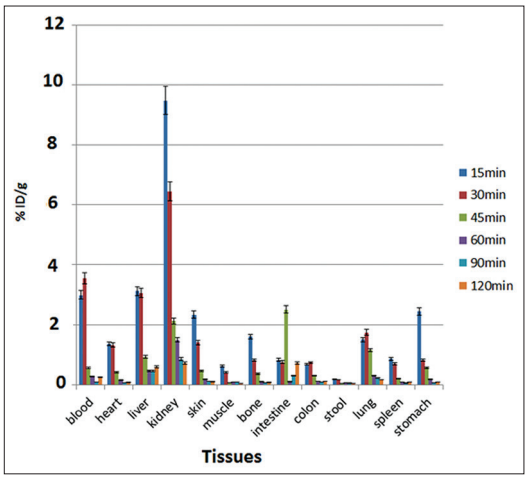

Figure 6: Biodistribution of [^{68}Ga]-DOTA-amlodipine (37 MBq, 100 μCi) in wild-type rats 15–120 min after intravenous injection via tail vein (%ID/g: Percentage of injected dose per gram of tissue calculated based on the area under curve of 511 keV peak in gamma spectrum) (*n* = 5)

Imaging studies

^{68}Ga-DOTA-AMLO imaging in the wild type rats showed a highly comparable results with tissue dissection studies, 0.5 h after injection high intestine and kidney uptake was observed. 1 h postinjection, kidney and bladder uptake was observed; however, in 120 min most of the activity was at a baseline level and major activity detected in kidneys only as observe in a recent report [Figure 7].[10]

CONCLUSIONS

DOTA-AMLO was synthetized and characterized in this work. The developed conjugate demonstrated significant calcium channel blockade activity and also very low apoptosis/necrosis effect while showing toxicity. [^{68}Ga]-DOTA-AMLO was prepared in 7–10 min at 95°C in >99%, radiochemical purity and specific activity of 1.9–2.1 GBq/mmol with water soluble properties and acceptable stability. The tracer excreted through kidneys

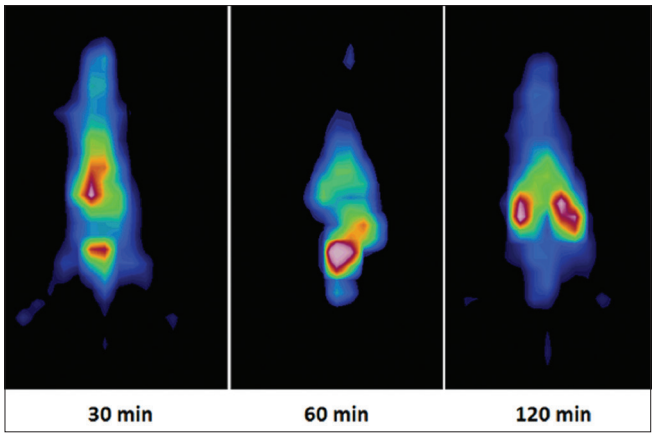

Figure 7: Static images of [^{68}Ga]-DOTA-amlodipine in normal rats 30, 60 and 120 min after injection. The injected doses were 4.4 MBq for each rat

and liver as expected for dihydropyridines. Excluding excretory organs, calcium channel rich tissues including myocardium and smooth muscle cells including colon, intestine, and lungs demonstrated significant uptake. Co-incidence images supported the biodistribution data up to 2 h. Initial biodistribution results showed significant uptake in calcium channel rich organs using. Further biological studies in animal models are needed to demonstrate the further efficacy of the tracer for imaging of L-type calcium channels.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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