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Original article

Synthesis and biological evaluation of newly designed phosphonate based bone-seeking agent

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ABSTRACT

A cyclic tetraaza based bifunctional triphosphonate ligand 10-(2-aminoethyl)-1,4,7,10-tetraaza cyclododecane-1,4,7-tris(methylenephosphonic acid) (DO3MP-EA) was synthesized as bone-seeking theranostic agent. The compound was characterized by spectroscopic techniques and labelled with ^{99m}Tc with more than 97% purity. Blood clearance of 99mTc labelled compound a quick wash out from the circulation. The compound was excreted mainly via kidneys and accumulation of ^{99m}Tc-DO3MP-EA in bone was 9.53 ± 1.06% of injected dose per gram of bone at 1 h. The preliminary CADD analysis confirms the efficacy of DO3MP-EA (G Score −7.005) as better binding agent for osteocalcin (pdb 1Q8H) rather than other known clinical agents. Subsequently stability constant of chelate with Ga(III) was found to be 18.6 which confirms its efficacy as ^{68}Ga labelled PET radiopharmaceutical for bone.

1. Introduction

Efficient and early tumour and metastases imaging is important in the determination of malignancy and its proper treatment which can be diagnosed and treated using nuclear medicine [1]. However, the introduction of radionuclide metal ions directly in vivo is limited by toxicity. Therefore, pharmaceutical are needed for carrying the metal ion to the body. Key factors for any molecule to act as pharmaceuticals are rate of complex formation, complex stability and tissue localization in vivo [2]. For better management of bone metastases treatment radiopharmaceutical should have good affinity to bone and higher affinity to the matrix associated with metastatic growth [3]. In vivo stability of radiopharmaceutical is the crucial issue for its clinical application. It should exhibit long-term stability at pH ~4.5 as microenvironment in the kidney is acidic. In this aspect 1,4,7,10-tetracarboxymethyl-1,4,7,10-tetraazacyclododecane (DOTA) like ligands are kinetically more inert as compared to open-chain ligands [3].

Earlier researchers have tried many techniques to diagnose the bone lesion at different surface levels. One of the renowned techniques from late 70s was ^{99m}Tc based 'Single Photon Emission Tomography' (SPECT) in which phosphonate was the main group for targeting bone as its presence facilitates the specificity of agent. Wide range of non-specific bone imaging agents has been used in clinical applications. However, there is requirement of functionalized and targeted ligand that evaluates the physiological parameters and localized anomalies.

Though bisphosphonate containing ligands show much higher affinity for bone as compared to monophosphonate containing ligands, monophosphonate containing ligands are equally important due to the presence of different absorption sites at the hydroxyapatite (HAP) surface [4].

The sensitivity and resolution of diagnostic agents were increased with the invention of 'Positron Emission Tomography' (PET) imaging, specifically by use of ^{18}F and ^{68}Ga isotopes [5–7]. ^{18}F ($t_{1/2}$ = 110 min, $E_{max,\beta+}$ = 634 keV) is the most simple approach as it inherently possesses a high affinity to bone. But the need of cyclotron for the production of ^{18}F limits its use [8]. Generator produced radionuclide for PET application e.g. ^{68}Ga, can fill this gap. In addition to this, chelate for carrying ^{68}Ga radionuclide can be designed for therapeutic and targeted applications [9–11].

The coordination position of Ga in the periodic table is such that it can bind strongly with macrocyclic/cyclic systems similar to lanthanides [12,13]. Ga based DOTA systems have stability in vivo conditions, a prerequisite for designing of radiopharmaceuticals [14–19].

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To overcome the limitations of the currently used bone diagnostic agents, the design of ligand and coordination properties of isotope are important to determine how the novel agent is going to bind with hydroxyapatite/calcium binding protein for better detection.

In the present work we have taken three phosphonate groups along with tetraazamacrocyclic system which can tightly bind with required isotopes $^{99m}Tc/^{68}Ga$. The fourth arm of the macrocylic system has been appended with amine group having ability to bind qaat target site in bone imaging [20,21]. This has been confirmed by the docking studies of DO3MP-EA with protein (1Q8H). The amine arm of tetraazamacrocyclic compound can be used to load cytotoxic drugs for therapy compared to its analogue DOTMP which is used for bone palliation with Sm/Ho/Eu/Ce [22–26].

For designing the new bone imaging agent we have selected osteocalcin, which is most abundant non-collagenous protein in bone. This acts as a biological marker for the clinical assessment of bone disease. Osteocalcin also affects bone mineralization because it has high affinity to the mineral component of bone hydroxyapatite [27]. We have put osteocalcin structure as target protein for various macrocyclic/acyclic systems to check the binding efficacy.

2. Materials and methods

2.1. Chemicals

1,4,7,10-Tetraazacyclododecane (purity 99%), phosphorous acid (purity 98%), phosphorous trichloride (purity 99%), bromo ethyl amine (purity 99%), *tert*-butyl bromoacetate (purity 98%), trifluoroacetic acid (TFA) (purity 99%), common solvents and reagents were purchased from Sigma–Aldrich Co. ^{99m}Tc was procured from Regional Centre for Radiopharmaceuticals (Northern Region), Board of Radiation and Isotope Technology (BRIT), Department of Atomic Energy, India. The metal salt gallium trichloride hexahydrate ($GaCl_3.6H_2O$) was purchased from Aldrich. Stock solution of the metal cation was prepared by dissolving $GaCl_3 \cdot 6H_2O$ in water, and the concentration was determined by complexometric titration with standardized Na_2H_2EDTA xylenol orange as indicator. The stock solution of hydrochloric acid (0.03 mol dm^{-3}) was prepared from 35% aqueous solution ("puriss" Fluka, Switzerland). Deionized water was used in all experiments. Column chromatography was carried out using silica MN60 (60–120 mesh), thin layer chromatography (TLC) on aluminium plates coated with silica gel 60 F_{254} (Merck).

2.2. Instrumentation

^1H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz respectively using Bruker Avance II 400 MHz system (Switzerland). Mass spectroscopy was done on 6310 system of Agilent (Germany) using ESI positive mode. γ-scintigraphic studies were done on Hawkeye Camera (Wisconsin) and a γ-scintillation counter was used for counting radioactivity.

2.3. Animal models

Animal protocols were approved by the Institutional Animal Ethics Committee (INM/DASQA/IAEC/09/015). New Zealand rabbits (2–3 kg) and BALB/c mice (22–28 g) were used for blood clearance as well as imaging and biodistribution studies.

2.4. Docking studies

To design improved bone imaging agent, drug design studies were performed through Schrödinger Masetro LLC 9.1 software in which GLIDE is used for ligand–protein binding studies. The proteins which were taken from protein data bank RCSB-PDB having resolution around 2.0 Å. All the ligands docked were prepared in .mol format. The molecules taken were known good bone seekers from literature (Alendronate, Pamidronate, Zolendronate, and DOTMP) (Fig. 1). All these molecules were docked with known calcium/metal binding proteins available in pdb crystallographic forms (Osteocalcin (1Q8H), Osteopontin (3CXD), Osteonectin (1SRA), and BMP2).

2.5. Ligand synthesis and characterization

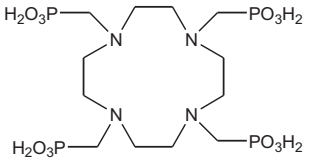
2.5.1. Synthesis of N-boc-2-aminoethyl bromide (**1**)

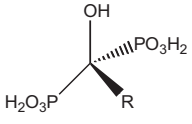
2-Bromoethylaminehydrobromide (2.5 g, 12.2 mmol) and triethyl amine (2.552 ml, 18.3 mmol) were dissolved in ethanol (30 ml) and stirred at 0 °C. Di-t-butyl-dicarbonate (2.66 g, 12.2 mmol) was dissolved in ethanol (30 ml) and added drop wise to the reaction mixture at 0 °C. Reaction mixture was further stirred for 1 h at 0 °C and 4 h at room temperature. The solvent was removed in vacuo and residue was dissolved in chloroform. The organic layer was washed with water (3 × 50 ml). The organic layer was dried over $MgSO_4$ and evaporated to get the compound. Yield: 84%

^1H NMR ($CDCl_3$, 400 MHz), δ(ppm): 1.47 (s, 9H); 3.46–3.49 (t, 2H, $J = 5.2$ Hz); 3.53–3.57 (t, 2H, $J = 5.2$ Hz); 4.97 (br, NH). ^{13}C NMR ($CDCl_3$, 100 MHz), δ(ppm): 28.35; 32.85; 42.37; 79.85; 155.58.

2.5.2. Synthesis of 1,4,7-tris(carbotertbutoxymethyl)-1,4,7,10-tetraazacyclododecane (^{t}Bu-DO3A) (**2**)

1,4,7,10-Tetraazacyclododecane (1 g, 5.81 mmol) was dissolved in dry acetonitrile (20 ml) under nitrogen at 0 °C. $NaHCO_3$ (1.464 g, 17.44 mmol) was added with stirring. *tert*-Butyl bromoacetate (3.4 g, 17.44 mmol) in 10 ml acetonitrile was added slowly from a dropping funnel in 1 h. The reaction was stirred at 0 °C for another 3 h and at room temperature for 45 h. The progress of the reaction was monitored by TLC using dichloromethane (DCM):methanol (9:1) (R_f 0.67). On completion of reaction, reaction mixture was filtered and filtrate was evaporated to dryness. Crude compound





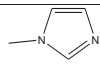
Compound	R
Alendronate	$(CH_2)_3NH_2$
Paridronate (APD)	$(CH_2)_2NH_2$
Zolendronate	

Fig. 1. Structure of DOTMP and known bone imaging agents.