SYNTHESIS OF RADIOPHARMACEUTICAL LIGANDS AND THEIR APPLICATIONS

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Abstract:
The tremendous role of radiopharmaceuticals in disease diagnosis and therapy, and the associated benefits can be expanded by in-house development of radiopharmaceuticals in a cost-effective way. Ligands play a pivotal and integral role in the specificity of radiopharmaceuticals, and an indigenous development of ligands are the need of the hour. Bio-Organic Division (BOD), BARC is committed to develop in-house synthetic protocols for important radiopharmaceutical ligands, which imparts a huge impact on societal benefits via providing cheaper and affordable healthcare.

1. Introduction:

Radiopharmaceutical is a targeted module, which relies upon the delivery of a radionuclide to the diseased site, using a delivery vehicle that accumulates or binds specifically to the target, resulting in a highly specific diagnostic or therapeutic modality (**Fig. 1**).[1] The major difference between a conventional therapy and radiopharmaceutical therapy is, that, unlike conventional drugs, radiopharmaceuticals do not show any pharmacological effects and dose-response relationships since they are usually used in trace quantities.

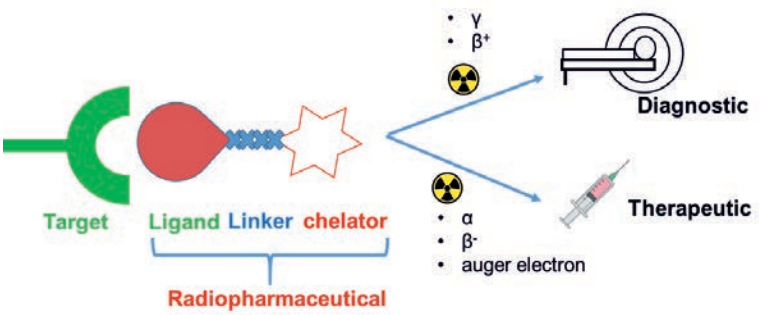


Fig 1. Design and use of a radiopharmaceutical.

Radiopharmaceuticals containing radionuclides which emit pure gamma radiation (e.g. ^{99m}Tc, ^{67}Ga, ^{123}I, ^{111}In etc.) or pure positron emission (e.g. ^{18}F, ^{68}Ga, ^{89}Zr etc.), are used for diagnostic radiopharmaceutical, where no therapeutic effects are intended (**Fig. 2**). The diagnosis is done using either single photon emission computed tomography (SPECT) or positron emission tomography (PET) depending on the nature of the radionuclide used.[2] Among these, ^{99m}Tc is regarded as the main workhorse of SPECT imaging.[3] Even today, more than 70% of all the diagnostic SPECT scans relies on this isotope. Among PET radiopharmaceuticals, [^{18}F]FDG is considered as a gold standard, and is being used as a first-hand diagnostic agent for last 30 years.[4] However, the non-specificity of [^{18}F]FDG towards cancer detection had led researchers to find alternatives. Introduction and availability of ^{68}Ga via a ^{68}Ge/^{68}Ga generator has opened many alternative paths for designing new PET tracers.[5]

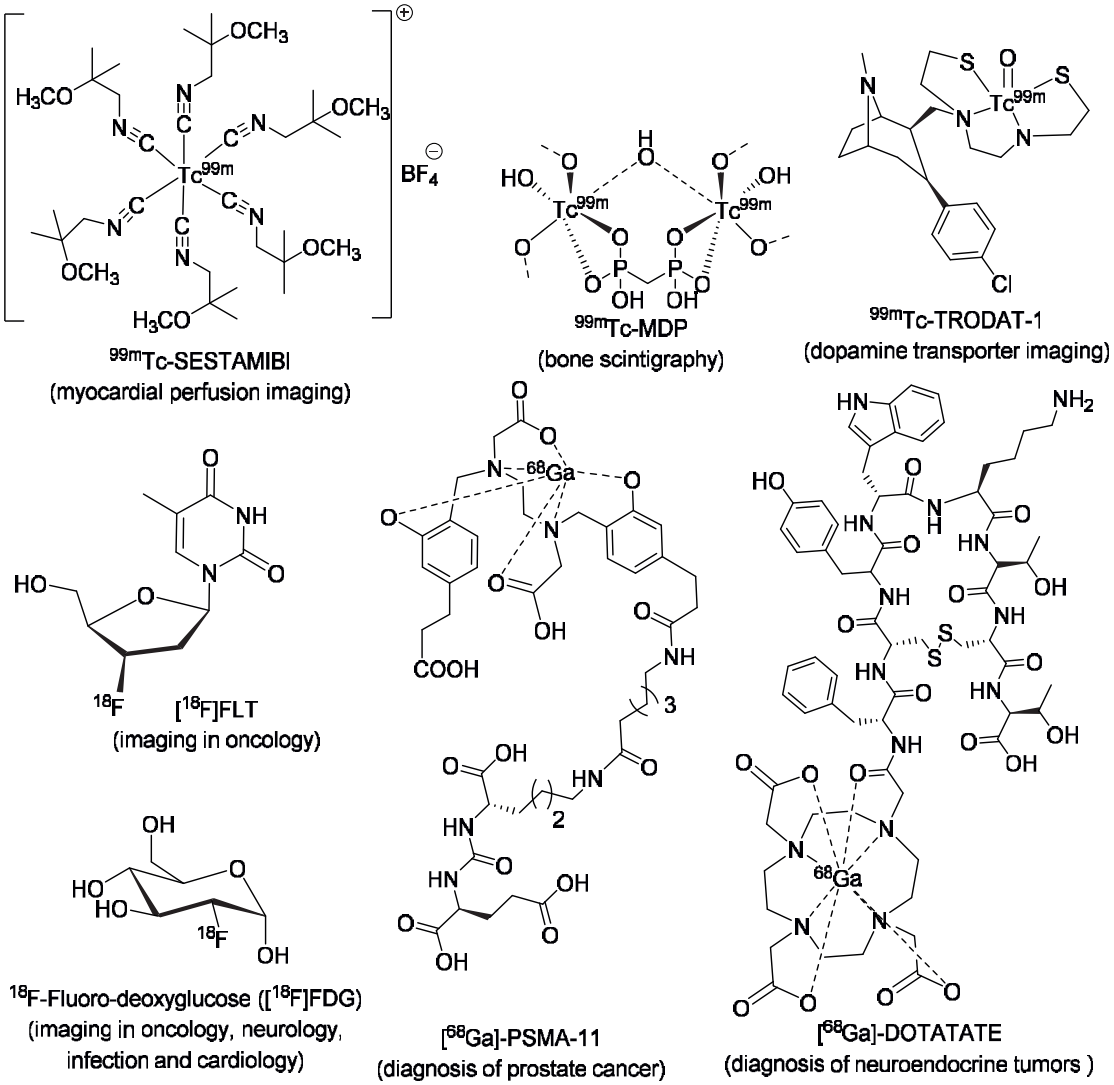


Fig 2. Selected examples of diagnostic SPECT and PET radiopharmaceuticals.

On the other hand, radionuclides which emit beta- particles (e.g. ^{131}I, ^{153}Sm, ^{177}Lu etc.) or alpha- particles (e.g. ^{223}Ra, ^{225}Ac, ^{227}Th etc.) or Auger electrons, are used for therapeutic radiopharmaceuticals, where a therapeutic effect is intended (**Fig. 3**).[6] Therapeutic radiopharmaceuticals are advantageous compared to the conventional external radiotherapy in cases of secondary or metastatic cancers, which requires treatment of disseminated diseased sites. In addition, recently, the concept of theranostics, where an integration of therapeutics with diagnostics in a single module allows image-guided therapy, have gained attention. Theranostics for somatostatin receptor positive neuroendocrine tumors with [$^{68}Ga/^{177}Lu$]DOTATATE and norepinephrine transporter (NET) positive neuroblastoma tumors with [$^{123/131}I$]meta-iodobenzylguanidine ([$^{123/131}I$]mIBG) have been developed and are in clinical use.[7]

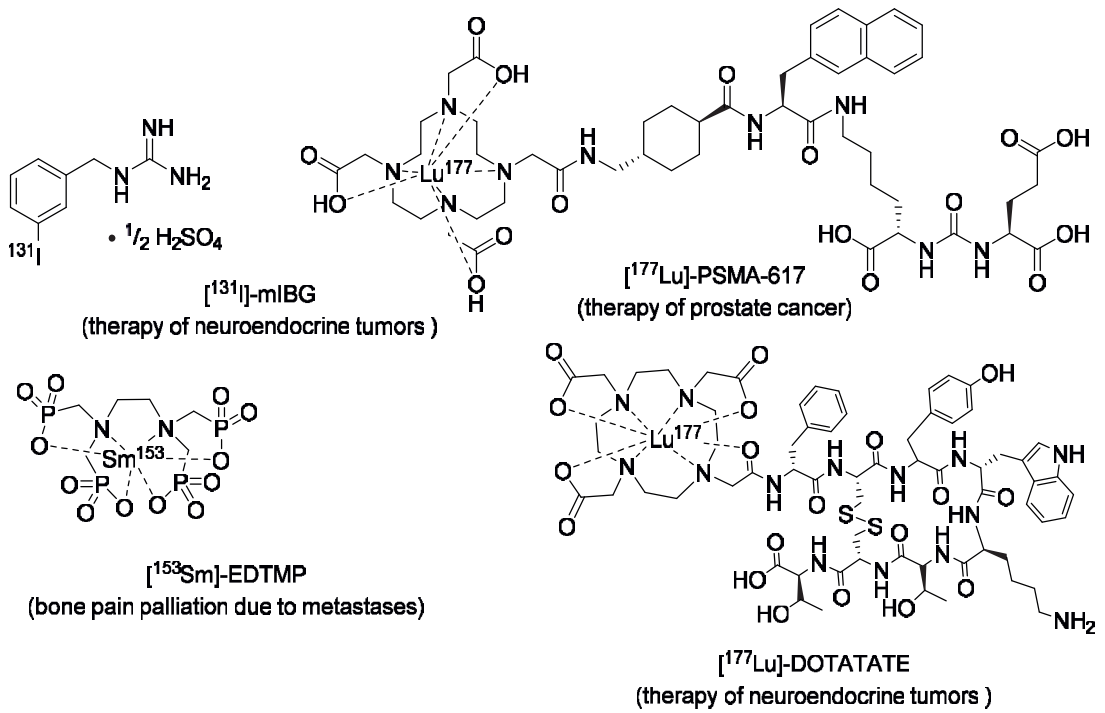


Fig 3. Selected examples of therapeutic radiopharmaceuticals.

2. Design and development of ligands:

The main roles of the ligands, both in metallic or nonmetallic radiopharmaceuticals, are to impart specificity to the agent towards diseased sites, with a minimal accumulation at normal sites. In addition, ligand also plays a role in binding the radionuclide and in imparting stability to the metal-essential radiopharmaceuticals. Hence, while designing a ligand, its pharmacokinetics, pharmacodynamics as well as co-ordination chemistry is to be properly considered. Ligand synthesis is usually accomplished via multi-step, tedious organic synthesis. Till date, various kinds of ligands have been established, viz. small molecule ligands like mIBG, EDTMP; peptide-based ligands like DOTA-TATE, PSMA-11, PSMA-

617; monoclonal antibodies etc. for use in diagnostic, therapeutic as well as theranostic radiopharmaceuticals. In recent years, researchers are also coming out with ligands that specifically target the cellular components of the tumor stroma and microenvironment. The process of ligand development is quite cumbersome, as it requires a combination of knowledges from chemistry, biology and physics. It starts from the target identification, target validation and designing of possible structural leads via in-silico studies. Next, the lead molecule is synthesized, purified, properly characterized using multiple techniques, radiolabeled and are subjected to iterative in-vitro and in-vivo preclinical assays. In general, the outcome from the preclinical assays results in the disqualification of many lead molecules, requiring structural modification of the lead molecule. This again goes through the same route for synthesis and evaluation. Finally, after satisfying pre-clinical assays, an initial proof-of-concept studies are done in patients.[8]

Design and development of radiopharmaceutical ligands mostly depends on two major criteria, (i) specific binding of the ligand to the overexpressed markers or receptors on the cancer site, and, (ii) specific interaction with tumor site via participating in the physiological processes of the particular organ/tissue.[9] The ligand design utilizing the specific binding to the overexpressed receptors on the malignant cell surface has been the most common design strategy, since it requires much less stringent parameters. In general, such a ligand is composed of a targeting biomolecule, pharmacokinetic modifying linker and a bifunctional chelating agent to carry the radionuclide.[10] The targeting biomolecules or the antibodies, both miniature and monoclonal, are based on the proposition that the large proteins which are overexpressed in the cancer cell surface interacts specifically with their complementary epitopes. However, the complementary epitopes have certain limitations like poor blood clearance, very high molecular weight etc. which had limited their clinical applications in the early days. Subsequently, identification of the epitope-binding smaller domain, followed by molecular engineering to improve immune recognition, have resulted in the development of many antibodies which are in clinical use. These biomolecules are often tethered to a bifunctional chelator, e.g. DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid), NOTA (1,4,7-Triazacyclononane-1,4,7-triacetic acid), HYNIC (Hydrazinonicotinic acid), HBED-CC (N,N′-bis-[2-hydroxy-5-(carboxyethyl)benzyl]ethylenediamine-N,N′-diacetic acid) etc., to complex metallic radionuclides with high thermodynamic stability, and to avoid hydrolysis of the radionuclide. Apart from these, few small molecule ligands e.g. TRODAT-1, mIBG, EDTMP, MDP etc. have also been developed for targeting specific receptors or binding sites, and being used in clinics. Table 1 lists some of the representative antibodies and small molecules which have been developed for this purpose.

Table 1. Some representative examples of receptor specific radiopharmaceutical ligands.

Type of target	Ligand	Radiopharmaceutical	Used for
Somatostatin receptor	DOTATATE	^{177}Lu-DOTATATE	Therapy of somatostatin + neuroendocrine tumors
Prostate specific membrane	PSMA-11 PSMA-617	^{177}Lu-PSMA-617	Diagnosis and therapy of prostate cancer.

antigen		^{68}Ga-PSMA-11	
Norepinephrine Transporter	*m*-Iodobenzylguanidine	[^{131}I]mIBG	Therapy of neuroblastoma.
CD45	aCD45 antibody	[^{131}I]aCD45	Bone marrow transplant preparation.
B cells	aCD20 antibody	[^{90}Y]aCD20	Radioimmunotherapy.
Angiogenesis associated integrin	Cyclic RGD peptides	^{99m}Tc-3P-RGD_2 ^{18}F-Alfatide-I	early cancer detection
Hydroxyapatite	Ethylenediamine tetra (methylenephosphonic acid)	^{153}Sm-EDTMP	Bone pain palliation due to metastases

The second strategy includes the design of ligands based on their higher demand in rapidly proliferating malignant cells. The up-regulation of many transport and synthetic mechanisms for sugar, amino acids and nucleosides in the energy-demanding cancer cells have distinguished them from the normal cells, and this have been utilized for the design and development of many ligands. For example, fluorodeoxyglucose (FDG), a glucose analogue, have been used as a ligand in the PET radiopharmaceutical, [^{18}F]FDG, which is clinically utilized for staging of cancer. Being a glucose analog, [^{18}F]FDG is taken up by cells via glucose transporters and retained via phosphorylation. Another PET imaging agent, [^{18}F]-FLT, derived from the ligand 3′-fluoro-3′-deoxythymidine (FLT), is a nucleoside-analog which accumulates during the S-phase of the cell cycle through the action of cytosolic thymidine kinase, TK1. Its uptake is essentially limited to dividing cells, making [^{18}F]FLT an effective imaging agent of cell proliferation.[11] Another important ligand, DTPA, although lipophobic, can cross blood-brain barrier in case of abnormalities via passive diffusion, and is being used for brain imaging in the form of ^{99m}Tc-DTPA.[12] Table 2 depicts some of the radiopharmaceuticals that have been developed using ligands capable of accumulating in malignant cells.

Table 2. Some representative examples of radiopharmaceutical ligands based on preferential accumulation.

Accumulation pathway	Ligand	Radiopharmaceutical	Used for
Glucose transporter	FDG	[^{18}F]FDG	Cancer imaging
Thymidine kinase	FLT	[^{18}F]FLT	Cancer imaging
Reduction	Fluoromisonidazole	[^{18}F]FMISO	Hypoxia imaging
membrane electrical potential	2-Methoxyisobutylisonitrile	^{99m}Tc-sestamibi	Myocardial perfusion imaging

Amino acid transport system l	Fluoroethyl-l-tyrosine	[^{18}F]FET	Imaging of brain tumor
Caspase enzymes	Peptide YDEVDG-NH_2	^{131}I-YDEVDG-NH_2	Apoptosis Imaging

2.1. *In-house Synthesis of some important radiopharmaceutical ligands:*

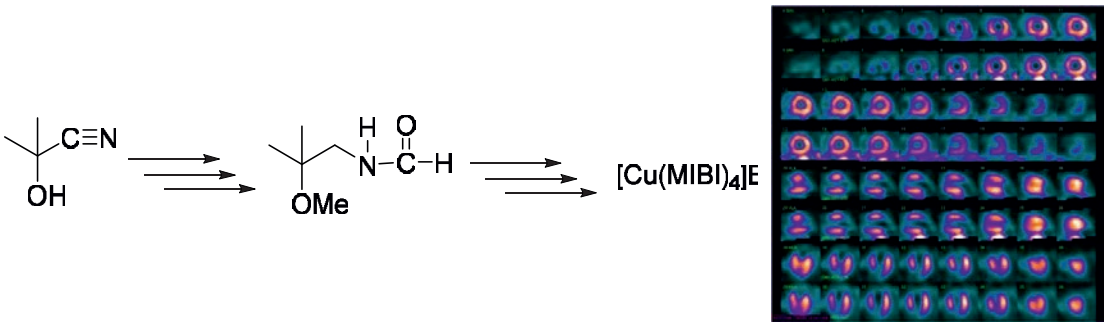
In Department of Atomic Energy (DAE), Bhabha Atomic Research Centre (BARC) and Board of Radiation and Isotope Technology (BRIT) collaborates to develop affordable radiopharmaceuticals, which, after proper validation and DAE-Radiopharmaceutical Committee (DAE-RPC) approval, are provided to hospitals pan-India via BRIT. Bio-Organic Division (BOD) in Bio-Science Group (BSG), BARC has pioneered the synthesis of several radiopharmaceutical ligands, and via collaborations with BRIT, Radiopharmaceuticals Division (RPhD, BARC), Radiation Medicine center (RMC, BARC), and Tata Memorial Hospital (TMH) has succeeded in obtaining the approval of DAE-RPC for clinical use. These in-house developed radiopharmaceuticals are commercially available via BRIT. Apart from these, some radiopahrmaceuticals ligands, designed and developed in-house at BOD, BSG, have also shown promising results. All of these have been discussed separately in the following sections.

2.1.1. Tetrakis(2-Methoxyisobutylisonitrile)Copper(I) tetrafluroborate ([Cu(MIBI)$_4$]BF$_4$):

Tetrakis(2-Methoxyisobutylisonitrile)Copper(I) tetrafluroborate ([$Cu(MIBI)_4$]BF_4) is a precursor in the synthesis of [^{99m}Tc$(MIBI)_6$]BF_4, which is commonly used as a myocardial perfusion imaging agent for evaluation and risk stratification of patients with known or suspected coronary artery disease.[13] Being a cationic complex, it diffuses passively through the capillary and cell membrane, and finally captured by myocyte cells of the heart. Myocardial perfusion imaging is mostly used in diagnosis of ischemic heart diseases, where more than 75% cases are diagnosed with [^{99m}Tc$(MIBI)_6$]BF_4. Hence, [^{99m}Tc$(MIBI)_6$]BF_4 has become one of the most important diagnostic radiopharmaceuticals. It was developed in 1981 by Alan Davison, received FDA approval in 1990, and is being sold commercially since February 1991.[14] [$Cu(MIBI)_4$]BF_4 serves as the main ingredient in the radiopharmaceutical freeze dried kit, which can be radiolabeled using ^{99m}Tc milked out from the ^{99}Mo/^{99m}Tc generator, and hence has a high demand in hospitals which perform a MIBI scan.

BOD, BSG has designed and developed a synthetic method for the synthesis of [$Cu(MIBI)_4$]BF_4 using commercially available starting materials and reagents (**Fig. 5A**). First, 2-Methoxyisobutylisonitrile (MIBI) is synthesized in four steps with an overall yield of 85%. Next, MIBI is treated with Cuprous chloride (prepared under inert atmosphere) in the presence of ethanol to obtain tetra (2-Methoxyisobutylisonitrile)Copper(I) chloride, [$Cu(MIBI)_4$]Cl, which on further treatment with sodium tetrafluoroborate in aqueous medium yielded [$Cu(MIBI)_4$]BF_4. This is purified via crystallization (purity >99% using HPLC), and characterized via ^{1}H NMR, ^{13}C NMR and elemental analysis. This was used for synthesis of [^{99m}Tc$(MIBI)_6$]BF_4, evaluated in-vitro and in-vivo, and DAE-RPC approval for clinical use was obtained in 2009. Since then, BOD caters the overall need of BRIT to prepare the lyophilized kits, which are supplied to hospitals pan-India. These kits are listed in BRIT website as TCK-50.[15] Annually, more than 5000 kits are prepared by BRIT using the in-house synthesized ligand, and are delivered to more than 60 hospitals pan-India, which caters to

more than 20000 patients. To fulfil this, BOD produces approx. 8 g of $[Cu(MIBI)_4]BF_4$ annually. The demand has escalated from 2 g/year to 8 g/year in the last 5 years, and the synthetic route has been modified accordingly to scale up the synthesis up to 5 g/batch.



A **B**

Fig. 5. (A) Synthesis of $[Cu(MIBI)_4]BF_4$; (B) Typical myocardial perfusion scan images using $[^{99m}Tc(MIBI)_6]BF_4$ (image courtesy BRIT, Vashi).

2.1.2. Prostate-specific membrane antigen (PSMA)-11:

PSMA-11 is an API for the preparation of ^{68}Ga-PSMA-11, which is used for the diagnosis of prostate cancer. Prostate cancer is one of the most fatal cancers worldwide, and accounts for nearly 10% of all tumors in men.[16] Diagnosis of prostate cancer at an early stage is the need of the hour, and a sensitive, targeted diagnostic radiopharmaceutical has an edge over other modalities. Diagnostic radiopharmaceutical for prostate cancer relies on the overexpression of Prostate Specific Membrane Antigen (PSMA) on the prostate cancer cell surface. Although, radiolabeled PSMA antibodies would had been a natural choice for development of diagnostic radiopharmaceuticals, their significant kidney uptake, poor blood clearance and accompanied complications had led researchers to find an alternative. Among several trials, a Glu-urea-Lys based ligand PSMA-11 got a breakthrough, and the radiolabeled ^{68}Ga-PSMA-11 was ultimately approved by FDA in 2020 for use in patients for diagnosis of prostate cancer.[17] As on today, this is the most used diagnostic modality for prostate cancer worldwide.

Even after its FDA approval, diagnosis of prostate cancer using ^{68}Ga-PSMA-11 had been severely limited in India due to high cost of the diagnosis, which, in turn, was mostly due to the high commercial cost of the API, PSMA-11. Towards this, BOD designed and executed a cost-effective, indigenous synthesis of PSMA-11, which benefitted Indian patients by lowering the cost of diagnosis.[18]

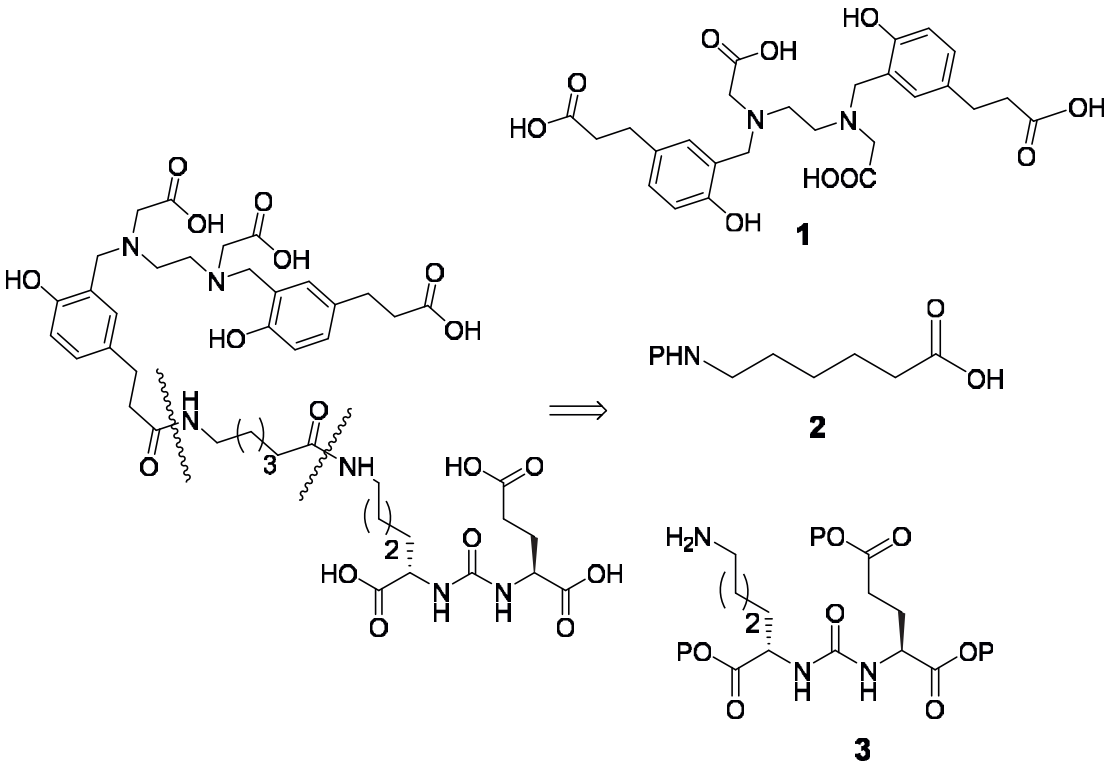


Fig. 6. Convergent retrosynthetic approach for PSMA-11.

Structurally, the ligand PSMA-11 is made up of three parts (**Fig. 6**), (i) Glu-urea-Lys derivative (binding motif), (ii) a six membered linear hydrocarbon linker with suitable functionalities, and (iii) specific chelator for ^{68}Ga called as HBED-CC. Initially the Glu-urea-Lys derivative **1** was prepared using a flow reactor. Next, the linker was synthesized, joined to derivative **1** via an amide bond, and finally linked to the HBED-CC chelator. The purity of the synthesized PSMA-11 was determined by HPLC analysis, and was characterized using LC-MS and NMR. The analytical data of in-house synthesized PSMA-11 was found to be identical with that of the commercial equivalent.

After radiolabeling with ^{68}Ga, preclinical studies, and proper QC studies, in-house synthesized ^{68}Ga-PSMA-11 was approved by DAE-RPC in 2023 for clinical use. This has been one of the most successful and effective strategy to cut-down the cost of diagnosis for prostate cancer patients in India. This will soon be available to hospitals pan-India via BRIT.

2.1.3. ^{18}F-FLT (3'-deoxy-3'-^{18}F-fluorothymidine):

The thymidine analog, 3′-deoxy-3′-^{18}F-fluorothymidine (^{18}F-FLT) has been clinically used as an alternative to ^{18}F-FDG for in vivo imaging of proliferating tissues and malignant tumors. BOD has developed a protocol for synthesis of the precursor of ^{18}F-FLT via two alternative routes, i) using carbohydrate as a starting material,[19a] and ii) using thymidine as a starting

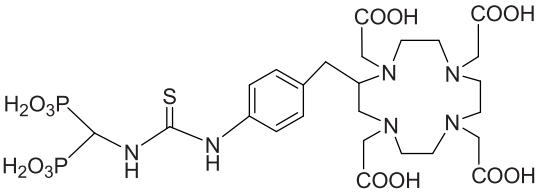
material.[19b] The route employing thymidine was proven to be more efficient in terms of yields and atom-economy, and was followed to prepare the ligand in gm scale. The ligands are under evaluation at RMC, BARC.

2.1.4. Bifunctional chelator for early detection of bone metastasis:

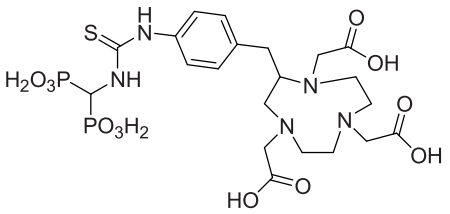
Tumors, particularly of breast and prostate origin, metastasize into bones leading to poor prognosis of cancer. Bone metastasis is associated with severe pain, loss of mobility, etc., and lead to a very poor quality of life. Bone metastasis is clinically diagnosed using SPECT radiopharmaceuticals. However, more sensitive diagnosis using PET radiopharmaceuticals are warranted to enable clinicians for early detection, which can lead to early therapy. Apart from the cyclotron derived $Na^{18}F$, although a number of ligands viz. BPAMD, EDTMP, bifunctional bisphosphonates etc. have been designed, radiolabeled with ^{68}Ga, and studied as PET diagnostic radiopharmaceuticals for the similar purpose, there is still need of an ideal ligand with optimal accumulation in skeleton which can be regularly used in clinics. Towards this, we have designed bifunctional chelators using a macrocyclic chelator conjugated to a geminal bisphosphonate as a radiopharmaceutical ligand with high selectivity for hydroxyapatites in bone.[20]

Fig. 7 depicts the structures of two bifunctional bisphosphonates which were designed, synthesized and evaluated. Both the ligands were prepared in-house using multistep organic synthesis involving a novel 3-component reaction, and subsequent conjugation to the macrocyclic chelator DOTA or NOTA via a thiourea linker. Both the ligands were radiolabeled with ^{68}Ga in high yield (>98%). Both the radiolabeled complexes showed excellent *in vitro* stability in physiological saline and human serum, and also showed high affinity for hydroxyapatite particles *in vitro*. Pre-clinical biodistribution studies in normal Wistar rats demonstrated rapid and almost exclusive skeletal accumulation of the complex.

It has been well established that NOTA, a hexadentate ligand capable of chelating ^{68}Ga with high thermodynamic stability [$\log\beta = 30.98$] by virtue of its matching cavity size with the hydrodynamic radius of Ga^{+3}, has an advantage in in-vitro stability over similar DOTA derivatives. Keeping this in mind, six male patients suffering from prostate cancer in their advanced stages and likely to give rise to skeletal metastasis, were subjected to PET scan using our in-house synthesized NOTA-bisphosphonate after acquiring required ethical clearance for clinical studies from the local Institutional Ethics Committee (IEC) of the hospital as well as written informed consents from the patients before enrolment in this clinical study. A representative whole-body PET scan of a patient (65 y, male) with skeletal metastases from primary carcinoma prostate recoded 30 min post-administration of ^{68}Ga-NOTA-biaphosphonate is shown in **Fig. 7B**. The PET scan shows that the synthesized radiotracer could effectively detect small metastatic skeletal lesions of the patient, as indicated by arrows, due to its site-specific localization in the micro-metastatic sites in the skeleton.



DOTA-bisphosphonate



NOTA-bisphosphonate

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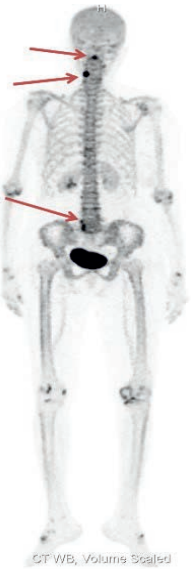

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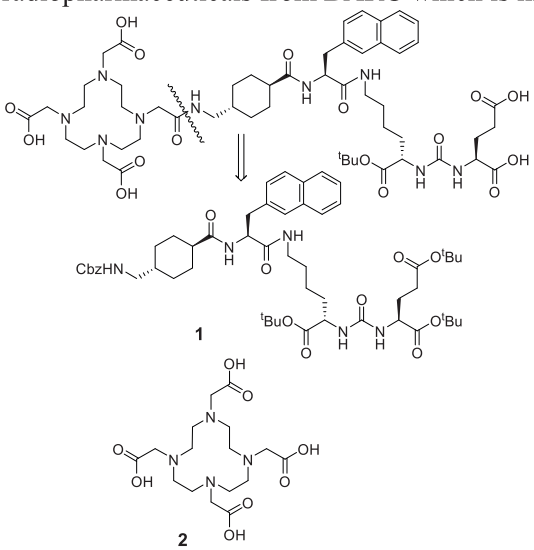
Fig. 7. (A) Structures of in-house synthesized bifunctional bisphosphonates; (B) Whole body PET image of a 65y male with primary carcinoma prostate and suffering from bone pain recorded 30 min post-administration. Arrows indicate site-specific localization of the PET tracer in the small metastatic lesion site (Image courtesy Molecular Group of Companies, Puthuvype, Ernakulam, Kerala, India).

2.1.5. Prostate-specific membrane antigen (PSMA)-617:

The importance of PSMA targeting ligand for detection and therapy of prostate cancer using radiopharmaceuticals is well established, and attempts have been made to develop diagnostic and therapeutic ligands based on PSMA targeting ligands. Radiopharmaceuticals viz. ^{68}Ga-PSMA-11 and ^{177}Lu-PSMA-617 have been clinically approved for diagnosis and therapy of prostate cancer. In fact, ^{177}Lu-PSMA-617 is considered as the latest advance in the treatment of advanced prostate cancer called as metastatic Castration Resistant Prostate Cancer (mCRPC). However, the unaffordability of ^{177}Lu-PSMA-617 to a majority of the Indian patients due to its high cost and irregular availability in India has led us to find out a cost-effective synthetic route of the ligand PSMA-617.[21]

The synthesis of the ligand PSMA-617 was achieved by both linear and convergent approach. The fragments **1** and **2**, (**Fig. 8**) could be assembled to yield the desired product PSMA-617. We envisaged that the fragments could be built from the macrocyclic ligand DOTA and the cyclohexyl carboxylic acid. The convergent approach gave a slightly better yield of the target compound compared to the linear route, and the synthetic complications were less. Thus, a cost-effective route of synthetic route was designed and standardized. The quality of the in-house synthesized ligand was compared to the commercial one via spectroscopic and chromatographic techniques, and they were found to be identical. The in-house synthesized PSMA-617 was radiolabeled with ^{177}Lu, and after quality control and pre-clinical studies, the radiopharmaceutical ^{177}Lu-PSMA-617 was approved by DAE-RPC for clinical use. As on date, BOD produces and supplied approx. ~150 mg of PSMA-617 annually to BRIT for

radiolabeling, kit formation and subsequent supply to hospitals pan-India. More than 6000 prostate cancer patients are benefitted annually from our in-house synthesized PSMA-617 all over India. This is one of the most important in-house developed therapeutic radiopharmaceuticals from BARC which is in clinical use.



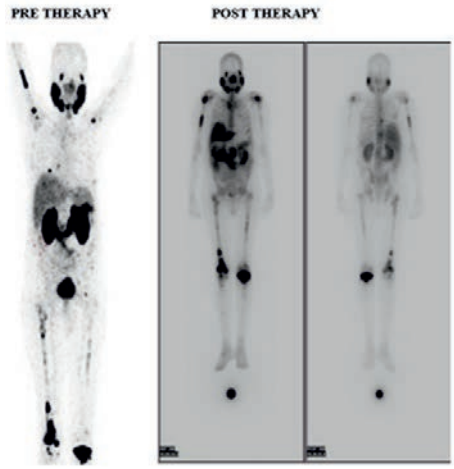


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Fig. 8. (A) Retrosynthetic approach for PSMA-617; (B) Representative images of a prostate cancer patient before and after therapy using in-house synthesized ^{177}Lu-PSMA-617 (image courtesy BRIT).

3. Conclusion and future outlook:

The success of radiopharmaceuticals in cancer therapy is undoubtable. BARC and BRIT play a major role in popularizing radiopharmaceuticals in clinics. This can only be attained via regular, uninterrupted supply of popular and important radiopharmaceuticals to hospitals in India in affordable cost. Indigenous synthesis of ligands can not only bring down the cost of therapy, but also can regularize the production of radiopharmaceuticals. For example, the following table illustrates how our synthesized radiopharmaceuticals have brought down the cost of diagnosis and therapy in clinics.

Sl. no.	Product	Application	Cost (Import)	Cost (indigenous)
1	$[^{99m}Tc(MIBI)_6]BF_4$	Myocardial perfusion imaging	Rs. 30000/-	Rs. 7000/-
2.	^{177}Lu-PSMA-617	Prostate cancer therapy	Rs. 200000/-	Rs. 50000/-
3.	^{68}Ga-PSMA-11	Diagnosis of prostate cancer	Rs. 28000/-	Rs. 8000/-*

*Expected to be launched by Dec. 2023.

Apart from this, new and more effective ligands can be designed and synthesized based on the experience of organic synthesis. For example, envisaging that the diagnosis using ^{68}Ga-PSMA-11 is restricted only to PET diagnostic centers, which are limited and costly, we have started developing ^{99m}Tc-HYNIC-PSMA-11, which can be used in more accessible SPECT-CT centers. Additionally, the high cost of ^{131}I-mIBG hemisulfate and ^{177}Lu-DOTATATE, which are two of the mostly used radiopharmaceuticals, have led us to venture into the design and in-house development of synthetic protocol for both the ligands. BOD has a rich experience in organic synthesis, and this can be expanded more into the field of radiopharmaceuticals towards an ultimate societal benefit in terms of cheaper and affordable healthcare.

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