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GCPII Imaging and Cancer

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Abstract

Glutamate carboxypeptidase II (GCPII) in the central nervous system is referred to as the prostate-specific membrane antigen (PSMA) in the periphery. PSMA serves as a target for imaging and treatment of prostate cancer and because of its expression in solid tumor neovasculature has the potential to be used in this regard for other malignancies as well. An overview of GCPII/PSMA in cancer, as well as a discussion of imaging and therapy of prostate cancer using a wide variety of PSMA-targeting agents is provided.

Keywords

Prostate-specific membrane antigen; PSMA; prostate cancer; molecular imaging; PET; SPECT

PSMA IN CANCER

As has been discussed in previous chapters, enzymatic activity of glutamate carboxypeptidase II (GCPII) is observed in a number of normal and diseased tissues inside and outside of the central nervous system (CNS). GCPII expression within the brush border of the jejunum is one of two locations for which the function of GCPII activity is definitely known. The other is GCPII expression on astrocytes, which cleaves *N*-acetylaspartylglutamate (NAAG) into NAA and glutamate, as discussed in Chapter 6. On the luminal side of brush border cells in the jejunum GCPII is known as folate hydrolase I, which cleaves all γ-linked glutamates from food-derived folate species, enabling folate transport across the intestine and loading onto folate carrier proteins for distribution throughout the body [1–3]. Once these γ-linked monoglutamyl folates are imported into cells through folate receptors, they are then reduced and re-poly-γ-glutamated by folylpoly-γ-glutamate synthetase [4] for storage. GCPII is also expressed in prostate adenocarcinomas [5–9], where it is referred to as the prostate-specific membrane antigen (PSMA), as well as within a few subtypes of bladder carcinoma [10], schwannoma [11] and on the tumor neovasculature of many solid tumors [12–15]. It is also present in the neoendothelium of benign tissues such as endometrium and keloid scar vasculature [16], for which the endogenous substrate(s) and function(s) remain unknown, although new hypotheses have been introduced and will be outlined below.

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PSMA Expression in Prostate Cancer

PSMA expression and localization in the normal human prostate is associated with the cytoplasm and apical side of the epithelium surrounding prostatic ducts but not basal epithelium, neuroendocrine or stromal cells [17]. Cytoplasmic PSMA is an *N*-terminally truncated form of PSMA called PSM′, which has no folate hydrolase activity or capacity to hydrolyze NAAG [18–20]. It appears to be the product of post-translational modification rather than mRNA splicing. The function of PSM′ is unknown. Dysplastic and neoplastic transformation of prostate tissue results in the targeting of PSMA from the apical membrane to the luminal membrane of the ducts [21,22]. Further transformation eventually leads to expression on the plasma membrane of less differentiated epithelial cells, which is associated with the transition into and achievement of androgen growth independence [23–25]. As tumor cells advance in Gleason grade, the ratio of PSMA/PSM′ reliably increases [18] and PSMA expression is thought to circumvent decreased androgen signaling by contributing to an unknown ligand-induced alternative signal transduction pathway [17]. Indeed, androgen receptor (AR) signaling down-regulates the expression of full-length PSMA [5], which may contribute to increasing expression of PSMA by tumor cells following androgen ablation therapy [26,27] and to heterogeneous expression within tumor cells of increasing malignancy in intact males.

Interestingly, dogs are the only other mammalian species known to express PSMA in the prostate (by RT-PCR) and also are the only other species known to develop prostate cancer spontaneously, which is also frequently PSMA-positive [28,29]. Canine prostate cancer, like human prostate cancer, also becomes more aggressive following castration and exhibits greater PSMA expression following castration. The biological function of PSMA expression on prostate cancer cell membranes remains unknown. Speculations range from alternative folate transporter mechanisms [30,31], a receptor for an as yet unknown endogenous ligand or possibly as a source of glutamate. Because nearly 100% of distant metastases express PSMA, PSMA activity is very likely contributing to successful travel to and coloniziation of distant sites such as bone [32,33].

PSMA has both sequence homology and biological behavior similar to that of the transferrin receptor [34,35]. Both receptors are membrane bound and both bind their ligands as a dimer before internalization through clathrin-coated pits [36,37]. PSMA also exists on the membrane surface as a monomer but is enzymatically active only as a dimer [5]. Following substrate, small-molecule antagonist or specific antibody binding, PSMA-bound ligands are then internalized within the cell and are either retained in lysosomal compartments along with the degrading PSMA receptor [5], or bound ligands may be released to distribute within the cell or diffuse out of the cell as labile metabolites [38,39]). Some internalized PSMA proteins are also recycled intact back to the membrane surface through the recycling endosomal compartment (REC) [5, 40]. The fate of bound ligands through that pathway remains unknown. It is possible that bound ligands might be delivered intact to the cytosol, as is the case for endogenous folates [31]. The cytoplasmic tail of PSMA contains consensus sequences for protein kinase C (PKC) recognition and phosphorylation. Phosphorylation of various Thr and Tyr residues by PKC may direct PSMA interaction with selected adaptor proteins, which control receptor sorting into either lysosomes or the REC for recycling [5].

PSMA contains ten putative glycosylation sites and is highly glycosylated [41]. Glycosylation is necessary for enzymatic activity as well as for protein folding and membrane targeting [42] within prostate cancer cells. Interestingly, different human prostate cancer lines and tumors within patients have been found to express PSMA with different glycosylation patterns and correspondingly different enzymatic activity profiles [43,42]. Control of glycosylation for membrane targeting and enzymatic activity is likely involved in metastasis. Indeed, various human prostate cancer cell lines reflecting androgen sensitive and insensitive growth differed in their PSMA enzymatic activity (using *N*-acetylaspartylglutamate as the substrate) where the androgen insensitive C4-2B cell line, having the highest metastatic potential in mouse models, had 109 times the carboxypeptidase activity of LAPC-4, an androgen sensitive non-metastatic cell line [44], and three to five times the activity of two common androgen insensitive and one sensitive, but less aggressive cell lines. Differential glycosylation of PSMA may be controlled by residual androgen signaling since three of four cell lines described above that displayed growth-related androgen insensitivity also possess mutant ARs with disparate residual signaling capacity. Growth sensitivity to androgens is not always tied to AR transcriptional control of other prostate cancer markers, such as for prostate-specific antigen (PSA) or kallikrein 2 expression, and differs by cell line [44]. Notably, the only two isolated prostate cancer cell lines devoid of AR expression (PC-3 and DU-145) are also similarly devoid of PSMA expression [45].

PSMA in the Neovasculature

PSMA is also expressed on the apical and luminal surface of new blood endothelial cells associated with most solid tumors as well as the neovasculature of some normal proliferative tissues such as endometrium and wounds within normal tissues such as heart valve injuries, pleural lesions and some keloid scars [16]. PSMA expression in tumor neovasculature has so far only been reported in humans although the search for PSMA-expressing neovasculature in tumors from murine hosts continues. The function of PSMA expression on solid tumor neovasculature remains unknown although two biochemical pathway interactions augmented by PSMA folate hydrolase activity have been reported as important to vasculogenesis. One report describes a feedback loop connection between PSMA, γ1-integrin, and p21-associated kinase (PAK) signaling in which PSMA activity activates γ1-integrin (coordinates cell adhesion to and movement across laminin, a component of the extracellular matrix) and γ1-integrin activates PAK, which phosphorylates and deactivates filamin A. Deactivation of filamin A promotes outreach of invasive filopodia by the cytoskeleton, which is important for endothelial cell invasion [46]. Gordon *et al*. [16] speculate that the folate hydrolase activity of PSMA on endothelial plasma membranes scavenges released extracellular poly-γ-glutamyl folates and internalizes these folates, which are stored and converted into 5-methyl-tetrahydrofolate (THF). THF reduces and recycles the tetrahydrobiopterin (BH4) cofactor of the endothelial isoform of nitric oxide synthase (eNOS), allowing eNOS catalyzed production of NO, a potent stimulator of angiogenesis [47,48].

Various folate receptors that enable import of all species of extracellular folate are ubiquitous on all cell types [49–51] and intracellular pools of stored folates are frequently

adequate due to intake from fortified dietary sources [52,53]. When considering the effect of glutamate carboxypeptidase/folate hydrolase activity, it is important to remember that PSMA enzymatic activity upon endogenous folates results in more than just folic acid. Cleavage also results in the generation of multiple glutamates, which activate glutamate receptors.

PSMA and Glutamate Signaling

It has recently been recognized that functional glutamate signaling occurs in several cancer types outside of the CNS: melanoma [54], head and neck cancer [55], osteosarcoma [56], colorectal cancer [57], squamous cell carcinoma [58] and in prostate cancer [59]. Signaling can involve ionotropic (*N*-methyl-D-aspartate receptor, NMDAR) or metabotropic glutamate receptors (mGluR) as well as glutamate transporters [60]. The synthesis of NO by eNOS is particularly sensitive to intracellular calcium concentration, where increasing calcium elevates production of NO [61,62]. Intracellular calcium concentration is increased by excitatory NMDAR and group I mGluR signaling, both of which are activated by glutamate binding. PSMA is co-expressed with mGluR5, a group I mGluR, in prostate cancer cells [59] (C.A. Foss unpublished results) and also mGluR1 [59] and the NMDAR is expressed in prostate cancer of increasing grade [63]. The functional significance and therapeutic implications of possible PSMA-augmented excitatory glutamate signaling is the subject of current work by the authors. Currently, NMDAR vascular presence has been reported only in brain microvasculature [64,65] while excitatory group I mGluRs have so far only been reported in brain and dermal microvasculature [66–68]. It is possible that excitatory glutamate signaling in the neovasculature of any origin has not been investigated due to the continuing focus of glutamate transmission on CNS and PNS neuronal and glial cell functioning. However, PSMA-gated glutamate-evoked calcium-dependant NO generation in pre-vascular endothelial cells to stimulate angiogenesis is an intriguing mechanistic possibility. PSMA enzymatic cleavage would simultaneously supply a folate moiety to facilitate eNOS cofactor BH4 turnover while raising intracellular calcium concentration through NMDAR and/or mGluR1/5 glutamate signaling, leading to increased NO generation and angiogenesis (Fig. 1).

PSMA expression alone in prostate cancers has been shown to be ineffective in predicting the course of the disease, although metastatic tumors almost universally express large amounts of PSMA [26,69]. It is possible that the combination of expression of membrane-bound PSMA with excitatory glutamate receptors, which may express differentially according to environment and accumulated genetic changes, shape malignancy and/or metastatic potential in prostate tumors. Serum and tissue glutamate levels are typically 30–40 times higher in the periphery [70] than in resting synapses (40–60 μM vs. 1–2 μM), where potentially toxic signaling is prevented by control of synaptic glutamate [71]. Peripheral NMDARs are not voltage dependant and can be activated in the presence of both glycine and glutamate [72]. Peripheral group I mGluRs are constitutively active in the presence of glutamate [73] and are regulated in part by frequent internalization *via* clathrin coated pits [74]. PSMA is also internalized *via* clathrin coated pits and has been postulated to interact with mGluR5 through filamin A binding [59.] It is possible that poly-γ-glutamyl folate binding and/or cleavage by PSMA may cause a conformational change that activates

adjacent mGluR5 and/or mGluR1, enabling folate-released or tissue glutamate binding and subsequent specific downstream signaling events such as NF-γB activation, which is known to be constitutively active in prostate cancers, possibly through this mechanism (Fig. 2). Similarly, glutamate signaling through group I mGluRs in astrocytes has been shown to activate NF-γB signaling [75]. More work in this area is needed to illuminate these interactions and functional consequences of PSMA and excitatory glutamate receptor co-expression.

Because the expression of membrane-bound PSMA is very restricted in normal tissues and is abundantly expressed in prostate cancers and the neovasculature of most solid tumors, it is an attractive target for both diagnostic imaging of metastatic tumors and targeted therapies for these tumors, which will be described further below. Increased knowledge concerning the connection between PSMA folate hydrolase activity and glutamate signaling in both prostate tumors and in neoangiogenesis would also be helpful in terms of drug development, diagnostic and prognostic significance. Radiolabeled small molecule probes for the NMDAR [76], mGluR5 [77–82] and mGluR1 [83–85] have been developed to allow patient selection for targeted therapies to augment or disrupt interactions between these proteins. Radiolabeled small molecule probes targeting PSMA for positron emission tomography (PET) and single photon emission computed tomography (SPECT) imaging have also been developed and will be described in the following sections.

MOLECULAR IMAGING OF CANCER

Molecular imaging broadly defined is the non-invasive detection and measurement of cellular and molecular processes in whole living beings using a variety of existing modalities including PET, SPECT, magnetic resonance (MR), computed tomography (CT), ultrasound, fluorescence, or bioluminescence [86–88]. Imaging has become an indispensable tool in cancer research, clinical trials and medical practice. In the era of molecular oncology and personalized medicine, development of molecular imaging methodologies can detect processes related to metabolism, angiogenesis, and hypoxia as well as image other cellular processes such as gene expression, receptor expression, and signaling pathways. Molecular imaging in cancer promises to address the following issues in cancer management: (1) detection of the presence of malignancy or to direct biopsy; (2) staging; (3) therapeutic monitoring – particularly early after initiation of therapy; (4) provision of a prognostic biomarker differentiating aggressive from indolent disease; (5) improvement and acceleration of development of novel therapeutics. PET/CT imaging has emerged over the last decade as an important molecular imaging modality in oncology as evidenced by the rapid rise in the total number of [^{18}F]fluorodeoxyglucose (FDG) PET/CT scans performed for clinical use, with more than 1.5 million FDG PET/CT or PET scans in the United States in 2006 [89].

MOLECULAR IMAGING OF PROSTATE CANCER

Prostate cancer is the mostly commonly diagnosed cancer and the second leading cause of cancer death among men in the United States and second most common cancer in men worldwide [90,91]. Conventional imaging modalities, including bone scintigraphy (bone

scan), CT, ultrasound, and MR imaging, are currently used to detect primary prostate cancer and metastatic disease for staging and risk stratification. However, there is a need for imaging beyond current capabilities to improve management and selection of appropriate therapy in the following clinical scenarios: (1) *initial diagnosis* (accurate diagnosis and anatomic localization directly within the prostate to guide biopsy and determine the likely effectiveness of focal therapy; risk stratification to determine whether the lesion represents indolent versus aggressive disease; accurate staging); (2) *biochemical recurrence* after initial primary therapy (detection of local and metastatic disease); and, (3) *progressive metastatic disease* (detection and localization of metastases; assessment of overall metastatic burden; assessment of castrate-sensitive and -resistant disease; assessment of early treatment response). By targeting the biological mechanisms unique to prostate cancer, molecular imaging can help address many of the above mentioned unmet clinical needs and hasten the development of new therapeutics. PET is the current premier translational molecular imaging technique and will provide the basis for the following discussion. It is against the existing and more conventional molecular imaging agents discussed in this section that the newer agents that target PSMA must be judged.

FDG PET

Although PET as a functional imaging modality, in particular FDG PET, has played an increasingly important role in tumor detection, staging, and therapeutic monitoring in a variety of other cancers, the role of FDG PET has been limited in prostate cancer for detection of primary disease and soft tissue and osseous metastases [92–94]. FDG PET detection of metastatic prostate cancer can however be improved with optimal detection of metastatic disease based on receiver operating characteristic (ROC) analysis of PSA ≥ 2.4 and PSA velocity of 1.3 ng/mL/year [95]. When there is detectable FDG uptake in metastatic prostate cancer, increasing FDG uptake (> 33% increase in maximum standardized uptake value [SUVmax]) on chemotherapy proved to be a prognostic marker and outcome measure comparable to a combination of imaging and biochemical markers (serum PSA, bone scan, CT and/or MRI) in patients with advanced hormone-refractory metastatic disease [96].

[^{18}F]Fluoride PET

[^{18}F]Fluoride-labeled NaF ([^{18}F]NaF)has long been recognized as an excellent radiopharmaceutical for bone imaging, but fell out of use when more widely available, kit-based [^{99m}Tc]diphos-phonates emerged for skeletal scintigraphy [97]. [^{18}F]NaF uptake in osteoblastic activity is 2-fold greater than [^{99m}Tc]methylene diphosphonate (MDP). Through use of PET, which is of inherently higher sensitivity and resolution than SPECT, with its semi-quantitative capacity and the recent availability of hybrid (PET/CT imaging systems), [^{18}F]NaF has been revisited as a viable radiopharmaceutical for imaging bone metastasis [98]. A prospective study of [^{18}F]NaF PET/CT, [^{18}F]NaF PET, MDP SPECT, and MDP planar imaging for detection of bone metastases in patients with prostate cancer demonstrated the high sensitivity and specificity of [^{18}F]NaF PET/CT, with higher specificity than PET alone and higher sensitivity and specificity than standard MDP SPECT or planar bone scintigraphy [99]. However, the added clinical value of improved and earlier detection of bone metastasis by [^{18}F]NaF PET/CT must be balanced against providing

increased radiation exposure and cost. The National Oncology PET Registry (NOPR) sought and recently obtained approval from the Center for Medicare and Medicaid Services (CMS) to begin a registry to perform the necessary collection of data to allow for coverage of [^{18}F]NaF PET scans for detection of bony metastases in cancer [100].

Radiolabeled Choline Analogs

Choline uptake in prostate cancer cells is associated with cell membrane phospholipid metabolism, with extracellular uptake dependent on choline uptake through a choline transporter and phosphorylation through a choline kinase [101–103]. [^{11}C]Choline and [^{18}F]fluorocholine uptake mechanisms are similar with rapid tumor uptake. However, in addition to differences in physical half-life (20 min for ^{11}C versus 109 minutes for ^{18}F), there is substantial urinary excretion of [^{18}F]fluorocholine but only minimal urinary excretion of [^{11}C]choline [104]. [^{11}C]Choline and [^{18}F]fluorocholine are the most studied PET radiopharmaceuticals for prostate cancer, but due to issues inherent in what are usually small studies and the lack of histopathologic verification of imaging findings, the role of these agents in prostate cancer remains unclear and awaits further assessment in large, prospective, multi-center trials.

For detection of primary prostate cancer, [^{11}C]choline PET is limited due to significant overlap with uptake seen in prostatitis or benign prostatic hypertrophy. There has been no correlation between radiolabeled choline uptake and tumor histologic grade, volume of disease or PSA level [105–107]. However, other studies claim to be able to differentiate benign from malignant processes by visual evaluation or to differentiate aggressive prostate cancer (Gleason 4+3 or higher from 3+4 or lower) through use of tumor-to-background radiotracer uptake ratios [108,109]. When compared to pelvic MR imaging with 3D MR spectroscopy, [^{11}C]choline demonstrated lower sensitivity for localization of primary prostate cancer [110]. [^{18}F]Fluorocholine performed better for detection of primary prostate cancer, i.e., it was able to localize dominant areas of malignancy, but was unable to detect small lesions. Addition of dual time point imaging enabled differentiation between benign and malignant primary prostate lesions [111, 112]. However, another study did not recommend [^{18}F]fluorocholine for localizing prostate cancer, although in highly selected patients [^{18}F]fluorocholine PET/CT allowed the identification of neoplastic regions within the prostate [113].

For staging and detection of metastatic disease, an early study demonstrated relatively high sensitivity and specificity (80% and 96%, respectively) for detection of nodal metastases for [^{11}C]choline, although this technique possessed lower sensitivity and comparable specificity compared to MRI or CT for detection of LN metastases [114]. For detection of lymph node metastases at PSA relapse, [^{11}C]choline showed a high positive predictive value for detection of metastases in patients with PSA $\geq$ 2.0 with the detection rate directly related to serum PSA level. However, the negative predictive value and sensitivity were low, attributable to the inability to detect microscopic disease in subcentimeter lymph nodes due to PET detectability limits [115,116]. In patients with advanced metastatic disease, [^{11}C]choline PET/CT affected management in 24% of patients [117]. Notably, [^{11}C]choline uptake declined after anti-androgen therapy in primary and metastatic prostate cancer in one

study but reportedly did not significantly affect the rate of detection of metastases in two other studies [105–117]. [^{18}F]Fluorocholine could reliably detect metastases in PSA recurrence but only when PSA levels were> 4 ng/mL [118]. A systematic review and critical analysis of published results with [^{18}F]fluorocholine reported recommendation for its use for initial staging among intermediate- to high-risk populations (PSA > 10 ng/mL or Gleason score ≥ 7) and restaging for recurrent disease, optimally if PSA > 2 ng/mL or there are short PSA doubling times [119].

Acetate

[^{11}C]Acetate uptake in prostate cancer is associated with fatty acid metabolism and related to levels of expression of fatty acid synthase (FAS) [120]. Although less extensively studied than choline PET radiotracers, [^{11}C]acetate PET imaging is thought to have similar accuracy for detection of recurrent disease [121]. Other studies of [^{11}C]acetate at PSA relapse demonstrated detection of metastatic disease with findings influencing patient management in 28% of patients, but also demonstrated a false positive uptake in 15% of patients [122–124]. A review of several studies for detection of local, nodal and osseous metastases in recurrent prostate cancer was unable to recommend definitively between choline or acetate PET radiotracers for these indications [125]. [^{18}F]Fluoroacetate has also been synthesized and reported to have potential application in prostate cancer [126].

[^{18}F]FACBC

[^{18}F]FACBC (*anti*-1-amino-3-[^{18}F]fluorocyclobutane-1-carboxylic acid) is a synthetic, non-metabolized amino acid analog of L-leucine that demonstrated detection of primary prostate tumor and local pelvic nodal metastatic disease as well as suspected sites of recurrence in an initial pilot study of 15 patients [127]. A larger study of 50 patients comparing [^{18}F]FACBC with that of [^{111}In]capromab pendetide (ProstaScint™) in the detection of recurrent prostate cancer found [^{18}F]FACBC to be more sensitive for detection of intraprostatic and extraprostatic sites of disease [128]. The mechanism of uptake in prostate cancer is thought to be *via* the amino acid transporter system ASC transporter 2 (ASCT2), which sodium-dependent. [^{18}F]FACBC is not incorporated into proteins [129].

[^{18}F]FDHT

[^{18}F]FDHT (16β-fluoro-5α-dihydrotestosterone) is an [^{18}F]-labeled analog of dihydrotestosterone, the endogenous ligand for AR. Initial studies evaluating [^{18}F]FDHT PET imaging for AR imaging in patients with castrate-resistant prostate cancer confirmed receptor mediated uptake with diminished uptake after competition with testosterone treatment. [^{18}F]FDHT was present within 78% of lesions seen by conventional imaging in one study, demonstrating a sensitivity of 63% on a patient-by-patient basis and 86% sensitivity on a lesion-by-lesion basis [130, 131]. As a radiotracer designed against a well characterized receptor, a pharmacokinetic model of [^{18}F]FDHT behavior was able to provide a surrogate measure of free AR concentration [132].

Other

Other PET radiopharmaceuticals have also been studies in prostate cancer in a limited number of studies, including [^{11}C]methionine, [^{18}F]FMAU (1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) thymine), [^{18}F]FLT (3′-deoxy-3′-[^{18}F]fluorothymidine) [133–135].

IMAGING PSMA: ANTIBODIES

Monoclonal antibodies are widely used in basic research and are often used for targeted therapy in oncology. Aside from therapeutic use, monoclonal antibodies have also been applied to molecular imaging, enabling specific targeting *in vivo*. Radiolabeled antibodies are detected with PET and SPECT in analogy to the low molecular weight agents discussed above [136]. Several recent reviews have appeared that outline the use of monoclonal antibodies and antibody fragments for imaging, and the strategies used to obviate problems of non-specific binding and long circulation times that often accompany the use of antibodies for this indication [137].

As discussed above, PSMA is expressed in virtually all prostate cancers and that expression progressively increases with cancer grade [138]. Unlike prostate cancer-specific secretory molecules such as PSA and prostatic acid phosphatase (PAP), PSMA is an integral membrane protein [139]. Prostate cancer cells overexpress this protein by several orders of magnitude over normal prostate cells, making PSMA an ideal target for detection and treatment of prostate cancer. Currently, the only monoclonal antibody approved by the U.S. Food and Drug Administration (FDA) available for imaging prostate cancer is that based on 7E11/CYT-356.

7E11/CYT-356

7E11/CYT-356 is a monoclonal antibody against PSMA that binds to a six amino acid sequence on the intracellular surface of the protein. The ^{111}In-labelled version of 7E11/CYT-356, is what is commonly known as ProstaScint™, introduced above. ProstaScint™ was approved by the FDA in 1997 for human imaging [140]. As stated by the FDA, ProstaScint™ was approved for usage “...in newly diagnosed patients with biopsy-proven prostate cancer thought to be clinically localized... who are at high risk for pelvic lymph node metastasis” and “...in post-prostatectomy patients with a rising PSA and a negative or equivocal standard metastatic evaluation in whom there is a high clinical suspicion of occult metastatic disease.” ProstaScint™ is reported to stage primary prostate cancer with 68% accuracy, 62% sensitivity and 72% specificity [141], which suggests that this agent may be useful but suboptimal. ProstaScint™ is also sequestered to a large extent in liver, kidney, and other non-prostate sites [142]. Non-specific tissue uptake, especially that which occurs in the lower abdomen and pelvis, creates background signal that often masks the prostate. Long circulating times must also be addressed, usually through subtraction of a blood pool image of the pelvic vasculature.

One reason that images from ProstaScint™ are difficult to interpret may be due to the fact that the antibody binds to an intracellular epitope on prostate cancer cells, and has access to target only through destruction of the cell membrane as occurs in states of necrosis and apoptosis [143]. In tissues such as bone, where access to target is even more limited than in

soft tissue, ProstaScint™ has proved unable to detect metastases reliably [144]. ProstaScint™ is injected approximately one week prior to imaging to allow time for maximal delivery to target, and for clearance of non-specifically bound signal. Over the course of the week, the ^{111}In radiolabel signal decays significantly, due to its 2.8 day physical half-life. Decay correction of the signal generated one week post-injection has proved challenging [145]. That difficulty in image interpretation is magnified further by continued nonspecifically-bound signal even after allowing for one week of clearance [146]. Nevertheless, new techniques specifically designed to interpret images due to ProstaScint™ can overcome some of those limitations. Localization of the SPECT signal is aided by superimposing anatomic CT data on the SPECT image. Difficulties still obtain, however, with superimposing these two different data sets due to changes in the shape and volume of the rectum and/or bladder and due to patient movement between their acquisition [147]. While such efforts have greatly reduced the incidence of false positives in the diagnosis of primary prostate cancer, ProstaScint™ has proved less reliable than biopsies of nearby organs such as the seminal vesicles [148].

J591

J591 is a deimmunized monoclonal antibody that is specific for the extracellular domain of PSMA [149]. Unlike 7E11, which binds to an intracellular epitope, J591 binds viable cells expressing PSMA on the outside of the cell membrane. The antibody is prepared for imaging or therapy by conjugating it with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) a chelator for a variety of radiometals [150]. The J591 antibody has been radiolabeled with ^{111}In and ^{99m}Tc for imaging and with ^{90}Y and ^{177}Lu for therapy [149, 151, 152]. Also, although beyond the scope of this review, near-infrared (NIR) fluorophores have also been attached to J591 for use as imaging agents [153].

As in the case of ProstaScint™, [^{177}Lu]J591requires time to reach its target, also requiring about a week to reach metastatic lesions. Similar to ProstaScint™, this radiotracer also appears in other non-target organs, most noticeably in the liver, kidneys, and spleen, as is common to antibody based imaging agents. However, in comparison to ProstaScint™, [^{177}Lu]J591 demonstrates higher target-to-background ratios [149]. Most recently the J591 antibody, labeled with ^{64}Cu, has proved capable of reporting on AR signaling, opening up an entirely new use for PSMA-based imaging agents [25].

3/A12, 3/F11, 3/E7

Three monoclonal antibodies specific for PSMA, 3/A12, 3/F11, 3/E7,are currently under development as molecular imaging agents for prostate cancer. As for J591, 3/A12, 3/F11 and 3/E7 are all conjugated to DOTA [152]. The conjugated DOTA is then radiolabeled with ^{64}Cu, a radionuclide that decays through positron emission, γ-decay and electron capture, and is imaged using PET [154]. These antibodies all demonstrate specific uptake in PSMA-positive tumors, with 3/A12 displaying significantly more uptake in the kidneys than the others. In flow cytometry assays for competitive binding alongside J591, the antibodies showed comparable half-maximal saturation concentrations with 14 nM for 3/A12, 17nM for 3/E7, and 9nM for 3/F11, while J591 had a half-maximal saturation concentration of 16 nM [155]. In animal studies, combined PET/MR imaging allows for the easy identification

of tumors with low antibody uptake [156]. The high specificity of these antibodies seems to warrant further studies and cautionary optimism for use in prostate cancer.

IMAGING PSMA: LOW MOLECULAR WEIGHT AGENTS

As stated above, one of the drawbacks of using monoclonal antibodies for imaging is the long time required for the antibody to clear from non-target tissues, often a span of several days between injection of the agent and imaging. Strategies for addressing the poor pharmacokinetics of antibodies for imaging include the use of aptamers or small molecules as the PSMA targeting agent. Aptamers are either 8–15KDa oligonucleotides or peptides, isolated from combinatorial libraries, which can be selected for specific binding to target molecules through affinity maturation [157]. Their specificity and affinity for targets are similar to antibodies. ^{99m}Tc-Labeled aptamer TTA1 demonstrated uptake in breast, colon, glioblastoma, and lung tumor xenografts [158] and demonstrates the promise of this approach. Most work so far with PSMA-binding aptamers has concentrated on the delivery of therapeutics including short hairpin RNA (shRNA) [159]. Aptamer A10 [160] as well as aptamer-doxorubicin [161] showed specific binding to PSMA-positive cells *in vitro*. A10-Apatamer-siRNA and A10-Aptamer-shRNA chimera have demonstrated tumor regression in PSMA-positive tumor xenografts [162] and increased the sensitization of PSMA-positive tumor xenografts to external radiation [163]. Co-delivery of doxorubicin and shRNA against Bcl-xl using a PSMA aptamer-conjugated polyplex was more toxic to PSMA positive cells than a simple mixture of the drug and shRNA, which demonstrates the potential of targeted co-delivery of therapeutic agents [164]. Bi-specific apatamers consisting of a PSMA targeting aptamer and an agonistic 4-1BB aptamer inhibited PSMA-positive tumor xenograft growth *via* 4-1BB co-stimulation of tumor-specific CD8+ T cells [165] and could be useful in developing a targeted immune response to cancer.

The A10 aptamer has also been utilized as the PSMA targeting moiety (20 nM affinity, [166,167]) for aptamer-nanoparticle [164, 168–172] and aptamer-quantum dot [173] conjugates. Several of these preparations, when loaded with either doxorubicin or cisplatin, demonstrated either PSMA-specific cell growth inhibition *in vitro* [164, 170, 172, 173] or tumor regression from a single intratumoral injection [168]. Only recently has an aptamer to PSMA been radiolabeled. In this report, the positron emitter ^{64}Cu complexed to DOTA was conjugated to the aptamer. However, no *in vivo* images have yet been produced [174].

As discussed above, in addition to providing a target for monoclonal antibodies such as ProstaScint™ and J591, PSMA possesses an enzymatic site in its extracellular domain that cleaves endogenous substrates such as NAAG and poly-γ-glutamyl folic acid. The crystal structure of PSMA with and without inhibitors in the enzymatic site has been described [175–180]. The enzymatic site contains two zinc ions, and is composed of two pockets, the glutamate-sensing pocket (S1′ pocket) and the non-pharmacophore pocket (S1 pocket). A tunnel of an approximate length of 20 Å connects the enzymatic site to the surface. Many small molecule substrates and inhibitors for this enzyme have been prepared and tested, many prior to the reporting of the crystal structure of PSMA. This topic has been reviewed recently [181, 182]. Small molecule PSMA inhibitors are generally zinc binding compounds attached to a glutamate or glutamate isostere [183] and fall into three families of

compounds: (1) phosphonate-, phosphate-, and phosphoramidate compounds; (2) thiols; and (3) ureas. The initial work on the phosphonate and phosphate inhibitors, which included the potent GCPII inhibitor 2-(phosphonomethyl) pentanedioic acid, 2-PMPA (IC50 ~ 0.9 nM, [184]), as well as the thiol based GCPII inhibitors, came from research conducted at ZENECA and then Guilford Pharmaceuticals [185, 186]. Later, extensive studies with the phosphoramidate inhibitors came from Berkman's group (IC50s ~ 0.5–20 nM), [187–189]. The initial preparation and testing of urea-based inhibitors was reported by Kozikowski *et al*. [190]. Research on new imaging agents for prostate cancer based on small molecule PSMA inhibition have concentrated on the use of phosphoramidates and ureas. Of the agents derived from 2-PMPA the first optical imaging agent was prepared by conjugating IRdye78 to the known GCPII inhibitor GPI to form **1** (Fig. 4). That agent possessed a high binding affinity (low nanomolar) for PSMA positive cells and clearly stained PSMA positive cells *in vitro*. However, its rapid clearance from the blood limits the time for uptake in tumors [191]. Berkman's group reported compound **2**, which was also a potent PSMA inhibitor and demonstrated internalization of the fluorescent molecule [188]. Changing to the near-IR dye Cy5.5 to produce **3** also resulted in a potent inhibitor and may be useful for *in vivo* imaging although no *in vivo* images were reported [192]. Conjugation of a photodynamic therapeutic agent, pyropheophorbide-a to a phosphoramidate resulted in agent **4**,which was phototoxic to PSMA-positive cells *in vitro* and could eventually lead to a treatment for primary prostate cancer [192]. A [^{18}F]fluorobenzoyl phosphoramidate, compound **5**, demonstrated specific uptake in PSMA positive xenografts [193].

The first urea based small molecule PSMA inhibitor was [^{11}C]methyl-cysteine-glutamate urea **6** [194], followed by radioiodinated tyrosine-glutamate urea **7** [195] (Fig. 5). Other radiolabeled cysteine-glutamate ureas include 4-[^{18}F]fluorobenzyl-, and 4-[^{125}I]iodobenzyl-cysteine-glutamate ureas, compounds **8 [**196] and **9** [197]. The lysine glutamate urea **10** and its tri-esters are important platforms on which to add radiolabeled prosthetic groups. We have used it to prepare compounds **11**-**13** while Molecular Insight Pharmaceuticals, Inc. (Cambridge, MA) has used it to prepare **14** and **15 [**198–201]. All of those radiolabeled PSMA inhibitors (**6**-**15**) showed high affinity (very low nanomolar), selective binding to PSMA-positive cells and specific uptake in PSMA-positive xenografts. All compounds exhibited urinary excretion and high uptake in the kidneys due to the presence of PSMA in mouse kidneys. Clinical SPECT imaging with ^{123}I-labeled **14** and **15** detected metastatic [202] lesions while PET/CT imaging with [^{18}F]**8** (N-[N-[(*S*)-1,3-dicarboxypropyl]carbamoyl]-4-[^{18}F]fluorobenzyl-L-cysteine, DCFBC) detected both bone and lymph node metastases [203]. In the latter study several lesions in areas considered suspicious for metastases but not seen on either CT or bone scan were clearly visualized. Additional clinical studies using these new agents and possibly others will be required in order to determine which agent performs best in detecting metastatic disease and determining its usefulness in imaging primary disease and for staging.

We have also used compound **10** and its esters to add a linking group to tether a bulky radiometal chelate to the urea, allowing the urea to reach the enzymatic site while keeping the metal chelate on the exterior. This has produced inhibitors containing [^{99m}Tc]tricarbonyl, and [^{68}Ga]DOTA, compounds **16**, and **17** respectively (Fig. 6) as well as

a series of $^{99m}Tc^{V}O^{+3}$ complexes" [204–206], all which showed high specific uptake in PSMA positive xenografts. The use of the positron emitter ^{68}Ga is especially exciting because it is produced within a generator, which would eliminate the need for a local cyclotron. We have also used this tethered urea to form optical agents **18**-**21**, which all demonstrated high specific uptake in PSMA positive xenografts [201,207]. Recently we reported the dual modality agent **22**, in which a single injection of 1mCi (37 MBq) of [^{111}In]**22** permitted the visualization of tumor xenografts by both SPECT and near-IR optical imaging [208]. Finally, this tethered urea has been used to prepare PSMA-targeted antibody recruiting molecules [209] and may provide new platforms for radiolabeling and imaging of prostate cancer.

Bis-glutamate ureas are also important platforms on which to construct useful PSMA binding compounds. Low's group prepared a series of ^{99m}Tc-labeled agents, one of which is **23** (Fig. 7), and optical agents **24** and **25** [210, 211]. All of those compounds showed specific uptake in PSMA-positive xenografts. An ^{18}F-labeled version, **26**, has also been prepared, but no imaging or biodistribution data has yet been reported [212].

Like aptamers, conjugates of small molecule PSMA inhibitors are also being investigated for targeted delivery of therapeutics and radiotherapeutics. Tethered **10** has been used to deliver drug loaded nanoparticles to PSMA positive cells [213]. Kozikowski has reported a urea-doxorubicin conjugate that retained its high binding affinity but had poor anti-tumor activity most likely due to the fact that the agent lacked a mechanism to release the active drug [214]. To address the drug release problem, Low's group has prepared a series of urea–pro-drug conjugates, such as **27**, containing a cleavable disulfide bond. Several of their conjugates demonstrated high affinity binding to PSMA and low nanomolar cytotoxicity to PSMA positive cells [215]. MIP-1375, a ^{131}I-labeled version of **14** inhibited the growth of PSMA positive xenografts [216], which suggests that targeted radiotherapy using small molecule PSMA inhibitors may be possible either with [^{131}I] or linker-tethered radiometal complexes.

PERSPECTIVE

While PSMA itself may not be a critical therapeutic target for prostate cancer, as the biology and significance of PSMA in this disease is still being elucidated, PSMA represents a validated target for delivering important vehicles – such as imaging and therapeutic agents. It can also report on other, arguably more important therapeutic targets, such as AR signaling [25]. It has been used to enhance vaccines against prostate cancer [217]. PSMA is often used as a target for the proof-of-principle for nano-agents [218]. It has been the target for enzymatically activated prodrugs, with one product in clinical trials [219]. Its expression in tumor neovasculature has not been extensively explored in non-prostate tumor imaging and therapy. Accordingly, in addition to its well characterized presence as GCPII in the CNS, PSMA in the periphery will likely continue to be leveraged for imaging and treating prostate as well as a variety of PSMA-expressing cancers and other tissues.

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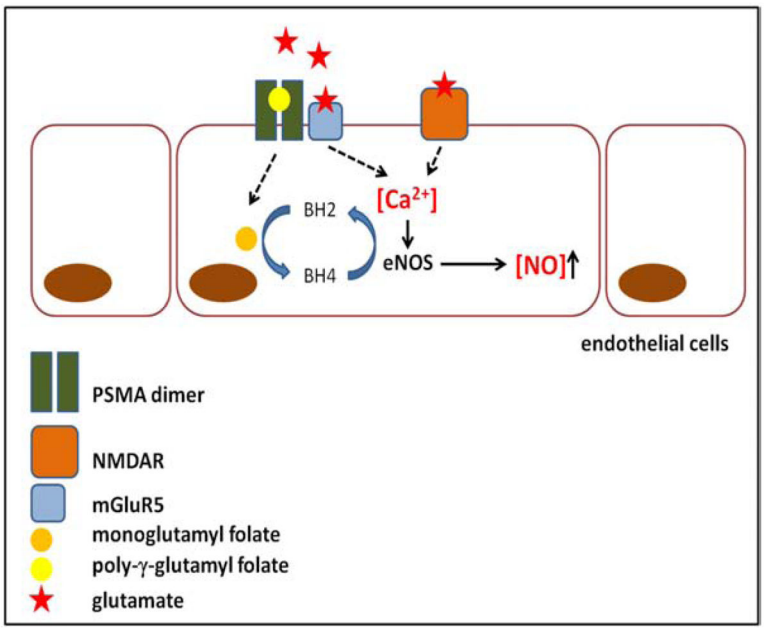


Fig. (1).

Diagram of PSMA-augmented generation of NO in nascent endothelial cells. PSMA folate hydrolase activity may boost NO production in two complementary ways including scavenging extra folate (orange circle) to increase BH4 eNOS cofactor turnover and generation of extra glutamate (stars), which raises intracellular calcium levels to increase expression of eNOS. NO is important for angiogenesis and expression of PSMA on neovasculature and may increase the efficiency of vessel formation.

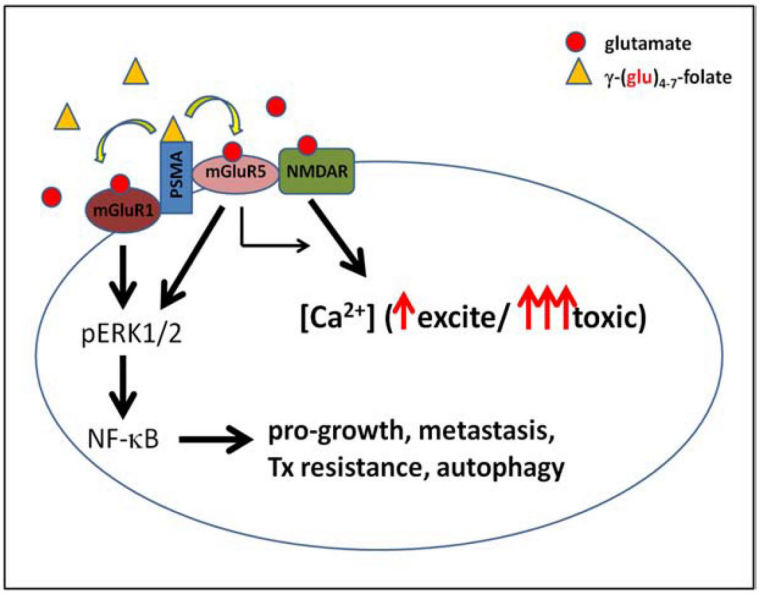


Fig. (2).

Diagram of the possible consequences of PSMA folate hydrolase activity in prostate cancer cells. Scavenged poly-γ-glutamyl folate substrates could feed nearby or interacting mGluR1/5 or NMDARs with released glutamates, maintaining both the downstream activation of NF-γB and raising intracellular calcium levels indirectly through mGluR5 signaling or directly through NMDAR channel opening. Small amounts of glutamate might be beneficial while large amounts of glutamate could cause excitotoxicity.

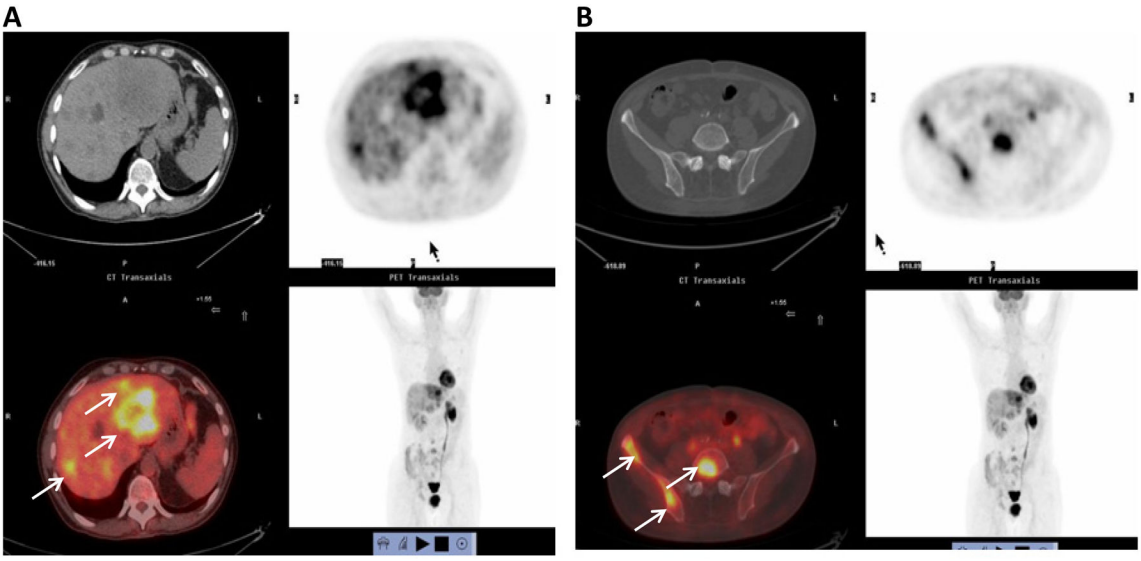


Fig. (3).

FDG PET showing FDG uptake at sites of widely metastatic disease in a patient with advanced castrate-resistant prostate cancer. (**A**) liver metastases (white arrows); (**B**) lumbar and right pelvic bone metastases (white arrows). (Johns Hopkins PET center).

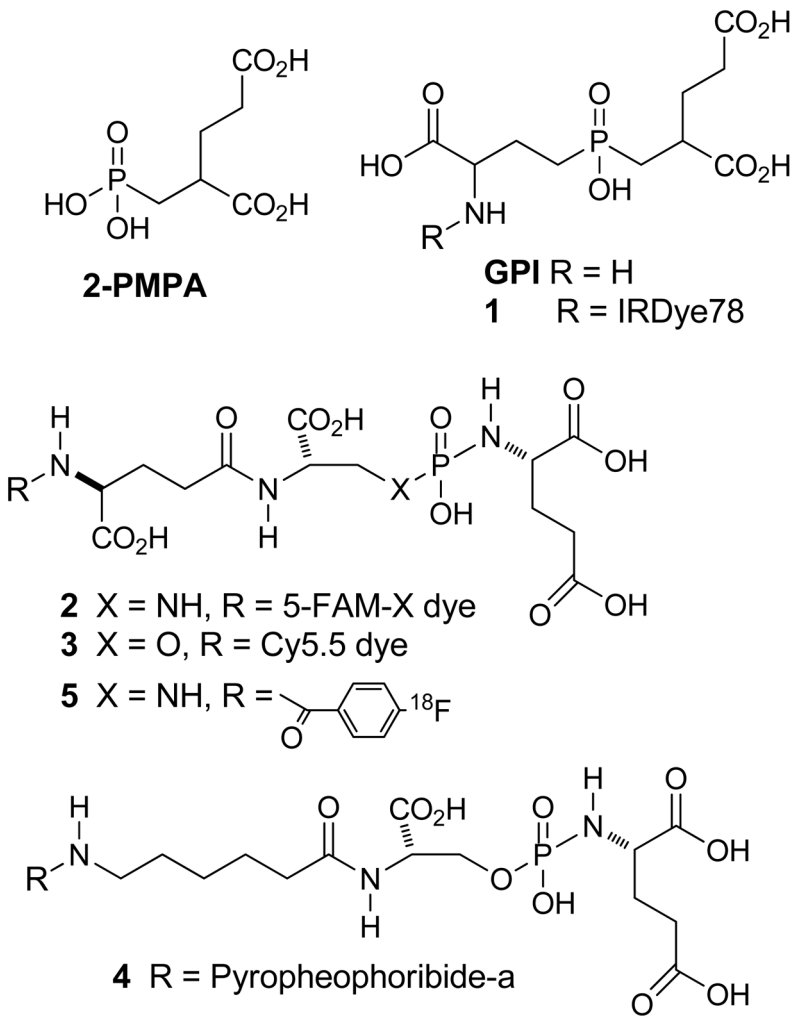


Fig. (4).
Phosphorus-containing GCPII imaging and therapeutic agents.

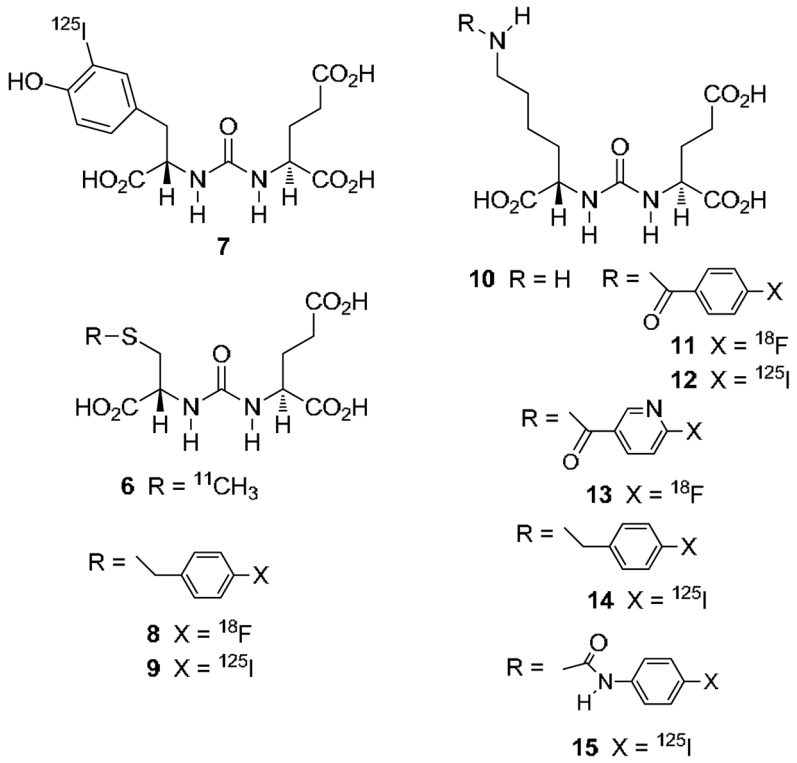
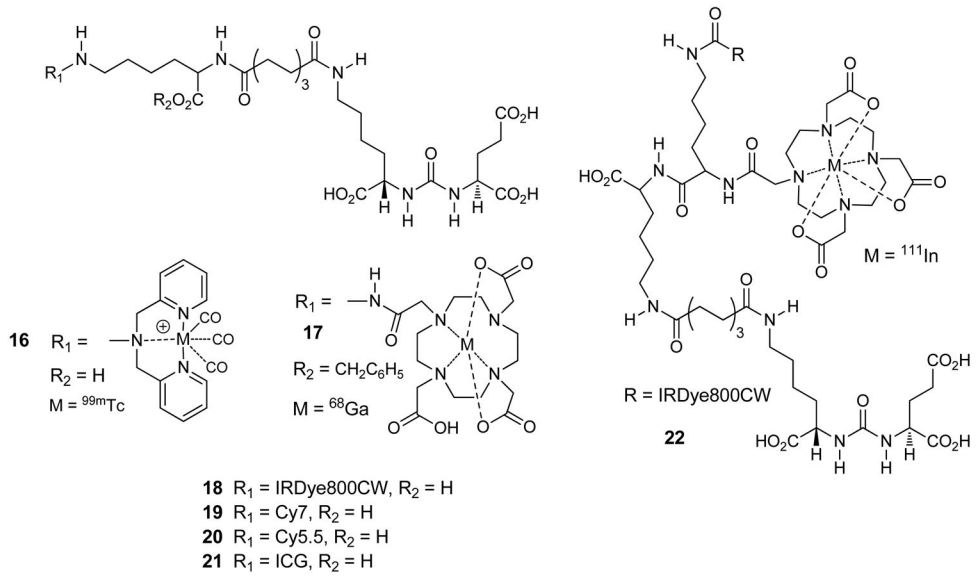


Fig. (5).
Urea-based GCPII imaging agents.



18 R_1 = IRDye800CW, R_2 = H
19 R_1 = Cy7, R_2 = H
20 R_1 = Cy5.5, R_2 = H
21 R_1 = ICG, R_2 = H

Fig. (6).
Optical and radiometallated GCPII imaging agents.

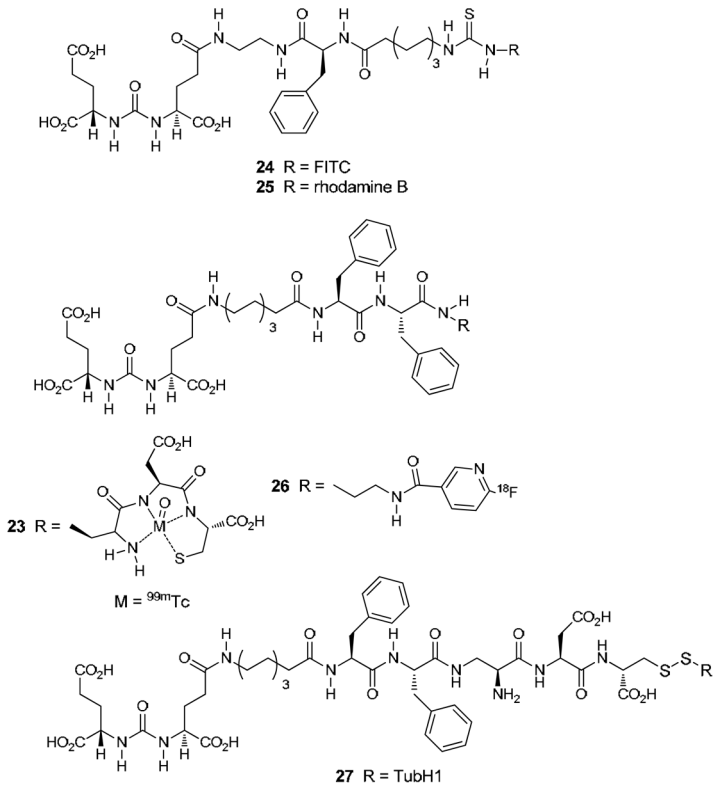


Fig. (7).
Additional urea-based imaging agents for GCPII.