Preclinical Evaluation of Novel Glutamate-Urea-Lysine Analogues That Target Prostate-Specific Membrane Antigen as Molecular Imaging Pharmaceuticals for Prostate Cancer

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Abstract

Prostate-specific membrane antigen (PSMA) is expressed in normal human prostate epithelium and is highly up-regulated in prostate cancer. We previously reported a series of novel small molecule inhibitors targeting PSMA. Two compounds, MIP-1072, (S)-2-(3-((S)-1-carboxy-5-(4-iodobenzylamino)pentylo)ureido)pentanedioic acid, and MIP-1095, (S)-2-(3-((S)-1-carboxy-5-(3-iodophenyl)ureido)pentylo)ureido)pentanedioic acid, were selected for further evaluation. MIP-1072 and MIP-1095 potently inhibited the glutamate carboxypeptidase activity of PSMA ($K_i = 4.6 \pm 1.6 \text{ nmol/L}$ and $0.24 \pm 0.14 \text{ nmol/L}$, respectively) and, when radiolabeled with $^{123}$I, exhibited high affinity for PSMA with a half-life of 166 minutes for LNCaP cells ($K_d = 3.8 \pm 1.3 \text{ nmol/L}$ and $0.81 \pm 0.39 \text{ nmol/L}$, respectively). The association of $^{123}$I-MIP-1072 and $^{123}$I-MIP-1095 with PSMA was specific. There was no binding to human prostate cancer PC3 cells, which lack PSMA, and binding was abolished by coincubation with a structurally unrelated NAALADase inhibitor, 2-(phosphonomethyl)pentanedioic acid (PMPA). $^{123}$I-MIP-1072 and $^{123}$I-MIP-1095 internalized into LNCaP cells at 37°C. Tissue distribution studies in mice showed 17.3 ± 6.3% of injected dose per gram of LNCaP xenograft tissue, for $^{123}$I-MIP-1072 and $^{123}$I-MIP-1095, respectively. $^{123}$I-MIP-1095 exhibited greater tumor uptake but slower washout from blood and nontarget tissues compared with $^{123}$I-MIP-1072. Specific binding to PSMA in vivo was observed in the LNCaP human adenocarcinoma xenograft model. PSMA-specific radiopharmaceuticals should provide a novel molecular targeting option for the detection and staging of prostate cancer.

Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in men in the United States (1). In 2009, it is estimated that 192,000 men will be diagnosed with prostate cancer and 27,000 men will die of the disease (1). Current diagnosis is typically through digital rectal exam and blood prostate-specific antigen (PSA) testing. Since the introduction of serum PSA screening, prostate cancer incidence rates have increased dramatically as have the number of men being treated for the disease (2). However, 20% to 30% of men with prostate cancer have serum PSA levels within the reference range, resulting in false negatives (3, 4), whereas others have elevated serum PSA levels due to conditions other than prostate cancer (i.e., benign prostatic hyperplasia), resulting in false positives and unnecessary biopsies (5). Because elevated serum PSA levels do not always correlate with disease, there is skepticism regarding the value of broad-based PSA testing with regard to predicting surgical cures (6). Initial results from the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial showed that annual PSA testing for 6 years and annual digital rectal exam testing for 4 years (performed in the same years as the first four PSA tests) did not reduce the number of deaths from prostate cancer through a median follow-up period of 11.5 years. These results suggest that many men were diagnosed with, and treated for, cancers that would not have been detected in their lifetime without screening and, as a consequence, were exposed to the potential harms of unnecessary treatments, such as surgery and radiation therapy (7). Therefore, accurate initial diagnosis and determination of the extent of disease continues to be a major challenge for selecting appropriate treatment options, monitoring the effects of therapeutic interventions, and detecting disease after recurrence. New agents that will more accurately diagnose and stage prostate cancer, as well as monitor therapy, will enable improved treatment planning and result in improved patient outcome.

Prostate-specific membrane antigen (PSMA), also known as folate hydrolase I or glutamate carboxypeptidase II, is a transmembrane, 750 amino acid, type II glycoprotein that is primarily expressed in prostate cancer, including metastatic disease (8–10). PSMA is an $\alpha$-linked acidic dipeptidase (NAALADase) with reactivity toward poly-$\gamma$-glutamyl folates and has the capability of sequentially removing the poly-$\gamma$-glutamyl terminus of dipeptides (11, 12). Because PSMA is expressed by virtually all prostate cancers and its expression is further increased in poorly differentiated, metastatic and hormone-refractory carcinomas (9), it is a very attractive target for prostate cancer imaging and therapy.

PSMA was originally identified as the ligand of the monoclonal antibody 7E11-C5, marketed as ProstaScint (Capromab Pendetide), with a histologic profile that showed a high degree of specificity for the LNCaP human adenocarcinoma cell line (10). ProstaScint is not in widespread use in part because it targets the intracellular domain of PSMA (amino terminus) and is believed to bind mostly necrotic...
portions of prostate tumors and not to viable tumor cells (13, 14). More recently, radiolabeled monoclonal antibodies that bind to the extracellular domain of PSMA have been developed and have been shown to accumulate in PSMA-positive prostate tumor models in animals (15). Early promising results from clinical trials have shown the utility of PSMA as a diagnostic and therapeutic target (16, 17). Although monoclonal antibodies hold promise for tumor detection and therapy, there have been limited clinical successes outside of lymphoma because of their long circulating plasma half-lives and low permeability in solid tumors, particularly in metastases to the bone. Lower molecular weight small molecules, with higher permeability in solid tumors, will likely have an advantage. In addition, small molecules will likely display improved pharmacokinetics in normal tissues compared with intact immunoglobulins, making lesion detection more conspicuous.

Recently, Maresca and colleagues (18) described the design and synthesis of a series of small molecule inhibitors of PSMA with the potential to diagnose and stage prostate cancers through commonly used molecular imaging modalities such as single photon emission computed tomography (SPECT). Here, we evaluate two of the most potent radiiodinated compounds, \(^{[123]}\)MIP-1072 and \(^{[123]}\)MIP-1095, for their ability to bind to PSMA and localize to PSMA expressing tumors in vivo.

**Materials and Methods**

Synthesis and radiolabeling of MIP-1072 and MIP-1095. The synthesis of MIP-1072 (S)-2-(3-(S)-1-carboxy-5-(4-iodobenzylamino)pentyl)ureido)pentanenitric acid and MIP-1095 (S)-2-(3-(S)-1-carboxy-5-(4-iodophenyl)ureido)pentanenitric acid, along with the radiolabeling precursors trimethyliminiumMIP-1072 and trimethyliminiumMIP-1095, and their subsequent radiolabeling with \(^{123}\)I were described previously (18). Briefly, radiolabeling was accomplished by iododestannylation of the trimethylstannyl precursors (S)-di-tert-butyl 2-(3-(S)-1-tert-butoxy-1-oxo-6-(4-(trimethylstannyl)benzylamino)hexan-2-yl)ureido)pentanodoate and (S)-di-tert-butyl 2-(3-(S)-1-tert-butoxy-1-oxo-6-(4-(trimethylstannyl)phenyl)ureido)hexan-2-yl)ureido)pentanodoate with 50 to 100 mCi of \(^{123}\)I NaI using acidic oxidizing conditions to form \(^{123}\)MIP-1072 and \(^{123}\)MIP-1095, respectively, in moderate radiochemical yields (50–70%) in as little as 10 min. The radiiodostannylidene afforded the \(^{123}\)I-labeled tri-tert-butyl esters that were purified using simple C18 Sep Pak columns and deprotected with trifluoroacetic acid to afford the desired radiiodinated inhibitors in >95% radiochemical purity. The specific activity was determined to be 2–4,000 mCi/μmol.

**NAAADase inhibition by MIP-1072 and MIP-1095.** The ability of nonradiolabeled MIP-1072 and MIP-1095 to inhibit the NAAADase activity of PSMA was tested in LNCaP cell lysates as previously described (19) with minor modifications. Briefly, LNCaP cells were collected, washed in 0.32 mol/L sucrose, and lysed in cold 50 mmol/L Tris-HCl (pH 7.4), 0.5% Triton X-100. The lysate was centrifuged at 20,000 × g for 10 min at 4°C. The radioiodostannylidene was reisolated in triplicate, incubated with 1 mol/L nonradiolabeled MIP-1072 or MIP-1095. Cells were then washed and the amount of radioactivity was measured on a γ-counter. Specific binding was calculated as the difference between total binding and nonspecific binding. The \(K_d\) and maximum number of binding sites (\(B_{max}\)) were determined by nonlinear regression analysis using GraphPad Prism software.

Internalization. LNCaP cells (3 × 10⁶ cells per well in 12-well plates in duplicate) were incubated in HBS [50 mmol/L HEPES (pH 7.5), 0.9% sodium chloride] at 4°C. Nonspecific binding was determined by adding 10 μmol/L nonradiolabeled MIP-1072 or MIP-1095. Cells were then washed and the amount of radioactivity was measured on a γ-counter. Specific binding was calculated as the difference between total binding and nonspecific binding. The \(K_d\) and maximum number of binding sites (\(B_{max}\)) were determined by nonlinear regression analysis using GraphPad Prism software.

Mouse tissue distribution. A quantitative analysis of the tissue distribution of \(^{[123]}\)MIP-1072 and \(^{[123]}\)MIP-1095 was performed in separate groups of male NCr-nu/nu mice bearing LNCaP or PC3 xenografts administered via the tail vein as a bolus injection (1.25 μCi/mouse at a specific activity of >1,000 mCi/μmol) in a constant volume of 0.035 ml. The animals (n = 5/time point) were euthanized by asphyxiation with carbon dioxide at 0.25, 1, 2, 4, 8, and 24 h after injection. To examine specificity, other mice (n = 5) were injected with 0.5 ml/mouse averatin (19 mg/ml). For in vivo biodistribution studies, the mice were anesthetized by an IP injection of 0.15 ml/mouse ketamine/xylazine (1:1). Mice were injected at the right flank in the right 0.25 ml of the cell suspension. Mice were used for tissue distribution studies when the tumors reached approximately 100 to 400 mm³. Male severe combined immunodeficient mice (Charles River Laboratories) were implanted with 1 × 10⁶ cells/mL suspended in HBSS (Sigma-Aldrich) behind the left shoulder (PC3 PIP) and right shoulder (PC3 fla).

**Mice tissue distribution.** A quantitative analysis of the tissue distribution of \(^{[123]}\)MIP-1072, \(^{[123]}\)MIP-1095, or ProstaScint (Cardinal Health) was performed in separate groups of male NCr-nu/nu mice bearing LNCaP or PC3 cell xenografts administered via the tail vein as a bolus injection (1.25 μCi/mouse at a specific activity of >1,000 mCi/μmol) in a constant volume of 0.035 ml. The animals (n = 5/time point) were euthanized by asphyxiation with carbon dioxide at 0.25, 1, 2, 4, 8, and 24 h after injection. To examine specificity, other mice (n = 5) were inoculated with 50 mg/kg PMAA and sacrificed at 2 h. Tissues were dissected, excised, weighed wet, and counted in an automated γ-counter. Tissue biodistribution results expressed as percent injected dose per gram of tissue (ID%/g) were determined.

**SPECT/CT imaging.** All in vivo experimental procedures were undertaken in compliance with United States laws governing animal experimentation and were approved by the Johns Hopkins University IACUC. Male Fox Chase severe combined immunodeficient mice were each implanted with either 5 × 10⁶ LNCaP cells, or 5 × 10⁶ PC3 PIP (PSMA-)
and PC-3 flu (PSMA-) cells on opposite flanks. When the tumors reached approximately 5 to 7 mm in diameter, mice were anesthetized using 1% isoflurane gas in oxygen flowing at 0.6 L/min before and during radiopharmaceutical injection. Mice were injected via the tail vein with 1 mCi of either [123I]MIP-1072 or [123I]MIP-1095 at a specific activity of >1,000 mCi/A mol. Mice bearing LNCaP tumors were imaged 4 h postinjection and mice bearing PC-3 PIP or flu tumors were imaged at 2 h postinjection. A Gamma Medica X-SPECT scanner equipped with two opposing low-energy 0.5-mm aperture pinholes and tunable computed tomography (CT) was used for all scans. Mice were scanned over 180° in 5.5°, 45-s increments. A CT scan was performed before scintigraphy for both anatomic coregistration and attenuation correction. Data were reconstructed and fused using commercial software from Gamma Medica, which includes a 2D-OSEM algorithm.

Results

MIP-1072 and MIP-1095 are potent inhibitors of NAALADase. The ability of MIP-1072 and MIP-1095 to inhibit the glutamate carboxypeptidase activity of PSMA was tested in LNCaP cellular lysates by monitoring the hydrolysis of [3H]-NAAG. The \( K_i \) values for MIP-1072 and MIP-1095 were 4.6 ± 1.6 nmol/L and 0.24 ± 0.14 nmol/L, respectively, indicating that both MIP-1072 and MIP-1095 are potent inhibitors of NAALADase enzymatic activity. The greater potency of MIP-1095 compared with MIP-1072 is consistent with competitive binding data (18). The structurally unrelated PSMA inhibitor, PMPA, was included as a positive control and displayed a \( K_i \) of 2.1 ± 0.1 nmol/L, in agreement with the \( K_i \) determined by Tiffany and colleagues (23).

\[^{123}I\]MIP-1072 and \[^{123}I\]MIP-1095 bind to cells expressing PSMA and are internalized. LNCaP and PC3 cells were incubated with \[^{123}I\]MIP-1072 or \[^{123}I\]MIP-1095 to examine the specificity for PSMA-expressing prostate cancer cells. Both compounds bound to LNCaP cells but not to the PSMA-deficient PC3 cells. Binding to LNCaP cells was inhibited by either nonradiolabeled compound or the structurally unrelated PSMA inhibitor, PMPA (Fig. 1A). Saturation binding analysis was conducted to determine the affinity of \[^{123}I\]MIP-1072 and \[^{123}I\]MIP-1095 for PSMA expressed on LNCaP cells. Cells were incubated with 30 to 300,000 pmol/L \[^{123}I\]MIP-1072 or \[^{123}I\]MIP-1095 to determine \( K_d \) and \( B_{max} \) (Fig. 1B). Consistent with the order of potency of the NAALADase inhibition assay, MIP-1095 was found to have greater affinity for PSMA than MIP-1072 (\( K_d \) = 0.81 ± 0.39 nmol/L and 3.8 ± 1.3 nmol/L, respectively). The \( B_{max} \) obtained with MIP-1072 was found to be 1,490 ± 60 fmol/10^6 cells (0.9 × 10^6 sites/cell) and the \( B_{max} \) obtained with MIP-1095 was found to be 1,680 ± 110 fmol/10^6 cells (1 × 10^6 sites/cell), consistent with the value obtained with the ProstaScint antibody (12).

Figure 1. A, binding of \[^{123}I\]MIP-1072 or \[^{123}I\]MIP-1095 to LNCaP and PC3 cells. Cells were incubated for 1 h with each compound in the absence or presence of unlabeled compound or PMPA. B, saturation binding analysis of \[^{123}I\]MIP-1072 and \[^{123}I\]MIP-1095. LNCaP cells were incubated at 4°C for 1 h with 30 to 300,000 pmol/L \[^{123}I\]MIP-1072 or \[^{123}I\]MIP-1095. The \( K_d \) and \( B_{max} \) were determined by nonlinear regression analysis. C, LNCaP cellular internalization of \[^{123}I\]MIP-1072 and \[^{123}I\]MIP-1095. LNCaP cells were incubated with 100 nmol/L radiolabeled compound for the indicated time, washed, and treated with a mild acid buffer to separate total bound (dashed lines) from internalized material (solid lines). The results are representative of two independent experiments.
To determine if $[^{123}\text{I}]$MIP-1072 and $[^{123}\text{I}]$MIP-1095 are internalized into LNCaP cells by endocytosis, cells were incubated with $[^{123}\text{I}]$MIP-1072 or $[^{123}\text{I}]$MIP-1095 for up to 2 h at 4°C and 37°C, and washed with a mild acid buffer to remove compound that is bound to the cell surface. Figure 1C depicts the total binding (dashed lines) of $[^{123}\text{I}]$MIP-1072 and $[^{123}\text{I}]$MIP-1095 and the acid insensitive binding, or internalized compound (solid lines) to LNCaP cells. The results showed a time-dependent increase in radioactivity associated with the cellular pellet at 37°C but not at 4°C, indicating internalization in a temperature-dependent manner. These results were confirmed by a saturation binding analysis at 37°C, which showed an elevation in the apparent $B_{\text{max}}$ of both compounds, indicating internalization (data not shown).

Table 1. Tissue distribution of $[^{123}\text{I}]$MIP-1072 and $[^{123}\text{I}]$MIP-1095 in NCr nude mice bearing LNCaP xenografts

<table>
<thead>
<tr>
<th>Tissue</th>
<th>0.25</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>2.59 ± 0.24</td>
<td>0.47 ± 0.15</td>
<td>0.21 ± 0.04</td>
<td>0.06 ± 0.03</td>
<td>0.06 ± 0.03</td>
<td>0.01 ± 0.0</td>
</tr>
<tr>
<td>Heart</td>
<td>1.25 ± 0.22</td>
<td>0.31 ± 0.08</td>
<td>0.19 ± 0.07</td>
<td>0.01 ± 0.02</td>
<td>0.05 ± 0.04</td>
<td>0.01 ± 0.05</td>
</tr>
<tr>
<td>Lungs</td>
<td>2.34 ± 0.61</td>
<td>0.86 ± 0.24</td>
<td>0.4 ± 0.19</td>
<td>0.08 ± 0.04</td>
<td>0.06 ± 0.03</td>
<td>0.05 ± 0.06</td>
</tr>
<tr>
<td>Liver</td>
<td>2.26 ± 0.56</td>
<td>2.22 ± 0.43</td>
<td>2.64 ± 0.63</td>
<td>2.17 ± 0.67</td>
<td>1.78 ± 0.4</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.28 ± 1.67</td>
<td>1.91 ± 0.88</td>
<td>0.75 ± 0.36</td>
<td>0.16 ± 0.12</td>
<td>0.18 ± 0.11</td>
<td>0.14 ± 0.1</td>
</tr>
<tr>
<td>Kidneys</td>
<td>90.1 ± 20.2</td>
<td>151 ± 26</td>
<td>159 ± 46</td>
<td>358 ± 18.7</td>
<td>21.5 ± 25.5</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.69 ± 0.17</td>
<td>0.26 ± 0.03</td>
<td>0.21 ± 0.06</td>
<td>0.11 ± 0.05</td>
<td>0.21 ± 0.08</td>
<td>0.11 ± 0.05</td>
</tr>
<tr>
<td>Lg intestine</td>
<td>0.41 ± 0.08</td>
<td>0.15 ± 0.04</td>
<td>0.28 ± 0.12</td>
<td>0.48 ± 0.13</td>
<td>0.72 ± 0.22</td>
<td>0.37 ± 0.18</td>
</tr>
<tr>
<td>Sm intestine</td>
<td>0.83 ± 0.1</td>
<td>0.41 ± 0.12</td>
<td>0.35 ± 0.1</td>
<td>0.24 ± 0.07</td>
<td>0.27 ± 0.06</td>
<td>0.07 ± 0.02</td>
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<tr>
<td>Testes</td>
<td>0.91 ± 0.24</td>
<td>0.58 ± 0.19</td>
<td>0.43 ± 0.13</td>
<td>0.26 ± 0.17</td>
<td>0.18 ± 0.14</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>Sk muscle</td>
<td>0.8 ± 0.18</td>
<td>0.35 ± 0.17</td>
<td>0.2 ± 0.1</td>
<td>0.08 ± 0.1</td>
<td>0.02 ± 0.04</td>
<td>0.08 ± 0.04</td>
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<tr>
<td>Bone</td>
<td>0.69 ± 0.15</td>
<td>0.25 ± 0.07</td>
<td>0.17 ± 0.08</td>
<td>0.1 ± 0.18</td>
<td>0.04 ± 0.04</td>
<td>0.03 ± 0.05</td>
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<tr>
<td>Brain</td>
<td>0.08 ± 0.02</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0 ± 0</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
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<tr>
<td>Adipose</td>
<td>1.45 ± 0.27</td>
<td>0.74 ± 0.28</td>
<td>0.46 ± 0.27</td>
<td>0.12 ± 0.16</td>
<td>0.04 ± 0.11</td>
<td>0.01 ± 0.11</td>
</tr>
<tr>
<td>Tumor</td>
<td>12.9 ± 3.3</td>
<td>17.4 ± 6.3</td>
<td>14.0 ± 6.9</td>
<td>13.7 ± 3.1</td>
<td>9.74 ± 3.48</td>
<td>5.03 ± 1.35</td>
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$[^{125}\text{I}]$MIP-1072 and $[^{125}\text{I}]$MIP-1095 localize to PSMA-expressing tissues in vivo. The tissue distribution of $[^{123}\text{I}]$MIP-1072 and $[^{123}\text{I}]$MIP-1095 was assessed in NCr-nu/nu mice bearing LNCaP xenografts and the results are illustrated in Table 1. The radiolabel was detected at varying levels in all tissues examined and generally decreased over time. At 24 hours, blood and nontarget tissues were at or below the limits of detection in mice injected with $[^{123}\text{I}]$MIP-1072 or $[^{123}\text{I}]$MIP-1095. As anticipated, $[^{123}\text{I}]$MIP-1072 and $[^{123}\text{I}]$MIP-1095 uptake and exposure was greatest in the kidney, which has been shown to express high levels of NAALADase (9), and in PSMA-positive LNCaP xenografts. Peak kidney accumulation for $[^{123}\text{I}]$MIP-1072 was 159 ± 46%ID/g at 2 hours and peak LNCaP xenograft accumulation was 17.4 ± 6.3%ID/g at 1 hour. Peak kidney accumulation for $[^{125}\text{I}]$MIP-1095 was 88.7 ± 23.8%ID/g at 2 hours.

NOTE: Data are %ID/g, expressed as mean ± SD.
Abbreviations: Lg intestine, large intestine; Sm intestine, small intestine; Sk muscle, skeletal muscle.
The tissue distribution of the radiolabeled antibody, ProstaScint, was compared with \([^{123}I]\)MIP-1072 and \([^{123}I]\)MIP-1095 (Table 2). Clearance of ProstaScint from blood and nontarget tissues was much slower than what was observed for the small molecules. LNCaP xenograft tumor tissue continued to accumulate ProstaScint over time with peak accumulation of 31.4 ± 20.7%ID/g at the 72-hour time point (the latest time point studied). Unlike \([^{123}I]\)MIP-1072 and \([^{123}I]\)MIP-1095, ProstaScint did not accumulate in kidney tissue to an appreciable extent consistent with previously reported data (15, 26) despite the fact that immunohistochemistry and reverse transcription-PCR have shown PSMA to be expressed in the proximal tubules of kidney (9, 27). This may be due to the fact that ProstaScint binds an internal epitope of PSMA (13, 14), or, because of their size, antibodies do not penetrate tissues well and are not filtered by glomeruli. Despite the high accumulation in the LNCaP xenograft, the widespread use of ProstaScint as a diagnostic tool for prostate cancer has been limited in part by the lack of tissue specificity for the antibody, thereby giving rise to high non-specific background accumulation in other tissues. The LNCaP xenograft between 1 and 24 hours was slower with \([^{123}I]\)MIP-1095 than with \([^{123}I]\)MIP-1072 (Table 2). Clearance from the LNCaP xenograft between 1 and 24 hours was 34.3 ± 12.7%ID/g at 4 hours. Clearance from the LNCaP xenograft between 1 and 24 hours was slower with \([^{123}I]\)MIP-1095 than with \([^{123}I]\)MIP-1072 (P < 0.05). There was no significant difference in the level of \([^{123}I]\)MIP-1095 in the LNCaP xenograft between 1 and 24 hours (P > 0.06). \([^{123}I]\)MIP-1095 showed a slower clearance from blood and most organs compared with \([^{123}I]\)MIP-1072 (P < 0.05 for blood, heart, lungs, liver, spleen, kidneys, stomach, intestines, and testes between 1 and 8 hours) with a greater proportion of \([^{123}I]\)MIP-1095 cleared via the hepatobiliary route when compared with \([^{123}I]\)MIP-1072. Little uptake was detected in the brain, which exhibits high NAALADase activity (23), indicating that \([^{123}I]\)MIP-1072 and \([^{123}I]\)MIP-1095 do not cross the blood-brain barrier. Minimal deiodination was observed for both compounds as the thyroid contained <1% of the total injected dose at all time points. Because mouse prostate tissue does not express PSMA (24, 25), their prostates were not included in the analysis.

### Table 2. Tissue distribution of ProstaScint in NCr nude mice bearing LNCaP xenografts

<table>
<thead>
<tr>
<th>Tissue</th>
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<th>24</th>
<th>48</th>
<th>72</th>
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<tbody>
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<td>Blood</td>
<td>30.8 ± 7.6</td>
<td>29 ± 5.2</td>
<td>15.5 ± 5</td>
<td>11.8 ± 0.9</td>
<td>10.8 ± 7.5</td>
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<tr>
<td>Heart</td>
<td>8.47 ± 1.17</td>
<td>7.87 ± 0.99</td>
<td>4.39 ± 1.08</td>
<td>2.7 ± 0.37</td>
<td>1.76 ± 0.36</td>
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<td>Lungs</td>
<td>19.46 ± 2.65</td>
<td>15.07 ± 2.85</td>
<td>9.04 ± 2.52</td>
<td>6.57 ± 1.89</td>
<td>4.75 ± 0.73</td>
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<tr>
<td>Liver</td>
<td>16.7 ± 4.5</td>
<td>14.1 ± 2</td>
<td>10.2 ± 1.8</td>
<td>8.31 ± 1.8</td>
<td>7.87 ± 1.31</td>
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<tr>
<td>Spleen</td>
<td>9.96 ± 2.77</td>
<td>8.91 ± 2.44</td>
<td>8.61 ± 1.44</td>
<td>8.62 ± 1.87</td>
<td>8.39 ± 3.81</td>
</tr>
<tr>
<td>Kidneys</td>
<td>8.7 ± 1.5</td>
<td>8.7 ± 1.1</td>
<td>9 ± 1.2</td>
<td>9.8 ± 1</td>
<td>10.9 ± 3.4</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.05 ± 0.25</td>
<td>1.26 ± 0.53</td>
<td>1.08 ± 0.41</td>
<td>0.82 ± 0.13</td>
<td>0.77 ± 0.25</td>
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<tr>
<td>Lg intestine</td>
<td>0.76 ± 0.12</td>
<td>1.47 ± 0.34</td>
<td>2.68 ± 0.89</td>
<td>1.49 ± 0.91</td>
<td>1.01 ± 0.34</td>
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<tr>
<td>Sm intestine</td>
<td>2.44 ± 0.5</td>
<td>3.23 ± 0.23</td>
<td>2.07 ± 0.44</td>
<td>1.82 ± 0.91</td>
<td>1.49 ± 0.29</td>
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<tr>
<td>Testes</td>
<td>1.73 ± 0.35</td>
<td>2.82 ± 0.39</td>
<td>1.96 ± 0.56</td>
<td>1.55 ± 0.2</td>
<td>1.45 ± 0.54</td>
</tr>
<tr>
<td>Sk.muscle</td>
<td>0.85 ± 0.57</td>
<td>0.66 ± 0.18</td>
<td>0.96 ± 0.28</td>
<td>0.84 ± 0.08</td>
<td>0.76 ± 0.19</td>
</tr>
<tr>
<td>Bone</td>
<td>2.42 ± 0.59</td>
<td>2.58 ± 0.86</td>
<td>2.04 ± 0.47</td>
<td>1.64 ± 0.36</td>
<td>1.34 ± 0.4</td>
</tr>
<tr>
<td>Brain</td>
<td>0.66 ± 0.13</td>
<td>0.96 ± 0.69</td>
<td>0.38 ± 0.13</td>
<td>0.26 ± 0.02</td>
<td>0.23 ± 0.09</td>
</tr>
<tr>
<td>Adipose</td>
<td>2.13 ± 0.49</td>
<td>1.54 ± 0.71</td>
<td>2.16 ± 0.31</td>
<td>1.93 ± 0.52</td>
<td>1.57 ± 0.46</td>
</tr>
<tr>
<td>Tumor</td>
<td>3.33 ± 1.13</td>
<td>7.92 ± 3.17</td>
<td>21.8 ± 10.2</td>
<td>27.8 ± 10.4</td>
<td>31.4 ± 20.1</td>
</tr>
</tbody>
</table>

NOTE: Data are %ID/g, expressed as mean ± SD.

### Table 3. Tumor to tissue ratios of \([^{123}I]\)MIP-1072, \([^{123}I]\)MIP-1095, and ProstaScint in NCr nude mice bearing LNCaP xenografts

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ratio</th>
<th>0.25</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>([^{123}I])MIP-1072</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor/blood</td>
<td>4.97</td>
<td>37.1</td>
<td>65.4</td>
<td>220</td>
<td>176</td>
<td>411</td>
<td></td>
</tr>
<tr>
<td>Tumor/skeletal muscle</td>
<td>16.2</td>
<td>50.0</td>
<td>70.2</td>
<td>182</td>
<td>446</td>
<td>64.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>([^{123}I])MIP-1095</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor/blood</td>
<td>2.10</td>
<td>10.6</td>
<td>24.3</td>
<td>40.6</td>
<td>57.5</td>
<td>174</td>
<td></td>
</tr>
<tr>
<td>Tumor/skeletal muscle</td>
<td>8.79</td>
<td>49.3</td>
<td>80.8</td>
<td>133</td>
<td>121</td>
<td>304</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ratio</th>
<th>1</th>
<th>4</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProstaScint</td>
<td>0.11</td>
<td>0.27</td>
<td>1.41</td>
<td>2.37</td>
<td>2.92</td>
<td></td>
</tr>
<tr>
<td>Tumor/skeletal muscle</td>
<td>3.93</td>
<td>12.0</td>
<td>22.8</td>
<td>33.1</td>
<td>41.2</td>
<td></td>
</tr>
</tbody>
</table>
imaging agent is confounded by the high accumulation in non-target tissues. This is evident in the poor tumor/blood and tumor/skeletal muscle ratios at the time points measured (Table 3). $^{[123]}$I-MIP-1072 and $^{[123]}$I-MIP-1095 exhibit tumor/blood and tumor/skeletal muscle ratios greater than 100 after only a few hours, whereas the tumor/blood ratio of ProstaScint reached 3 only after 72 hours, the final time point of the analysis.

Consistent with the tissue distribution studies, SPECT/CT imaging at 4 hours after injection of $^{[123]}$I-MIP-1072 and $^{[123]}$I-MIP-1095 revealed high uptake and selectivity for PSMA-expressing tissues: kidney and LNCaP tumor (Fig. 2, top). Additionally, $^{[123]}$I-MIP-1072 and $^{[123]}$I-MIP-1095 detected PC3 PIP (PSMA +) but not PC3 flu (PSMA −) tumors by SPECT/CT at 2 hours after injection (Fig. 2, bottom), indicating that the uptake is specific to PSMA and is not related to blood flow or permeability differences between cell lines. As anticipated, high uptake was also observed in the kidneys, which express PSMA.

$^{[123]}$I-MIP-1072 and $^{[123]}$I-MIP-1095 bind specifically to PSMA in vivo. To examine the specificity of targeting PSMA in vivo, NCr-nu/nu mice bearing either LNCaP or PC3 xenografts were coinjected with $^{[123]}$I-MIP-1072 or $^{[123]}$I-MIP-1095 and 50 mg/kg of the PSMA inhibitor, PMPA. Both $^{[123]}$I-MIP-1072 and $^{[123]}$I-MIP-1095 localized to PSMA-expressing LNCaP tumors but not to the PSMA-deficient PC3 tumors. In addition, binding to the LNCaP tumor xenografts and the kidneys was blocked by coinjecting the mice with 50 mg/kg PMPA (Fig. 3).

**Discussion**

We describe here the preclinical evaluation of two novel potential radiopharmaceuticals, $^{[123]}$I-MIP-1072 and $^{[123]}$I-MIP-1095, which...
were designed to target PSMA in prostate cancer cells and tissue. PSMA is expressed in normal prostate, brain, kidney proximal tubules, and intestinal brush border membranes. Importantly, expression is dramatically up-regulated in poorly differentiated, metastatic and hormone-refractory carcinomas (9) as well as after androgen deprivation therapy (28) and in lymph node metastases (29). The function of PSMA in prostate cancer is unclear, although it is reported to play a role in tumor invasiveness (30). It has been reported that increased expression of PSMA in primary prostate cancer correlates with other adverse traditional prognostic factors and independently predicts disease outcome (31, 32). Numerous studies have shown its utility as a diagnostic marker and therapeutic target with a >90% prevalence in disease (9, 33). Additionally, PSMA is expressed in the endothelial cells of tumor neovascularure of many solid tumors (21, 27), indicating that it may have utility as a diagnostic or therapeutic molecular target in cancers other than prostate.

Several imaging modalities are currently being collectively used for the diagnosis, staging, and prognosis of prostate cancer metastases. Conventional cross-sectional imaging with CT and magnetic resonance imaging rely on anatomic changes (lesions, >1 cm) often resulting in missed lymph node metastases. Nodal enlargement due to metastases occurs relatively late in the progression of prostate cancer, and therefore, neither CT nor magnetic resonance imaging is effective at detecting the often-microscopic lymph node metastases. Radionuclide bone scans are commonly used for monitoring bone metastases. However, false positives are common as a result of inflammation, previous bone injuries, and arthritis, and are especially problematic in older men (34). Therefore, the need exists for new methodologies of not only detecting the primary tumor, but metastatic lesions as well.

Molecular imaging, which relies on signal from a radiotracer that binds specifically to a biochemical marker on tumor cells rather than anatomic features, may provide a means to detect both primary cancer and metastases. SPECT and positron emission tomography are two methods commonly used to provide biochemical information through molecular imaging. $^{18}$F-fluorodeoxyglucose is a glucose analogue that is readily taken up by hypermetabolic cancer cells and is an efficient means to detect many solid tumors (35–37), but is not effective in most primary prostate tumors and metastases due to the low glycolytic rate of prostate cancer (38, 39). Thus, we have undertaken an effort to improve the diagnosis and staging of prostate cancer by developing molecules that target a cancer-specific biochemical marker, PSMA, for imaging by conventional SPECT technology.

$[^{123}I]$MIP-1072 and $[^{123}I]$MIP-1095 were shown here to bind specifically and with high affinity to PSMA (+) LNCaP cells but not to PSMA (-) PC3 cells. Both compounds internalized in prostate cancer cells that express PSMA in a time-dependent, acid-insensitive manner. Because cellular internalization was shown at $37\text{ }^\circ\text{C}$ but not $4\text{ }^\circ\text{C}$, it is believed that it occurs via the endocytotic pathway. Recently, other PSMA-specific inhibitors (40) and antibodies (12) have been reported to be internalized through endocytosis as well. The saturation binding and NAAADase inhibition studies revealed an ~5-fold greater affinity of MIP-1095 over MIP-1072. This is likely a result of additional hydrophobic contacts outside of the PSMA binding pocket. The elucidation of the cocrystal structure of PSMA with both substrates and inhibitors revealed that electrostatic interactions between the carboxylic acids of the glutamic and aspartic acid residues and Arg 534/Arg536, and Asn519 of PSMA are critical for binding (41). In addition, there is a hydrophobic pocket accessory to the active site that may be exploited in the rational design of inhibitors. This information led us to design inhibitors based on a glutamate-urea-X heterodimer structural motif where X corresponds to an α amino acid. These molecules contain the three carboxylic acid groups...
required for binding to PSMA. The urea functional group interacts with the Zn²⁺ containing active site and the side chains of Tyr552 and His553. Substantial differences in affinity have been reported for other halogenated glutamate-urea-lysine heterodimers of this series as a consequence of the nature of the halogen and position of the halogen on the aryl ring (18). Several other glutamate-urea-X dimers with high affinity and selectivity for PSMA-expressing cells and xenografts have also been described (42–48).

[123I]MIP-1072 and [123I]MIP-1095 exhibited peak LNCaP tumor uptake of 17.3 ± 6.3 (at 1 hour) and 34.3 ± 12.7 (at 4 hours) % ID/g, respectively. However, high uptake was also observed in the mouse kidneys, which could be blocked by the structurally unrelated PSMA inhibitor, PMPA, indicating that the uptake was mediated by specific binding to PSMA. Several reports have confirmed that PSMA is expressed in the mouse kidneys (25, 49), and similar results have been shown with other agents targeting PSMA (43–46). Neither compound accumulated in the brain to an appreciable extent indicating that they do not cross the blood-brain barrier and are unlikely to interfere with the physiologic NAALADase activity of glutamatergic neurotransmission. In this regard, most imaging radiopharmaceuticals do not elicit pharmacologic responses as the actual mass of compound administered is typically at tracer levels. In addition, although PSMA is expressed in the human prostate and kidneys, a defined physiologic role has yet to be established so it is difficult to predict the effect that [123I]MIP-1072 and [123I]MIP-1095 may have a priori.

As ProstaScint is the only Food and Drug Administration–approved imaging agent used exclusively for the detection of prostate cancer, we sought to compare the tissue distribution of [123I]MIP-1072 and [123I]MIP-1095 with ProstaScint in LNCaP-bearing xenograft mice. All three compounds localized to PSMA-expressing LNCaP xenografts but with very different pharmacokinetic profiles. [123I]MIP-1072 is cleared more rapidly from target and nontarget tissues and primarily through urinary excretion, whereas [123I]MIP-1095 is cleared by both urinary and hepatobiliary routes. The differences in the clearance profiles do not seem to be related to metabolism as both compounds are stable in liver microsomes and blood plasma (data not shown). ProstaScint, like most antibodies, clears from the blood very slowly with peak accumulation in the LNCaP xenograft at the latest time point studied, resulting in a low tumor/background ratio and prolonged total body exposure to radiation. One other disadvantage of ProstaScint is that it targets the intracellular domain of PSMA (13, 14), and because antibodies do not readily cross the cell membrane; it is thought that it binds only to necrotic cells of prostate tumors. More recently, however, anti-PSMA monoclonal antibodies that target an extracellular domain of PSMA, e.g., J591, have entered into clinical trials (50). We have designed small molecules with affinities similar to that of ProstaScint and J591, but with enhanced ability to diffuse into the extravascular space and with faster blood clearance. [122I]MIP-1072 and [122I]MIP-1095 retain high accumulation in PSMA-expressing xenografts as evident by the tissue distribution results and the SPECT/CT images. Thus, we believe radiolabeled small molecule radiotherapeutics that bind PSMA offer the preferred approach. Nonetheless, ProstaScint and J591 have validated PSMA as an excellent target for the molecular imaging of prostate cancer.

Although the initial focus here is on using [125I]MIP-1072 and [125I]MIP-1095 for the detection of prostate cancer, it may be possible to substitute [131I] for targeted radiotherapy of prostate cancer. [131I] is commonly used in the treatment of thyroid cancer as it emits high-energy (606 keV) β particles capable of ablating tumors. Of course, the potential for radiotherapy will depend upon the dose to nontarget tissues, in particular, the kidneys, which based on the data presented here are likely to be dose limiting. PSMA has been shown by immunohistologic techniques to be expressed in human kidneys (9, 27). However, there is no information on the level of expression in human kidneys compared with mouse kidneys. A phase I clinical trial is currently under way to evaluate [123I]MIP-1072 and [123I]MIP-1095 in patients with metastatic prostate cancer. If the extrapolated dosimetry data from this trial implies that the radiation to nontarget organs and tissues is tolerable, radiolabeling either MIP-1072 or MIP-1095 with [131I] could be a powerful tool in the eradication of prostate cancer, whereby patients with positive [125I]MIP-1072 or [125I]MIP-1095 diagnostic scans may then be treated with the same compound radiolabeled with [131I].

In conclusion, there currently exists a grave unmet medical need for new imaging modalities to assist physicians in selecting appropriate treatment regimens for prostate cancer and improving patient outcomes. We believe that the widespread availability of low-molecular-weight radiotherapeutics such as [125I]MIP-1072 and [125I]MIP-1095, which may be capable of detecting both the primary prostate cancer as well as soft tissue and bone metastases, will not only satisfy this critical unmet need, but could alter the current paradigm for the detection and staging of prostate cancer and offer a unique opportunity to follow response to systemic therapies by noninvasive external imaging. Patient management would be significantly improved as these molecular imaging pharmaceuticals are designed to track specifically both the location and progression of prostate tumor metastases through their PSMA expression.

Disclosure of Potential Conflicts of Interest

This work was conducted at Molecular Insight Pharmaceuticals, Inc., and Johns Hopkins University, S.M. Hillier, K.P. Maresca, F.J. Femia, J.C. Marquis, C.N. Zimmerman, J.A. Barrett, J.L. Joyal, and J.W. Babich are employees of Molecular Insight Pharmaceuticals, Inc. W.C. Eckelman and M.G. Pomper are consultants for Molecular Insight Pharmaceuticals, Inc.

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