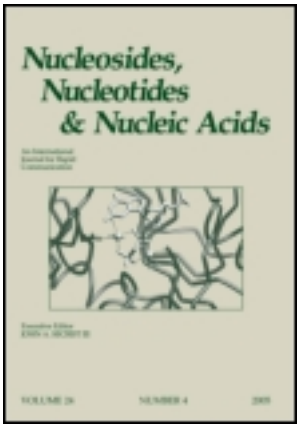


Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lncn20

Structure-Activity Relationships of 2′,5′-Oligoadenylate Analogue Modifications of Prostate-Specific Membrane Antigen (PSMA) Antagonists

Xinning Wang [a], Haibin Tian [b], Zhenghong Lee [b] & Warren D. W. Heston [a]

[a] Department of Cancer Biology, Cleveland Clinic, Cleveland, Ohio, USA

[b] Department of Radiology, Case Western Reserve University, Cleveland, Ohio, USA

Published online: 12 Apr 2012.

To cite this article: Xinning Wang , Haibin Tian , Zhenghong Lee & Warren D. W. Heston (2012): Structure-Activity Relationships of 2′,5′-Oligoadenylate Analogue Modifications of Prostate-Specific Membrane Antigen (PSMA) Antagonists, Nucleosides, Nucleotides and Nucleic Acids, 31:5, 432-444

To link to this article: http://dx.doi.org/10.1080/15257770.2012.671988

Nucleosides, Nucleotides and Nucleic Acids, 31:432–444, 2012

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257770.2012.671988

Taylor & Francis
Taylor & Francis Group

STRUCTURE-ACTIVITY RELATIONSHIPS OF 2′,5′-OLIGOADENYLATE ANALOGUE MODIFICATIONS OF PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA) ANTAGONISTS

Xinning Wang,[1] Haibin Tian,[2] Zhenghong Lee,[2] and Warren D. W. Heston[1]
[1]*Department of Cancer Biology, Cleveland Clinic, Cleveland, Ohio, USA*
[2]*Department of Radiology, Case Western Reserve University, Cleveland, Ohio, USA*

□ *Prostate-specific membrane antigen (PSMA) is an ideal biomarker for prostate cancer. A previously reported 2-5A conjugate RBI1033 (**3**) showed binding affinity more than 10 times higher than the parent urea-based compound (S)-2-(3-((S)-5-amino-1-carboxypentyl)ureido) pentanedioic acid (**1**). The purpose of this work is to further optimize the structure of **3** to identify highly selective ligands of PSMA. It was found that conjugates having 2-5A in their structure showed extraordinary improved binding affinity to PSMA compared with compound **1**. Removal of 2-5A significantly reduced its biological activity. The results will provide a path to agents for targeted imaging and treatment of prostate cancer.*

Keywords PSMA; 2′, 5′-oligoadenylate conjugate; structure-activity relationship

ABBREVIATIONS

PSMA	prostate-specific membrane antigen
Rt	retention time
r.t.	room temperature
SMCC	succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate
2-5A	2′,5′-oligoadenylate

INTRODUCTION

According to the American Cancer Society, prostate cancer is the most prevalent cancer in American males and represents their second leading

Received 16 September 2011; accepted 27 February 2012.
This work was supported by grants from Department of Defense (DOD) (W81XWH-07-1-0656, PI: W. D. W. Heston; W81XWH-05-1-0600, PI: X. Wang), and in part by grant NIH/NCI U24 CA110943 (PI: J. Duerk). The authors also thank Dr. Steve Shin-lin Huang at Cleveland Clinic for help with the manuscript.
Address correspondence to Warren D. W. Heston, Department of Cancer Biology, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195, USA. E-mail: hestonw@ccf.org.

cause of cancer-related death. Prostate cancer kills more than 200,000 men annually worldwide. About 241,740 new cases of prostate cancer will be diagnosed, and about 28,170 men will die of the disease in the United States in 2012.[1] Prostate-specific membrane antigen (PSMA) is a type II transmembrane protein consisting of 750 amino acids and having a molecular weight of ~110,000.[2,3] Immunohistochemical analysis of pathological specimens indicates that PSMA is expressed by virtually all prostate cancers, and its expression is further increased in poorly differentiated, metastatic, and hormone-refractory carcinomas.[2,4,5] PSMA expression has also been found in a variety of tumor, but not normal, vascular endothelium, which further broadens its interest and potential applications in anti-angiogenic therapies.[6,7] In contrast to other well-known prostate-restricted molecules such as prostate-specific antigen (PSA) that are secretory proteins, PSMA is a type II integral cell-surface membrane protein that is not secreted.[6] With its abundant expression in most prostate cancers, PSMA would therefore appear to be an ideal target for diagnosis and therapy.

The development of small molecules for targeted imaging and treatment of prostate cancer has been based on the identification of enzymatic activity of PSMA. PSMA cleaves alpha-linked glutamates from *N*-acetylaspartylglutamate.[8] It also has a unique folate hydrolase activity which can remove the gamma-linked terminal glutamates from folate in a sequential fashion.[9] Highly potent ligands targeting PSMA have been described recently.[10–14] To date, some of the best low weight inhibitors are highly charged water-soluble agents which include the di-acid of glutamic acid as part of their structure. One of the leading compounds is (S)-2-(3-((S)-5-amino-1-carboxypentyl)ureido)pentanedioic acid (**1** in Figure 1) containing a central urea group.[11] It can be easily modified without compromising activity through the –SH group. Selective imaging of PSMA-expressing tumor xenografts has been reported by radiolabeling of the urea-based PSMA inhibitors.[15–20] Recently, a 2′,5′-oligoadenylate (2-5A) containing urea-based analogue *RBI1033* (**3** in Figure 1) has been synthesized,[21] which showed binding activity 10 times better than compound **1** itself. The purpose of this study is to perform structure-activity relationship studies of **3** to identify highly selective ligands of PSMA that can be subsequently coupled to imaging agents for systemic delivery to localized, primary and disseminated, metastatic prostate tumors.

RESULTS AND DISCUSSION

Chemistry

Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, **5** in Scheme 1) is a heterobifunctional linker. It can covalently conjugate to amine- and sulfhydryl-containing molecules through its

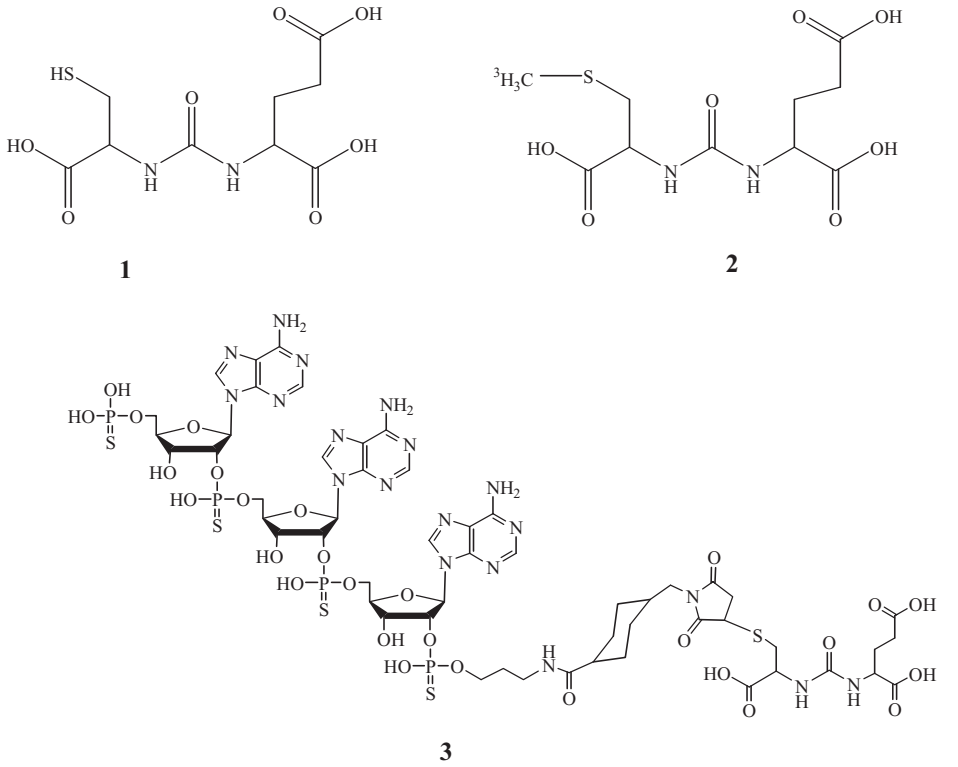
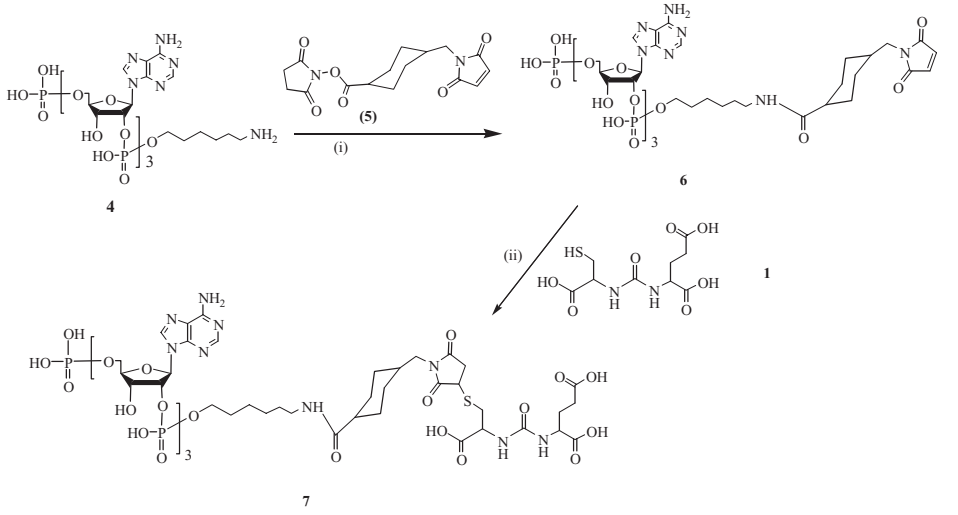
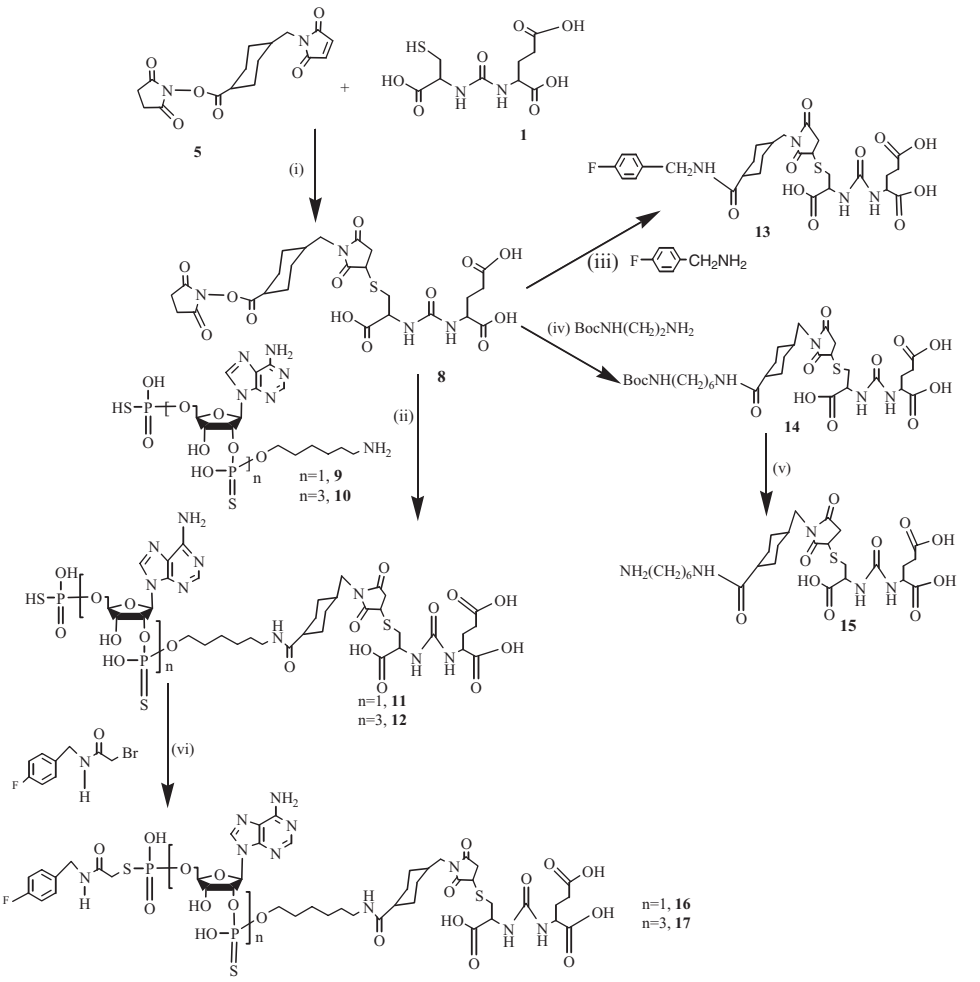


FIGURE 1 Structures of urea-based PSMA inhibitors **1–3**.



SCHEME 1 Synthetic pathway of compound **7**. (i) SMCC (**5**), 100 mM phosphate buffer, pH 7.0, r.t. overnight; (ii) (S)-2-(3-((S)-5-amino-1-carboxypentyl)ureido)pentanedioic acid (**1**), 100 mM phosphate buffer, pH 7.0, 1 hour at r.t.

N-hydroxysuccinimide (NHS) ester and maleimide group. Conjugation reaction can be carried out either by reaction with amine group first (see Scheme 1) or by reaction with sulfhydryl group first (see Scheme 2). Conjugate **7** was synthesized through reaction of 3′-C6-amine modified 2-5A trimer **4** (see Scheme 1) with excess amount of **5** in phosphate buffer to form intermediate **6** first. After purification, **6** was reacted with sulfhydryl containing compound **1** to get **7** (see Scheme 1). This method is the same as the one used for the preparation of previously reported ligand **3**.[21]



SCHEME 2 Synthetic pathway of compounds **11–17**. (i) DMF, 1 hour at r.t.; (ii) phosphorothioate 2-5A monomer (**9**) or trimer (**10**), DMF, r.t. overnight; (iii) 4-fluoro-benzylamine; (iv) *N*-Boc-1,6-diaminohexane, DMF, r.t. overnight; (v) 4 M hydrochloric acid/dioxane, 1 hour at r.t.; (vi) *N*-(4-fluorobenzyl)-2-bromoacetamide, methanol/phosphate buffer, pH 8.0, 120°C, 30 minutes.

Phosphorothioate-containing conjugates **11** and **12** were prepared by the reaction of **5** with excess amount of sulfhydryl-containing compound

1 at room temperature (r.t.) for 1 hour followed by direct addition of C6-amine modified phosphorothioate 2-5A monomer (**9**) or trimer (**10**) (see Scheme 2). To avoid hydrolysis of NHS-ester group in **5**, anhydrous *N*,*N*-dimethylformamide (DMF) was used as a solvent instead of phosphate buffer. Using the method as shown in Scheme 2, side reaction of 5′-phosphorothioate group with maleimide group of **5** was successfully prevented and compounds **11** and **12** were obtained at good yield. Another advantage of the method is that it did not require purification of the intermediate reaction and therefore was more time saving.

To help investigate the importance of the 2-5A for the binding affinity, compounds **13** and **15** omitting 2-5A part were synthesized. Both compounds can be made by using either method as shown in Scheme 1 or 2 and no purification is needed after first step coupling. Cleavage of the *N*-Boc group in **14** was achieved by the addition of 4 M HCl in dioxane to get desired compound **15** (see Scheme 2).

To get further evidence to support that the new ligands designed here can be further terminal modified without losing their binding affinity, cold compounds **16** and **17** of future ^{18}F-radiolabeled analogues were synthesized. Terminal labeling of **11** and **12** was performed by reaction with *N*-(4-fluorobenzyl)-2-bromoacetamide (see Scheme 2). This is a common method used to label oligonucleotides. The reaction happened quickly at 120°C and gave **16** or **17** as the highly predominant product. Compared with **11** and **12**, compounds **16** and **17** have longer retention time (Rt) due to increased lipophilicity following the addition of the halogenobenzyl substitution.

Competitive Binding Studies

All new compounds synthesized were evaluated in a competitive binding assay using tritium labeled S-methylated derivative of **1** (*N*-[*N*-[(*S*)-1,3-dicarboxypropyl]carbamoyl]-*S*-[^{3}H]-methyl-L-cysteine, **2**, in Figure 1) as the radioligand to examine the ability of the novel compounds to compete binding with **2** on PSMA-expressing LNCaP cells. Parent compound **1** was used as positive control. The results are summarized in Table 1.

Previously, we have reported that inclusion of a C3-amine modified 2-5A trimer into **1** (compound **3**) improved its binding affinity 10 times.[21] Here we confirmed the observation by preparation of a series of analogues of compound **3**. In all the newly synthesized conjugates, a C6 linker was used to replace the original C3 linker in compound **3** because our initial study found that extension of the linker from C3 (compound **3**) to C6 (compound **12**) improved the binding activity more than 100-fold. Our study showed that compounds with 2-5A in their structures (compounds **7**, **11**, **12**, **16**, and **17**) had significantly improved binding activity compared with those omitting 2-5A (compounds **13** and **15**). These results indicated that the integration

TABLE 1 Summary of competitive binding results of new conjugates. IC_{50} is the concentration required to inhibit the binding of 3H-ZJ24 (**2**) by 50%. Values are mean ± *SD* of three replicates

Compounds	IC_{50} (nM)
1	13.9 ± 0.12
3	1.5 ± 0.12
7	1.58 ± 0.26
11	0.11 ± 0.06
12	0.012 ± 0.003
13	96.2 ± 5.4
15	163.1 ± 6.2
16	1.93 ± 0.11
17	3.36 ± 0.15

of 2-5A plays a crucial role in PSMA binding, and that part cannot be eliminated from structure. It was also found that the binding affinity increased when the number of 2-5A increased from one (compound **11**) to three (compound **12**). Another interesting finding was that free 5′-phosphorothioate contributed to the activity greatly; replacement of 5′-phosphorothioate group (**12**) with phosphate group (**7**) or substitution of the free 5′-thioate group in **11** and **12** decreased their binding activity (**16** and **17**) dramatically.

The crystal structure of PSMA has been well elucidated.[22–29] The active site contains two zinc atoms, a smaller pharmacophore binding site (S1′) and a larger nonpharmacophore pocket (S1). According to this elucidated crystal structure of PSMA, the glutamate portion (P1′) of our compounds should be undoubtedly located in the S1′ pocket of PSMA, while the remainder of the molecule (P1) should be oriented in the S1 pocket. The S1 pocket contains a ~20 Å deep tunnel that narrows gradually. Extension of C3 (**3**) to C6 (**12**) might have optimized the steric hindrance, thus leading to better fit and binding characteristic. Similar observations have been reported by Kularatne et al. recently.[30] They included an 8-aminooctanoic acid spacer in their ligands and found that the linker improved binding potency more than 10-fold. The S1 pocket of PSMA has an adjacent hydrophobic pocket and it is arginine rich. It has been reported that introduction of a phenyl group can improve the binding activity.[30,31] Similar to a phenyl group, an adenine group from 2-5A can form hydrophobic binding with the pocket. On the other side, the negatively charged phosphorothioate groups of 2-5A can form strong ion pairs with the positively charged guanidine groups of arginine. These two factors working together offer the 2-5A containing compounds excellent affinity. At this time, it is still not clear how the 5′-terminal phosphorothioate affects the binding. One hypothesis is that it might interact with the sulfhydryl group of cysteines in PSMA and form S–S bond, thus increasing

its binding affinity. Our future work will be to perform docking studies to help understand the binding characteristics of our conjugates.

As mentioned before, prostate cancer is the leading cancer in men and the second most common cause of cancer-related death. Furthermore, by the time of diagnosis, only half of the tumors are clinically localized and half of these represent extracapsular spread. Sensitive, functional imaging techniques are required to identify the extent of disease prior to therapy. PSMA provides an excellent target for prostate tumor imaging and therapy because of its transmembrane location and the fact that it is significantly upregulated in prostate cancer metastasis relative to the few normal tissues in which it resides at least 100-fold lower levels. Compounds **11** and **12** contain a 5′-terminal phosphorothioate ester group that is active in nucleophilic reaction. *N*-(4-fluorobenzyl)-2-bromoacetamide is suitable as the carrier of a positron emitter to label phosphorothioate.[32] This terminal labeling offers minimal structural perturbation, and more importantly, protects 2-5A from terminal enzymatic degradation due to various exonuclease in vivo. After labeling, the corresponding cold compounds (**16** and **17**) lost 10- to 100-fold of activity compared with unlabeled **11** and **12**, but still had binding activities much better than parent compound **1**. Parent compound **1** has been reported to have a Ki at 1 nM in inhibiting PSMA's enzymatic activity. Other groups have tried *N*-[*N*-(*S*)-1,3-dicarboxypropyl]-*S*-[^{11}C]methyl-L-cysteine ([^{11}C]-DCMC, Ki = 3.1 nM),[18] *N*-[*N*-(*S*)-1,3-dicarboxypropyl]carbamoyl]-4-[^{18}F]fluorobenzyl-L-cysteine ([^{18}F]-DCFBCA, IC50 = 13.9 nM),[20] and ^{123}I-labled glutamtate-urea-lysine analogues ([^{123}I]MIP-702, Ki = 4.6 nM; [^{123}I]MIP-1095, Ki = 0.24 nM).[19] They all showed selectively high uptake on PSMA-expressing tumor xenografts. Our compounds resemble part of their structures and have better or comparable binding affinity to PSMA. In in vitro cytotoxicity assay, none of the compounds showed any toxic effect to LNCaP cells, which indicated their low toxicity (data not shown). These facts together suggested that the future of using these ligands for imaging should be very promising.

CONCLUSIONS

In summary, a series of novel urea-based PSMA ligand containing 4-(*N*-maleimidomethyl)cyclohexane-1-amidate linkage has been synthesized. Integration of 2-5A into the structure reveals outstanding activity in competitive binding assay. Compared to antibodies, these conjugates can be easily synthesized and are more cost effective. More importantly, as small molecules, they should have favorable pharmacokinetic behaviors, such as fast uptake and quick clearance. The results reported in this article provide supporting evidence for future 18-F radiolabeling as potential diagnostic imaging agents by targeting PSMA.

EXPERIMENTAL

General Methods

(S)-2-(3-((S)-5-amino-1-carboxypentyl)ureido)pentanedioic acid (**1**) was custom made by Bachem Bioscience Inc. (Torrance, CA, USA) 3′-C6-amine modified 2′,5′-oligoadenylate trimer{[(2R,5R)-5-(6-amino-9H-purin-9-yl)-4-[({[2R,5R-5-(6-amino-9H-purin-yl)-4-[({[2R,5R-5-(6-amino-9H-purin-yl)-4-({[(6-aminohexyl)oxy](hydroxy) phosphoryl} oxy)-3-hydroxyoxolan-2-yl]-methoxy}(hydroxy)phosphoryl)oxy]-3-hydroxyoxolan-2-yl]methoxy} (hydroxy) phosphoryl)oxy]-3-hydroxyoxolan-2-yl]methoxy}phosphonic acid (**4**), phosphorothioate 2′,5′-oligoadenylate monomer thiophosphoric acid O-(6-amino-hexyl) ester O′-[2-(6-amino-purin-9-yl)-4-hydroxy-5-thiophos phonooxymethyl-tetrahydro-furan-3-yl] ester (**9**), and {[(2R,5R)-5-(6-amino-9H-purin-9-yl)-4-[({[(2R,5R)-5- (6-amino-9H-purin-9-yl)-4-[({[(2R, 5R)-5-(6-amino-9H-purin-9-yl)-4-({[(6-amino-hexyl)-oxy](hydroxy)sulfany lidene-phosphanyl}oxy)-3-hydroxyoxolan-2-yl]methoxy} (hydroxy)sulfany lidene-phosphanyl)oxy]-3-hydroxyoxolan-2-yl]methoxy}(hydroxy) sulfanyli dene-phosphanyl)oxy]-3-hydroxyoxolan-2-yl]methoxy}(sulfanyl)phosphinic acid (**10**) were custom synthesized by GeneACT Inc., Fukuoka, Japan. Compound **2** was custom made by GE Healthcare Life Sciences (Piscataway, NJ, USA). SMCC was purchased from Pierce (Rockford, IL, USA). *N*-succimimidyl 4-fluorobenzoate was ordered from ABX Advanced Biochemical Compounds, Radeberg, Germany. All the other chemicals were purchased from Sigma-Aldrich Inc., St. Louise, MO, USA. Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was obtained from a Varian Inova NMR Spectrometer (Darmstadt, Germany) at a proton frequency of 600 MHz. Deuterated chloroform was used as a solvent for obtaining NMR spectra. High-resolution matrix-assisted laser desorption/ionization mass (MALDI-MS) spectra were obtained from an Applied Biosystems 4800 MALDI TOF/TOF Analyzer (Carlsbad, CA, USA) in positive ion mode.

High-Performance Liquid Chromatography (HPLC)

All reactions were monitored by HPLC, which was performed on a Shimadzu HPLC system (Columbia, MD, USA) equipped with a SPD-20V prominence UV/visible detector and monitored at a wavelength at 260 nm. The gradient used was 0%–55% B over 45 minutes (A was 10 mM triethylammonium acetate, TEAA, pH 7.0; B was acetonitrile). Preparative HPLC was achieved using SymmetryPrep C18 column (100 mm × 19 mm × 5 μm, Waters Corporation, Milford, MA, USA) at a flow rate of 3.0 mL/minute. Analytical HPLC was performed using an analytical Symmetry C18 column (150 mm × 4.6 mm × 5 μm, Waters Corporation) at a flow rate of 1 mL/minute.

Synthesis of {[(2R,5R)-5-(6-amino-9H-purin-9-yl)-4-[({[(2R,5R)-5-(6-amino-9H-purin-9-yl)-4-[({[(2R,5R)-5-(6-amino-9H-purin-9-yl)-4-[({[6-({4-[(2,

5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl]cyclohexyl}formamido)hexyl] oxy}(hydroxy)phosphoryl)-oxy]-3-hydroxyoxolan-2-yl]methoxy}(hydroxy) phosphoryl)oxy]-3-hydroxyoxolan-2-yl]methoxy}(hydroxy)phosphoryl)oxy]-3-hydroxyoxolan-2-yl]methoxy}phosphonic acid (6). To a solution of 3′-C6-amine modified 2-5A trimer **4** (900 μg, 0.8 μmol) in 100 uL of 100 mM phosphate buffer (pH 7.0) was added 10.7 mg of SMCC (**5**, 32 μmol) in 1 mL of 100 mM phosphate buffer (pH 7.0). After stirring at r.t. overnight, the mixture was purified by preparative HPLC to obtain the desired product at 72.8% yield (815 μg). Rt = 23.5 minutes. $C_{48}H_{65}N_{17}O_{25}P_4$, MALDI-MS m/z: 1404 (M+1).

Synthesis of 2-[({2-[(1-{[4-({6-[({[(2R,5R)-2-(6-amino-9H-purin-9-yl)-5-{[({[(2R, 5R)-2-(6-amino-9H-purin-9-yl)-5-{[({[(2R,5R)-2-(6-amino-9H-purin-9-yl)-4-hydroxy-5-[(phosphonooxy)methyl]oxolan-3-yl]oxy}(hydroxy) phosphoryl)oxy]methyl}-4-hy-droxyoxolan-3-yl]oxy}(hydroxy)phosphoryl) oxy]methyl}-4-hydroxyoxolan-3-yl]oxy} (hydroxy)phosphoryl)oxy]hexyl}carbamoyl)cyclohexyl]-methyl}-2,5-dioxopyrrolidin-3-yl)sulfanyl]-1-carboxyethyl}carbamoyl)amino] pentanedioic acid (7). To a solution of **6** (300 μg, 0.2 μmol) in 400 μL of 100 mM phosphate buffer (pH 7.0) was added 500 μg of **1** (1.0 μmol). After stirring at r.t. for 1 hour, the mixture was purified by preparative HPLC to get the final product at 89.8% yield (300 μg). Rt = 17.5 minutes. $C_{57}H_{79}N_{19}O_{32}P_4S$, MALDI-MS m/z: 1698 (M+1).

Synthesis of 2-[3-(2-{1-[4-(6-{[2-(6-Amino-purin-9-yl)-4-hydroxy-5-thiophos-phonooxymethyl-tetrahydro-furan-3-yloxy]-hydroxy-thiophosphoryl-oxy}hexylcarbamoyl)-cyclohexylmethyl]-2,5-dioxo-pyrrolidin-3-ylsulfanyl}-1-carboxyethyl)ureido]-pentanedioic acid (11). SMCC 10 mg (0.03 mmol) was dissolved in 500 uL DMF, then 17 mg of **1** (0.06 mmol) in 500 uL DMF was added. After stirring at r.t. for 1 hour, 0.2 mg of 3′-C6-amine modified phosphorothioate 2′,5′-oligoadenylate monomer **9** (0.0035 mmol) was added. The mixture was stirred at r.t. overnight, then applied to preparative HPLC to give the purified product at 90% yield (0.35 mg). Rt = 13.4 minutes. $C_{37}H_{55}N_9O_{18}P_2S_3$, MALDI-MS m/z: 1072 (M+1).

Synthesis of 2-[({2-[(1-{[4-({6-[({[(2R,5R)-2-(6-amino-9H-purin-9-yl)-5-{[({[(2R,5R)-2-(6-amino-9H-purin-9-yl)-5-{[({[(2R,5R)-2-(6-amino-9H-purin-9-yl)-4-hydroxy-5-({[hydroxy(sulfanyl)phosphoryl]oxy}methyl)oxolan-3-yl] oxy} (hydroxy)sulfanylidene-phosphanyl)oxy]methyl}-4-hydroxyoxolan-3-yl] oxy} (hydroxy)-sulfanylidene-phosphanyl)oxy]methyl}-4-hydroxyoxolan-3-yl]oxy} (hydroxy) sulfanylidene-phosphanyl)oxy]hexyl}carbamoyl) cyclohexyl]methyl}-2,5-dioxopyrrolidin-3-yl)sulfanyl]-1-carboxyethyl} carbamoyl)amino]pentanedioic acid (12). The compound was synthesized using the same method as **11** at a yield of 83%. Rt = 13.9 minutes. $C_{57}H_{79}N1_9O_{28}P_4S_5$, MALDI-MS m/z: 1762 (M+1).

Synthesis of 2-(3-{1-Carboxy-2-[1-(4-{[(4-fluoro-benzylamino)-methyl]-carbamoyl}-cyclohexyl)-2,5-dioxo-pyrrolidin-3-ylsulfanyl]-ethyl}-ureido)-pentanedioic acid (13). The compound was synthesized using the same method as **11**. Rt = 22.3 minutes. MALDI MS m/z: 639 (M+1).

Synthesis of 2-[3-(2-{1-[4-(6-tert-Butoxycarbonylamino-hexylcarbamoyl)-cyclohexyl]-2,5-dioxo-pyrrolidin-3-ylsulfanyl}-1-carboxy-ethyl)-ureido] pentanedioic acid (14). SMCC (1 mg, 0.003 mmol) was dissolved in 0.5 mL DMF, and then 1.7 mg of **1** (0.006 mmol) in 0.5 mL DMF was added. After stirring at r.t. for 1 hour, *N*-Boc-1,6-diaminohexane (1.3 μL, 0.006 mmol) was added. The mixture was stirred at r.t. overnight, then applied to preparative HPLC to get purified product with a yield of 84% (1.8 mg). Rt = 22.1 minutes. $C_{32}H_{51}N_5O_{12}S$, MALDI-MS m/z: 752 (M+Na); 768 (M+K).

Synthesis of 6-{[4-(3-{2-Carboxy-2-[3-(1,3-dicarboxy-propyl)-ureido]-ethylsulfanyl}-2,5-dioxo-pyrrolidin-1-yl)-cyclohexanecarbonyl]-amino}-hexyl ammonium chloride (15). One milligram of **14** (0.0014 mmol) was dissolved in 1 mL of 4 M HCl/dioxane. After stirring at r.t. for 1 hour, the solvent was removed to give the dry product with a yield of 97% (0.9 mg). Rt = 13.5 minutes. $C_{27}H_{44}ClN_5O_{10}S$, MALDI-MS m/z: 666 (M+1).

Synthesis of *N*-(4-fluorobenzyl)-2-bromoacetamide.[32] To a solution containing 4-fluorobenzylamine (1.25 g, 10 mmol) and *N*-methylmorpholine (1.0 g, 10 mmol) in 25 mL of dichloromethane was added bromoacetyl bromide (2.0 g, 20 mmol). After addition, the reaction mixture was stirred at r.t. overnight. The mixture was then filtered and concentrated to dryness, and the residue was purified on a silica column. Elution with hexane/ethyl acetate (9:1) gave the pure product as light yellow solid (2.1 g, 87.5%). ^{1}H NMR ($CDCl_3$) δ 7.25 (d, 2H), 7.04 (d, 2H), 4.45 (d, 2H), 3.93 (s, 2H).

Synthesis of 2-[({2-[(1-{[4-({6-[({[(2R,5R)-2-(6-amino-9H-purin-9-yl)-5-[({[({[(4-fluorophenyl)methyl]carbamoyl}methyl)sulfanyl](hydroxy) phosphoryl}oxy) methyl]-4-hydroxyoxolan-3-yl]oxy}(hydroxy)sulfanylidene-phosphanyl)oxy]hexyl} carbamoyl)cyclohexyl]methyl}-2,5-dioxopyrrolidin-3-yl)sulfanyl]-1-carboxyethyl} carbamoyl)amino]pentanedioic acid (16).[32] To a solution of **11** (200 μg, 0.19 μmol) in 1 mL of methanol-phosphate buffer (100 mM, pH 8.0) was added excess amount of *N*-(4-fluorobenzyl)-2-bromoacetamide (240 μg, 0.95 μmol). The mixture was stirred at 120°C for 30 minutes, then the solvent was evaporated and the products were purified by preparative HPLC (214 μg, 91.3%). Rt = 27.5 minutes. $C_{46}H_{63}FN_{10}O_{19}P_2S_3$, MALDI-MS m/z: 1237 (M+1).

Synthesis of 2-{[(1-carboxy-2-{[1-(2-methylbutyl)-2,5-dioxopyrrolidin-3-yl]sulfanyl}ethyl)carbamoyl]amino}pentanedioic acid; {[(2R,5R)-5-(6-amino-9H-purin-9-yl)-4-[({[(2R,5R)-5-(6-amino-9H-purin-9-yl)-4-[({[(2R,5R)-5-(6-amino-9H-purin-9-yl)-3-hydroxy-4-{[hydroxy(sulfanylidene)-phosphanyl]oxy} oxolan-2-yl] methoxy}(hydroxy)sulfanylidene-phosphanyl)oxy]-3-hydroxyoxolan-2-yl]methoxy} (hydroxy)-sulfanylidene-phosphanyl)oxy]-

3-hydroxyoxolan-2- yl]methoxy}[({[(4- fluorophenyl)-methyl]-carbamoyl} methyl)sulfanyl]phosphinic acid (17). The compound was synthesized using the same method as **16** with a yield at 86.3%. Rt = 11.6 minutes. $C_{66}H_{87}FN_{20}O_{29}P_4S_5$, MALDI-MS m/z: 1927 (M+1).

Cell Culture

Prostate cancer cell line LNCaP was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). LNCaP cells were grown at 37°C and 5% CO_2 under a humidified atmosphere. Cells were maintained in RPMI1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovin serum.

Competitive Binding Assay

Briefly, LNCaP cells (5×10^5) were incubated with different concentrations of ligands in the presence of 12 nM **2** in a total volume of 300 μL for 1 hour at 37°C. The mixture was centrifuged at 3000 g for 5 minutes at 4°C, then washed three time with 500 μL of cold PBS. Finally, 4 mL of EcoLume cocktail (MP Biomedicals, Solon, OH, USA) was added, and radioactivity was counted by scintillation counter. The concentration required to inhibit 50% of binding is determined (IC_{50}) by GraphPad Prism 3.0.

REFERENCES

1. Siegel, R.; Naishadham, D.; Jemal, A. Cancer statistics, 2012. *CA Cancer J. Clin.* **2012**, 62, 10–29.
2. Israeli, R.S.; Powell, C.T.; Corr, J.G.; Fair, W.R.; Heston, W.D. Expression of the prostate-specific membrane antigen. *Cancer Res.* **1994**, 54, 1807–1811.
3. Tasch, J.; Gong, M.; Sadelain, M.; Heston, W.D. A unique folate hydrolase, prostate-specific membrane antigen (PSMA): a target for immunotherapy? *Crit. Rev. Immunol.* **2001**, 21, 249–261.
4. Wright, G.L., Jr.; Grob, B.M.; Haley, C.; Grossman, K.; Newhall, K.; Petrylak, D.; Troyer, J.; Konchuba, A.; Schellhammer, P.F.; Moriarty, R. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology* **1996**, 48, 326–334.
5. Bostwick, D.G.; Pacelli, A.; Blute, M.; Roche, P.; Murphy, G.P. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 184 cases. *Cancer* **1998**, 82, 2256–2261.
6. Troyer, J.K.; Beckett, M.L.; Wright, G.L., Jr. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int. J. Cancer* **1995**, 62, 552–558.
7. Chang, S.S.; Reuter, V.E.; Heston, W.D.; Bander, N.H.; Grauer, L.S.; Gaudin, P.B. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. *Cancer Res.* **1999**, 59, 3192–3198.
8. Carter, R.E.; Feldman, A.R.; Coyle, J.T. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc. Natl. Acad. Sci. U SA* **1996**, 93, 749–753.
9. Pinto, J.T.; Suffoletto, B.P.; Berzin, T.M.; Qiao, C.H.; Lin, S.; Tong, W.P.; May, F.; Mukherjee, B.; Heston, W.D. Prostate-specific membrane antigen: a novel folate hydrolase in human prostatic carcinoma cells. *Clin. Cancer Res.* **1996**, 2, 1445–1451.

10. Jackson, P.F.; Tays, K.L.; Maclin, K.M.; Ko, Y.S.; Li, W.; Vitharana, D.; Tsukamoto, T.; Stoermer, D.; Lu, X.C.; Wozniak, K.; Slusher, B.S. Design and pharmacological activity of phosphinic acid based NAALADase inhibitors. *J. Med. Chem.* **2001**, 44, 4170–4175.
11. Kozikowski, A.P.; Nan, F.; Conti, P.; Zhang, J.; Ramadan, E.; Bzdega, T.; Wroblewska, B.; Neale, J.H.; Pshenichkin, S.; Wroblewski, J.T. Design of remarkably simple, yet potent urea-based inhibitors of glutamate carboxypeptidase II (NAALADase). *J. Med. Chem.* **2001**, 44, 298–301.
12. Majer, P.; Jackson, P.F.; Delahanty, G.; Grella, B.S.; Ko, Y.S.; Li, W.; Liu, Q.; Maclin, K.M.; Polakova, J.; Shaffer, K.A.; Stoermer, D.; Vitharana, D.; Wang, E.Y.; Zakrzewski, A.; Rojas, C.; Slusher, B.S.; Wozniak, K.M.; Burak, E.; Limsakun, T.; Tsukamoto, T. Synthesis and biological evaluation of thiol-based inhibitors of glutamate carboxypeptidase II: discovery of an orally active GCP II inhibitor. *J. Med. Chem.* **2003**, 46, 1989–1996.
13. Slusher, B.S.; Vornov, J.J.; Thomas, A.G.; Hurn, P.D.; Harukuni, I.; Bhardwaj, A.; Traystman, R.J.; Robinson, M.B.; Britton, P.; Lu, X.C.; Tortella, F.C.; Wozniak, K.M.; Yudkoff, M.; Potter, B.M.; Jackson, P.F. Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury. *Nat. Med.* **1999**, 5, 1396–1402.
14. Tang, H.; Brown, M.; Ye, Y.; Huang, G.; Zhang, Y.; Wang, Y.; Zhai, H.; Chen, X.; Shen, T.Y.; Tenniswood, M. Prostate targeting ligands based on *N*-acetylated alpha-linked acidic dipeptidase. *Biochem. Biophys. Res. Commun.* **2003**, 307, 8–14.
15. Banerjee, S.R.; Foss, C.A.; Castanares, M.; Mease, R.C.; Byun, Y.; Fox, J.J.; Hilton, J.; Lupold, S.E.; Kozikowski, A.P.; Pomper, M.G. Synthesis and evaluation of technetium-99m- and rhenium-labeled inhibitors of the prostate-specific membrane antigen (PSMA). *J. Med. Chem.* **2008**, 51, 4504–4517.
16. Chandran, S.S.; Banerjee, S.R.; Mease, R.C.; Pomper, M.G.; Denmeade, S.R. Characterization of a targeted nanoparticle functionalized with a urea-based inhibitor of prostate-specific membrane antigen (PSMA). *Cancer Biol. Ther.* **2008**, 7, 974–982.
17. Chen, Y.; Foss, C.A.; Byun, Y.; Nimmagadda, S.; Pullambhatla, M.; Fox, J.J.; Castanares, M.; Lupold, S.E.; Babich, J.W.; Mease, R.C.; Pomper, M.G. Radiohalogenated prostate-specific membrane antigen (PSMA)-based ureas as imaging agents for prostate cancer. *J. Med. Chem.* **2008**, 51, 7933–7943.
18. Foss, C.A.; Mease, R.C.; Fan, H.; Wang, Y.; Ravert, H.T.; Dannals, R.F.; Olszewski, R.T.; Heston, W.D.; Kozikowski, A.P.; Pomper, M.G. Radiolabeled small-molecule ligands for prostate-specific membrane antigen: in vivo imaging in experimental models of prostate cancer. *Clin. Cancer Res.* **2005**, 11, 4022–4028.
19. Hillier, S.M.; Maresca, K.P.; Femia, F.J.; Marquis, J.C.; Foss, C.A.; Nguyen, N.; Zimmerman, C.N.; Barrett, J.A.; Eckelman, W.C.; Pomper, M.G.; Joyal, J.L.; Babich, J.W. Preclinical evaluation of novel glutamate-urea-lysine analogues that target prostate-specific membrane antigen as molecular imaging pharmaceuticals for prostate cancer. *Cancer Res.* **2009**, 69, 6932–6940.
20. Mease, R.C.; Dusich, C.L.; Foss, C.A.; Ravert, H.T.; Dannals, R.F.; Seidel, J.; Prideaux, A.; Fox, J.J.; Sgouros, G.; Kozikowski, A.P.; Pomper, M.G. *N*-[*N*-[(*S*)-1,3-Dicarboxypropyl]carbamoyl]-4-[18F]fluorobenzyl-L-cysteine, [18F]DCFBC: a new imaging probe for prostate cancer. *Clin. Cancer Res.* **2008**, 14, 3036–3043.
21. Cramer, H.; Okicki, J.R.; Rho, T.; Wang, X.; Silverman, R.H.; Heston, W.D. 2-5A ligands—a new concept for the treatment of prostate cancer. *Nucleosides Nucleotides Nucleic Acids* **2007**, 26, 1471–1477.
22. Davis, M.I.; Bennett, M.J.; Thomas, L.M.; Bjorkman, P.J. Crystal structure of prostate-specific membrane antigen, a tumor marker and peptidase. *Proc. Natl. Acad. Sci. USA* **2005**, 102, 5981–5986.
23. Mesters, J.R.; Henning, K.; Hilgenfeld, R. Human glutamate carboxypeptidase II inhibition: structures of GCPII in complex with two potent inhibitors, quisqualate and 2-PMPA. *Acta Crystallogr. D Biol. Crystallogr.* **2007**, 63, 508–513.
24. Hlouchova, K.; Barinka, C.; Konvalinka, J.; Lubkowski, J. Structural insight into the evolutionary and pharmacologic homology of glutamate carboxypeptidases II and III. *Febs J.* **2009**, 276, 4448–4462.
25. Barinka, C.; Byun, Y.; Dusich, C.L.; Banerjee, S.R.; Chen, Y.; Castanares, M.; Kozikowski, A.P.; Mease, R.C.; Pomper, M.G.; Lubkowski, J. Interactions between human glutamate carboxypeptidase II and urea-based inhibitors: structural characterization. *J. Med. Chem.* **2008**, 51, 7737–7743.
26. Barinka, C.; Hlouchova, K.; Rovenska, M.; Majer, P.; Dauter, M.; Hin, N.; Ko, Y.S.; Tsukamoto, T.; Slusher, B.S.; Konvalinka, J.; Lubkowski, J. Structural basis of interactions between human glutamate carboxypeptidase II and its substrate analogs. *J. Mol. Biol.* **2008**, 376, 1438–1450.

27. Barinka, C.; Rovenska, M.; Mlcochova, P.; Hlouchova, K.; Plechanovova, A.; Majer, P.; Tsukamoto, T.; Slusher, B.S.; Konvalinka, J.; Lubkowski, J. Structural insight into the pharmacophore pocket of human glutamate carboxypeptidase II. *J. Med. Chem.* **2007**, 50, 3267–3273.
28. Barinka, C.; Starkova, J.; Konvalinka, J.; Lubkowski, J. A high-resolution structure of ligand-free human glutamate carboxypeptidase II. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **2007**, 63, 150–153.
29. Mesters, J.R.; Barinka, C.; Li, W.; Tsukamoto, T.; Majer, P.; Slusher, B.S.; Konvalinka, J.; Hilgenfeld, R. Structure of glutamate carboxypeptidase II, a drug target in neuronal damage and prostate cancer. *Embo J.* **2006**, 25, 1375–1384.
30. Kularatne, S.A.; Wang, K.; Santhapuram, H.K.; Low, P.S. Prostate-specific membrane antigen targeted imaging and therapy of prostate cancer using a PSMA inhibitor as a homing ligand. *Mol. Pharm.* **2009**, 6, 780–789.
31. Maung, J.; Mallari, J.P.; Girtsman, T.A.; Wu, L.Y.; Rowley, J.A.; Santiago, N.M.; Brunelle, A.N.; Berkman, C.E. Probing for a hydrophobic a binding register in prostate-specific membrane antigen with phenylalkylphosphonamidates. *Bioorg. Med. Chem.* **2004**, 12, 4969–4979.
32. Kuhnast, B.; Dolle, F.; Terrazzino, S.; Rousseau, B.; Loc'h, C.; Vaufrey, F.; Hinnen, F.; Doignon, I.; Pillon, F.; David, C.; Crouzel, C.; Tavitian, B. General method to label antisense oligonucleotides with radioactive halogens for pharmacological and imaging studies. *Bioconjug. Chem.* **2000**, 11, 627–636.