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Design and Synthesis of a PSMA Inhibitor–Doxorubicin Conjugate for Targeted Prostate Cancer Therapy

Sarva Jayaprakash,[a] Xinning Wang,[b]
Warren D. Heston,[b] and Alan P. Kozikowski*[a]

Prostate cancer is the most commonly diagnosed cancer and the second leading cause of death in men in the United States after lung cancer.[1] It is estimated that prostate cancer affects approximately 180 000 and kills 40 000 men in the United States each year. As long as the cancer is confined to the prostate, it can be successfully controlled by surgery or radiation, but there is little effective treatment available for the metastatic disease, particularly if androgen-deprivation therapy fails.[2,3] Advances in our understanding of tumor immunology have, however, led to new approaches for treatment of prostate and other cancers, including tumor antigen-specific immunotherapy. Several prostate antigens have now been identified that are attractive candidates for both prostate-tumor imaging and therapy. One of the most interesting among these is the prostate-specific membrane antigen (PSMA).[4,5] PSMA is considerably upregulated in prostate cancers, metastatic disease, and in hormone-refractory prostate cancers.[6,7]

Capromab pendetide (ProstaScint) is a commercially available monoclonal antibody in clinical use for detecting prostate cancer, but it has been cited for complexities associated with its administration and the interpretation of the images obtained.[8–10]

PSMA is a 100 kDa, type II membrane glycoprotein highly expressed by all prostate cancers as well as by nonprostatic tumor neovasculature and the vascular endothelium of virtually all solid sarcoma and carcinoma tumors.[11–14] PSMA is highly homologous to the neuropeptidase NAALADase (GCP II) that releases the neurotransmitter glutamate from the neuronal peptide NAAG (*N*-acetyl-L-aspartyl-L-glutamate). PSMA also exhibits folate hydrolase activity whereby it cleaves terminal glutamates from γ-linked polyglutamates.[15,16] A 3.5 Å resolution crystal structure of the PSMA ectodomain has been disclosed recently.[17] The homodimer contains a binuclear zinc site, catalytic residues, and a proposed substrate-binding arginine patch, which is similar to our previously modeled three-dimensional structure of the GCP II extracellular domain obtained through homology-based modeling methods.[18] Based upon mechanistic considerations together with the modeling studies, we have developed an array of potent, urea-based inhibitors of NAAG peptidase/PSMA.[19,20] Since PSMA is significantly upregulated in prostate cancer and metastasis, and is strongly expressed in the neovasculature of solid tumors, we have explored the use of our designed small-molecule inhibitors as PET imaging agents.[21] As these imaging applications proved successful, we turned our attention to the possibility of using these same PSMA inhibitors for the targeting of anticancer drugs to prostate cancer cells.

Anticancer drugs, including doxorubicin and others, have limited utility in prostate cancer therapy due to their poor target selectivity and systemic toxicities, such as cardiotoxicity and immunosuppression.[22] Targeted therapy for cancer offers potential improvements over existing chemotherapy regimens since the drug is delivered preferentially to the cancer tissue and side effects can be minimized. Research in this area has produced several cytotoxic conjugates with improved selectivity and drug effects.[23–25] We sought to synthesize a conjugate between one of our PSMA inhibitors and the anticancer drug doxorubicin in order to test whether such a hybrid molecule would lead to a drug with an improved therapeutic index. We report here the design, synthesis, and biological activity of PSMA–doxorubicin conjugate **1** targeted for prostate cancer therapy.

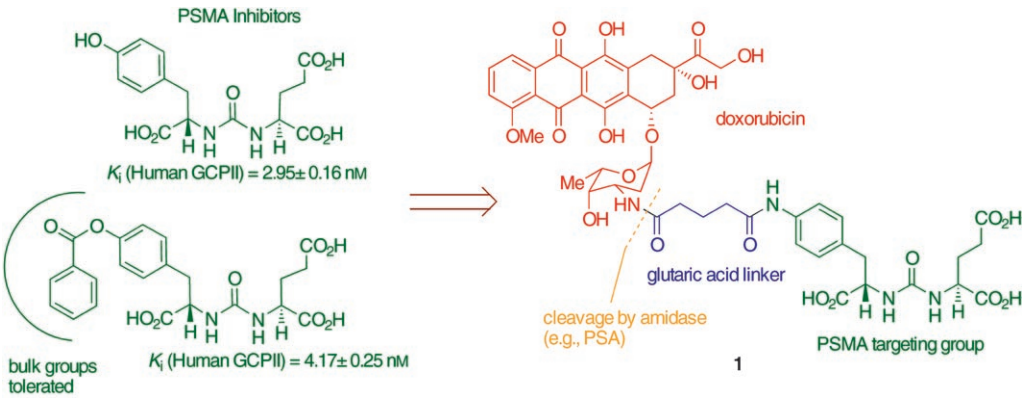
In the design of the PSMA–doxorubicin conjugate, it was important to define the optimal sites on both the doxorubicin and the PSMA inhibitor for attachment of a linker. It is known that acylation of the amino group present in the pyranose ring of doxorubicin results in a decrease in the cytotoxicity of this drug.[26] Our previous SAR work on the NAAG peptidase inhibitors had revealed that the urea formed between glutamate and a variety of phenylalanine derivatives containing diverse substituents on the *para*-position of the aromatic ring serve as potent (nM) peptidase inhibitors.[27] We elected to join a urea-based PSMA inhibitor made up of *p*-aminophenylalanine through a glutaric acid linker to doxorubicin (Scheme 1). The amino groups present in these two moieties thus became the key connection points. In pursuing this approach, we imagined that the PSMA inhibitor would serve to target the doxorubicin to the prostate cancer cells. Once it was localized there and internalized, other amidase/peptidase activities (e.g., PSA, but not the extracellular PSMA, as this would be inhibited by the bioconjugate) would free the doxorubicin. In support of the possibility for internalization, we refer to the recent article by Langer et al. that demonstrates that a nanoparticle–aptamer bioconjugate comprising RNA aptamers that target PSMA can be internalized into LNCaP cells after a 2 h incubation period.[28] Moreover, a previous report has revealed an antibody-mediated enhancement in the rate of PSMA endocytosis in LNCaP cells.[29]

With the above considerations in mind, we began the synthesis of our designed, small-molecule–PSMA inhibitor-based bioconjugate with the preparation of the urea derivative **6**, as shown in Scheme 2.

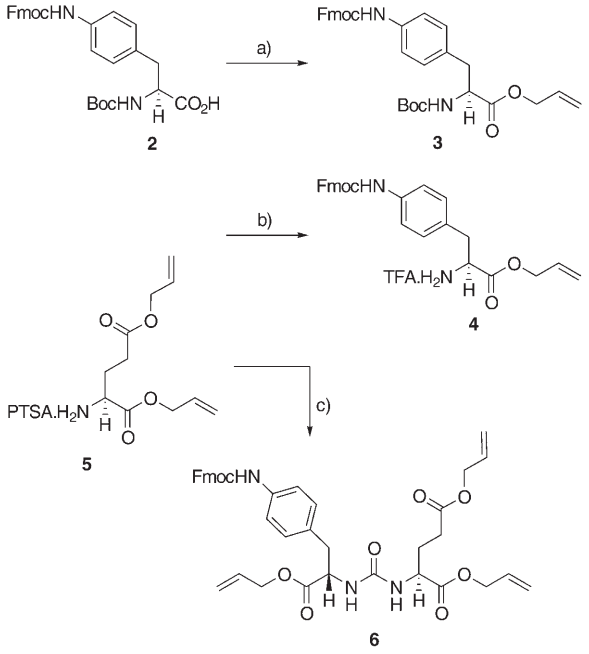
[a] *Dr. S. Jayaprakash, Prof. A. P. Kozikowski*
Drug Discovery Program
Department of Medicinal Chemistry and Pharmacognosy
833 South Wood Street, Chicago, IL 60612 (USA)
Fax: (1) 312-996-7107
E-mail: kozikowa@uic.edu

[b] *Dr. X. Wang, Dr. W. D. Heston*
The Cleveland Clinic Foundation
Department of Cancer Biology
9500 Euclid Avenue, Cleveland, OH 44195 (USA)

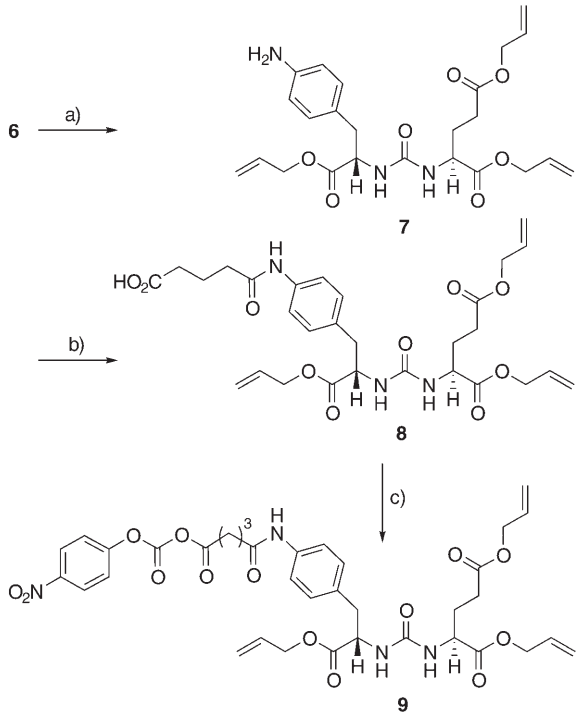
*Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author: experimental procedures and characterization data for compounds **1**, **3**, **4**, **6**–**10**.*



Scheme 1. Design of PSMA inhibitor–doxorubicin conjugate **1**.



Scheme 2. Synthesis of urea intermediate **6**. a) allyl bromide, K_2CO_3, DMF, RT, 2 h, 92%, b) TFA, CH_2Cl_2, 0 °C, 2 h, quantitative, c) triphosgene, Et_3N, −78 °C, 1.5 h, **4**, Et_3N, DCM, −78 °C RT, 12 h, 54%.

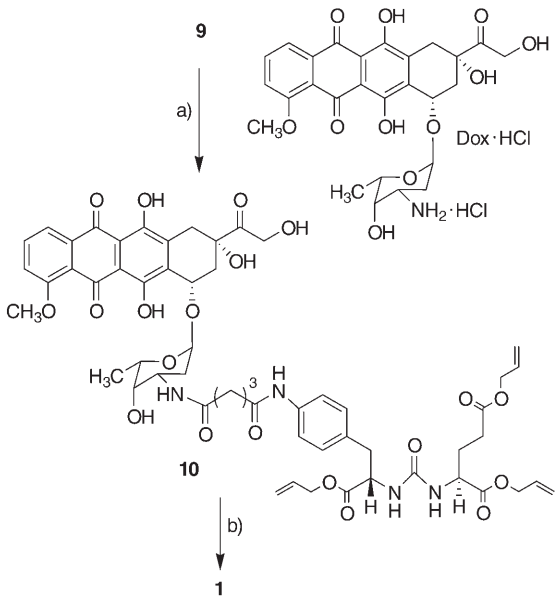


Scheme 3. Synthesis of active ester intermediate **9**. a) Et_2NH, CH_3CN, RT, 40 min, 94%, b) glutaric anhydride, DCM, RT, 2 h, 84%, c) *p*-nitrophenyl chloroformate, Et_3N, DCM, 0 °C to RT, 3 h, 65%.

Esterification of the known Boc-Phe(4-NHFmoc)-OH (**2**)[30] with allyl bromide, followed by treatment with trifluoroacetic acid, yielded the allyl ester **4** in 95% yield (two steps). Next, urea **6** was obtained in 54% yield by treating the glutamic acid diallyl ester (**5**)[30] with triphosgene followed by in situ trapping of the isocyanate intermediate by amine **4**. With the required protected PSMA-targeted urea in hand, we then needed to append the glutaric acid residue to the anilino nitrogen. The Fmoc protecting group in **6** was cleaved by treatment with diethylamine in acetonitrile to give the free amine intermediate **7** in 94% yield (Scheme 3). Compound **7** was then treated with glutaric anhydride in DCM at room temperature to provide the carboxylic acid **8** in 84% yield. This reaction proceeded well under mild conditions without the addition of a base. In order to activate the free carboxyl group of **8** for coupling with doxorubin, it was treated with 4-nitrophenyl chloroformate in the presence of triethylamine to afford the mixed anhydride **9** in 65% yield, as shown in Scheme 3.

With the anhydride **9** in hand, we then explored its reaction with doxorubicin. Much to our satisfaction, the penultimate prodrug intermediate **10** was readily formed in 68% yield.[31] Finally, all protecting groups were removed by treatment with $Pd(PPh_3)_4$ and the allyl scavenger morpholine to furnish the required bioconjugate **1** in 43% yield (Scheme 4).

To investigate the activity of this novel conjugate, we first evaluated its binding affinity towards the active dimeric form of soluble recombinant human PSMA. The binding affinity of **1** relative to our previously reported PSMA inhibitor ZJ24[10a] was measured through competition assays by using tritated ZJ24 (^{3}H-ZJ24). (C-ZJ24 has proven valuable for imaging PSMA-posi-



Scheme 4. Final steps in the synthesis of bioconjugate **1**. a) Et_3N, DMF, 0 °C to RT, 3 h, 68%, b) $Pd(PPh_3)_4$, morpholine, DMF, 0 °C to RT, 8 h, 43%.

tive prostate cancer cells.[11]) Different final concentrations of the inhibitor were incubated with recombinant PSMA dimer in the presence of ^{3}H-ZJ24 (12 nM), and the concentration required to inhibit 50% of binding (IC_{50}) was determined. We found that the conjugate **1** blocked the binding of ^{3}H-ZJ24 with an IC_{50} value of 40.8 ± 1.6 nM compared to ZJ24 (IC_{50} = 15.3 ± 2.3 nM). However, doxorubicin itself however failed to block ^{3}H-ZJ24 even at 10 μM (Figure 1). These data demonstrate the validity of our design concept in that we have created a bioconjugate with high binding affinity to PSMA.

We also tested the in vitro cytotoxicity of the new conjugate against PSMA-positive C4-2 and PSMA-negative PC3 prostate cancer cells using the colorimetric CellTiter 96 Aqueous Cell Proliferation Assay (Promega). Cells were seeded in 96-well culture plates the day before treatment. Test substances were added after serial dilution and exposed to the cells for 72 h.

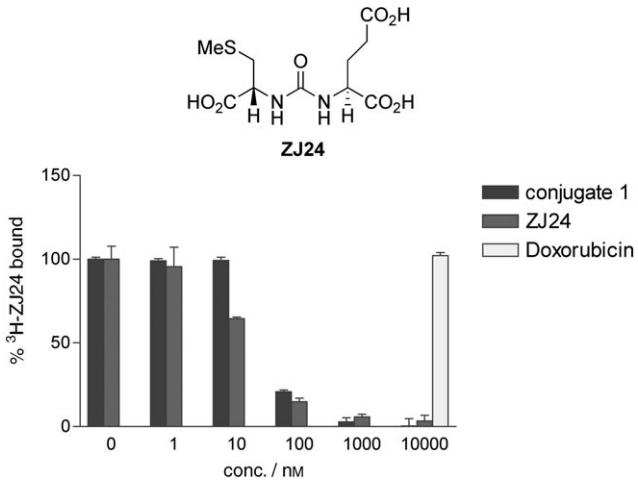


Figure 1. Competition binding studies of conjugate **1**.

After that, CellTiter 96 aqueous reagent was added to each well. After a 3-hour incubation period at 37 °C, the absorbance at 490 nm was measured with a 96-well plate reader, and the IC_{50} values were determined. We found that the PSMA–doxorubicin conjugate was much less potent than doxorubicin both to the C4-2 and PC3 cells. Doxorubicin exhibited IC_{50} values of 32 nM against the C4-2 cells and 223 nM against the PC3 cells. On the other hand, conjugate **1** inhibited only 30% of C4-2 cell growth, even at a relatively high concentration of 5 μM, while no effect was observed against the PC3 cells (Figure 2). It is possible that the conjugate is not undergoing the appropriate enzymatic processing required to release the active moiety; the metabolism of **1** is therefore currently under study.

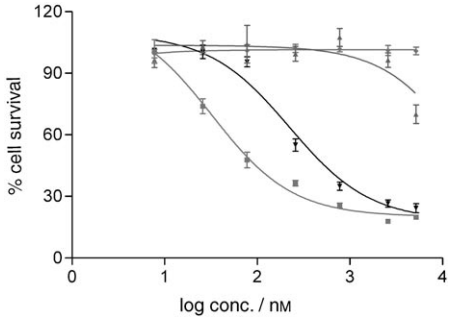


Figure 2. Cytotoxic studies of conjugate **1**. ■: Dox (C4-2), ▲: conjugate **1** (C4-2), ▼: Dox (PC3), ◆: conjugate **1** (PC3).

In summary, this work outlines a possible strategy for directing anticancer drugs to prostate cancer cells by targeting a protein, PSMA, that is over-expressed in prostate cancers. This strategy is based upon our discovery of highly potent urea-based PSMA inhibitors that have proven effective in prostate-tumor imaging with PET. We have developed a conjugate that demonstrates high binding affinity for PSMA, however, its in vitro antitumor activity is poor. While studies are underway to obtain a better understanding of the reason for the poor antiproliferative action of **1**, we believe that its high binding affinity is encouraging, and suggests that the other conjugates should be explored. This work further reveals that fairly large groups can be incorporated into these urea-based inhibitors without compromising binding affinity; this suggests the potential to employ such chemistry in the design of optical imaging agents.

Acknowledgements

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Keywords: doxorubicin · inhibitors · neurotransmitters · prostate cancer · PSMA

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[30] Chemicals purchased from Chem-Impex International, Inc. were used.
[31] Compound **10** was fully characterized by NMR and LC-MS data.

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