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PSMA-targeted contrast agents for intraoperative imaging of prostate cancer^{†,‡}

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Abstract

Prostate-specific membrane antigen (PSMA) can serve as a molecular cell surface target for the detection and treatment of prostate cancer. Near-infrared (NIR) fluorescence imaging enables highly sensitive, rapid, and non-radioactive imaging of PSMA, though specific targeting still remains a challenge because no optimized contrast agents exist.

PSMA is a glycosylated type-II membrane protein that primarily expresses at high levels in normal prostate and prostate cancer, and its expression increases with clinical stage.^{1,2} Many efforts have been made to detect and treat primary and metastatic prostate cancers using PSMA-based targeting such as computed tomography, nuclear imaging, and magnetic resonance imaging.^{3–7} Despite the recent advancement in nuclear medicine-based therapy, surgical resection remains a definitive treatment for prostate cancer.^{1,8} Compared to PSMA-targeted nuclear medicine, optical imaging could provide real-time guidance during prostate resection with highly sensitive, rapid, and non-radioactive NIR fluorescence light, and importantly, highlight areas of extracapsular extension or in-transit metastases.^{7–9} However, surgery is performed without intraoperative image guidance because there are no clinically available optical contrast agents to help the surgeon ensure clean tumor margins and find small occult metastases in the surgical field.^{9–11}

Recently, we reported zwitterionic (ZW) fluorophores for efficient targeting of vital tissues and tumors with optimized biodistribution and renal clearance.^{12–16} Particularly, the bio-distribution and clearance patterns are predominantly governed by the overall molecular charges and hydrophobicity of NIR fluorophores when conjugated to small molecule

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ligands.¹⁵ ZW fluorophores are designed to form balanced charges and biologically inherent surface after conjugation with targeting agents by preventing interactions with biological tissues and proteins, yet improving targeting efficiency.^{17,18}

Lysine-urea-glutamate (KUE) is a low molecular weight, urea-containing PSMA ligand (Da: 319, Ki: 498 nM), which has been widely studied for imaging prostate cancers.^{5–7,19–22} When KUE was conjugated directly with ZW800-1, however, no membrane binding was observed on LNCaP cells *in vitro* and *in vivo* (data not shown). This result indicates that the conjugated fluorophore agitates the favorable pharmacokinetic property of targeted ligand.¹⁷ To evaluate the influence of effector domain on the targeting efficiency of bioconjugates, in this study, a series of KUE-conjugated NIR fluorophores were synthesized and their PSMA-binding was tested in terms of net charges, spacer length, hydrophobicity, and polarity. Since the size of KUE is smaller than that of effector domain, we hypothesized that the targeting efficiency as well as biodistribution and clearance patterns of KUE conjugates would be strongly governed by the conjugated NIR fluorophores.^{17,23} To systematically compare the influence of physicochemical properties of fluorophores on PSMA targeting efficiency, as shown in Fig. 1, a series of polymethine fluorophores were selected and conjugated with various length of polyethylene glycol (PEG): a pentamethine cyanine (*i.e.*, Cy5.5) and 800 nm heptamethine fluorophores (*i.e.*, Cy7, ZW800+3C, ZW800-1, and ZW800-3C).^{14,24–26}

Bioconjugation of NIR fluorophores to KUE was completed by the conventional NHS ester chemistry.¹⁴ The final KUE-PEG4-conjugated NIR fluorophores were purified by preparative HPLC to 90% as measured using a photodiode array (PDA) at 210 nm absorbance (Fig. S1–S4, ESI[†]).

The conventional method for creating targeted contrast agents is to conjugate separate targeting and effector domains with/without spacers.^{17,18} Given the depth of the S1 pocket under the surface of PSMA, therefore, a suitable length of flexible linkers should be considered between a targeted ligand and a fluorophore.^{5,27} To find the optimal length for PSMA-specific binding, we introduced a set of PEGs with varying EG repeating units from 2 to 24 (Fig. 1b). Prior to the measurement of optical properties and PSMA targeting efficiency, all KUE conjugates were purified by preparative HPLC and analyzed by mass spectrometry (ESI[†]).

We hypothesized that *in vitro* and *in vivo* PSMA targeting would be mediated by the physicochemical properties of conjugated fluorophores by modulating targeting affinity, biodistribution, and clearance. The physicochemical and optical properties of KUE conjugates are summarized in Fig. 2a and Fig. S1 (ESI[†]). Distribution coefficient (log *D* at pH 7.4), topological polar surface area (TPSA), net charges at pH 7.4, and 3D energy minimized conformer were calculated by using MarvinSketch 16.12.12 software (ChemAxon). As shown in Fig. 2a, KUE is a negatively charged small molecule with TPSA of 189.16 Å² and log *D* of −10.15 at pH 7.4. KUE has surface charges of −3/+1 and adds −3 charges after peptide bond formation with the carboxylic group of NIR fluorophores, which significantly increases the overall hydrophilicity and polarity of the final conjugates. 3D

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energy-minimized conformer structures display the difference in hydrophobicity and charge distributions over the molecular surface. By varying the substitutions on the indole rings of the polymethine core, it was possible to systematically modify the hydrophobicity, polarity, and electron-resonance properties without affecting the optical performance (Fig. 2b). Overall, PEG4 spacer ($-\text{CH}_2-\text{CH}_2-\text{O}-$; C: sp^3 hybridization) provided sufficient flexibility for KUE to overcome spatial limitations in order to effectively interact with the PSMA on the cell membrane.^{10,11}

To determine the effect of charges, hydrophobicity, and polarity of the KUE conjugates on *in vitro* PSMA targeting, LNCaP cells (PSMA+) and PC3 cells (PSMA-) were treated with 2 μM of KUE-PEG4-conjugated Cy5.5, ZW800+3C, Cy7, ZW800-1, or ZW800-3C. To avoid any endocytosis-mediate uptake, all live cell binding assays were performed at 4 °C for 60 min. NIR fluorescence microscopy revealed that LNCaP cells treated with Cy5.5 (net charge = -6, $\log D = -13.84$, TPSA = 474.71) and ZW800+3C (net charge = -6, $\log D = -13.65$, TPSA = 483.20) conjugates were highly fluorescent than those treated with Cy7 (net charge = -4, $\log D = -8.91$, TPSA = 378.03), ZW800-1 (net charge = -2, $\log D = -8.80$, TPSA = 365.97), or ZW800-3C (net charge = 0, $\log D = -2.03$, TPSA = 254.40) conjugates (Fig. 3a). In contrast, almost no fluorescence was found in PC3 cells treated with the same series of KUE conjugates (Fig. S5, ESI[†]). This proves that high negative charges (net charge -3), hydrophilicity ($\log D$ at pH 7.4 > -10), and polarity (TPSA > 365) contribute to the binding affinity of KUE (net charge = -3, $\log D = -10.15$, TPSA = 189.16) on the PSMA.³⁻⁵ Despite the similar affinity of KUE-PEG4-Cy5.5 and KUE-PEG4-ZW800+3C on PSMA binding, ZW800+3C emits 800 nm fluorescence where much lower autofluorescence could be achieved in the body. Therefore, ZW800+3C conjugates were used for further *in vitro* optimization and *in vivo* intraoperative imaging.

To investigate the influence of linker space on PSMA binding, different lengths of PEG $_x$ ($x = 0, 2, 4, 12$, or 24) were applied between KUE and ZW800+3C. PEG spacers can provide sufficient flexibility for a targeting ligand to overcome spatial limitations in order to effectively interact with a corresponding target protein or receptor.^{28,29} As shown in Fig. 3b, *in vitro* PSMA targeting efficiency revealed that PEG2 (10 atoms, ≈ 11 Å) and PEG4 (16 atoms, ≈ 18 Å) provided an optimum length for keeping the potent PSMA specificity of KUE.^{10,11} Interestingly, LNCaP cells treated with KUE-ZW800+3C showed negligible signals on the cell membrane because the S1 pocket of PSMA is a tunnel-like region, about 20 Å towards the surface of the enzyme.^{5,27} On the other hand, PEG12 (40 atoms, ≈ 47 Å) and PEG24 (76 atoms, ≈ 89 Å) resulted in diminished NIR signals on the membrane of LNCaP cells due to the steric hindrance of long, flexible linkers, where the KUE moiety buried with limited exposure to PSMA.^{30,31} In all cases, only negligible fluorescence signals were observed in the PSMA-negative PC3 cells treated with the same series of KUE-PEG $_x$ -ZW800+3C fluorophores (Fig. S5, ESI[†]).

Since KUE-PEG4-ZW800+3C exhibited the highest PSMA affinity on the cell membrane, we tested this optimized targeted agent for *in vivo* biodistribution and PSMA targeting. 10 nmol of KUE-PEG4-ZW800+3C was administered intravenously into xenograft tumor mice inoculated with LNCaP (PSMA+) and PC3 (PSMA-) cells. As shown in Fig. 4a, of KUE-PEG4-ZW800+3C distributed quickly into bloodstream and peripheral tissues ($t_{1/2\alpha} = 3.89$

min), while relatively long elimination half-life ($t_{1/2\beta}$) was found (≈ 5 h). Because of this prolonged blood circulation and PSMA-specific binding, KUE-PEG4-ZW800+3C provided high targetability on PSMA positive tumors 4 h post-intravenous injection (Fig. 4b and c). More importantly, this optimized targeted agent showed reduced nonspecific uptake in major organs and tissues except for relatively high uptake in kidneys because of active renal clearance (Fig. S6, ESI[†]). H&E histological analysis and NIR fluorescence microscopy revealed the specific targeting of KUE-PEG4-ZW800+3C on the PSMA positive tumors with minimal uptake in PSMA negative tumors ($**P < 0.01$; Fig. 4d). These results indicate that physicochemical properties of targeted agents such as low log *D* and high polarity in addition to negative surface charges and hydrophilicity play the key role in biodistribution, clearance, and tumor targeting after a single intravenous injection.

In summary, a series of PSMA-targeted contrast agents with varying net charges, spacer length, hydrophobicity, and polarity were developed systematically by conjugating KUE on the backbone of different NIR fluorophores. Since the targeting moiety KUE is a highly negatively charged small molecule, the overall *in vivo* PSMA targeting, biodistribution, and clearance were mediated by the physicochemical properties of conjugated fluorophores. Particularly, KUE-PEG4-ZW800+3C carrying –6 net negative charges and 18 Å linker spacer showed an optimized performance in both *in vitro* and *in vivo* PSMA targeting assays. Because of the low log *D* values and high polarity, this optimized PSMA-targeted contrast agent excreted to urine after active uptake into the PSMA positive tumors. These results suggested that the selection of effector domain and linker length plays the key role in the targeting affinity of small molecule ligands.¹⁷ Current study, however, is limited to reveal the effect of effector domain on the cellular binding and *in vivo* targeting efficiency of small molecule ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Notes and references

1. Murphy G, Ragde H, Kenny G, Barren R, Erickson S, Tjoa B, Boynton A, Holmes E, Gilbaugh J, Douglas T. *Anticancer Res.* 1995; 15:1473–1479. [PubMed: 7544569]
2. Murphy GP, Elgamal AA, Su SL, Bostwick DG, Holmes EH. *Cancer.* 1998; 83:2259–2269. [PubMed: 9840525]
3. Misra P, Humblet V, Pannier N, Maison W, Frangioni JV. *J Nucl Med.* 2007; 48:1379–1389. [PubMed: 17631555]
4. Mease RC, Dusich CL, Foss CA, Ravert HT, Dannals RF, Seidel J, Prideaux A, Fox JJ, Sgouros G, Kozikowski AP, Pomper MG. *Clin Cancer Res.* 2008; 14:3036–3043. [PubMed: 18483369]
5. Banerjee SR, Foss CA, Castanares M, Mease RC, Byun Y, Fox JJ, Hilton J, Lupold SE, Kozikowski AP, Pomper MG. *J Med Chem.* 2008; 51:4504–4517. [PubMed: 18637669]

6. Hillier SM, Maresca KP, Femia FJ, Marquis JC, Foss CA, Nguyen N, Zimmerman CN, Barrett JA, Eckelman WC, Pomper MG, Joyal JL, Babich JW. *Cancer Res.* 2009; 69:6932–6940. [PubMed: 19706750]
7. Chen Y, Pullambhatla M, Banerjee SR, Byun Y, Stathis M, Rojas C, Slusher BS, Mease RC, Pomper MG. *Bioconjugate Chem.* 2012; 23:2377–2385.
8. Neuman BP, Eifler JB, Castanares M, Chowdhury WH, Chen Y, Mease RC, Ma R, Mukherjee A, Lupold SE, Pomper MG, Rodriguez R. *Clin Cancer Res.* 2015; 21:771–780. [PubMed: 25501577]
9. Humblet V, Lapidus R, Williams LR, Tsukamoto T, Rojas C, Majer P, Hin B, Ohnishi S, De Grand AM, Zaheer A, Renze JT, Nakayama A, Slusher BS, Frangioni JV. *Mol Imaging.* 2005; 4:448–462. [PubMed: 16285907]
10. Nakajima T, Mitsunaga M, Bander NH, Heston WD, Choyke PL, Kobayashi H. *Bioconjugate Chem.* 2011; 22:1700–1705.
11. Watanabe R, Sato K, Hanaoka H, Harada T, Nakajima T, Kim I, Paik CH, Wu AM, Choyke PL, Kobayashi H. *ACS Med Chem Lett.* 2014; 5:411–415. [PubMed: 24900850]
12. Choi HS, Liu W, Liu F, Nasr K, Misra P, Bawendi MG, Frangioni JV. *Nat Nanotechnol.* 2010; 5:42–47. [PubMed: 19893516]
13. Choi HS, Nasr K, Alyabyev S, Feith D, Lee JH, Kim SH, Ashitate Y, Hyun H, Patonay G, Strekowski L, Henary M, Frangioni JV. *Angew Chem, Int Ed Engl.* 2011; 50:6258–6263. [PubMed: 21656624]
14. Choi HS, Gibbs SL, Lee JH, Kim SH, Ashitate Y, Liu F, Hyun H, Park G, Xie Y, Bae S, Henary M, Frangioni JV. *Nat Biotechnol.* 2013; 31:148–153. [PubMed: 23292608]
15. Bao K, Nasr KA, Hyun H, Lee JH, Gravier J, Gibbs SL, Choi HS. *Theranostics.* 2015; 5:609–617. [PubMed: 25825600]
16. Hyun H, Henary M, Gao T, Narayana L, Owens EA, Lee JH, Park G, Wada H, Ashitate Y, Frangioni JV, Choi HS. *Mol Imaging Biol.* 2016; 18:52–61. [PubMed: 26084246]
17. Owens EA, Henary M, El Fakhri G, Choi HS. *Acc Chem Res.* 2016; 49:1731–1740. [PubMed: 27564418]
18. Owens EA, Lee S, Choi J, Henary M, Choi HS. *Wiley Interdiscip Rev: Nanomed Nanobiotechnol.* 2015; 7:828–838. [PubMed: 25645081]
19. Chandran SS, Banerjee SR, Mease RC, Pomper MG, Denmeade SR. *Cancer Biol Ther.* 2008; 7:974–982. [PubMed: 18698158]
20. Eder M, Schafer M, Bauder-Wust U, Hull WE, Wangler C, Mier W, Haberkorn U, Eisenhut M. *Bioconjugate Chem.* 2012; 23:688–697.
21. Huang B, Otis J, Joice M, Kotlyar A, Thomas TP. *Biomacro-molecules.* 2014; 15:915–923.
22. Weineisen M, Simecek J, Schottelius M, Schwaiger M, Wester HJ. *EJNMMI Res.* 2014; 4:63. [PubMed: 26116124]
23. Lee JH, Park G, Hong GH, Choi J, Choi HS. *Quant Imaging Med Surg.* 2012; 2:266–273. [PubMed: 23289086]
24. Hyun H, Bordo MW, Nasr K, Feith D, Lee JH, Kim SH, Ashitate Y, Moffitt LA, Rosenberg M, Henary M, Choi HS, Frangioni JV. *Contrast Media Mol Imaging.* 2012; 7:516–524. [PubMed: 22991318]
25. Hyun H, Owens EA, Narayana L, Wada H, Gravier J, Bao K, Frangioni JV, Choi HS, Henary M. *RSC Adv.* 2014; 4:58762–58768. [PubMed: 25530846]
26. Njiojob CN, Owens EA, Narayana L, Hyun H, Choi HS, Henary M. *J Med Chem.* 2015; 58:2845–2854. [PubMed: 25711712]
27. Mesters JR, Henning K, Hilgenfeld R. *Acta Crystallogr, Sect D: Biol Crystallogr.* 2007; 63:508–513. [PubMed: 17372356]
28. Liu T, Nedrow-Byers JR, Hopkins MR, Berkman CE. *Bioorg Med Chem Lett.* 2011; 21:7013–7016. [PubMed: 22018464]
29. Peng ZH, Sima M, Salama ME, Kopeckova P, Kopecek J. *J Drug Targeting.* 2013; 21:968–980.
30. Sawant RR, Sawant RM, Kale AA, Torchilin VP. *J Drug Targeting.* 2008; 16:596–600.
31. Wang M, Thanou M. *Pharmacol Res.* 2010; 62:90–99. [PubMed: 20380880]

Fig. 2. Physicochemical and optical properties of KUE-conjugates varying in net charges, spacer length, hydrophobicity, and polarity: (a) physicochemical properties of each fluorophore. log D = distribution coefficient; TPSA = topological polar surface area. (b) 3D conformer distributions (top) and optical properties (bottom) of KUE-PEG4-fluorophores. Absorbance and fluorescence emission spectra were measured in 100% FBS supplemented with 50 mM HEPES, pH 7.4.

Fig. 3. *in vitro* PSMA targeting assay on living prostate cancer cells: A series of (a) KUE-PEG4-linked NIR fluorophores and (b) KUE-PEG x -linked ZW800+3C conjugates ($x = 0, 2, 4, 12$, or 24) were incubated at a concentration of $2 \mu\text{M}$ in PSMA-positive LNCaP cells at 4°C for 60 min prior to washing and image acquisition. Fluorescence images have identical exposure time and normalization. Scale bars = $100 \mu\text{m}$.

Fig. 4. Selective *in vivo* targeting of KUE-PEG4-ZW800+3C into 20 g xenograft tumor mice (10 nmol; 0.4 mg kg^{-1}): (a) blood clearance and pharmacokinetics for 4 h. (b) Biodistribution based on SBR (organ vs. muscle) at 4 h post-injection ($n = 3$, mean $\pm$ s.d., $**P < 0.01$). Abbreviations used are: Du, duodenum; He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung; Pa, pancreas; Sp, spleen; $T(+)$, positive tumor; $T(-)$, negative tumor. (c) Intraoperative imaging of tumors 0.5 h and 4 h post-injection. Scale bars = 1 cm. (d) NIR fluorescence microscopy and H&E histology of resected tumors at 4 h post-injection. Solid circle, tumor microenvironment; dashed circle, tumor necrosis. Scale bars = 100 μm .