Published in final edited form as:

Bioorg Med Chem Lett. 2011 December 1; 21(23): 7013–7016. doi:10.1016/j.bmcl.2011.09.115.

Spacer length effects on *in vitro* imaging and surface accessibility of fluorescent inhibitors of prostate specific membrane antigen

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Abstract

Prostate-specific membrane antigen (PSMA), a type II transmembrane protein, has been becoming an active target for imaging and therapeutic applications for prostate cancer. Recently, the development of its various chemical inhibitor scaffolds has been explored to serve as carriers for therapeutic or diagnostic payloads targeted to PSMA-positive tumor cells. However, there have been few efforts to definitively determine the optimal length of linker between PSMA inhibitor cores and their payload molecules with regard to the affinity to PSMA and *in vitro* performance. In our present model study, three spacer-length varied fluorescent inhibitors (FAM-CTT-54, FAM-X-CTT-54 and FAM-PEG₈-CTT-54) were synthesized, and further enzymatic inhibition studies displayed linker length-dependent changes in: inhibitory potency (IC₅₀ = 0.41 nM, 0.35 nM, 1.93 nM), modes of binding (reversible, slowly reversible, irreversible), respectively. Furthermore, cell-labeling imaging revealed the spacer length-related change of fluorescence intensity (FAM-X-CTT-54 > FAM-PEG₈-CTT-54 > FAM-CTT-54). These results suggest that selection of linkers and their lengths will be important considerations in the development of next-generation prostate tumor-targeted imaging probes and therapeutic agents that specifically home to PSMA on tumor cells.

Keywords

Tumor-targeting imaging; PSMA; Confocal microscopy; Fluorescein conjugate; PEG linker

The cell-surface enzyme prostate-specific membrane antigen (PSMA) is perhaps the most important enzyme-biomarker and target in prostate cancer research and is being aggressively pursued as a target for the selective delivery of novel diagnostic and therapeutic agents. PSMA is up-regulated and strongly expressed on prostate cancer cells, including those that are metastatic.¹ Endothelial-expression of PSMA in the neovasculature of a variety of non-prostatic solid malignancies has also been detected.^{2,3} The unique enzymatic activity of PSMA^{4–6} and the resolution of its crystal structure^{7–9} have enabled the development of various high-affinity chemical inhibitor scaffolds for this enzyme-biomarker.^{10–17} Recently,

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the successful deployment of PSMA inhibitors as targeting motifs for imaging and therapeutic agents suggests that such constructs can serve as pharmacokinetic alternatives to antibodies.^{18–23}

Although we previously reported that phosphoramidate peptidomimetic PSMA inhibitors were capable of both cell-surface labeling of prostate tumor cells for imaging^{18, 19, 24} and intracellular delivery for targeted photodynamic therapy,^{20, 25, 26} there have been few efforts to definitively determine the optimal spacer length between a PSMA inhibitor core and its diagnostic or therapeutic payload with respect to inhibitory potency and *in vitro* performance. The polyethylene glycol (PEG) spacer has been widely applied in nanotechnology to covalently couple small-molecule ligands or antibodies onto the surfaces of nanoparticles for targeted imaging or drug delivery.^{27–30} In general, PEG spacers can lead to improved plasma circulation and biocompatibility of nanoparticles due to their enhanced hydrophilicity, and provide sufficient flexibility for a targeting molecule to overcome spatial limitations in order to effectively interact with a corresponding target protein or receptor.²⁸ In the present study, we examined the effect of the spacer length between a representative phosphoramidate PSMA inhibitor core (CTT-54) and fluorescein-based dye (Fig. 1) upon both the inhibitory potency against PSMA and the cell-labeling of PSMA+ cells. The preparation of both the phosphate PMSA inhibitor and its fluorescein conjugates is provided in the Supplementary data.

In this study, a series of fluorescent PSMA inhibitor conjugates (FAM-CTT-54, FAM-X-CTT-54 and FAM-PEG₈-CTT-54) were synthesized according to a previously reported method.¹⁸ As shown in the Supplementary data (Fig. S1, S2), the absorption spectra (400 ~ 800 nm) and fluorescence emission (at ~520 nm) of free fluorescein dye and the fluorescent PSMA inhibitor conjugates displayed similar absorbance spectra and fluorescence intensity. These data suggest that that conjugation of CTT-54 through various spacer lengths had little impact on the spectral properties.

In Figure S3A–D, PSMA inhibition studies confirmed that conjugation of CTT-54^{19, 20, 31} to fluorescein-based dyes through various spacer lengths (FAM-CTT-54, IC₅₀ = 0.41 nM; FAM-X-CTT-54, IC₅₀ = 0.35 nM;¹⁹ FAM-PEG₈-CTT-54, IC₅₀ = 1.93 nM) had no adverse effect upon the inhibitory potency of the parent inhibitor core CTT-54 (IC₅₀ = 14 nM).¹⁹ To understand the impact of spacer length on the fluorescent inhibitor conjugates, we examined the enzymatic activity recovery profiles for PSMA inhibition by CTT-54, FAM-CTT-54, FAM-X-CTT-54 and FAM-PEG₈-CTT-54, according to our previously reported method.^{16, 18} Both CTT-54 and FAM-PEG₈-CTT-54 were shown to be irreversible inhibitors, while FAM-CTT-54 was completely irreversible and FAM-X-CTT-54 exhibited characteristics of slowly reversible inhibitors (Fig. 2). These data suggest that the placement of the fluorophore too close to the PSMA active may prevent essential conformational changes necessary for irreversible inhibition.

To determine whether the spacer length would affect the *in vitro* imaging of PSMA-positive prostate cancer cells (LNCaP), these cells were treated with each of the fluorescent inhibitor conjugates in the presences of 0.2% NaN₃ to block energy-dependent PSMA internalization.^{19, 32} Confocal microscopy revealed that the surfaces of LNCaP cells treated with FAM-X-CTT-54 and FAM-PEG₈-CTT-54 were considerably more fluorescent than cells treated with FAM-CTT-54 (Fig. 3). This data suggested quenching of the fluorophore when bound deeper into PSMA due to the absence of a spacer to link the fluorophore and the inhibitor core.

An anti-fluorescein antibody-coupled to AlexaFluor 594 (red) was used to probe the surface accessibility of the fluorophore on the fluorescent inhibitor conjugates when bound to

PSMA on LNCaP cells (Fig. 4). Red fluorescence was only observed on the surface of LNCaP cells treated first with FAM-PEG₈-CTT-54. This data suggested that unlike the shorter spacers, a spacer length such as PEG₈ would allow the fluorophore to be sufficiently remote from the PSMA surface and accessible to its antibody binding. This data were consistent with the finding above indicating that with the shorter linker, the fluorophore was likely buried in the PSMA binding cavity resulting in fluorescence quenching.

In order to gain further insight on the mode of binding, fluorescence quenching, and decrease of the antigenicity for some of the fluorescent inhibitor conjugates, molecular docking was performed for CTT-54, FAM-CTT-54, FAM-X-CTT-54 and FAM-PEG₈-CTT-54 in a crystal structure of the extracellular domain of PSMA (PDB: 3BI1).³³ Due to limitations of the software, the molecular docking of FAM-PEG₈-CTT-54 failed. The surface images for the docking of PSMA-CTT-54, PSMA-FAM-CTT-54 and FAM-X-CTT-54 (Fig. S4A–C) revealed that CTT-54 was buried in the active site of PSMA and the fluorescein moieties of FAM-CTT-54 and FAM-X-CTT-54 were localized in a cleft in the spillway toward the active site of the enzyme. This latter observation suggests that the fluorescein group would be inaccessible to the anti-fluorescein antibody, which is consistent with the decreased antigenicity observed in the anti-fluorescein antibody labeling experiments above. In Figure S4D–E, it was noted that in contrast to FAM-X-CTT-54, the fluorescein moiety of FAM-CTT-54 was in close proximity to the indole group of PSMA's Trp541, indicating a possible π -interaction and supporting the putative fluorescence quenching observed above for FAM-CTT-54 bound in PSMA on LNCaP cells.

FAM-CTT-54, FAM-X-CTT-54 and FAM-PEG₈-CTT-54 were confirmed to be potent inhibitors of PSMA with no loss of inhibitory potency due to conjugation of the parent core scaffold, CTT-54. This trend has been observed in our previous work¹⁹ and is consistent with what we observed previously for other dye conjugates of PSMA inhibitors.^{18, 24} All these studies suggest that an additional interaction between the conjugated fluorescent dye and PSMA may contribute to the enhanced potency of these fluorescent inhibitors.

In addition to the S1 and S1' substrate-binding sites of PSMA that have been shown to accommodate P1 and P1' residues of various substrates or inhibitors,^{7–9} recent structural data support the presence of an arene binding site remote from PSMA's active site.³⁴ The S1' site specifically defines the critical structural features (C-terminal glutamate or analogs) of substrates or inhibitors required for the recognition and binding to PSMA.³³ On the other hand, S1 site is flexible and can accommodate both polar and nonpolar molecules. The arrangement of both the S1 and recently identified remote arene binding sites in addition to the "open" or "closed" conformation of the entrance lid (Trp541-Gly548) appear to be dependent on a ligand's structure.³³ Molecular docking results (Figure S4A–C) suggest that the inhibitor core CTT-54 should allow for a "closed" conformation of the entrance lid. In contrast, docking FAM-CTT-54 or FAM-X-CTT-54 would be more conducive to an "open" conformation. The correlation of the entrance lid conformation and the mode of binding is consistent with the observation that CTT-54 exhibits irreversible inhibition of PSMA while FAM-CTT-54 completely reversible inhibitor or FAM-X-CTT-54 slowly reversible inhibitor (Fig. 2). Based on this trend, it was expected that FAM-PEG₈-CTT-54 (irreversible PSMA inhibitor) should allow for a "closed" entrance lid conformation by allowing the fluorescein group to be positioned closer to the protein surface and beyond the landscape of the entrance lid.

Based on the relatively low IC₅₀ value of FAM-CTT-54 (0.41 nM), the poor performance in the *in vitro* cell imaging studies of FAM-CTT-54 compared to either FAM-X-CTT-54 or FAM-PEG₈-CTT-54 was unexpected. Previously reported studies on fluorescein-quenching were mainly focused on biotin-streptavidin or anti-fluorescein antibody-fluorescein binding

pairs. When all four biotin-binding sites of streptavidin are bound with biotin-4-fluorescein (B4F), approximately 88% of the B4F fluorescence is quenched.³⁵ Both free and conjugated fluorescein has also been found to be quenched by an anti-fluorescein antibody.³⁶ The crystal structure of the fluorescein-bound antibody 4-4-20 demonstrated that the aromatic residues Tyr37 (light chain) and Trp33 (heavy chain) were key to the observed fluorescence quenching.³⁷ In contrast to FAM-X-CTT-54, the molecular docking of FAM-CTT-54 in PSMA revealed that the fluorescein group of FAM-CTT-54 could be positioned near the indole group of Trp541 for a possible π -interaction (Fig. S4D–E). Therefore, we hypothesize that Trp541 is an important contributor to the fluorescein-quenching of FAM-CTT-54 in the *in vitro* cell imaging studies (Fig. 3).

Although S1 and arene binding sites in PSMA exhibit considerable flexibility in accommodating aromatic and polyaromatic groups, we have concluded that there may be size limitations. For example, in our previous work^{20, 26} we observed that a Pyropheophorbide-a conjugated PSMA inhibitor ($IC_{50} = 102$ nM) exhibited considerably weaker affinity for PSMA compared to its inhibitor core CTT-54 ($IC_{50} = 14$ nM). It is expected that the installation of as spacer of sufficient length could allow a diagnostic or therapeutic payload conjugated to a PSMA inhibitor to bypass the arene binding site, project beyond the protein surface, and enable a “closed” conformation of the entrance lid. These are particularly important considerations in developing drug conjugates to PSMA inhibitors while preserving both affinity and mode of binding to PSMA. A structural study of PSMA has revealed that an approximately 20 Å deep funnel-shaped cavity leads from the surface of the enzyme to its active site.⁹ In the present study, fluorescein-based immunofluorescence imaging provided the experimental proof that a PEG₈ spacer was sufficiently long enough to escape the PSMA protein surface and render the fluorescein accessible to its antibody binding. In addition, we have found that unlike biotin-PEG₁₂-CTT-54, biotin-PEG₄-CTT-54 labeled cells cannot be detected by extravidin-conjugated fluorescein (unpublished data). Furthermore, crystal structure studies have revealed that PEG linkers are highly dynamic and absent of specific interactions with PSMA.³⁴ Therefore, the current study together with these previous findings suggest that a PEG_{8–12} linker between an irreversible PSMA inhibitor core and a therapeutic payload should be sufficiently long enough to retain both the binding affinity of inhibitor core and mode of binding in subsequent tumor-targeted therapeutic applications.

In conclusion, the present study revealed that fluorescent PSMA inhibitor conjugates exhibited spacer length-dependent effects on inhibitory potency, mode of inhibition, and fluorescence-quenching. Therefore, the results presented herein suggest that length-optimal spacers will be critical in the development of novel drug conjugates of high-affinity, small-molecule inhibitors for both PSMA-based targeted prostate tumor and tumor neovasculature chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors extend their gratitude for technical assistance to G. Helms and W. Hiscox at the WSU Center for NMR Spectroscopy, as well as C. Davitt and V. Lynch-Holm at the WSU Franceschi Microscopy and Imaging Center. This work was supported in part by the Washington State Life Sciences Discovery Fund (LSDF 08-01 2374880) and the National Institutes of Health (5R01CA140617-02).

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Figure 1.
Structures of PSMA inhibitor core CTT-54, and its fluorescein conjugates: FAM-CTT-54, FAM-X-CTT-54, and FAM-PEG₈-CTT-54.

Figure 2.

The enzymatic activity recovery profiles for PSMA inhibited by FAM-CTT-54, FAM-XCTT-54, and FAM-PEG8-CTT-54 and CTT-54. On the basis of recovery profiles, CTT-54 and FAMPEG₈-CTT-54 are irreversible; FAM-CTT-54 is completely reversible and FAM-X-CTT-54 is slowly reversible. Uninhibited PSMA served as a control.

Figure 3.

Direct fluorescent labeling of PSMA-positive cells with fluorescent inhibitors. Live LNCaP cells were labeled with 5 μ M each of fluorescent inhibitors (green) for 30 min at 37 $^{\circ}$ C: (A) FAM-CTT-54, (B) FAM-X-CTT-54, and (C) FAM-PEG₈-CTT-54. All cells were fixed and nuclei stained with Hoechst 33342 (blue). Distance scale is 20 μ m.

Figure 4.

Indirect immunofluorescence imaging of PSMA-positive cells with an anti-fluorescein antibody. LNCaP cells were incubated with (A) 5 μ M FAM-CTT-54, (B) 5 μ M FAM-X-CTT-54, (C) 5 μ M FAM-PEG₈-CTT-54, and (D) no inhibitor all followed by incubation with anti-fluorescein antibody-coupled to AlexaFluor 594 (red). All cells were fixed and nuclei stained with Hoechst 33342 (blue). Distance scale is 20 μ m.