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Article

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Modulating the cellular uptake of fluorescently tagged substrates of prostate-specific antigen before and after enzymatic activation.

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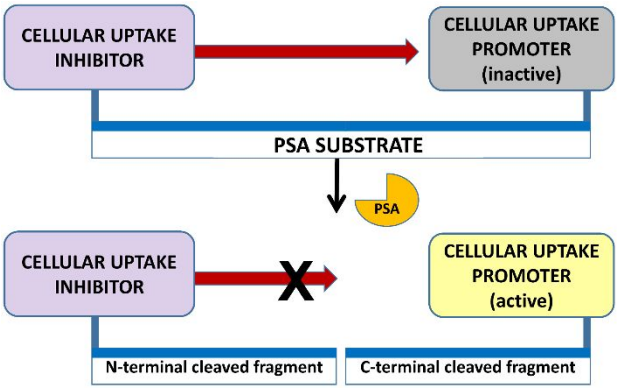
Supporting Information

ABSTRACT: A series of peptides based on the prostate-specific antigen (PSA) specific sequence histidine-serine-serine-lysine-leucine-glutamine were functionalised with an anthraquinone fluorophore at the C-terminal residue side chain using the copper(I) catalyzed azide-alkyne cycloaddition reaction. The effect of incorporating a negatively charged N-terminal tetra-glutamic acid group to the substrate and the effect of masking the negatively charged C-terminal carboxylic acid functionality of the substrate was investigated using confocal fluorescence microscopy in two cell lines (DLD-1 and LnCaP). The addition of a tetra-glutamic acid group to the N-terminus of the intact sequence was shown to reduce cellular uptake of the intact substrate prior to activation by PSA. In contrast, masking the C-terminal carboxylic acid group of the substrate as a methyl ester was shown to improve cellular uptake of the peptide fragment after activation by PSA. The synthesized C-terminal methyl ester substrates with the anthraquinone attached to the side chain were confirmed to be cleaved by PSA in LC-MS analysis, and the cytotoxicity of the substrates was shown to increase in the presence of PSA, consistent with cleavage and uptake of the C-terminal fragment. The results indicate that C- and N- terminal functionalisation of peptide substrates targeting PSA can be used to modulate the cellular uptake of peptides before and after enzymatic activation, and may thus be an important consideration in the design of tumour activated prodrugs.



INTRODUCTION

The tumour activated prodrug ('TAP') strategy has been recognised as a possible means of reducing the side effects and systemic toxicity of chemotherapy.(*1*) The improved selectivity of chemotherapy afforded by the TAP strategy is typically achieved by targeting a differentiating feature of the tumour tissue. In the treatment of prostate cancer, the serine protease prostate-specific antigen ('PSA') is a possible and ideal target for TAPs.(*2*) The presence of enzymatically active PSA is largely limited to prostate tissue, where it is expressed in high concentration.(*3-6*) PSA expression is often maintained in androgen resistant and metastatic tumours, indicating that PSA remains a viable TAP target even at the later stages of disease progression.(*7, 8*)

The PSA specific substrate histidine-serine-serine-lysine-leucine-glutamine ('HSSKLQ') has previously been utilised to deliver TAP bound cytotoxins to prostate cancer cells with promising results.(*9, 10*) Generally, to ensure selectivity, the TAP strategy requires that the cellular uptake of prodrugs in non-targeted tissue is minimal. This requirement is of particular importance for prodrugs based on the HSSKLQ sequence, as intracellular proteases have been shown to cleave and hence activate derivatives of the sequence.(*11*) Although cellular internalisation of TAPs in non-targeted tissues is undesirable, rapid internalisation subsequent to activation in the targeted site is generally a requirement of the TAP strategy; both for the generation of a chemotherapeutic effect and also to reduce the possibility of migration of the activated agent to distant non-targeted tissues.(*12*)

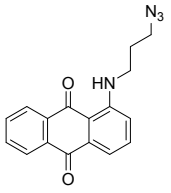
It is well established that electrostatic charge affects the cellular uptake of peptides and other materials on account of the slight negative charge of the cell surface.(*13, 14*) TAPs incorporating negatively charged poly-glutamate sequences that serve to reduce cellular uptake in non-targeted cells have been designed and reported.(*15*) Similarly, the design of a suitable TAP may include strategies or functionalisation to promote cellular uptake after activation, such as the inclusion of a cell-penetrating poly-arginine sequence.(*15*) It has previously been demonstrated that the methyl ester derivatives of amino acids, dipeptides, and tripeptides are freely able to diffuse into cells, and therefore the methyl ester functionality might also be exploited to improve cellular uptake of prodrugs once activated.(*16, 17*)

Utilising the previously described PSA-selective HSSKLQ peptide sequence, we here describe the design, synthesis, and *in vitro* testing of two model tumour activated prodrugs targeting PSA. Two C-terminal fragment peptides were also synthesised for testing. For all peptides, the C-terminal residue side chain of the peptides was functionalised with a fluorescent anthraquinone tag using a copper(I) catalysed azide-alkyne cycloaddition ('CuAAC') strategy that is orthogonal to standard peptide chemistry techniques. Fragment peptides were synthesised with and without a C-terminal methyl ester functionality and the substrates with and without a negatively charged tetra-glutamic acid group as a proposed means of modulating the cellular internalisation properties of the model TAPs. The cellular localisation of the C-terminal fragments and the model TAPs before and after activation by PSA was subsequently studied *in vitro* using confocal fluorescence microscopy.

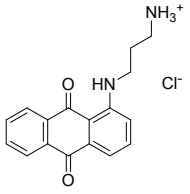
RESULTS AND DISCUSSION

Synthesis. The CuAAC compatible fluorescent tag AQAZ **(1)** was synthesised as a crystalline solid in good yield by a diazo transfer reaction of the amine parent compound 1C3 **(2)** (Chart 1). The precursor 1C3 **(2)** has previously been synthesised and utilised as a fluorescent tag for *in vitro* microscopy applications.(*18*)

Chart 1. Anthraquinone fluorescent tag and precursor.



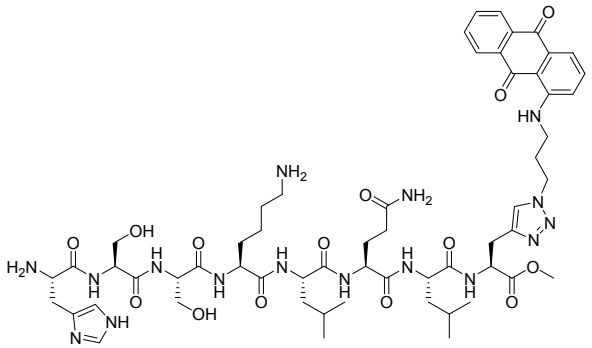
AQAZ **(1)**



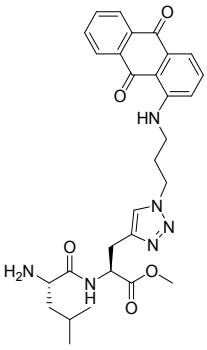
1C3 **(2)**

The fluorescently tagged peptides synthesised using AQAZ **(1)** are presented in Chart 2. Yields, analytical HPLC retention times ('RT'), and HRMS characterisation for the peptides are reported in the Methods section.

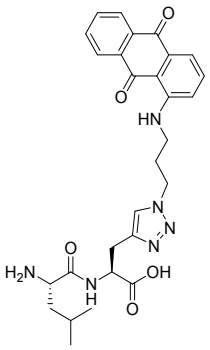
Chart 2. Fluorescently tagged peptide substrates and fragments.



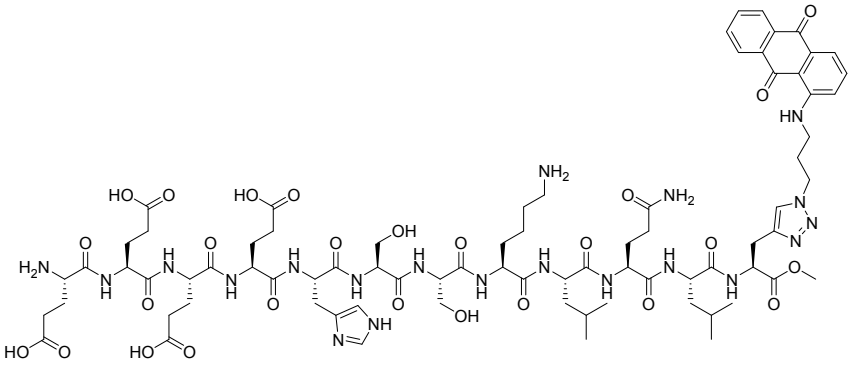
PSA-LAQMe **(3)**



LAQMe **(5)**



LAQ **(6)**



4EPSA-LAQMe **(4)**

PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)** are proposed substrates for PSA based on the HSSKLQ sequence. Both are functionalised at the C-terminal residue side chain with the fluorescent anthraquinone tag attached via CuAAC conjugation. The fluorescent anthraquinone tag serves as a model of a cytotoxic agent, and enables the localisation of the model prodrugs to be visualised *in vitro* before and after activation by PSA. Both substrates feature the C-terminal methyl ester functionality as a proposed post-activation cellular uptake promoter. 4EPSA-LAQMe **(4)** is further functionalised to feature a negatively charged tetra-glutamic acid sequence at the N-terminus as a proposed cellular uptake inhibitor of the intact substrate. For each substrate, the expected site of PSA cleavage is the glutamine-leucine bond.

Two peptide fragments were also synthesised for *in vitro* testing. LAQMe **(5)** is the expected C-terminal fragment obtained from cleavage of PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)**. LAQ (**6)** is the C-terminal carboxylic acid analogue of the expected C-terminal fragment.

Wang resin was used for the synthesis of LAQMe **(5)** and LAQ **(6)**; whilst HMBA-AM (4-hydroxymethylbenzoic acid aminomethyl) resin was used for the synthesis of the C-terminal methyl ester peptides PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)**. The C-terminal methyl ester fragment LAQMe **(5)** was synthesised via off-resin esterification of LAQ **(6)**. In all instances, side chain functionalisation via orthogonal CuAAC reaction on the solid phase support was found to produce the expected species as the major product.

Peptides were cleaved from the Wang resin support according to standard protocol,(*19*) whereas cleavage from the HMBA-AM support was performed by treatment of the resin with methanol at room temperature. Yields from Wang resin cleavage was 45% for LAQ **(6)**, indicating that CuAAC is an effective means of functionalising peptides in reasonable yield. In contrast, the yield of PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)** obtained from the HMBA-AM resin was only 4% and 5%, respectively. Very little information regarding cleavage of peptides from the HMBA-AM resin is available in the literature; the mechanism of base-mediated cleavage is recognised to be poorly understood, and crude yields of 13-32% have been reported as optimised.(*19, 20*) Cleavage of peptides from the HMBA-AM resin using standard reported methods resulted in no obtainable yield or degradation of the products.(*19*) The mechanism of successful cleavage using methanol alone was not investigated, but as the conditions used were relatively mild, this method may possibly be optimised to be suitable for the synthesis of a variety of other fluorophore or cytotoxin functionalised C-terminal methyl ester peptides in improved yields.

Fluorescence Spectra. The fluorescence spectra of the four peptides and the free anthraquinone fluorophore, 1C3 **(2)**, are presented in Figure 1. As for 1C3 **(2)**, the absorption and emission maxima for each of the peptides are approximately 522 and 644 nm, respectively. The fluorescence intensites of the peptide fragments LAQMe **(5)** and LAQ **(6)** were similar, though were both reduced relative to the intact peptide substrates, possibly due to differences in intermolecular interactions between the peptide and solvent as a result of sequence length.

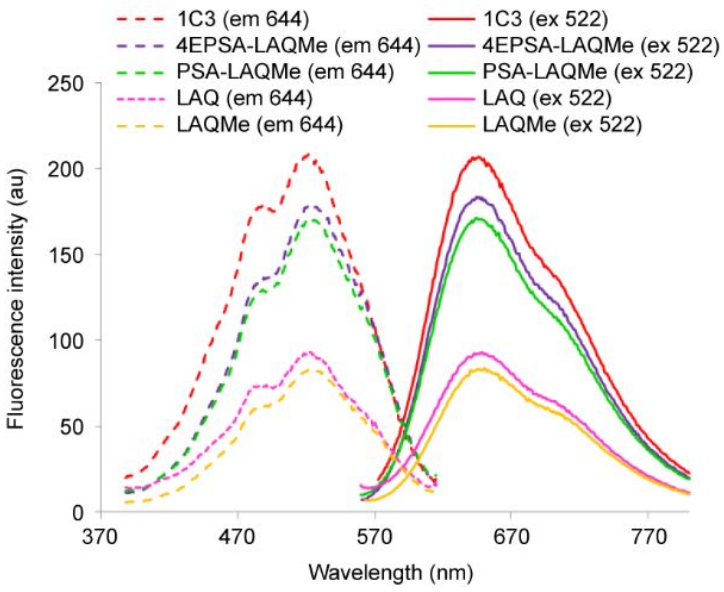


Figure 1. Fluorescence excitation and emission spectra for the fluorescently tagged peptide substrates, fragments, and free fluorophore.

Enzyme Assays. Analytical LC-MS traces for the PSA cleavage assays of PSA-LAQMe **(3)**, 4EPSA-LAQMe **(4)**, and the fluorogenic substrate standard (Mu)HSSKLQ-AFC (where Mu is 4-morpholinecarbonyl; AFC is 7-amino-4-trifluoromethylcoumarin) are presented in Figure 2. Mass spectrometry data for all experiments are included in the Supporting Information.

Cleavage at the expected site by PSA was observed for all substrates but was still incomplete after 24 h of incubation. No evidence of cleavage at the expected glutamine-leucine site was observed for any of the substrates in the absence of PSA. In each instance, the N-terminal fragment peak was partly shielded by co-elution with an LC-MS contaminant, likely N-butyl benzenesulfonamide ($m/z = 214$).

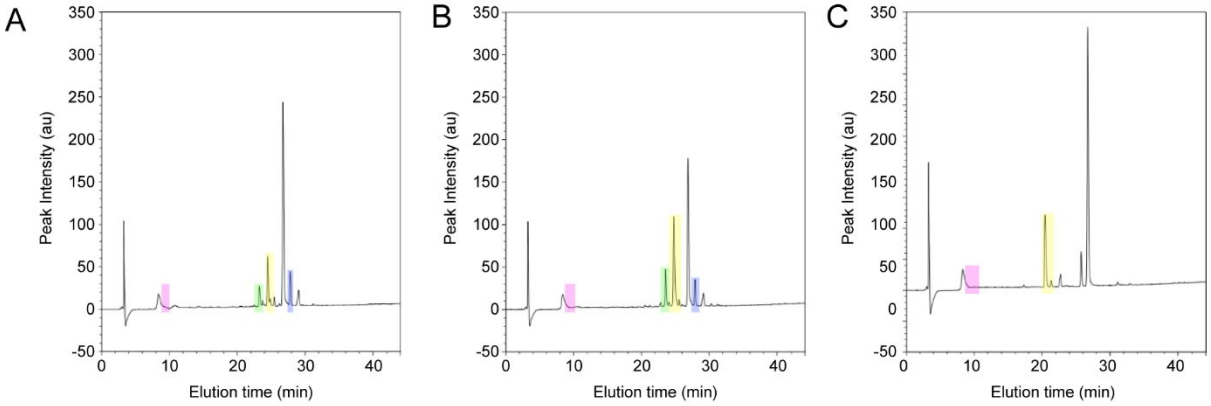


Figure 2. 24 h PSA Cleavage assays for model TAPs. The large peak in each profile at 26.2 min corresponds to Tris buffer. **(A)** Cleavage assay for PSA-LAQMe **(3)**. N-terminal fragment RT = 8.5 min, pink; intact peptide with C-terminal carboxylic acid RT = 23.5 min, green; intact peptide RT = 24.7 min, yellow; C-terminal fragment RT = 27.9 min, blue. **(B)** Cleavage assay for 4EPSA-LAQMe **(4)**. N-terminal fragment RT = 9.2 min, pink; intact peptide with C-terminal carboxylic acid RT = 23.7 min, green; intact peptide RT = 25.0 min, yellow; C-terminal fragment RT = 28.1 min, blue. **(C)** Cleavage assay for (Mu)HSSKLQ-AFC. N-terminal fragment RT = 10.1 min, pink; intact peptide RT = 20.6 min, yellow.

LC-MS revealed that the C-terminal methyl ester substrates PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)** were not completely stable in the experimental conditions used, with some formation of the C-terminal carboxylic acid analogue observed. A small amount of formation of the C-terminal carboxylic acid was also observed in PSA free controls, suggesting the instability of the C-terminal methyl ester is not entirely enzyme-mediated. Whilst it is possible that the instability may be specific to the experimental conditions used and not relevant *in vivo*, the nature of this instability requires additional investigation in future studies as it may affect the efficacy of prodrugs based on these sequences. Other minor peaks were present in the LC-MS trace but could not be identified based on the mass spectrometry data; these peaks may indicate cleavage of the peptide at other locations prior or subsequent to initial PSA cleavage, degradation of the enzyme, or non-enzyme mediated degradation of the substrates.

Cleavage of the peptide substrates was still incomplete after 24 h, which indicates that cleavage is proceeding more slowly than the rate that has previously been reported for the HSSKLQ substrate.(*11*) Whilst this may suggest that functionalisation of the substrate has hindered essential binding interactions with PSA, cleavage of the fluorogenic substrate standard (Mu)HSSKLQ-AFC was also incomplete after 24 h. The reduction in cleavage efficiency from the expected rate may

indicate there is a considerable variation in PSA activity between batches, or that the cleavage efficiency of PSA for the HSSKLQ sequence may be highly sensitive to experimental conditions. Nevertheless, despite a slower than expected rate of cleavage, the results of the cleavage assays indicate that both of the synthesised substrates are cleavable by PSA.

Confocal Microscopy. Fluorescence images of cells treated with the anthraquinone compounds are presented in Figures 3-7. Corresponding bright field images are provided in the Supporting Information. Experiments were performed in two cell lines (LnCaP and DLD-1) to best elucidate the effect of PSA on the distribution of intracellular fluorescence. The DLD-1 cell line is derived from colorectal adenocarcinoma tissue, and does not express PSA. LnCaP cells are derived from the lymph node metastasis of a prostate carcinoma, and are reported to express PSA.(*21*)

Confocal fluorescence images of cells treated with either 1C3 **(2)** for 1 h, LAQ **(6)** for 1 or 4 h, LAQ **(6)** co-incubated with the membrane permeabilising agent Triton X-100 for 10 min, or LAQMe **(5)** for 1 or 4 h are presented in Figure 3. Whilst the free fluorophore 1C3 **(2)** was found to readily accumulate in the cytoplasm of cells after 1 h, low intracellular fluorescence was observed for cells treated with the peptide LAQ **(6)**, even after 4 h. This low intracellular fluorescence is despite utilising a 20% increase in photomultiplier tube detector voltage and a five-fold increase in dosing concentration for images acquired of LAQ **(6)** treated cells relative to those acquired of cells treated with 1C3 **(2)**. In contrast, utilising the same image acquisition settings and dosing concentration for LAQ **(6)** treated cells, intracellular fluorescence was readily visible in both cell lines treated with LAQMe **(5)** at both time points.

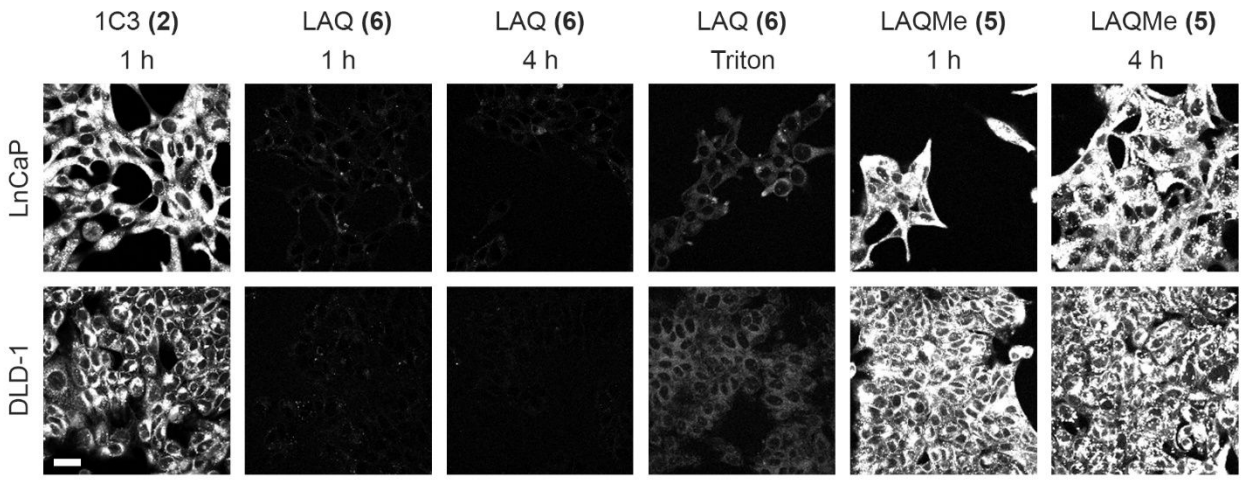


Figure 3. Fluorescence microscopy images of LnCaP and DLD-1 cells treated with 1C3 **(2)**, LAQ **(6)** for 1 or 4 h, LAQ **(6)** and Triton X-100 for 10 min, and LAQMe **(5)** for 1 or 4 h. The scale bar represents 30 μm.

Co-incubation of LAQ **(6)** and Triton X-100 was performed to test the hypothesis that the low intracellular fluorescence observed for cells treated with LAQ **(6)** is due to low cellular uptake. As seen in Figure 3, intracellular fluorescence was readily observed after 10 min of co-incubation of LAQ **(6)** with Triton X-100 in both cell lines. These results therefore suggest that cell penetration of the LAQ **(6)** peptide is inhibited by the C-terminal carboxylic acid functionality, perhaps as a result of electrostatic repulsion between the negative charge of the carboxylic acid functionality and the cell surface.

LnCaP and DLD-1 cells were then treated with the C-terminal methyl ester peptides LAQMe **(5)**, PSA-LAQMe **(3)**, or 4EPSA-LAQMe **(4)** in the presence or absence of introduced PSA. Images were acquired after 1, 4, and 24 h. Cells were treated with PSA at a concentration of 20 μg/ml. On account of the bright fluorescence for LAQMe **(5)** treated cells observed previously, images of all treated cells in these subsequent figures were acquired with a 10% reduction in photomultiplier tube detector voltage relative to those acquired for the C-terminal carboxylic acid peptide LAQ **(6)** in Figure 3.

As shown in Figure 4, LnCaP cells treated with PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)** displayed intracellular fluorescence in the presence or absence of introduced PSA at all time points. The pattern of fluorescence observed is consistent with PSA-mediated cleavage of the substrates

and subsequent uptake of the fragment occurring. However, the extent of the uptake in the absence of added PSA is not as great as might have been expected for a PSA producing cell line and could indicated that a substantial amount of the PSA present is inactive. In order to better understand this we have used the DLD-1 cell line which does not produce any PSA, enabling us to quantify the effect of the added PSA.

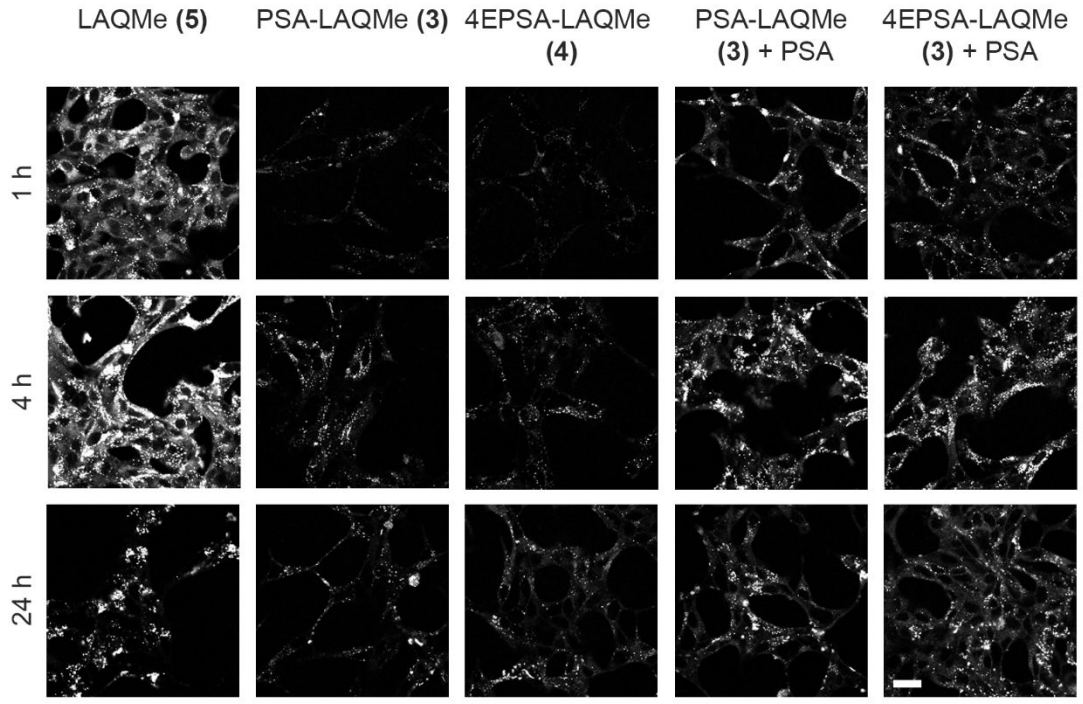


Figure 4. Confocal fluorescence microscopy images of LnCaP cells treated with LAQMe **(5)**, PSA-LAQMe **(3)**, or 4EPSA-LAQMe **(4)** for 1, 4, or 24 h in the presence or absence of exogenous PSA. The scale bar represents 30 µm.

As shown in Figure 5, intracellular fluorescence was not readily apparent for DLD-1 cells treated with either PSA-LAQMe **(3)** or 4EPSA-LAQMe **(4)** for 1 or 4 h in the absence of introduced PSA. This suggests that the HSSKLQ sequence itself serves to inhibit cellular uptake relative to the LAQMe **(5)** fragment. Faint fluorescence was observed after 24 h for cells treated with PSA-LAQMe **(3)** but not 4EPSA-LAQMe **(4)**. In the presence of PSA, intracellular fluorescence was readily visible for cells treated for 4 or 24 h with either PSA-LAQMe **(3)** or 4EPSA-LAQMe **(4)**. Fluorescence was also apparent for cells treated with PSA-LAQMe **(3)** or 4EPSA-LAQMe **(4)** in the presence of PSA after 1 h, but was quite faint, particularly for cells treated with 4EPSA-

LAQMe **(4)**. These results are consistent with gradual cleavage of the substrates by PSA, and may indicate that the tetra-glutamic acid has slowed cleavage of the substrate. The uptake following addition of PSA is extensive and after 24 hrs achieves levels similar to those seen for LAQMe **(4)** at 4 hrs indicating that any hydrolysis of the methyl ester has not significantly interfered with uptake.

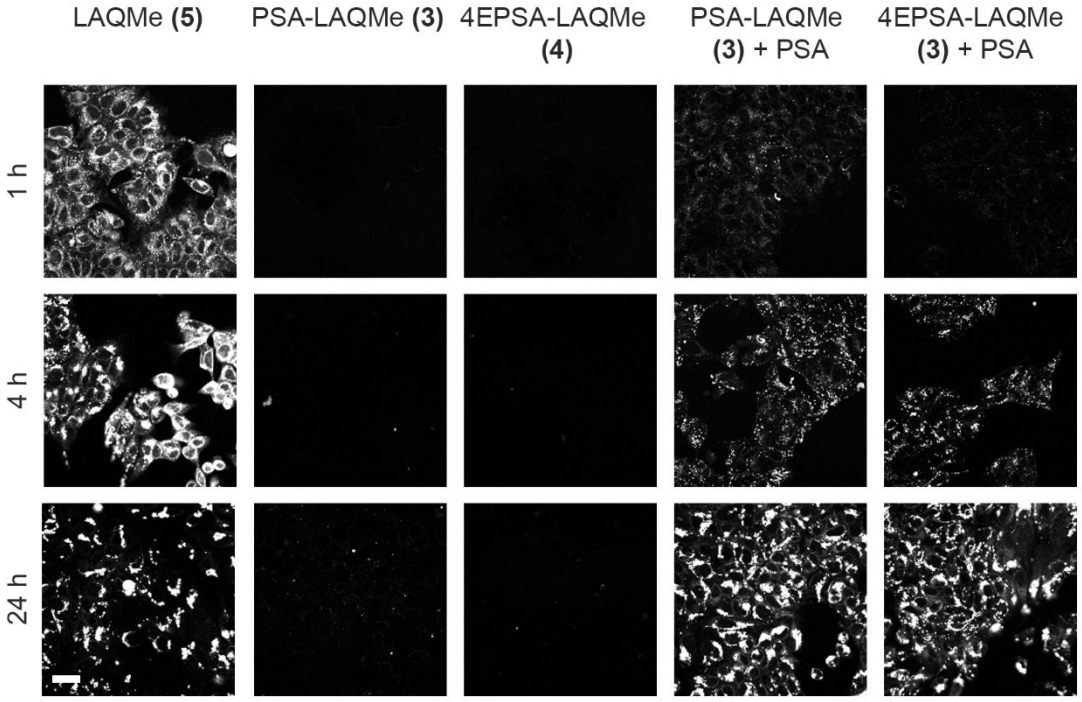


Figure 5. Confocal fluorescence microscopy images of DLD-1 cells treated with LAQMe **(5)**, PSA-LAQMe **(3)**, or 4EPSA-LAQMe **(4)** for 1, 4, or 24 h in the presence or absence of exogenous PSA. The scale bar represents 30 µm.

On account of the low overall intracellular fluorescence observed for DLD-1 cells treated with PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)** in the absence of PSA, the effect of the tetra-glutamic acid group on cell uptake could not readily be determined. To better visualise any existing difference, identical contrast enhancement settings (Image J) were applied to images of DLD-1 cells treated with either substrate for the two longer time points, 4 or 24 h, as presented in Figure 6. Relative to PSA-LAQMe **(3)**, cells treated the tetra-glutamic acid containing sequence, 4EPSA-LAQMe **(4)**, appeared to exhibit lower intracellular fluorescence, particularly after 24 h.

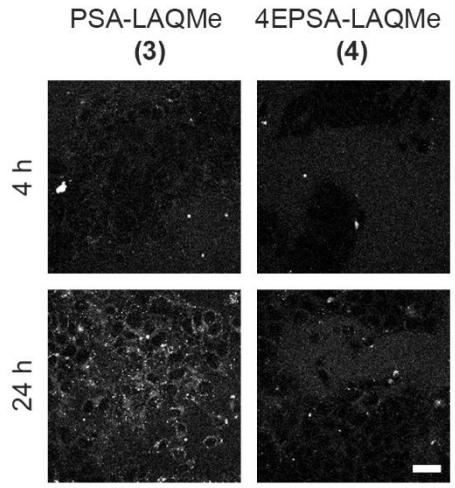


Figure 6. Contrast enhanced images of DLD-1 cells treated with PSA-LAQMe **(3)** or 4EPSA-LAQMe **(4)** at 4 and 24 h. The scale bar represents 30 µm.

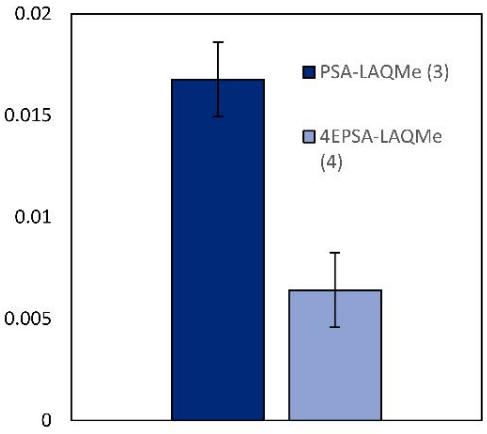


Figure 7. Corrected total cell fluorescence for DLD-1 cells treated with PSA-LAQMe **(3)** or 4EPSA-LAQMe **(4)**. Error bars represent SD.

To better quantify this difference, unenhanced images of PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)** treated cells were subsequently analysed for quantitative fluorescence using Image J.(*22*) Differences in mean corrected total cell fluorescence ('CTCF') for cells treated with PSA-LAQMe **(3)** (n = 9) or 4EPSA-LAQMe **(4)** (n = 9) were found to be significant in the analysed sets (unpaired t-test p value <0.05), with the CTCF of 4EPSA-LAQMe **(4)** treated cells being less than half of that of cells treated with PSA-LAQMe **(3)** (Figure 7). This suggests that the tetra-glutamic group further reduces cellular uptake of the intact model TAP.

Cytotoxicity. IC_{50} values for DLD-1 cells treated with PSA-LAQMe **(3)**, 4EPSA-LAQMe **(4)**, LAQMe **(5)**, 1C3 **(2)**, or cisplatin are presented in Table 1. IC_{50} values for the peptides co-incubated in the presence of PSA (at a concentration of 10 µg/mL) are also provided. Cisplatin was included as a standard, however the IC_{50} of cisplatin was found to be higher than values reported elsewhere. This may be due to the absence of serum used for the assays, as serum starvation has previously been reported to reduce to the cytotoxicity of cisplatin.(*23*) 1C3 **(2)** was also included as a standard, being a simple model for an anthraquinone with a side chain terminating with an amine, and the

results are similar to those obtained previously.[17] Calculated p-value results of unpaired t-test statistical analyses are provided in the Supporting Information.

Table 1. IC_{50} values of compounds in DLD-1 cells in the presence or absence of PSA.

	PSA-LAQMe **(3)**	4EPSA-LAQMe **(4)**	LAQMe **(5)**	1C3 **(2)**	Cisplatin
IC_{50} (μM) ± SD	296 ± 13	>500	102 ± 8	9 ± 0.5	26 ± 1
IC_{50} (μM) ± SD (+ PSA)	189 ± 11	202 ± 16	114 ± 2	-	-

Whilst the cytotoxicity of PSA-LAQMe **(3)** appeared to be greater than that of 4EPSA-LAQMe **(4)**, the significance of this difference could not be determined as the IC_{50} value of 4EPSA-LAQMe **(4)** was found to be beyond the range of concentrations tested (>500 μM) in all experimental replicates. The cytotoxicities of PSA-LAQMe **(3)** and 4EPSA-LAQMe **(4)** increased with the addition of PSA, consistent with cleavage of the substrates and subsequent uptake of the generated LAQMe **(5)** fragment. The cytotoxicity of both substrates was still less than that of LAQMe **(5)** itself, likely due to slow and incomplete cleavage of the substrates by PSA. Cytotoxicity of the substrates was comparable, with no significant difference in IC_{50} values for PSA-LAQMe **(3)** or 4EPSA-LAQMe **(4)** in the presence of PSA. This suggests that whilst a difference in cleavage efficiency between the substrates may exist, it does not result in a difference in *in vitro* efficacy over the course of 72 h. Finally, whilst LAQMe **(5)** was the most cytotoxic of all the peptides tested, the addition of PSA did not significantly affect its cytotoxicity.

Although the anthraquinone moiety was included in the design of these peptides to serve as a fluorophore, anthraquinones have previously been shown to be cytotoxic on account of their ability to intercalate with DNA and inhibit topoisomerase I.(*24*) The cytotoxicity of LAQMe **(5)** was lower than that found for 1C3 **(2)** despite the observed high cellular uptake of the LAQMe **(5)** fragment. The reduced cytotoxicity relative to that of 1C3 **(2)** may be due to the peptide or triazole functionality interfering with binding interactions of the anthraquinone to enzyme or DNA targets.

Discussion. This work has described the synthesis of a series of substrates for PSA functionalised with an anthraquinone fluorophore via CuAAC chemistry. Confocal fluorescence imaging has enabled the fate of the model prodrugs prior to and after activation by PSA to be visualised providing new insights into the potential of enzymatically cleaved drug delivery agents. The results obtained demonstrate the complementary utility of the tetra-glutamic acid and C-terminal methyl ester functionalities as a means to inhibit and promote cellular uptake, respectively. In combination with the PSA-specific sequence, HSSKLQ, these functionalities can be utilised to modulate the activity of prodrugs inside and outside of the targeted tumour site.

To date, previously reported prodrugs based on the HSSKLQ sequence typically feature attachment of the cytotoxic agent directly to the C-terminus of the substrate. We have instead described the use of CuAAC techniques to attach a fluorophore to the C-terminal residue side chain rather than the C-terminus itself. Side chain conjugation using CuAAC techniques is expected to be a useful strategy for synthesising a variety of functionalised peptides in a simple and predictable methodology, each carrying a different agent and therefore being appropriate for use against cancer cells exhibiting different phenotypes. However, the results obtained in this study indicate the substantial inhibitory effect of the unmasked negatively charged C-terminal carboxylic acid on cellular uptake of the activated fragment, despite the presence of the highly lipophilic anthraquinone. Strategies which involve side chain functionalisation of peptides should therefore involve some consideration to the masking of the C-terminal carboxylic acid to overcome the inhibitory effect on cell uptake observed.

For microscopy and cytotoxicity assays, PSA concentrations of 20 μg/mL or 10 μg/mL were used for experiments, respectively. These concentrations are lower than those typically found within the extracellular environment of prostate cancer tissue, which are reported to be in the 50-68 μg/mL range, of which 80-90% is enzymatically active.(*4, 25*) Although the use of higher PSA concentrations would be favourable for cleavage efficiency, conservative concentrations were instead chosen to establish proof of principle of cleavage. The results suggest that the levels of activity of endogenous PSA in the LnCaP fall well below this.

There are several possible limitations with respect to the design of these prodrugs that must be acknowledged. Firstly, the partly punctate pattern of localisation observed in the cytoplasm of cells treated with LAQMe **(5)** may indicate an endosomal or lysosomal accumulation. Further investigation is required to elucidate the subcellular fate of the C-terminal methyl ester peptides and determine if endocytic internalisation and lysosomal trafficking is involved. Depending on the intended cytotoxin or chemotherapeutic used, lysosomal localisation of an activated prodrug may be advantageous or a hindrance to activity. For example, lysosomal trapping may result in degradation or sequestration of the cytotoxic agent before reaching the intended target elsewhere within the cell; alternatively, lysosomal accumulation may be exploited as a means to avoid recognition by efflux transporters and hence circumvent cell resistance mechanisms.(*26, 27*)

Secondly, the presence of peptidases and esterases in human plasma may limit the clinical implementation of peptide and ester based prodrugs. Assays of human plasma have determined the significant presence of only three esterases: BChE, PON1, and albumin, but not carboxylesterase.(*28*) In this study, the stability of the model prodrugs in human serum was not investigated, but is an important consideration prior to further development of these compounds. Nevertheless, in our experiments, 4EPSA-LAQMe **(4)** was highly resistant to cellular uptake in cells, and LAQ **(6)** was also found to exhibit poor cellular uptake on account of the C-terminal carboxylic acid functionality. In light of these results, plasma esterase hydrolysis of a prodrug based on the 4EPSA-LAQMe **(4)** sequence would be expected to result in the formation of a compound which is even further resistant to cellular uptake compared to the parent compound. It would therefore be reasonable to expect that inadvertent hydrolysis of a C-terminal methyl ester based prodrug is not likely to directly produce toxicity to healthy tissues *in vivo*, but could be expected to reduce efficacy of the prodrug following activation. As esterase and peptidase recognition is stereochemistry dependent, incorporation of D-amino acids into the prodrugs may be an appropriate and simple strategy to stabilise the peptide sequences against peptidase and esterase activity.(*29*)

With the same rationale, the non-enzyme mediated instability of the C-terminal methyl ester functionality observed in the cleavage assays may result in a reduction in efficacy but not necessarily selectivity of the prodrugs. Whilst the results obtained indicate the utility of the C-

terminal methyl ester in promoting cellular uptake of the fragment, the development of an alternative functional group to mask the C-terminus of the peptides may be required to overcome the limitation of poor stability of the C-terminal methyl ester group.

Stability limitations of potential prodrugs based on the HSSKLQ sequence might be further minimised by modifications which promote more rapid cleavage by PSA, thus minimising the time in which the prodrugs are susceptible to degradation. This may be particularly important for sequences featuring the tetra-glutamic acid group, as whilst quantitative cleavage assays were not performed, the microscopy studies revealed that there may be some reduction in cleavage efficiency imparted by the tetra-glutamic acid functionality. Initial studies of newly identified sequences such as SSKYQ (serine-serine-lysine-tyrosine-glutamine) indicate that improved efficacy of cleavage can be achieved with simple modification of the HSSKLQ sequence.(*30*) Thus, emerging sequences targeting PSA with improved activity might be used to overcome the possible limitation of slow cleavage observed.

MATERIALS AND METHODS

Synthesis of 1C3 (2). The method used to synthesise 1C3 ([(3-aminepropyl)amine]anthracene-9,10-dione) **(2)** was based on several previously described methods for the synthesis of 1C3 (2) and for similar compounds.(*24, 31, 32*) Di-*tert*-butyl dicarbonate (21.8 g, 100 mmol, 1 eq) was dissolved in 1,4-dioxane (330 mL) and added drop-wise with stirring to a solution of propane-1,3-diamine (47.0 mL, 0.57 mol, 5.7 eq) in 1,4-dioxane (330 mL) over 5 h. After evaporation of the solvent, water (450 mL) was added and the resulting precipitate was removed by filtration. The aqueous filtrate was extracted with dichloromethane (3 × 200 mL) and the combined organic layers washed with brine and dried over sodium sulfate to yield *tert*-butyl (3-aminepropyl)carbamate as a yellow oil. A portion of the oil (3.6 g, 21 mmol, 1 eq) was combined with 1-chloroanthraquinone (5.0 g, 21 mmol, 1 eq), and potassium carbonate (5.8 g, 42 mmol, 2 eq) in dry acetonitrile (150 mL) and refluxed under nitrogen for 12 h. The solvent was removed under reduced pressure and the residue re-suspended in chloroform (400 mL) and washed with water (4 × 200 mL). The organic phase was concentrated to ~5 mL and purified using column chromatography on a silica solid phase by isocratic elution with chloroform. Fractions containing pure N-boc protected 1C3

(2) were identified using TLC (R_f silica, 1:1 EtOAc:Hexanes = 0.75) and were combined and concentrated to dryness. A portion of the material (5.0 g, 13 mmol) was dissolved in a mixture of hydrochloric (10 mL) and acetic (50 mL) acids and the solution was stirred for 1.5 hr. After removal of the acid via rotary evaporation, the solid residue was re-dissolved in chloroform, washed with water, and concentrated to dryness. The product was dissolved in a minimum volume of water (~ 2 mL) with heating (~ 80 ^oC), and subsequently precipitated from solution by drop-wise addition of ethanol (~ 6 mL). The solid was collected by filtration, washed with ethanol (2 × 5 mL) and diethyl ether (2 × 10 mL) to yield 1C3 **(2)**. Yield: 3.0 g, 8.8 mmol. ^{1}H NMR (300 MHz, $(CD_3)_2SO$, ppm): δ 9.64 (t, J = 5.1 Hz, 3H, NH_3), 8.19 (bs, 1H, NH), 8.16-8.07 (m, 2H, =C-H), 7.90-7.79 (m, 2H, =C-H), 7.63 (dd, J = 7.6, 8.4 Hz, 1H, =C-H), 7.41 (d, J = 7.6 Hz, 1H, =C-H), 7.27 (d, J = 8.4 Hz, 1H, =C-H), 3.51-3.45 (m, 2H, CH_2), 2.92-2.91 (bm, 2H, CH_2), 1.97 (dt, J = 6.7 Hz, 2H, CH_2). ^{13}C NMR (75 MHz, $(CD_3)_2SO$, ppm): δ 184.0, 182.8, 151.1, 135.6, 134.5, 134.2, 133.9, 133.5, 132.3, 126.3, 126.2, 118.6, 115.1, 112.2, 39.0, 36.6, 26.6. MS (ESI^+) ($MeOH/H_2O$): m/z = 281.44 ($[M + H]^+$). Elemental analysis (for $C_{17}H_{16}ClN_2O_2$): C 64.29%, H 5.34%, N 8.62% (found); C 64.66%, H 5.11%, N 8.87% (calculated).

Synthesis of AQAZ (1). AQAZ **(1)** was synthesised using 1C3 **(2)** and the diazo transfer reagent imidazole-1-sulfonyl azide, '1AZ'.(*33*) 1AZ (500 mg, 2.4 mmol, 1.1 eq) was added to a suspension of 1C3 **(2)** (708 mg, 2.2 mmol, 1 eq), potassium carbonate (699 mg, 5.1 mmol, 1.1 eq relative to moles of hydrochloride), and copper (II) sulfate pentahydrate (5 mg, 20 μmol, 0.01 eq) in methanol (10 mL) and the mixture was stirred at room temperature for 16 h. The mixture was subsequently concentrated to dryness, diluted with water (30 mL), and extracted with dichloromethane (3 × 50 mL). The organic layers were combined, dried over magnesium sulfate, filtered, and concentrated to dryness. Flash chromatography on an alumina solid phase by elution with 10% ethyl acetate in hexane afforded the product as a red solid. The solid was subsequently recrystallised from slow evaporation of a mixture of dichloromethane (12 mL), acetone (10 mL), and dimethyl sulfoxide (0.2 mL) to produce red crystalline needles. Yield: 478 mg, 1.6 mmol, 78%. ^{1}H NMR (300 MHz, $CDCl_3$, ppm): δ 9.69 (s, 1H, NH), 8.20-8.16 (m, 2H, =C-H), 7.73 – 7.63 (m, 2H, =C-H), 7.54 – 7.44 (m, 2H, =C-H), 6.97 (dd, J = 8.3, 1.2 Hz, 1H, =C-H), 3.51 (t, J = 6.7 Hz, 2H, CH_2), 3.40 (t, J = 6.7 Hz, 2H, CH_2), 2.00 (dt, J = 6.7 Hz, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$, ppm): δ 185.1, 183.6, 151.5, 135.4, 134.9, 134.7, 134.0, 133.0, 126.7, 117.6, 115.9, 113.2,

49.1, 40.0, 28.6. MS (ESI^+): *m/z* = 307.30 ($[M + H]^+$). Elemental analysis (for $C_{17}H_{14}N_4O_2$): C 66.87%, H 4.62%, N 18.20% (found); C 66.66%, H 4.61%, N 18.29% (calculated). R_f (silica, 1:1 EtOAc:Hexanes) = 0.8.

Peptide Synthesis. Peptides were synthesised manually using a preloaded solid-phase resin support and the Fmoc protection strategy. The OtBu, Trt, Boc, and tBu protecting groups were utilised for the side chains of Glu, His and Gln, Lys, and Ser residues, respectively. Quantities of reagents specified are for 0.125 mmol equivalents of peptide. The pre-loaded resin was allowed to swell with gentle shaking in DMF (5 mL) for 30 min in a 10 mL polypropylene syringe fitted with a porous polypropylene disc and cap (Torviq). Removal of the Fmoc group was afforded by treatment with a solution of 20% piperidine in DMF (3 ml) in a 2 × 2 min and 1 × 15 min sequence, utilising fresh solution for each step. After thorough washing with DMF (7 ml × 5), a solution of the amino acid to be coupled (5 eq), combined with HOBt (5.5 eq), HBTU (5 eq), and DIPEA (12 eq) in DMF (7 ml) was added to the syringe. All couplings were allowed to proceed for 1 h and performed in duplicate. The reaction solution was expelled and the resin was rinsed with DMF (5 ml × 10). The procedure was repeated for all subsequent deprotection and coupling steps, as required.

Fmoc-PropagylGlycine-Wang resin (0.78 mmol/g) was used for the synthesis of the peptides PSA-LAQ **(3)**, LAQ **(6)**, and LAQMe **(7)**. Fmoc-PropargylGlycine-HMBA-AM resin (0.70 mmol/g) was used for the synthesis of the C-terminal methyl ester peptides PSA-LAQMe **(4)** and 4EPSA-LAQMe **(5)**. The C-terminal methyl ester fragment LAQMe **(7)** was synthesised from LAQ **(6)** via amino acid esterification using trimethylsilyl chloride according to a published method.(*34*)

In brief, trimethylsilyl chloride (7.1 µL, 0.056 mmol, 2 eq) was added to pure LAQ **(6)** (15 mg, 0.028 mmol, 1 eq) and the mixture was stirred in a micro scintillation vial (0.5 mL capacity) at room temperature for 2 min. Methanol (100 µL) was added and the mixture was stirred for an additional 24 hr. The solvent and volatiles were removed by gentle blowing with N_2(g) to give the C-terminal methyl ester product as a hydrochloride salt. The peptide was subsequently isolated and purified as for the other synthesized peptides.

Attachment of Fluorophores. Attachment of each fluorophore via CuAAC was performed manually on the solid resin support using a method based on those reported previously.(*35, 36*) Attachment was through conjugation of the azide functionalized fluorophore to the propargyl functionalised peptide. All reagent quantities specified are for a 0.125 mmol equivalent of peptide. CuAAC reactions were performed using the Fmoc protected peptide in order to reduce inadvertent coordination of the catalytic copper ions to the N-terminus. To the swollen resin-bound peptide, 2 equivalents of AQAZ **(1)** together with DIPEA (50 eq for Wang resin; 20 eq for HMBA-AM resin), CuI (2 eq) and DMF (9 ml) was added. The syringe was gently shaken for 16 h after which the solution was drained. The resin was washed with DMF (5 ml × 10), the copper chelating agent diethyldithiocarbamic acid sodium trihydrate (0.02 M in DMF, 5 ml, 1 × 10 min), and DMF again (5 ml × 10). The N-terminal Fmoc group was deprotected as described previously and the resin washed again with DMF (8 ml × 10), DCM (5 ml × 10), and methanol (5 ml × 5).

Cleavage of peptides from Wang Resin. The peptide-bound resin was dried under vacuum for 12 h. It was subsequently gently shaken for 3 h with a cocktail consisting of TFA, TIS, and water prepared in a 9.5:0.25:0.25 ratio (3 mL). After collection of the filtrate, the resin was rinsed once with neat TFA (1 mL) and the combined filtrates were added to cold diethyl ether (20 mL). The resulting suspension was centrifuged and the pellet was washed with cold diethyl ether (3 x 20 mL). The crude peptide was purified by HPLC to yield the peptide with C-terminal carboxylic acid functionality.

Cleavage of peptides from the HMBA-AM resin. After attachment of the fluorophore and N-terminal Fmoc deprotection as described previously; sufficient methanol was added to cover the resin-bed (~1 mL) and the syringe left to stand uncapped for 16 h. The solution was filtered and the resin rinsed with additional methanol (5 × 5 mL) to collect residual peptide. The combined filtrates were evaporated to dryness and treated with 95% aqueous TFA solution (15 mL) for 2 h without shaking. The solution was evaporated to dryness and the peptide purified by HPLC to yield the peptide with a C-terminal methyl ester functional group.

Peptide Purification. Peptides were purified to ≥ 95% purity via reverse phase HPLC using a Waters 2535 HPLC system. Signals were monitored at 280 nm with a Waters 2489 UV/Visible

detector. Either a Waters Sunfire C18 column (5µm, 19 × 150 mm) at 7 mL/min or Waters XBridge Prep OBD C18 column (5µm, 30 × 150 mm) at 17 mL/ min was used. Water and acetonitrile containing 0.1% TFA was used as the eluent in gradients as specified. Relevant fractions were collected manually and all peptides lyophilised.

PSA-LAQ **(3)**. Purification: Waters XBridge, 10-50% over 40 min, RT = 28.34 min. PSA-LAQMe **(4)**. Purification: Waters Sunfire, 5-100% over 90 min. 4EPSA-LAQMe **(5)**. Purification: Waters Sunfire, 5-100% over 90 min, RT = 35.23 min. LAQ **(6)**. Purification: Waters Sunfire, 10-50% over 40 min, RT = 22.52 min. LAQMe **(7)**. Purification: Waters Sunfire, 10-50% over 40 min, RT = 23.91 min.

Peptide Characterisation. Peptides were characterised by HRMS and Analytical HPLC. Positive ion ESI HRMS was performed using a Bruker Apex Qe 7T Fourier Transform Ion Cyclotron Resonance mass spectrometer. Reverse phase analytical HPLC at a flow rate of 0.2 ml/min was performed using a Shimadzu LC-MS-2020 system and XBridge BEH300 Peptide Separations Technology C18 column (5µm, 2.1 × 150 mm); or an Alliance-Waters 2695 HPLC system and XTerra RP18 column (5µm, 2.1 × 150 mm). Water and acetonitrile with either 0.1% TFA (Alliance-Waters) or 0.1% formic acid (Shimadzu) was used as the eluent. Signals were monitored at 254 nm with a Waters 2998 Photodiode Array Detector or a Shimadzu SPD-M20A Prominence diode array detector. Analytical traces are presented in the Supporting Information.

PSA-LAQMe **(3)**. Yield 6.5 mg, 0.005 mmol, 4%. ESI: m/z = 614.31713 $(M + 2H)^{2+}$. Analytical: Shimadzu, 0-100% over 60 min, RT = 27.02 min. 4EPSA-LAQMe **(4)**. Yield: 11.6 mg, 0.007 mmol, 5%. ESI: m/z = 872.40155 $(M + 2H)^{2+}$. Analytical: Shimadzu, 0-100% over 60 min, 27.43 min. LAQMe **(5)**. Yield: 11 mg, 0.021 mmol, 74%. ESI: m/z = 547.26609 $(M + H)^{+}$. Analytical: Alliance-Waters, 0-100% over 60 min, RT = 32.60 min. LAQ **(6)**. Yield: 30 mg, 0.056 mmol, 45%. ESI: m/z = 533.25160 $(M + H)^{+}$. Analytical: Alliance-Waters, 0-100% over 60 min, 28.71 min.

Fluorescence Spectra. Fluorescence spectra of compounds were obtained at room temperature using a 6Q quartz fluorescence cuvette (pathlength = 10 mm) and a Varian Cary Eclipse

fluorescence spectrophotometer (PMT voltage = 600 V; slit width = 20 nm). All solutions were prepared in water at a concentration of 50 μM.

PSA Cleavage Studies. Methods and conditions used for the PSA cleavage assays were based on those previously reported.(*11*) Enzymatically active prostate-specific antigen from human seminal fluid was obtained from GenWay BioTech (2.19mg/mL solution in PBS at >95% purity by SDS-PAGE, 30 kDa, filter sterilised). Each substrate (40,000 pmol, 8 μL of a 5 mM stock solution prepared in water) was co-incubated at 37 °C with PSA (200 pmol, 6 μg, 2.1 μL of PSA solution) in PSA buffer (50 mM Tris and 150 mM NaCl, sterile filtered) in a total volume of 200 μL. 20 μL aliquots were taken after 24 h and diluted to 100 μL with the addition of 0.2% TFA in a 1:1 mixture of water and acetonitrile. 20 μL of each centrifuged sample was analysed using a Shimadzu LC-MS-2020 system with an XTerra MS C18 column (5 μM 2.1 × 150 mm) and gradient of 0-100% acetonitrile in water over 60 min with 0.1% formic acid as buffer. The resulting PDA trace was analysed for peaks at 254 nm in the 200-1000 m/z range. The cleavage of a fluorogenic substrate standard, (Mu)HSSKLQ-AFC, was also tested in the same conditions. Control incubations containing only substrate in buffer were also performed and analysed in the same manner to confirm that observed cleavage at the glutamine-leucine bond was enzyme mediated.

Cell Lines. The human-derived DLD-1 and LnCaP-FGC ('LnCaP') cell lines were obtained from The American Type Culture Collection (ATCC) and were used from passages 4-20 within 3 months of resuscitation. Cells were maintained in exponential growth in Adv DMEM supplemented with either 5% (for DLD-1) or 10% (for LnCaP) fetal bovine serum, 1% glutamine, and 1% combined antibiotic-antimycotic. No antibiotic-antimycotic was included in cells seeded for experiments. Cells were maintained in a humidified environment at 37.5 °C with 5% CO_2.

Confocal Fluorescence Microscopy. Approximately 9000 cells in 100 μL of supplemented media were seeded into each well of a 96-well glass bottom plate (MatTek). Prior to seeding of LnCaP cells, glass bottom plates were pre-treated with 50 μL of a 3.3 mg/L fibronectin in PBS solution for 45 min, after which the solution was discarded. Cells were allowed to adhere for 18 h in standard incubation conditions. The media in each well was then removed and the cells were gently

washed with Adv DMEM (3 × 100 μL). Adv DMEM supplemented with only 1% glutamine was added to each well (100 μL). LnCaP cells were allowed to reattach for an additional 12 h. Wells were dosed with compounds to a final concentration of 10 μM (for 1C3 **(2)**) or 50 μM (for all peptides), in the presence or absence of introduced PSA (2 μg per well, at a concentration of 20 μg/ml). Cells were incubated for 4 h, and images were subsequently acquired in a humidified live cell chamber at 37 ^{o}C using an Olympus FV1000 inverted fluorescence microscope with UPLSAPO 60XW objective lens and FV5-LDPSU NTT Opti λ 559 laser at 21% laser power, 1x gain, and 10% offset. The 559 laser was used at 635 V for Figure 3 images of cells treated with 1C3 **(2)**; at 780 V, for Figure 3 images of cells treated with LAQ **(6)** or LAQMe **(5)**; and at 695 V for all images in Figures 4 – 6.

Anthraquinone fluorescence emission was detected using collection in the 601-701 nm range and images acquired using Kalman averaging (3 frames), a scan rate of 4 μs/pixel, and a confocal aperture of 120 μm. Control images were acquired of untreated cells using identical settings, and all images were acquired in triplicate using cells from independent experiments. For experiments utilising Triton X-100, cells were treated to a final Triton X-100 concentration of 0.05% for 10 min. Control images of cells treated with Triton X-100 alone were also acquired to confirm any fluorescence was due to the tested compounds and not artefactual. Quantitative fluorescence imaging was performed according to a method described previously.(*22*) Corrected total cell fluorescence ('CTCF') was calculated using Image J and the formula CTCF = integrated density – (area of selected cells x mean background fluorescence). Measurements of cell fluorescence were obtained from readings of at least nine cells from separate experiments; and background fluorescence readings were calculated using three separate areas. Statistical analysis was performed using the unpaired t-test and GraphPad software.

Cytotoxicity. Approximately 10,000 cells in 100 μL of supplemented growth medium were seeded into each of the wells of a flat bottomed 96 well plate and allowed to adhere for 18 h. The media was gently removed and replaced with serum free media (1% glutamine) with or without PSA (1 μg per well, approximate concentration 10 μg/mL). Cells were subsequently dosed in triplicate with a range of compound concentrations ranging from 0 - 500 μM. The dosed cells were incubated

for 72 h, after which MTT (2.5 mg/mL, 20 μL) was added to each well. Cells were then incubated for a further 4 h after which the supernatant was gently removed via pipette and DMSO (200 μL) was added to each well to dissolve the formed formazan crystals. Dissolution of the crystals was aided through repeated agitation by pipetting of the DMSO in each well. Absorbance readings of each plate were subsequently conducted at 600 nm with a 1 min shake using a Perkin-Elmer Victor 3V microplate reader. Statistical analysis was performed using the unpaired t-test and GraphPad software. All IC_{50} values represent the mean ± SD obtained from the data of at least three independent experiments performed in triplicate.

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ASSOCIATED CONTENT

Supporting Information

^{1}H and ^{13}C NMR spectra, LC-MS and HPLC traces, HRMS profiles, bright field microscopy images, statistical analyses. Available free of charge via the Internet at http://pubs.acs.org.

ABBREVIATIONS

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CuAAC, copper(I) catalysed azide-alkyne cycloaddition; HSSKLQ, histidine-serine-serine-lysine-leucine-glutamine; PSA, prostate-specific antigen; RT, retention time; TAP(s), tumour activated prodrug(s).

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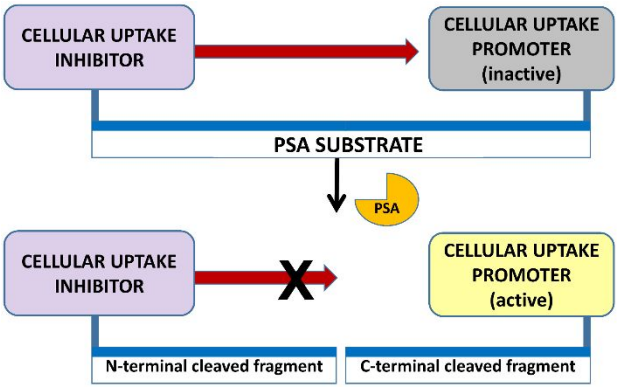
ToC Graphic


CELLULAR UPTAKE INHIBITOR
CELLULAR UPTAKE PROMOTER (inactive)
PSA SUBSTRATE
PSA
CELLULAR UPTAKE INHIBITOR
X
CELLULAR UPTAKE PROMOTER (active)
N-terminal cleaved fragment
C-terminal cleaved fragment