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Towards Enzyme Activated Antiprostatic Agents†

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Abstract—A prodrug conjugate designed to undergo activation by enzymatic prostate specific antigen has been synthesized. The prodrug system undergoes activation with PSA or α-chymotrypsin, and shows selective cytotoxicity in a PSA secreting cell line.

Despite improvements in local therapy and increased awareness, prostate cancer continues to be second only to lung cancer as a cause for cancer deaths in men.[1] Although prostate cancer in its advanced state is usually sensitive to hormonal therapy in the form of androgen ablation, this therapy is always of limited duration, thus there remains a dire need for new systemic therapies.[2] Prostate cancer is unique among solid tumors in its consistent secretion of prostate specific antigen (PSA) through all stages of the illness.[3] PSA is a serine protease, however, PSA in serum, but not in the prostatic microenvironment, is rapidly inactivated by binding to serum proteins.[4]

An attractive possibility would be to directly link a proteinogenic PSA substrate to a cytotoxic drug, such that a more active form of the drug is released following proteolysis. A peptide conjugate of the antiprostatic agent doxorubicin has been investigated, however in this case enzymatic hydrolysis resulted in release of a doxorubicin–amino acid conjugate, which had inferior activity to the parent drug.[5] We therefore sought to provide proof-of-principle for a three component PSA activated system comprised of a protease substrate and cytotoxin, coupled through an inert linker unit that chemically insulates the cytotoxic agent (Scheme 1).

Though a number of high-affinity substrates for PSA have been identified, we initially sought to evaluate enzymatic efficiency using a minimal substrate. Surveying known substrates reveals tyrosine conjugates to be particularly susceptible to hydrolysis, so conjugate **1a** was prepared and evaluated (Scheme 2).[6] Either using purified PSA (Cortex Biochem) or sonicates from the PSA active prostate cancer cell line, LNCaP, hydrolysis ensued on exposure, yielding tyrosine **2** (R^1 = Bz) and aniline **3** (R^2 = COOH). However, neither conditioned media from non-PSA secreting tumor cells (PC-3, MCF-7), nor their sonicates, had any effect on the substrate, even measured spectrophotometrically. Human serum also did not cleave the substrate. These results indicate that a protease with PSA-like activity is both secreted from, and exists within, LNCaP cells but not from non-PSA expressing cancer cells nor serum from normal donors.

Though derivatization of the carboxylate terminus of **1a/3a** (R^2) may be a means to introduce a prodrug, the product **3** would need to undergo rapid hydrolysis for efficient delivery of the cytotoxic agent. We therefore sought an alternative linker system which initiated spontaneous release of prodrug, and, based on the prior work of Katzenellenbogen, prepared benzyl alcohol **1b**.[7] As in the case of **1a**, release of free aniline (**3**, R = CH_2OH) occurred on exposure to PSA (Scheme 2). With this key finding in hand, we moved to assemble a prodrug conjugate that could became activated upon release. Katzenellenbogen successfully demonstrated enzymatic release (trypsin) of a *p*-aminobenzyl carbamate from a lysine conjugate, under which conditions the carbamate underwent spontaneous decarbamoylation, releasing free *p*-aminobenzyl alcohol and an amine (*p*-nitroaniline).[7] For our initial studies we postulated that a nitrogen mustard agent linked to the *p*-aminobenzyl alcohol via a carbamate function might also undergo spontaneous release,

†Dedicated in memory of Andrew H. Weinberg.

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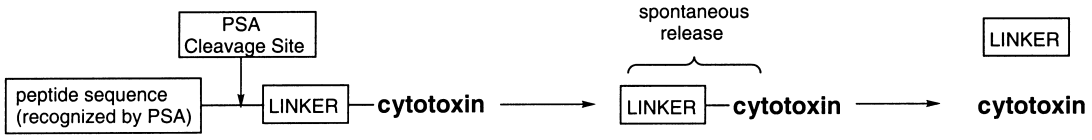
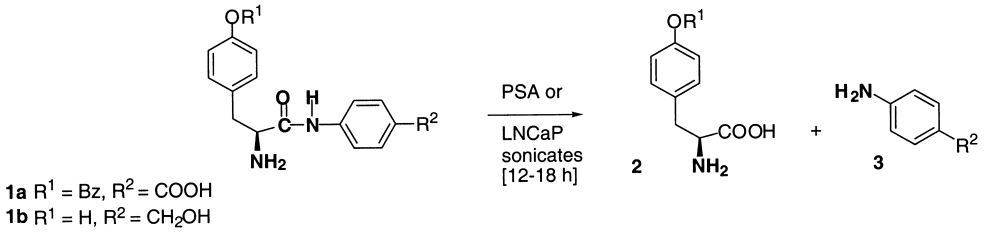
in the process liberating a cytotoxic species. Anchimeric assistance from the (basic) nitrogen atom plays a key role in the activation of mustard agents,[8] many of which have clinical application.[9]

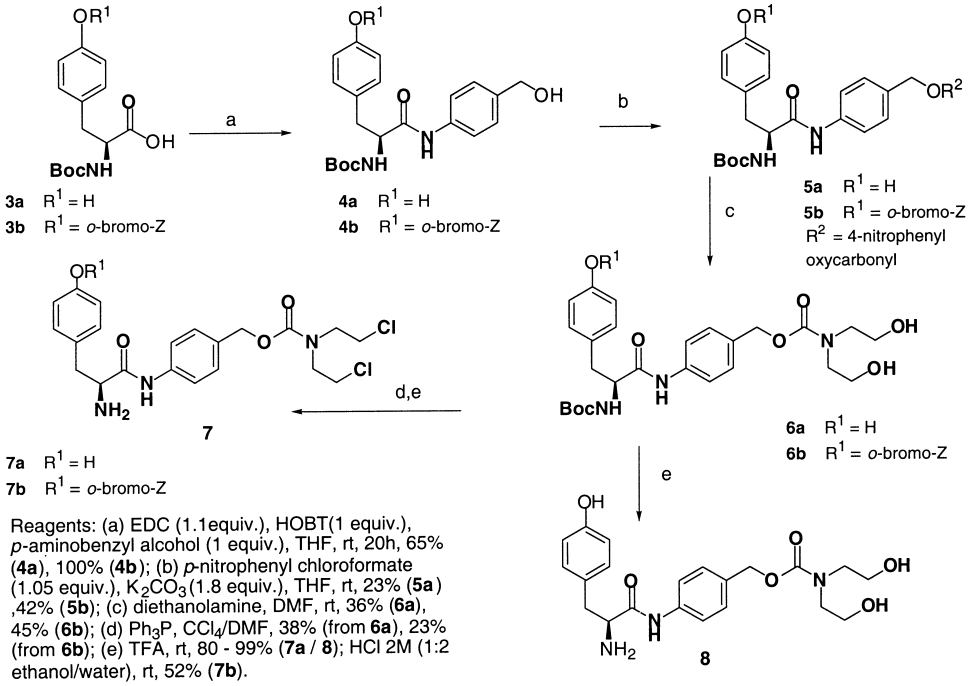
Accordingly, substrate **7** became our immediate target (Scheme 3). Boc protected tyrosine (or Tyr-O-Bz) was coupled with *p*-aminobenzyl alcohol to yield **4**. Coupling with *p*-nitrophenyl chloroformate gave key intermediate **5**, primed for coupling with the appropriate amine. Accordingly, reaction with diethanolamine gave **6**, which was converted to chloromustard and deprotected to give **7** in good yield. Though incubation of PSA with either **7a** or **7b** failed to induce release of the mustard agent, the endopeptidase α-chymotrypsin was effective with **7a**, giving products **10** and **11** (X = Cl) directly within 12 h (Scheme 4). However, as with PSA, α-chymotrypsin did not release **10/11** with substrate **7b** even after 24 h, which may reflect the poor solubility of this substrate. Significantly, when PSA mediated activation of **7a** is conducted in a 1:1 solution of buffer and DMSO, release of **10** is detected (~5%/18 h), suggesting solubility is a key factor. To address this issue further, we also prepared and investigated enzyme mediated activation of analogue **8**, which was freely soluble in the aqueous buffer used for the studies. In this case both α-chymotrypsin and PSA were able to promote release of **10**/**11** (X = OH) within 12 h.[10] Attempts to isolate the presumed intermediate **9** were unsuccessful, suggesting that once hydrolysis of the peptide bond is achieved, spontaneous decarboxylation ensues, as reported in the original example.[7]



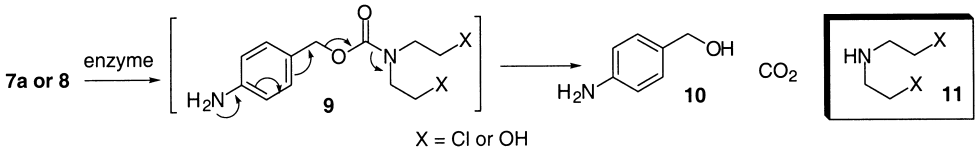
Scheme 1. Three component system for PSA activated cytotoxic prodrug.



Scheme 2. PSA mediated release of Tyr-linker conjugates.



Scheme 3. Preparation of tyrosyl mustard conjugates using *p*-amidobenzyl carbamate linker.



Scheme 4. Enzymatic activation of Tyr-linker-mechlorethamine conjugates.

Table 1. Cytotoxicity of conjugates [IC_{50}][a]

Entry	Substrate	LNCaP	MCF-7	HEK-293
1	**6a**	>500 μM ± 18	>900 μM ± 15	>600 μM ± 48
2	**7a**	33 μM ± 3	>900 μM ± 17	176 μM ± 14
3	Mechlorethamine[b]	64 μM ± 5	70 μM ± 6	
4	**7a**[c]	34 μM ± 3		173 μM ± 15

[a]Cells treated in sextuplicate with candidate compounds [72 h] and growth assessed by monitoring uptake of ^3H thymidine. IC_{50} expressed as concentration [μM] required for 50% reduction in cell growth.
[b]$CH_3N(CH_2CH_2Cl)_2$.
[c]Denotes PSA [1 μg] added to assay medium.

Cytotoxicity studies performed using a PSA secreting cell line (LNCaP) and non-PSA secreting lines (MCF-7, HEK-293) clearly demonstrated selective cytotoxicity for **7a** in the PSA rich cells, which may imply that enzymatic activation is plausible under cellular conditions (Table 1). However, when non-PSA secreting cells (HEK-293) are 'doped' with PSA, the cytotoxicity of **7a** remains unchanged (within standard error), pointing either to some alternate mode of activation unique to LNCaP cells, or PSA inactivation on binding to serum (entry 4). Opportunity to improve the therapeutic index of these agents exists, as a number of polypeptide sequences with high affinity for PSA have been reported,[5] which could be expected to reduce competing non-specific hydrolysis and improve prodrug solubility under aqueous conditions. Encouraged by the present findings, we are currently engaged in preparation of conjugates of other antitumoral agents including anthracyclines,[11] where this activation method, or related ADEPT directed strategies may prove promising,[12] and also enzyme activated imaging systems, where controlled release may be useful for mapping of the prostatic microenvironment.[13]

Representative Enzymatic Conditions

In duplicate, PSA (Cortex Biochem), 20 μL, 1.0 mg/mL, pH 7.4 0.01 M phosphate buffer saline [PBS]) or α-chymotrypsin (Sigma, TLCK-treated, 20 μL, 5.0 mg/mL, pH 7.4 0.01 M PBS), 160 μL PBS, and 20 μL substrate (1.0 mg/mL; 1:1, EtOH:H_2O) were incubated for 12–18 h at 37 °C. As a control, duplicate reactions containing PBS (180 μL) and substrate (20 μL) solutions were incubated under identical conditions. *p*-Aminobenzyl alcohol was quantified against authentic standards by HPLC (C18 μBondpak, 1 mL/min, 100% *i*PrOH, $t_R = 8.2$ min).

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