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A 5-Fluorodeoxyuridine Prodrug as Targeted Therapy for Prostate Cancer

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Abstract—A method for targeted delivery of the cytotoxic agent 5-fluorodeoxyuridine (FudR) (**1**) to sites of metastatic prostate cancer is described. The prodrug was synthesized by coupling the active drug (FudR) to the PSA-peptide via a self-cleaving diamino acid linker to produce HSSKLQ-Leu-Aib-FudR. This prodrug serves as a substrate for prostate specific antigen (PSA). This approach permitted efficient conversion of the inactive prodrug back to the active cytotoxic state by the enzymatic activity of PSA which is highly expressed by prostate cells.

One-third of all cancers in American males are of prostatic origin placing adeoncarcinoma of the prostate as the most commonly diagnosed malignancy in males in the United States.[1] Yearly, about 180,000 new prostate cancer cases are diagnosed with about 32,000 attributable deaths. These figures translate into the reality that 20% of all males will develop clinical prostate cancer during their lifetime lowering the average life span by about 9.2 years.[2,3] The five year cause-specific survival rates for patients that harbor localized prostate cancer approximate 88% whereas only 29% of patients with metastatic disease survive for five years.[3]

There is currently no curative therapy for men with metastatic prostate cancer who relapse after androgen ablation, even though numerous agents have been tested over the past 30 years.[4] Androgen ablation, although of substantial palliative benefit is never curative no matter how completely given.[5] Androgen ablative therapy ultimately fails due to the fact that metastatic PC within an individual patient is heterogeneously comprised of clones of both androgen independent and androgen dependent prostatic cancer cells.[6,7] The presence of these androgen independent prostatic cancer cells implies that the patient is no longer curable using androgen ablation. In addition, while emerging clinical data suggest that newer chemotherapeutic agents may prolong survival in a subset of men, the disease is uniformly fatal once it reaches the hormone-refractory state because current therapies are unable to completely eliminate these androgen independent prostate cancer cells. In addition, the dose and duration of administration of these newer agents is often limited by significant systemic toxicities.

Previously we have demonstrated that peptide-doxorubicin prodrugs can be selectively activated by prostate-specific antigen (PSA) both in vitro and in vivo.[8,9] The underlying hypothesis of this paper is that the proteolytic activity of prostate specific antigen (PSA) can be used to activate peptide bound 5-FudR prodrug substrates specifically to cytotoxic metabolites. These 5-FudR prodrugs could have potential use as systemic therapy for metastatic prostate cancer and could also be used as targeted radiation sensitizers for treatment of localized prostate cancer.

In this paper, we outline a plan of develop prodrug of very potent chemotherapeutic agent 5-fluoro-2′-deoxyuridine (5-FudR). In vivo, it is converted by thymidine kinase to FudR-5′phosphate which is cytotoxic by inhibiting the enzyme thymidylate synthetase.[10] The cytotoxicity of FudR, however, is not prostate cancer specific.

One approach to targeted delivery of FudR is to take advantage of the unique ability of PC cells to produce PSA. PSA, a serine protease with chymotrypsin-like activity, is secreted in high concentration (>1000 μg/mL) into the extracellular fluid by both normal and

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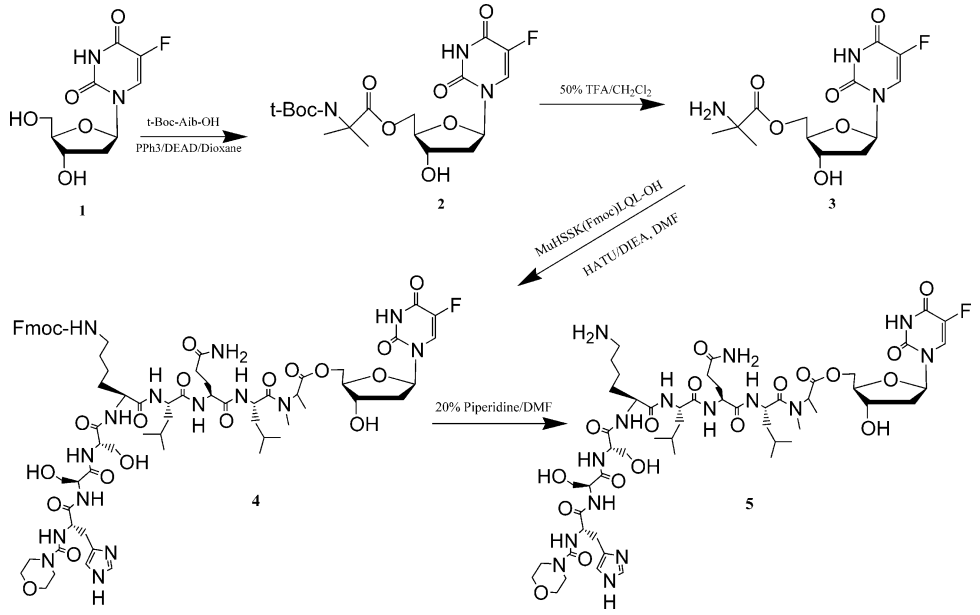
malignant prostate cells, which allows selective prostate drug targeting. The enzymatically active PSA eventually enters the bloodstream whereupon it is inactivated by binding of the major serum protease inhibitors α_1-antichymotrypsin and α_2 macroglubulin. The peptide His-Ser-Ser-Lys-Leu-Glu-OH (HSSKLQ) is PSA specific peptide substrate that is efficiently and selectively cleaved by the enzymatic activity of this protease.[11]

We now report the synthesis of the peptide-conjugate prodrug MuHSSKLQ-Leu-Aib-FudR (**5**). The prodrug was synthesized by the chemical coupling of active drug to PSA peptide via the self-cleaving diamino acid linker H-Leu-Aib-OH,[12] where Aib denotes a-methyl alanine. The synthetic procedure is outlined in Scheme 1. The most convenient synthetic route to *t*-Boc-Aib-FudR (**2**) was by direct condensation of FudR with *t*-Boc-Aib-OH in the presence of the Mitsunobo reagent. Although the reaction is slow, the target compound was generated in moderate yield. A particular advantage of this approach is that *t*-Boc-Aib-FudR could be generated under mild neutral conditions, and the need for protection and subsequent deprotection of 3′-OH protective group was avoided.

Briefly, *t*-Boc protected *N*-methyl alanine (*t*-Boc-Aib-OH) was coupled to the 5′-OH of FudR (**1**) via Mitsonobu condensation[13] to afford carboxylic ester **2**. Treatment of **2** with 50% TFA followed by condensation with peptide MuHSSK(fmoc)LQL-OH produced **4**. Treatment of **4** with base afforded prodrug (**5**) in about 50% yield.[14]

The newly synthesized peptide-FudR was assayed for PSA hydrolysis. In brief, the prodrug was incubated in PSA buffer with 5.7 g enzymatically active PSA derived from human seminal plasma. A control in PSA buffer alone was also carried out. HPLC monitoring demon-



Scheme 1.

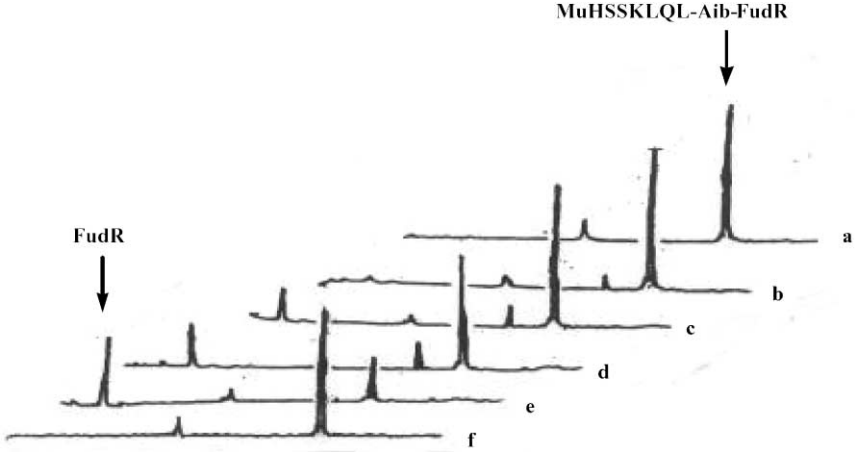


Figure 1. HPLC analysis (monitored at 275 nm) of prodrug cleavage at room temperature. (a) 50 nmol of **5** + PSA buffer (control); (b) 50 nmol **5** + PSA buffer + 20 μL PSA (0.57 mg/mL) 6 h; (c) 50 nmol of **5** + PSA buffer + 20 μL PSA 19 h; (d) 50 nmol of **5** + PSA buffer + 20 μL PSA 24 h; (e) 50 nmol of **5** + PSA buffer + 20 μL PSA 51 h; (f) 50 nmol of **5** + PSA buffer 51 h control. The column (C-18) was eluted with a linear gradient of 0.1% TFA/CH_3CN. The retention times for **5** and FudR, were 27.9 and 7.3 min, respectively.

strated release of the active drug and the peptide HSSKLQ after incubation with PSA. The HPLC trace at 275 nm demonstrates free FudR release at various time points (Fig. 1). Release of the peptide (not shown) was also demonstrated at 215 nm.

Colony survival assays were then performed to assess the efficacy and potency of the prodrug. In brief, non-PSA producing human cancer cells (TSU) were plated on 24-well plates, treated with drug or prodrug for 48 h. Viable colonies were counted and a dose–response curve constructed from which the IC_{50} value was determined. LNCaP cells did not produce reproducible colonies in colony survival assays. Therefore, to assess activation, cells were incubated with prodrug and cell counts determined as an indicator of antitumor effect. Treatment for 72 h with either drug or prodrug was followed by a 4-day growth period after which cell counts were carried out and IC_{50} values determined. The IC_{50} values for drug and prodrug are summarized in Table 1. More importantly, the prodrug had a 60-fold increase in cytotoxicity in LNCaP cells compared to TSU cells.

To confirm that LNCaP cells are not inherently sensitive to MuHSSKLQL-Aib-FudR and that the active entity resulting in prostate cancer cell death in PSA producing cells was indeed FudR, a hydrolysis assay was carried out. Briefly, 3×10^5 LNCaP cells were plated in a 6-well plate, permitted to attach overnight and treated with a 50 M solution of the prodrug. The time dependent hydrolysis of prodrug to drug in the LNCaP conditioned media was determined by constructing a standard curve correlating peak area to the amount of each compound present. As illustrated graphically in Figure 2, the prodrug, MuHSSKLQL-Aib-FudR was efficiently converted to the cytotoxin, FudR. At 120 h, approximately 75% of the prodrug was hydrolyzed. In parallel controls where the prodrug was incubated with media alone, non-specific hydroysis was $<1\%$ over a time period of 120 h.

In conclusion, we have demonstrated a novel approach to prostate targeting drug design, which takes advantage of the unique ability of PC cells to convert a biologically inactive prodrug into an active cytotoxin. This approach will allow for increased dose, duration, and therapy while avoiding systemic toxicity. The enzyme kinetic studies and activity of this 5-FudR prodrug as a targeted systemic toxin and as a radiosensitizer for prostate cancer is under study in our laboratories.

Table 1. IC_{50} values of drug and prodrug in non-PSA producing TSU cells and in PSA producing LNCaP cells

Compd	IC_{50} (nM)		Fold difference
	TSU cells	LNCaP cells	
FudR	58	69.2	0.84
MuHSSKLQ-Aib-FudR	7200	117.0	61.50

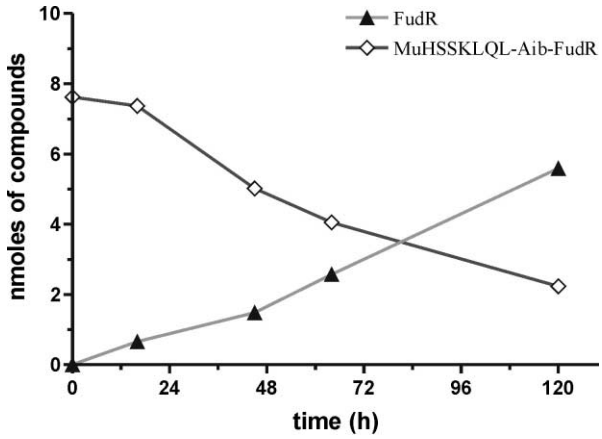


Figure 2. Hydrolysis of MuHSSKLQL-Aib-FudR to the cytotoxin FudR. PSA producing human prostate cancer cells (LNCaP) were incubated with media and 50 μM prodrug. At given time intervals equal aliquots of media were injected into HPLC and concentration of drug and prodrug were assessed.

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