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Article

Development of a Prostate-Specific Antigen Targeted Dual Drug Conjugate for Prostate Cancer Therapy

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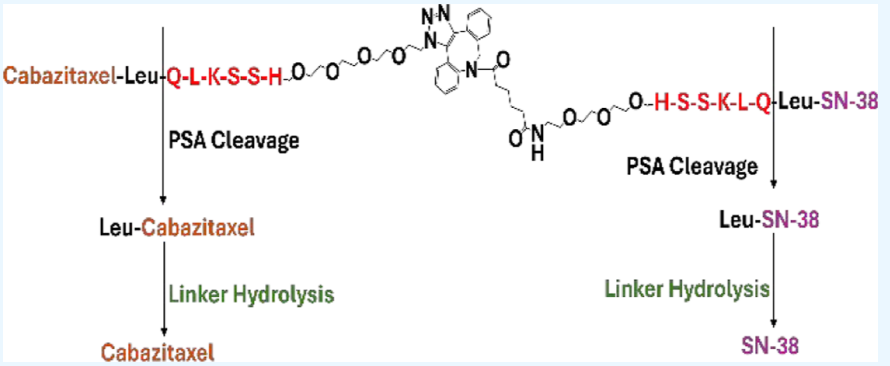

ABSTRACT: Prostate cancer (PC) is the most frequently diagnosed cancer and the second leading cause of cancer deaths in American men. While localized cases can be cured through surgery or local radiation, metastatic PC lacks curative therapy. The challenges to the success of treating advanced PC include the adverse effects of current treatment strategies, the continuous generation of cancer cells by cancer stem cells, and tumor heterogeneity. To overcome these challenges, researchers have explored the prodrug approach for targeted, combination drug delivery or release of cytotoxic agents specifically to sites of metastatic PC to improve therapeutic efficacy while decreasing systemic toxicity. The objective of this study is to develop a dual drug conjugate with a prostate-specific antigen (PSA) peptide recognition sequence for PC-specific combination SN-38 and cabazitaxel delivery in the treatment of advanced PC. To achieve this, His-Ser-Ser-Lys-Leu-Glu terminated with the dibenzocyclooctyne (DBCO) functional group conjugated to SN-38 via a leucine spacer was synthesized. Similarly, His-Ser-Ser-Lys-Leu-Glu terminated with azido poly(ethylene glycol) conjugated to cabazitaxel via a leucine spacer was synthesized. The conjugates were linked together via click chemistry to synthesize a dual drug conjugate. In vitro exposure to exogenous PSA was found to trigger the release of cytotoxic drugs. The dual drug conjugate exhibited time- and concentration-dependent cytotoxicity in PC-3 and LNCaP PC cells. The observed cytotoxicity was also dependent on PSA expression in the cellular models tested. This study demonstrates the potential of peptide−drug conjugates for the delivery of combination chemotherapeutics for selective cytotoxicity to PC cells.

INTRODUCTION

Prostate cancer (PC) is the most frequently diagnosed cancer and second leading cause of cancer deaths in American men.[1] Initial stages of PC are often androgen-dependent, responding well to androgen deprivation therapy.[2] However, a more aggressive form, known as androgen-independent prostate cancer[3,4] also referred to as metastatic castration-resistant prostate cancer (mCRPC) is ultimately observed with an average survival time of 16−18 months.[5] mCRPCs continue to produce and secrete prostate-specific antigen (PSA) into their extracellular fluid (ECF), even at later stages of disease progression. This characteristic offers a therapeutic opportunity to selectively activate targeted drug delivery systems that can be delivered to the tumor to exploit the abundant secretion of enzymatically active PSA by mCRPCs.[6]

One of the approaches that is extensively researched and found to be promising in the treatment of PC is the development of drug delivery systems to target PSA using the prodrug approach. This PSA-targeting approach has the added advantage of the "bystander effect", i.e., nearby cancerous cells that do not produce PSA can also be targeted, potentially killing all PC cells and other adjacent endothelial and stromal cells.[7−10] This "bystander effect" reduces the likelihood of tumor metastasis and recurrence.

PSA is a widely used PC biomarker that can assist in the diagnosis of disease.[11] PSA levels in the serum have been

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correlated with the presence and size of prostate tumor, including metastatic disease, as both normal and malignant prostate luminal epithelial cells produce and secrete PSA.[10] It has been reported that the ECF surrounding PC cells contain a high concentration of enzymatically active PSA. In contrast, PSA is rendered inactive in serum due to its complexation with protease inhibitors such as α-1-antichymotrypsin (ACT) and α-2-macroglobulin (A2M).[12,13] Within the serum, these inhibitors are found in elevated levels, effectively impeding the proteolytic function of PSA. This segregation of the presence or absence of PSA inhibitors between the serum and PC ECF establishes distinct environments that impact the enzymatic activity of PSA.[10,13] PSA expression is maintained even in androgen-resistant and metastatic tumors, indicating that PSA remains a viable target for cancer treatment.[13] Thus, the design of a PSA-targeted drug delivery system holds the potential for site specific delivery, thereby improving the efficiency of drug treatment and reducing the incidence of adverse effects.

In line with this hypothesis, researchers have developed targeted drug conjugates for PC therapy based on the identification of HSSKLQ as a PSA substrate that is capable of facilitating targeted delivery to PC.[8,9,13−15] The peptide sequence His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) was identified as a PSA-selective peptide substrate and has been reported to be a superior substrate for PSA due to its high specificity, water solubility, and serum stability.[14]

The first-line chemotherapy for treating PC is docetaxel (Taxotere), approved by the FDA in 2004 for CRPC that has progressed despite hormone therapy.[16] While docetaxel-based chemotherapy has shown significant clinical benefit in the treatment of mCRPC, it does have some limitations such as not offering a universal response in all patients and cases of disease progression or treatment resistance and other side effects.[17−19]

To address these limitations, cabazitaxel, a second-line taxane, was approved by the FDA in 2010 for the treatment of mCRPC patients who have previously received docetaxel-based chemotherapy. Cabazitaxel is a microtubule inhibitor that belongs to the taxane family of chemotherapy drugs, and it works by disrupting the cellular microtubule network.[19−22] Cabazitaxel interacts with the N-terminal amino acids of the beta-tubulin subunits to promote microtubule polymerization, hindering cell division during mitosis and arresting tumor cell cycle and proliferation.[20,23,24] Distinctively, cabazitaxel's molecular structure differs from docetaxel in its side chain, where methoxy side chains replace hydroxyl groups. These additional methyl groups counteract both inherent and acquired resistance to antitumor drugs by disrupting the adenosine-5′-triphosphate (ATP)-dependent efflux pump, encoded by the multidrug-resistant gene.[20,25] Thus, unlike other taxanes, which are prone to the *P*-glycoprotein (*P*-gp) efflux pump, cabazitaxel was specifically synthesized to be resistant to *P*-gp effects by decreasing its susceptibility to being recognized and pumped out by the *P*-gp efflux pump, leading to an improved intracellular concentration and allowing cabazitaxel to exert its cytotoxic effects more effectively. This makes cabazitaxel effective even in cases of docetaxel resistance as a second-line chemotherapy option for mCRPC in clinical practice.[17,18,20]

Despite the demonstrated efficacy of cabazitaxel in treating mCRPC, its clinical application is limited by its high toxicity and lack of selectivity.[26] It is hypothesized that novel delivery approaches that target tumor cells can improve their efficacy and minimize side effects. Also, the use of cabazitaxel in combination with other chemotherapeutic agents could be beneficial in improving the treatment of mCRPC.[27] The combination of two or more chemotherapy treatments has emerged as an alternative strategy in cancer therapy.[28] Combination chemotherapy leverages the distinct molecular mechanisms of multiple active agents to improve therapeutic efficacy and reduce individual toxicity from each drug while reducing drug resistance and minimizing overlapping toxicity.[29] Using this approach, the combination of taxanes and 7-ethyl-10-hydroxy-camptothecin (SN-38) has been explored in cancer.[30] A study by Wang et al., examined the synergistic effect of the use of docetaxel and SN-38 in human colon cancer cells. The results obtained from this study indicate a synergistic effect between both chemotherapeutic agents.[30] Similarly, a phase II study combining docetaxel and irinotecan (a prodrug of SN-38) showed good efficacy when both chemotherapeutic agents are combined.[31]

SN-38 is a topoisomerase 1 inhibitor and is the active metabolite of the anticancer drug, irinotecan hydrochloride (CPT-11).[32] SN-38 is 100- to 1000-fold more potent than CPT-11 in inducing antitumor activity.[33,34] SN-38 has been reported to have excellent cytotoxic effect on PC cell line PC-3.[35] While there is currently no specific study directly investigating the combination of SN-38 and cabazitaxel for the treatment of PC, the combination of these two chemotherapeutic agents may have potential synergistic effects in combating the disease. Most chemotherapeutic agents affect both healthy and cancerous cells, leading to adverse effects; hence, there is a need to develop a targeted approach to deliver these potent combination chemotherapeutic agents selectively to tumor cells.

In this study, a dual drug conjugate of two chemotherapeutic agents, SN-38 and cabazitaxel, for the PSA-targeted treatment of PC was developed. The peptide sequence HSSKLQ terminated with azido polyethylene glycol was synthesized and conjugated to SN-38. Similarly, HSSKLQ terminated with a dibenzocyclooctyne (DBCO) functional group was synthesized and conjugated to cabazitaxel. The two conjugates were linked together via click chemistry to synthesize a novel dual drug conjugate that is selectively activated by PSA within PSA-producing tumors. The cytotoxic potential of the SN-38-cabazitaxel conjugate was evaluated in vitro by using PC-3 and LNCaP models of PC.

MATERIALS

All of the starting reagents listed below were obtained from commercial sources and used without further purification. H-Gln-2-Cl-Trt resin was purchased from AAPPTEC (Louisville, KY, USA). N^{α}-Fmoc-N^{im}-trityl-L-histidine, Fmoc-L-leucine, Fmoc-*O*-*tert*-butyl-L-serine, Fmoc-*O*-trityl-L-serine, N^{α}-Fmoc-N^{ε}-allyloxycarbonyl-L-lysine, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU), *N*,*N*′-diisopropylethylamine (DIPEA), trifluoroacetic acid (TFA), 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC·HCl), 4-(dimethylamino)pyridine (DMAP), di*tert*-butyl dicarbonate, piperidine, and Z-leucine−OH were purchased from Chem Impex (Wood Dale, IL, USA). Boc-L-leucine−OH was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). 7-Ethyl-10-hydroxycamptothecin (SN-38) was purchased from Biosynth (San Diego, CA, USA). N_3−PEG_4-acid, DBCO-C6-acid, and cabazitaxel were

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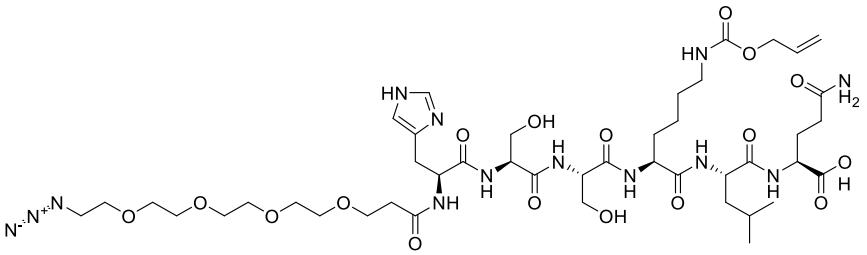


Figure 1. Chemical structure of N_3−PEG_4-HSSK(alloc)LQ-COOH (**1**).

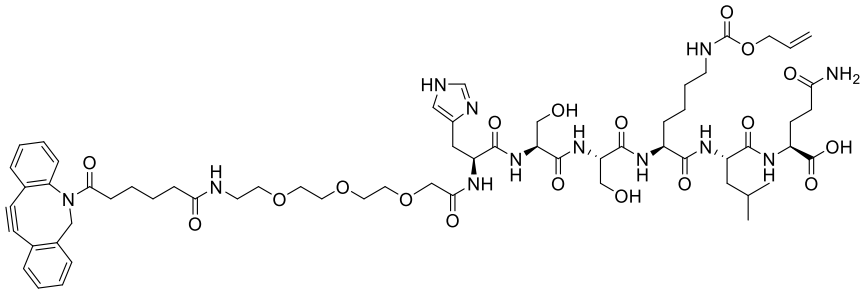
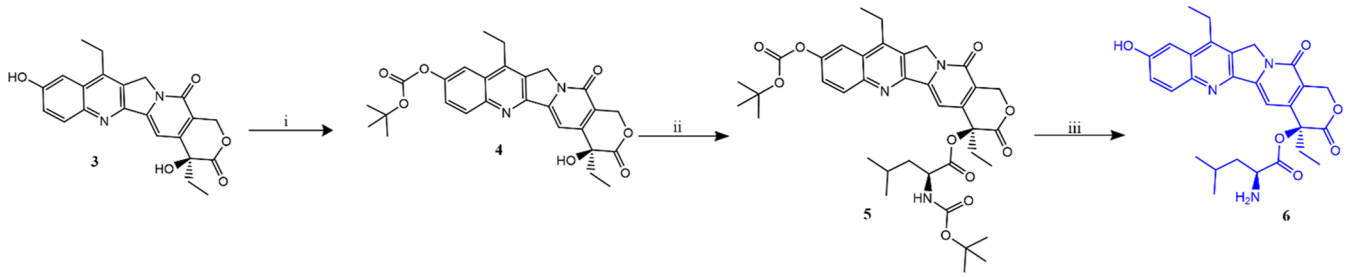


Figure 2. Chemical structure of DBCO-C_6−PEG_3-HSSK (alloc)LQ-COOH (**2**).

Scheme 1. Synthesis of NH_2-Leu−SN−38 (6): (i) Boc Anhydride, Pyridine; (ii) Boc-L-Leu-OH, EDC, DMAP in DCM; (iii) 20% TFA in DCM



purchased from Broad Pharm (San Diego, CA, USA). Purified native human PSA was purchased from Bio-Rad (Hercules, CA, USA). *N*,*N*-dimethylformamide (DMF), pyridine, tetrahydrofuran (THF), acetonitrile (ACN), methanol (MeOH), dichloromethane (DCM), dimethylbarbituric acid (DMBA), methanesulfonic acid, and diethyl ether were purchased from Sigma-Aldrich (Burlington, MA, USA) and used as received.

METHODS

Synthesis of N_3−PEG_4-HSSK(alloc)LQ-COOH (1). Azido-PEG-terminated peptide (N_3−PEG_4-HSSK(alloc)LQ-COOH) (**1**) (Figure 1) containing the PSA substrate (HSSKLQ), was synthesized by solid-phase peptide synthesis SPPS using a modified published method[36] (Supporting Information, Scheme S1). Briefly, N_3−PEG_4-HSSK(alloc)-LQ-COOH was synthesized on 2-Cl-Trt resin at the carboxy-terminal end of the peptide chain. H-Gln-2-Cl-Trt resin was swollen in DMF for 2 h followed by sequential peptide bond formation reactions in DMF using 2.5 equiv of Fmoc-L-leucine, N^{α}-Fmoc-N^{ϵ}-allyloxycarbonyl-L-lysine, Fmoc-*O*-*tert*-butyl-L-serine (coupled twice), N^{α}-Fmoc-N^{im}-trityl-L-histidine, and azido-PEG_4-acid. HATU (2.45 equiv) and DIPEA (5 equiv) were used as the coupling agent and base, respectively, for each step (Supporting Information, Scheme S1).

The amino acids were coupled for 45 min each using double coupling conditions. Following each amino acid coupling step, the Fmoc-protecting group on the N-terminal amine groups was removed using a solution of 20% piperidine in DMF. After coupling of all amino acids (HSSKLQ) on the resin, the N-terminal was capped with azide-terminated polyethylene glycol (N_3−PEG_4-COOH). Upon reaction completion, the peptide on the resin was washed consecutively with DMF, DCM, and methanol and vacuum-dried overnight at room temperature. Cleavage of the peptide from resin and removal of all acid labile protecting groups was achieved with 65% TFA solution in DCM. All solvents and volatiles were evaporated to concentrate, and the peptide was precipitated from solution in cold diethyl ether. The residue obtained was purified by preparative HPLC and lyophilized to obtain (3 g, 89%) N_3−PEG_4-HSSK(alloc)LQ-COOH (**1**) as an off-white powder.

Synthesis of DBCO-C_6−PEG_3-HSSK(alloc)LQ-COOH (2). A similar approach was used in the synthesis of DBCO-C_6 terminated peptide (DBCO-C_6−PEG_3-HSSK(alloc)LQ-COOH) (**2**) (Figure 2) with slight differences. Amino acids with trityl-protected side chains were used instead of amino acids with *tert*-butyl-protected side chains to facilitate cleavage from the resin using less than 5% TFA to ensure stability of the DBCO moiety. It has been reported that the DBCO group is not stable in solutions with high TFA concentrations.[37,38] In addition, after the coupling of all amino acids (HSSKLQ) on the resin, Fmoc-PEG_3-COOH (2.5 equiv) was coupled, followed by capping of the N-terminal with DBCO-C_6-acid

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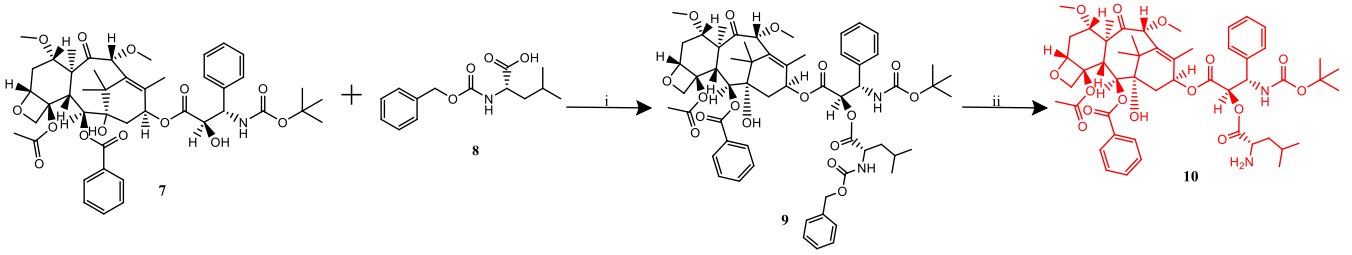
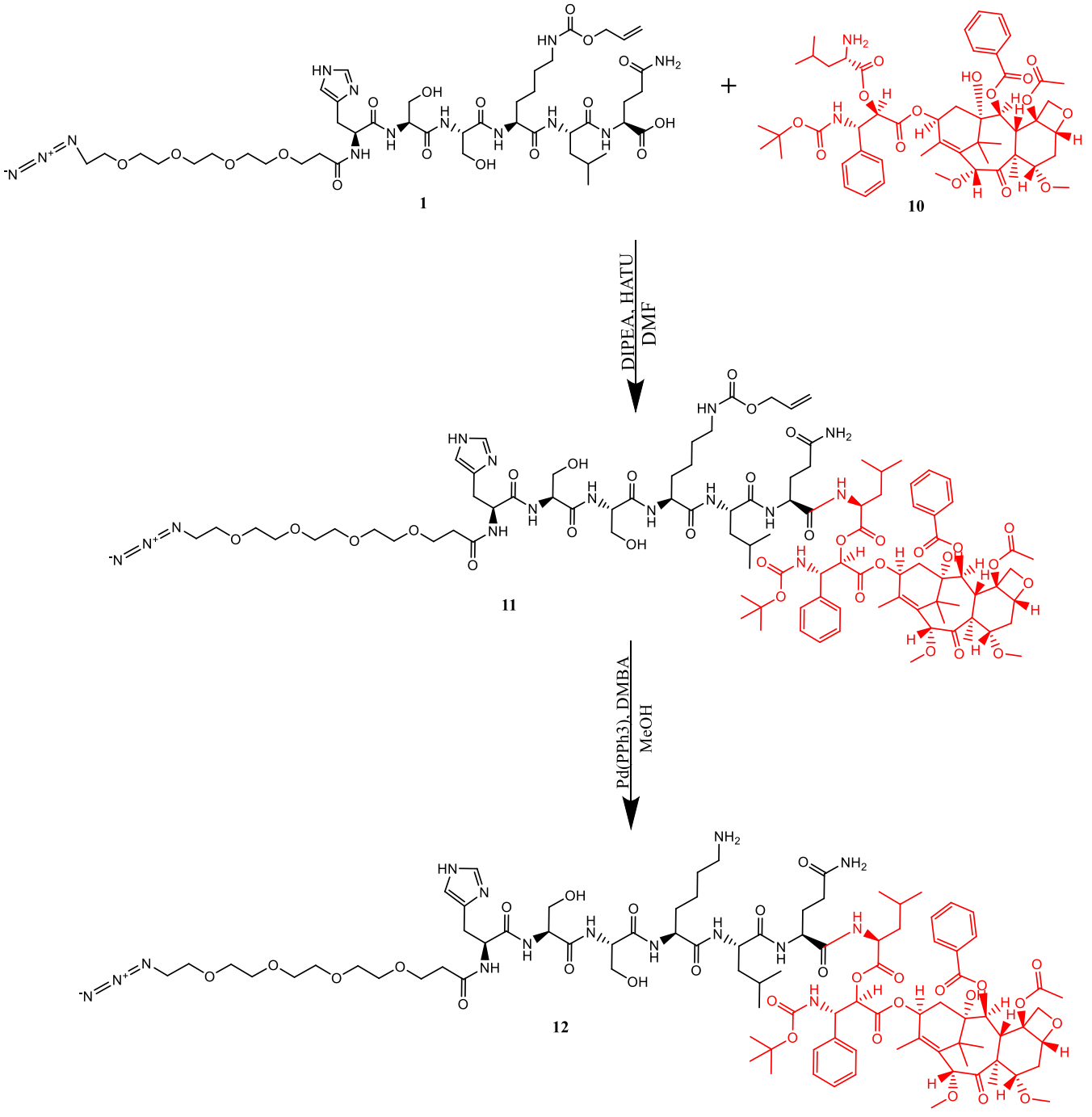
Scheme 2. Synthesis of NH_2-Leu-Cabazitaxel (10): (i) EDC, DMAP, DCM; (ii) MsOH, $H_{2(g)}$, Pd/C



Scheme 3. Synthesis of N_3−PEG_4-HSSKLQ-Leu-Cabazitaxel (12)



(DBCO-C_6−COOH). Cleavage of the peptide from resin and removal of all acid labile protecting groups was achieved using a solution of 5% TFA in DCM (Supporting Information, Scheme S2). Solvents and volatiles were evaporated to

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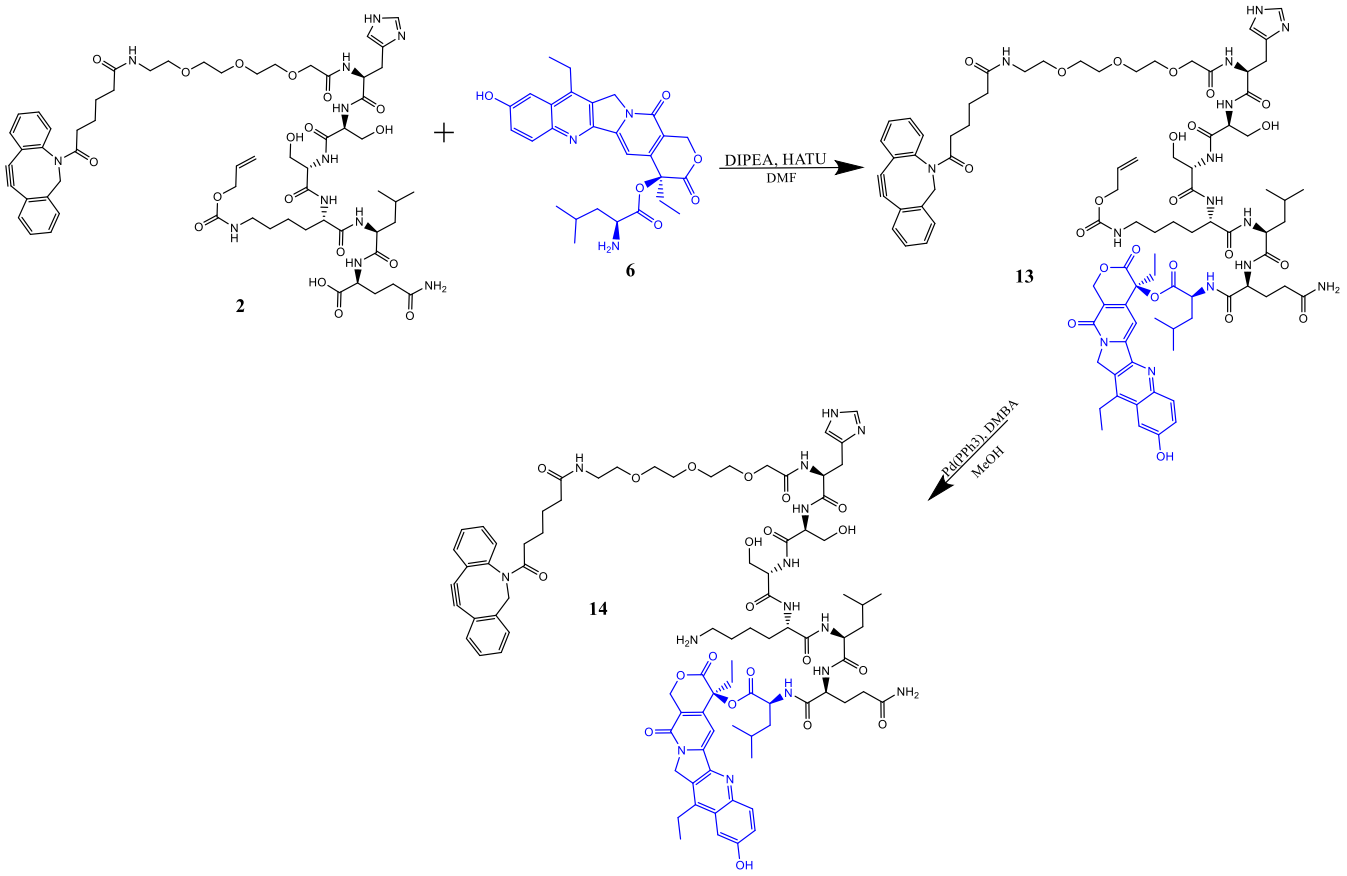
Scheme 4. Synthesis of DBCO-PEG$_3$-HSSKLQ-Leu−SN−38 (14)



concentrate, and the peptide was precipitated from the solution in cold diethyl ether. The residue obtained was purified by preparative HPLC and lyophilized to obtain DBCO-C$_6$−PEG$_3$-HSSK(alloc)LQ-COOH (**2**) (2.65 g; 68%) as off-white powder.

Synthesis of NH$_2$-Leu−SN−38 (6). To a suspension of SN-38 (**3**) (2.45 g, 6.25 mmol) in 250 mL of anhydrous DCM were added di*tert*-butyl dicarbonate (1.764 g, 8.1 mmol) and anhydrous pyridine (15.2 mL, 188 mmol). The suspension was stirred overnight at room temperature. The reaction solution was then filtered through Celite and was washed with 0.5 N HCl (3 × 150 mL) and saturated $NaHCO_3$ solution (1 × 150 mL). The organic phase was dried over sodium sulfate and evaporated under vacuum to give 2.8 g of Boc-protected SN-38 (**4**).[39] This approach gave selective SN-38 Boc protection at the 10-OH position (Scheme 1).

To a solution of **4** (1 g, 2.03 mmol) and Boc-L-Leu-OH (0.6 g, 2.59 mmol) in 20 mL of anhydrous DCM were added EDC·HCl (0.607 g, 3.17 mmol) and DMAP (0.077 g, 0.688 mmol). The mixture was stirred at 0 °C for 45 min and then warmed to room temperature. Reaction completion was monitored by electrospray ionization-mass spectrometry (ESI-MS) until the disappearance of starting materials, after which the reaction solution was washed with 1% $NaHCO_3$ (2 × 50 mL), water (50 mL), and 0.1 N HCl (2 × 50 mL). The organic phase was dried over anhydrous sodium sulfate, filtered, and purified using preparative HPLC. The resulting solid was dried under vacuum to give Boc-Leu-(Boc)−SN−38 (**5**) (1.2 g; 84%).

NH$_2$-Leu−SN−38 (**6**) was synthesized by modification of a published method.[40] Boc-Leu-(Boc)−SN−38 (**5**) (1.2 g, 1.7 mmol) was dissolved in a solution of 20% TFA in anhydrous DCM (40 mL). The solution was stirred for 3 h to remove the acid labile Boc-protecting groups. The solvent was evaporated under vacuum to concentrate, and the product was precipitated into 50 mL of diethyl ether. The resulting solid was dried under vacuum to give NH$_2$-Leu−SN−38 (**6**) (1.02 g, 85%). It was used without further purification (Scheme 1).

Synthesis of NH$_2$-Leu-Cabazitaxel (10). Cabazitaxel (**7**) (1 g, 1.2 mmol), Z-L-Leu-OH (**8**) (0.371 g, 1.4 mmol), and DMAP (0.177 g, 1.45 mmol) were dissolved in 10 mL of anhydrous DCM. EDC HCl (0.278 g, 1.45 mmol) was then added. The reaction mixture was stirred at room temperature overnight. The reaction was followed by ESI-MS until the disappearance of starting materials. The reaction mixture was diluted with 25 mL of DCM and washed with water (2 × 25 mL) followed by brine (25 mL). The solvent was removed under vacuum, and the residue was purified by preparative HPLC. The collected fraction was evaporated under vacuum to give Z-L-Leu-Cabazitaxel (1.2 g, 92%) (**9**).

NH$_2$-Leu-Cabazitaxel (**10**) was synthesized according to a modified published method.[41] Z-L-Leu-Cabazitaxel (**9**) (1.2 g, 1.1 mmol) was dissolved in MeOH/THF (3:14) followed by the addition of methanesulfonic acid (32 μL) and 10% palladium on carbon (Pd/C; 0.12 g) with stirring. The reaction mixture was flushed with hydrogen gas for 1 h. Solids were removed by filtration through a Celite pad, and the product was precipitated from heptanes at ambient temperature to give NH$_2$-Leu-Cabazitaxel (**10**) (1 g, 96%) (Scheme 2).

Synthesis of N$_3$−PEG$_4$-HSSKLQ-Leu-Cabazitaxel (12). N$_3$−PEG$_4$-HSSK-(alloc)LQ-COOH (**1**) (1 equiv) was dis-

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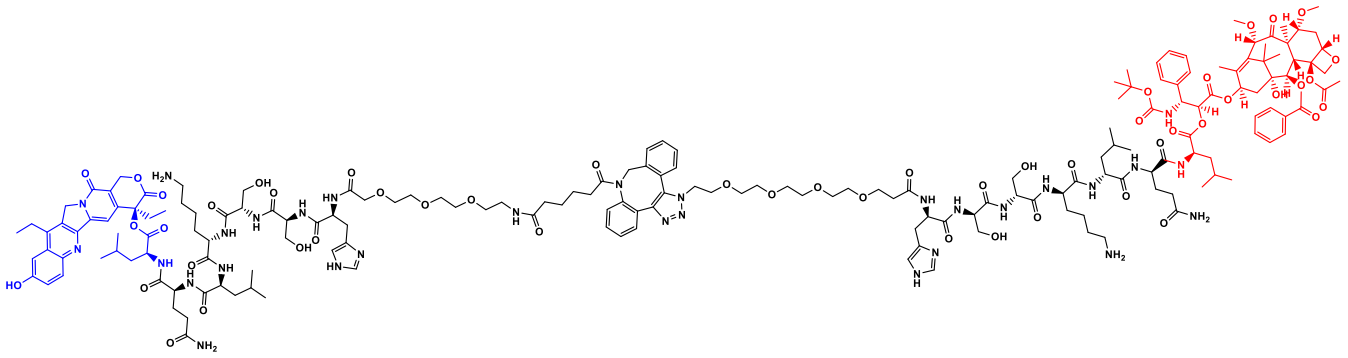

Figure 3. Structure of SN-38-Cabazitaxel conjugate (**15**).

solved in DMF, and DIPEA (4 equiv) was added. This was followed by the addition of HATU (1 equiv). The mixture was stirred for 10 min at room temperature followed by the addition of NH_2-Leu-Cabazitaxel (**10**) (0.5 equiv) per a modified published protocol.[10] The reaction mixture was stirred for 3 h, and the solvent was evaporated under reduced pressure. The crude product was purified by using preparative HPLC to give N_3−PEG_4-HSSK(alloc)LQ-Leu-Cabazitaxel (**11**). After purification, compound **11** was treated with 5 mol % of $Pd(PPh_3)$ and DMBA in methanol (5 equiv) according to a modified published method.[42] The reaction was monitored by using analytical HPLC and ESI-MS to confirm reaction completion. The solvent was evaporated, and the residue obtained was purified by preparative HPLC to obtain N_3−PEG_4-HSSKLQ-Leu-Cabazitaxel (**12**) (Scheme 3).

Synthesis of DBCO-C_6−PEG_3-HSSKLQ-Leu−SN−38 (14). DBCO-C_6−PEG_3-HSSK(alloc)LQ-COOH (**2**) (1 equiv) was dissolved in DMF, and DIPEA (4 equiv) was added. This was followed by the addition of HATU (1 equiv). The solution was stirred for 10 min at room temperature followed by the addition of NH_2-Leu−SN−38 (**6**) (0.5 equiv). The reaction mixture was stirred for 3 h according to a modified published method.[10] The solvent was removed under reduced pressure, and the crude was purified using preparative HPLC to give DBCO-C_6−PEG_3-HSSK(alloc)LQ-Leu−SN−38 (**13**). Compound (**13**) was treated with $Pd(PPh_3)_4$ (5 mol %) and DMBA (5 equiv) in DMF according to a modified published method.[42] The reaction was monitored using analytical HPLC and ESI-MS to confirm reaction completion. The solvent was evaporated, and the residue obtained was purified by preparative HPLC to obtain DBCO-PEG_3-HSSKLQ-Leu−SN−38 (**14**) (Scheme 4).

Synthesis of SN-38-Cabazitaxel Conjugate (15). N_3−PEG_4-HSSKLQ-Leu-Cabazitaxel (**12**) (2 equiv) was dissolved in 2 mL of DMSO in a vial and DBCO-PEG_3-HSSKLQ-Leu−SN−38 (**14**) (1 equiv) was added. The vial was clamped to a fixed angle rotator at 10 rpm and maintained at 37 °C overnight. The coupling by click chemistry to form the SN-38-cabazitaxel conjugate was confirmed by ESI-MS. The reaction was purified by using preparative HPLC to isolate the SN-38-cabazitaxel conjugate (**15**) (Figure 3).

Stability of SN-38-Cabazitaxel Conjugate. The stability of the cabazitaxel−SN−38 conjugate was evaluated in vitro using a modified published method.[36] This experiment is essential to show that cleavage is a result of enzyme-mediated hydrolysis and not poor aqueous stability. 100 μL of SN-38-cabazitaxel conjugate stock solution (10 mM in DMSO) was added to phosphate-buffered saline (PBS) buffer to make a final conjugate concentration of 200 μM containing 2% DMSO (v/v) at pH 7.4. The solution was incubated at 37 °C and rotated at 10 rpm using a Fisherbrand shaker, capable of 360-degree rotation. For HPLC analysis, 500 μL of the mixture was diluted with 500 μL of acetonitrile, filtered through a 0.2 μm syringe filter, and analyzed using analytical HPLC at preselected time points (0, 1, 2, 4, 8, and 24 h).

Cleavage Studies of SN-38-Cabazitaxel Conjugate. The cleavage studies were done by modifying published methods.[10,36] 100 μL of SN-38-cabazitaxel conjugate stock solution (10 mM in DMSO) was diluted in PSA buffer (50 mM Tris, 100 mM NaCl, pH 7.4) to give a final conjugate concentration of 200 μM containing 2% DMSO (v/v). Purified PSA was then added to give a final PSA concentration of 20 μg/mL. The solution was continuously mixed by rotation at 10 rpm on a Fisherbrand shaker at 37 °C for 24 h. To evaluate the extent of cleavage at different time points, 500 μL of the mixture was diluted with 500 μL of acetonitrile, filtered through a 0.2 μm syringe filter, and analyzed using analytical HPLC at preselected time points (0, 1, 2, 4, 8, and 24 h).

Cell Culture. PC cells PC-3 and LNCaP were obtained from College of Medicine, Howard University. The cells were cultured in RPMI (ATCC, Manassas, VA, USA) supplemented with 10% FBS (ATCC, Manassas, VA, USA) and 1% penicillin−streptomycin (Gibco, Thermo Fischer Scientific, Waltham, MA, USA). The cells were maintained at 37 °C in a humidified incubator with a 5% CO_2 atmosphere.

Cytotoxicity Studies of the SN-38-Cabazitaxel Conjugate and Combination Index Determination. PC-3 cells (3×10^3) and LNCaP cells (6×10^3) were seeded in 96-well plates in 100 μL of growth medium per well. The cells were incubated at 37 °C for 24 h to allow for cell attachment. At 24 h, the cells were treated with culture media containing different concentrations of pure drug equivalents (5, 10, 20, 40, 80, 160, and 320 nM) of the SN-38-cabazitaxel conjugate. Wells with culture media, DMSO solution in media, different concentrations of single drugs (at the same concentrations as in conjugate) in media, single Leu-coupled drugs (at the same concentrations in conjugate) in media, combination drug solution in media, and combination Leu-coupled drug solution in media were used as controls. Percent viability was calculated from cytotoxicity assay data using XTT assay per manufacturers protocol. At preselected time points (48 and 72 h) after treatment of cells, 70 μL of XTT solution was added to each cell well and incubated at 37 °C for 3 h. Subsequently, absorbance was measured at 450 nm using Biotek ELx808 absorbance microplate reader (Lonza, Walkersville, MD). Results are represented as the mean ± standard deviation of

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four replicates ($n = 4$). The combination index was determined using the CompuSyn software by the Chou-Talalay method. The combination index (CI) provides a quantifiable definition of synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1) in drug combinations.[43] The IC_{50} was calculated using AAT Bioquest IC_{50} calculator.[44] IC_{50} represents the concentration at which a substance exerts half of its maximal inhibitory effect.

RESULTS

Synthesis of N_3−PEG_4-HSSK(alloc)LQ-COOH (1) and DBCO-PEG_3-HSSK(alloc)LQ-COOH (2). Each of the drug-coupled intermediates (compounds 12 and 14) is composed of different components essential to the design and performance of the SN-38-cabazitaxel conjugate. The intermediates consist of (i) a cytotoxic agent (SN-38 or cabazitaxel); (ii) a Leu spacer; (iii) the HSSKLQ PSA substrate which confers site-specific drug release; (iv) and a short PEG sequence to impart water solubility terminated with DBCO and azide functional groups for "click" reactions.

Each of the intermediates was synthesized in a series of steps and characterized. N_3−PEG_4-HSSK (alloc)LQ-COOH (**1**) was synthesized by SPSS and characterized using ESI-MS m/z 1056.8379; ($[C_{44}H_{73}N_{13}O_{17} + H]^+$ calcd 1057.1500) (Supporting Information, Figure S1a). Analytical HPLC data revealed a single peak at retention time (RT) of 4.053 min, showing that the compound is pure (Supporting Information, Figure S1b). Using a similar approach, DBCO-PEG_3-HSSK-(alloc)LQ-COOH (**2**) was synthesized by SPSS and characterized using ESI-MS m/z 1287.9534; ($[C_{62}H_{86}N_{12}O_{18} + H]^+$ calcd 1288.4440) (Supporting Information, Figure S2a). The analytical HPLC spectrum showed a single peak at RT of 5.245 min, showing that the compound is pure (Supporting Information, Figure S2b).

Synthesis of NH_2-Leu−SN−38 (6). For the synthesis of Leu−SN−38, the hydroxyl group on position 10 of SN-38 was selectively protected with the *tert*-butyloxycarbonyl (Boc)-protecting group by reacting SN-38 with Boc anhydride in the presence of pyridine overnight.[40] ESI-MS m/z 493.3634, ($[C_{27}H_{28}N_2O_7+H]^+$ calcd 493.5360) (Supporting Information, Figure S3a). ^{1}H NMR (400 MHz, $CDCL_3$): δ 8.25 (d, J = 9.1 Hz, 1H), 7.93 (s, 1H), 7.71 (s, 1H), 7.39 (s, 1H), 5.71 (d, J = 17.2 Hz, 1H), 5.43 (d, J = 20.6 Hz, 2H), 5.31 (s, 3H), 4.42 (s, 1H), 2.21 (s, 9H), 2.04 (s, 3H), 1.64 (s, 2H), 1.28 (s, 1H), and 0.99 (s, 2H) (Supporting Information, Figure S3b). The analytical HPLC spectrum of (**4**) showed a single peak at RT of 12.127 min showing that the pure compound was isolated (Supporting Information, Figure S3c). This hydroxyl group protection is essential to facilitate the selective reaction of leucine with the hydroxy group on position 20 of SN-38. Thus, boc-L-leucine was reacted with boc−SN−38 (**4**) using EDC as the coupling agent and DMAP as the base to afford Boc-Leu-Boc−SN−38 (**5**). The reaction was confirmed by ESI-MS m/z 706.5236; ($[C_{38}H_{47}N_3O_{10} + H]^+$ calcd 706.8130) (Supporting Information, Figure S4a), and the analytical HPLC spectrum showed a single peak at RT of 16.922 min showing that the pure compound was isolated (Supporting Information, Figure S4b). After boc- removal, pure NH_2-Leu−SN−38 (**6**) was isolated by preparatory HPLC. ESI-MS m/z 506.5677; ($[C_{28}H_{31}N_3O_6+H]^+$ calcd 506.5790) (Supporting Information, Figure S5a). ^{1}H NMR (400 MHz, DMSO): δ 11.86 (s, 1H), 9.98 (s, 2H), 9.37 (d, J = 9.1 Hz, 1H), 8.95−8.64 (m, 2H), 8.58 (d, J = 22.0 Hz, 1H), 7.15−6.87 (m, 2H), 6.82−6.62 (m, 2H), 5.71 (s, 1H), 4.89 (s, 4H), 4.68−4.38 (m, 3H), 3.91 (d, J = 1.4 Hz, 6H), 3.71−3.55 (m, 2H), 3.26 (d, J = 6.8 Hz, 2H), 3.09 (s, 1H), 2.70 (t, J = 7.4 Hz, 3H), and 1.40 (s, 1H) (Supporting Information, Figure S5b). The analytical HPLC spectrum showed a single peak at RT of 5.139 min showing that the pure compound was isolated (Supporting Information, Figure S5c).

Synthesis of NH_2-Leu-Cabazitaxel (10). Leu-cabazitaxel was synthesized by reacting Z-protected leucine with cabazitaxel via an ester bond formation reaction to form Z-L-Leu-Cabazitaxel (**9**). This was confirmed using ESI-MS m/z 1083.8327; ($[C_{59}H_{74}N_2O_{17} + H]^+$ calcd 1084.2460) (Supporting Information, Figure S6a). The analytical HPLC spectrum showed a single peak at RT of 17.121 min, showing that the pure compound was isolated (Supporting Information, Figure S6b). The removal of the Z protecting group was done via hydrogenation using palladium on carbon and methanesulfonic acid as catalyst[41] to afford compound **10**. This was confirmed using ESI-MS m/z 949.7045; ($[C_{51}H_{68}N_2O_{15} + H]^+$ calcd 950.1120) (Supporting Information, Figure S7a). The analytical HPLC spectrum showed a single peak at RT of 7.331 min showing that the pure compound was isolated (Supporting Information, Figure S7b). ^{1}H NMR (400 MHz, DMSO): δ 8.93 (s, 2H), 8.38 (d, J = 9.4 Hz, 1H), 7.85 (d, J = 8.8 Hz, 5H), 7.55 (s, 1H), 6.18 (d, J = 1.5 Hz, 4H), 5.96 (s, 2H), 5.74 (s, 2H), 3.55 (d, J = 23.5 Hz, 2H), 2.93−2.88 (m, 25H), 2.66−2.51 (m, 6H), 2.49−2.41 (m, 5H), 2.49 (dd, J = 10.3, 8.3 Hz, 3H), 1.71 (d, J = 14.0 Hz, 5H), 1.77−1.48 (m, 4H), and 1.41 (dt, J = 14.5, 6.7 Hz, 3H) (Supporting Information, Figure S7c). This confirms that leucine as a spacer was successfully coupled to cabazitaxel, affording an amine group for conjugation with the PSA-targeting peptide.

Synthesis of Azide-PEG_4-HSSK (alloc)LQ-Leu-Cabazitaxel. N_3−PEG_4-HSSK(alloc)LQ-COOH (**1**) was coupled with NH_2-Leu-Cabazitaxel (**10**) via an amide bond to afford the conjugate, N_3−PEG_4-HSSK(alloc)LQ-Leu-Cabazitaxel (**11**). This was confirmed using ESI-MS m/z 1988.3837; ($[C_{95}H_{139}N_{15}O_{31} + H]^+$ calcd 1988.2390) (Supporting Information, Figure S8a). The analytical HPLC spectrum showed a single peak at RT 7.661 min, showing that the pure compound was isolated (Supporting Information, Figure S8b). To obtain the final pure azide-terminated cabazitaxel conjugate, the alloc protective group on **11** was removed in the presence of tetrakis (triphenylphosphine) palladium (0) and dimethylbarbituric acid (DMBA).[42] Alloc removal was followed by purification using preparative HPLC to afford conjugate N_3−PEG_4-HSSKLQ-Leu-Cabazitaxel (**12**). This was confirmed using ESI-MS m/z 1904.3836; ($[C_{91}H_{135}N_{15}O_{29} + H]^+$ calcd 1904.1650) (Supporting Information, Figure S9a). The analytical HPLC spectrum showed a single peak at RT 4.970 min showing that the pure compound was isolated (Supporting Information, Figure S9b).

*Synthesis of DBCO-PEG_3-HSSK (alloc)LQ-Leu−SN−38 (**13**).* The synthesized NH_2-Leu−SN−38 (**6**) was reacted with DBCO-PEG_3-HSSK(alloc)LQ-COOH (**2**) to form DBCO-PEG_3-HSSK(alloc)LQ-Leu−SN−38 (**13**). Compound 13 was characterized using ESI-MS m/z 1777.2411; ($[C_{90}H_{115}N_{15}O_{23} + H]^+$ calcd 1776.0000) (Supporting Information, Figure S10a). The analytical HPLC spectrum showed a single peak at RT at 7.055 min, showing that the pure compound was isolated (Supporting Information, Figure S10b). To obtain the final pure DBCO-PEG_3-HSSKLQ-Leu−SN−38 (**14**), the alloc protective group on **13** was removed as

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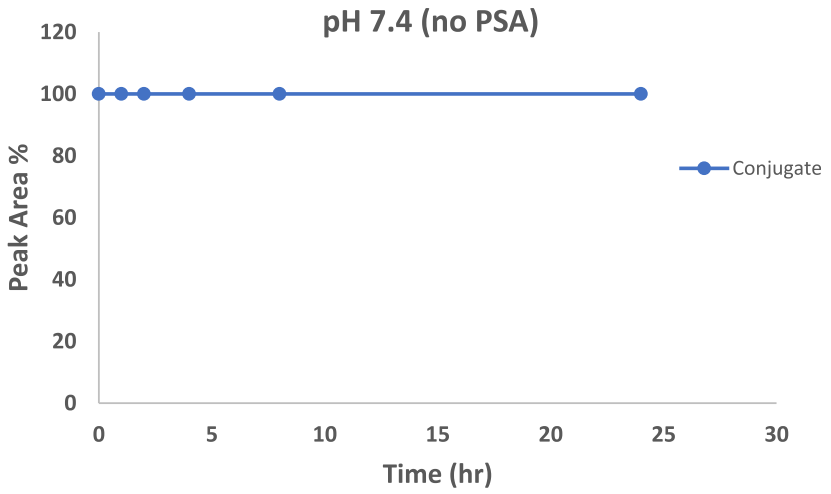

Figure 4. Hydrolytic stability study data of SN-38-cabazitaxel conjugate showing the area plot of the conjugate solution without exogenous PSA at pH 7.4. (SN-38-cabazitaxel conjugate; blue line, no appearance of parent drugs).

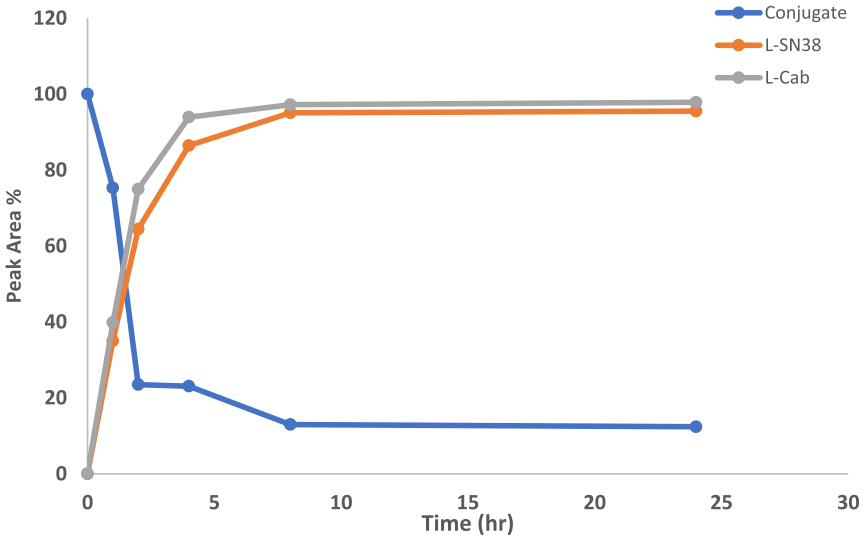

Figure 5. Cleavage studies data of SN-38-cabazitaxel conjugate showing the area plot of the conjugate solution in the presence of exogenous PSA at pH 7.4. (SN-38-cabazitaxel conjugate; blue line, appearance of L−SN−38; orange line, appearance of L-cabazitaxel; gray line).

reported for compound **12**. Characterization using ESI-MS *m*/*z* 1689.8154; ($[C_{86}H_{111}N_{15}O_{21} + H]^+$ calcd 1691.9260) (Supporting Information, Figure S11a). The analytical HPLC spectrum showed a single peak at RT 5.905 min showing that the pure compound was isolated (Supporting Information, Figure S11b).

Strain-Promoted Azide Alkyne Cycloaddition of the Drug Conjugates. Following the synthesis of N_3−PEG_4-HSSKLQ-Leu-Cabazitaxel (**12**) and DBCO-PEG_3-HSSKLQ-Leu−SN−38 (**14**), the intermediate conjugates were linked together using strain-promoted alkyne−azide cycloaddition (SPAAC) to afford the final dual drug SN-38-cabazitaxel conjugate (**15**). Characterization using ESI-MS *m*/*z* 3593.5984, M/2 1796.2992; ($[C_{177}H_{246}N_{30}O_{50} + H]^+$ calcd 3595.0830) (Supporting Information, Figure S12a). The analytical HPLC spectrum showed a single peak at RT 5.63 min, showing that the pure compound was isolated (Supporting Information, Figure S12b).

Measurement of Hydrolytic Stability of SN-38-Cabazitaxel Conjugate. The hydrolytic stability of the SN-38-cabazitaxel conjugate was analyzed in PBS (pH 7.4) at 37 °C for 24 h to mimic the pH of blood. It is important to verify the hydrolytic stability of the dual drug conjugate because the drugs are linked to the targeting peptide via hydrolytically degradable ester bonds. The data showed that at different time points, the area and retention time (RT = 5.62 min) of the conjugate peak at different time points remained the same when analyzed by HPLC, Figure 4 (Supporting Information, Figure S13a). These findings indicate that at physiological pH, the conjugate was hydrolytically stable with no decomposition or cleavage within the time evaluated.

In Vitro Cleavage Studies of SN-38-Cabazitaxel Conjugate. To evaluate the cleavage profile of the dual drug conjugate by PSA, exogenous PSA was added to different concentrations of the conjugate and the cleavage profile was monitored by analytical HPLC for 24 h. The percentage of conjugate remaining at any given time point was computed by taking the ratio of the conjugate peak area at predetermined times and the peak area at the 0 h time point.[45] The data shows that at 1 h, about 25% of the conjugate was cleaved to release Leu-Cabazitaxel and Leu−SN−38. At 24 h, over 85% of the conjugate had cleaved (Figure 5). Characterization of the 1

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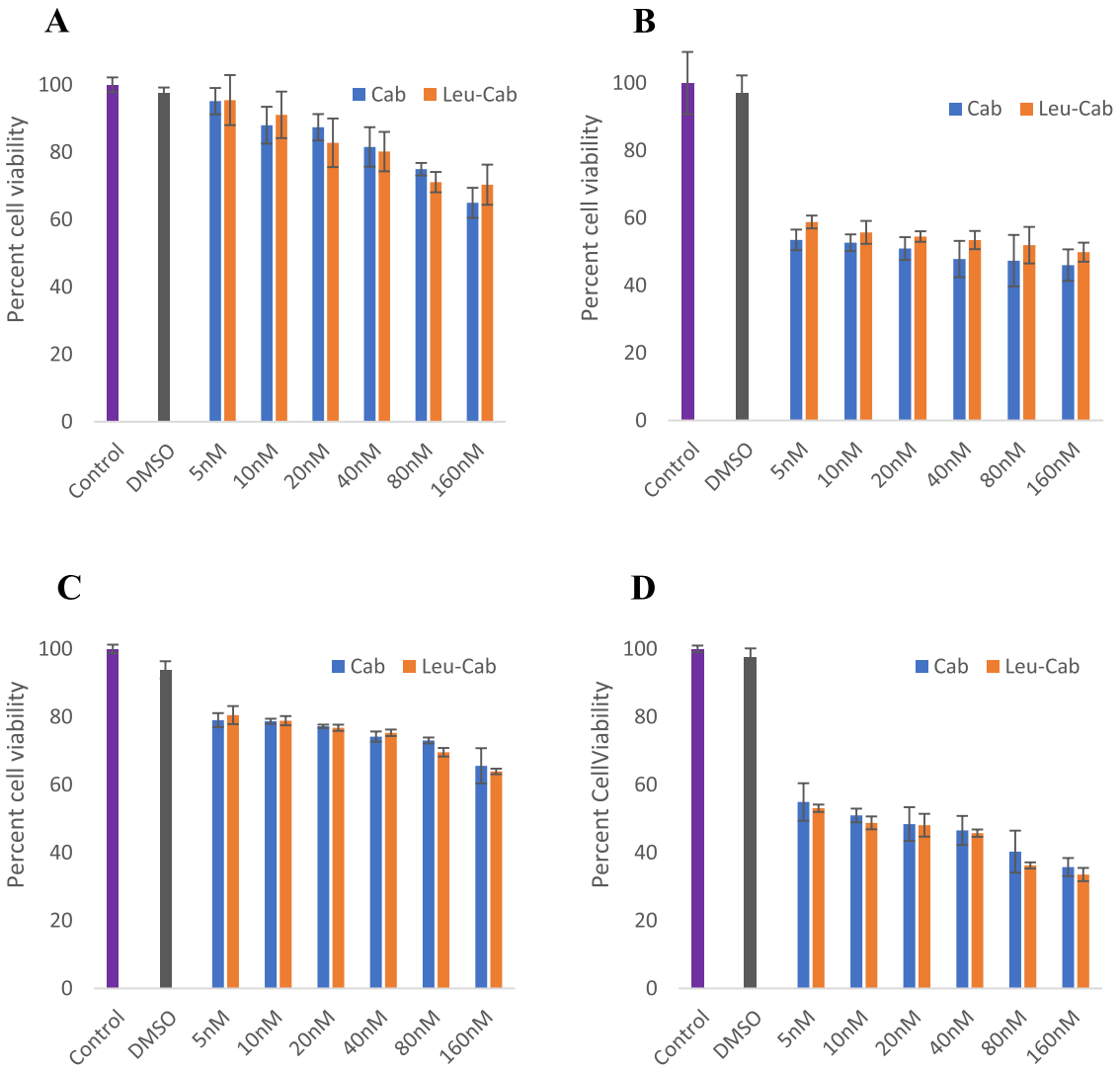

Figure 6. Cell viability data showing comparison of the effect of Cabazitaxel and L-cabazitaxel on (**A**) LNCaP cell line after 24 h exposure to treatment, (**B**) LNCaP cell line after 72 h exposure to treatment; (**C**) PC-3 cell line after 24 h exposure to treatment, and (**D**) PC-3 cell line after 72 h exposure to treatment. Data represent mean ± SD (n = 4). Controls represent 0.05% DMSO in medium (which represents the highest concentration of DMSO used to dissolve the drugs) and growth medium only.

h cleavage sample reveals the appearance of peaks consistent with the RT of the Leu-Cabazitaxel at 7.3 min, Leu−SN−38 at 5.1 min, and the RT of the dual drug conjugate at 5.6 min (Supporting Information, Figure S13b). Additional characterization of the 1 h sample by mass spectrometry confirmed the peaks to be Leu-Cabazitaxel and Leu−SN−38 (m/z = 949.36 and 506.22 respectively) (Supporting Information, Figure S13c). This result confirms literature reports that action of esterases or nonspecific hydrolysis is required to cleave the leucine spacer from Leu-drug to release the free drug compounds.[8,46]

Cytotoxicity of SN-38-Cabazitaxel Conjugate. Since PSA cleavage generates Leu-Cab and Leu−SN−38, a comparative evaluation of the cytotoxic potential of the drugs and their leucine conjugates is important. Thus, cytotoxicity studies were first done to compare the cytotoxic effect of SN-38 to that of Leu−SN−38 and cabazitaxel to that of Leu-cabazitaxel in LNCaP and PC-3 cells at 24 and 72 h, respectively, using the XTT assay. The results show that cabazitaxel and L-cabazitaxel have comparable cytotoxicity to LNCaP and PC-3 cells (Figure 6), respectively, at 24 h and 72 h. Similarly, SN-38 and L−SN−38 showed comparable cytotoxicity to LNCaP and PC-3 cells (Figure 7), respectively, at 24 and 72 h.

Data from the cell viability studies were used to determine the IC_{50}. For the mixtures of solutions of the individual drugs, i.e., SN-38 + Cabazitaxel at 72 h post-treatment, the IC_{50} value of 0.39 nM was generated for LNCaP cells while 0.18 nM was obtained for PC-3 cells. Similarly, mixtures of solutions of the leucine-coupled individual drugs, i.e., Leu−SN−38 + Leu-cabazitaxel had IC_{50} values of 0.12 nM in LNCaP cells and 0.03 nM in PC-3 cells at 72 h post-treatment (Table 1).

The CI values at the different concentrations of SN-38 and cabazitaxel evaluated in the LNCaP PC cell line at 24 h ranged from 0.04862 to 0.30784 (Table 2).

To evaluate the cytotoxicity of the synthesized SN-38-cabazitaxel conjugate (**15**), different concentrations of the conjugate ranging from 5 to 320 nM were used. Mixtures of solutions of the individual drugs, i.e., SN-38 + cabazitaxel, and mixtures of solutions of leucine-coupled drugs, i.e., Leu−SN−38 + Leu-cabazitaxel, at the same concentrations as the dual drug conjugate were used as controls. It should be noted that

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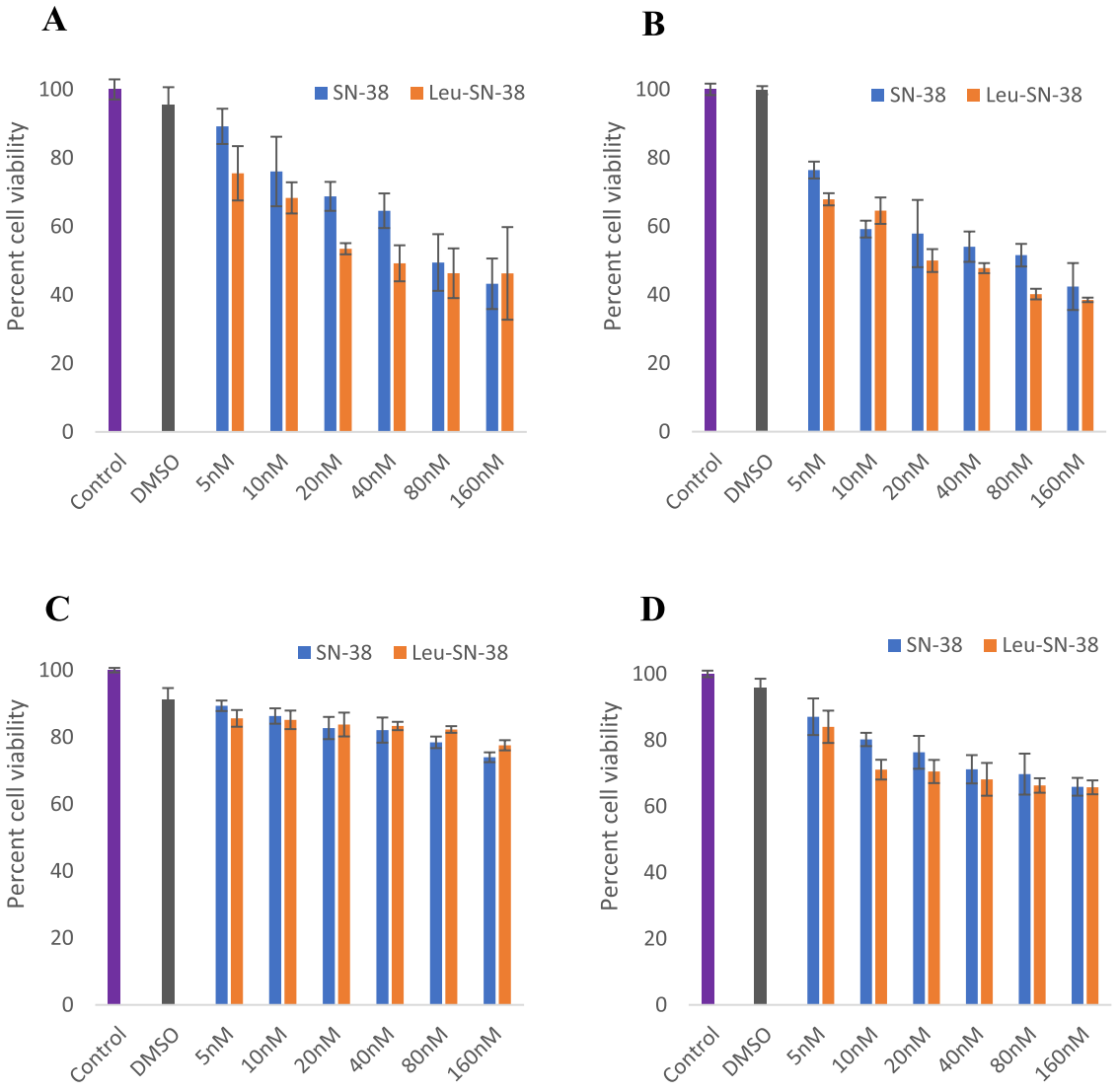

Figure 7. Cell viability data showing comparison of the effect of SN-38 and Leu−SN−38 on (**A**) LNCaP cell line after 24 h exposure to treatment; (**B**) LNCaP cell line after 72 h exposure to treatment; (**C**) PC-3 cell line after 24 h exposure to treatment, and (**D**) PC-3 cell line after 72 h exposure to treatment. Data represent mean ± SD (n = 4). Controls represent 0.05% DMSO in medium (which represents the highest concentration of DMSO used to dissolve the drugs) and growth medium only.

Table 1. IC_{50} Values of SN-38-Cabazitaxel Conjugate Solutions Compared to Controls in Different Cell Lines

Treatment	LNCaP (nM)	PC-3 (nM)
SN-38 + cabazitaxel solution	0.39	0.18
Leu−SN−38 + Leu-cabazitaxel solution	0.12	0.03
SN-38-cabazitaxel conjugate solution	0.71	6.35

Table 2. Concentration of 50:50 Combinations of SN-38 and Cabazitaxel and the Generated CI Values Using CompuSyn Software

	total dose (nM)	CI value
1	5	0.22191
2	10	0.20259
3	20	0.18227
4	40	0.10111
5	80	0.15921
6	160	0.30784
7	320	0.04862

in preparing the mixtures of the single drugs and mixtures of leucine-coupled drugs, the concentration of each drug in the mixture is one-half the concentration of their single drug counterparts. PC-3 and LNCaP cells were treated with the conjugate and controls and the percent viability of the cells was determined at 48- and 72 h using the XTT assay.

The percent cell viability for LNCaP cells treated for 48 and 72 h with different concentrations of the SN-38-cabazitaxel conjugate and controls is reported (Figure 8). The result shows concentration- and time-dependent cytotoxicity (Figure 8a,b). These data reveal that the conjugate is effective in inhibiting the growth of the cancer cells, though less effective when compared to the free drugs. It suggests that PSA expressed by the LNCaP cells was able to cleave the peptide carrier substrate (HSSKLQ) to release the free drugs in agreement with published literature.[10] The calculated IC_{50} value for the SN-38-cabazitaxel conjugate at 72 h post treatment was 0.71 nM in LNCaP cells and 6.35 nM in non-PSA expressing PC-3 cells (Table 1).

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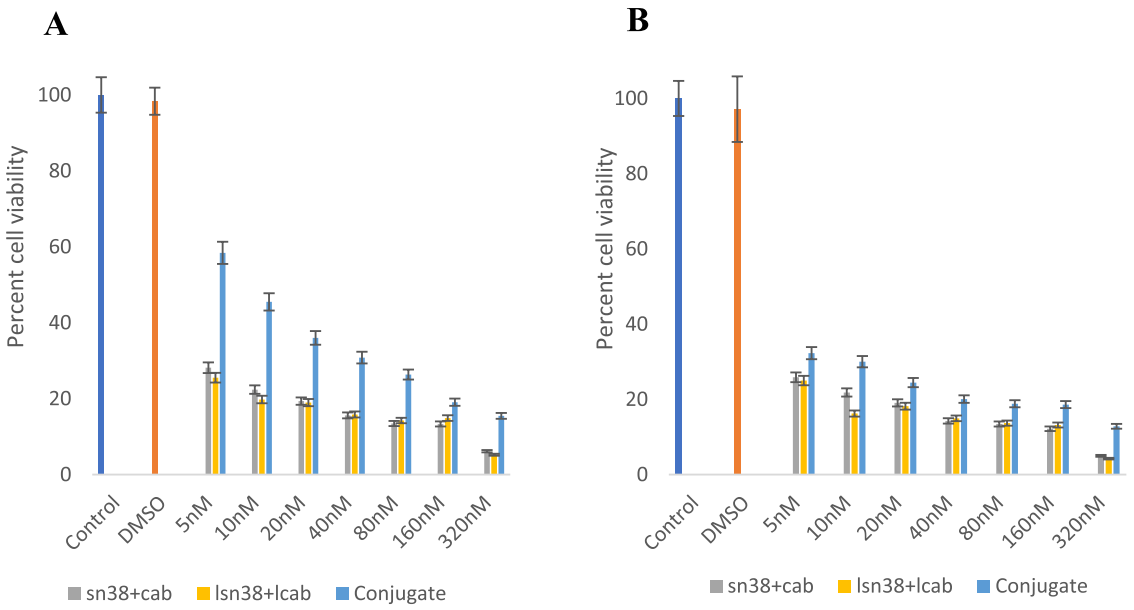

Figure 8. Cell viability data showing comparison of the effect of SN38 + cab, Leu-SN38 + Leu-cab, and the cabazitaxel−SN−38 conjugate on LNCaP cell line after (**A**) 48 h exposure to treatment and (**B**) 72 h exposure to treatment. Controls represent 0.05% DMSO in medium (which represents the highest concentration of DMSO used to dissolve the drugs) and growth medium only. Data represent mean ± SD (n = 4).

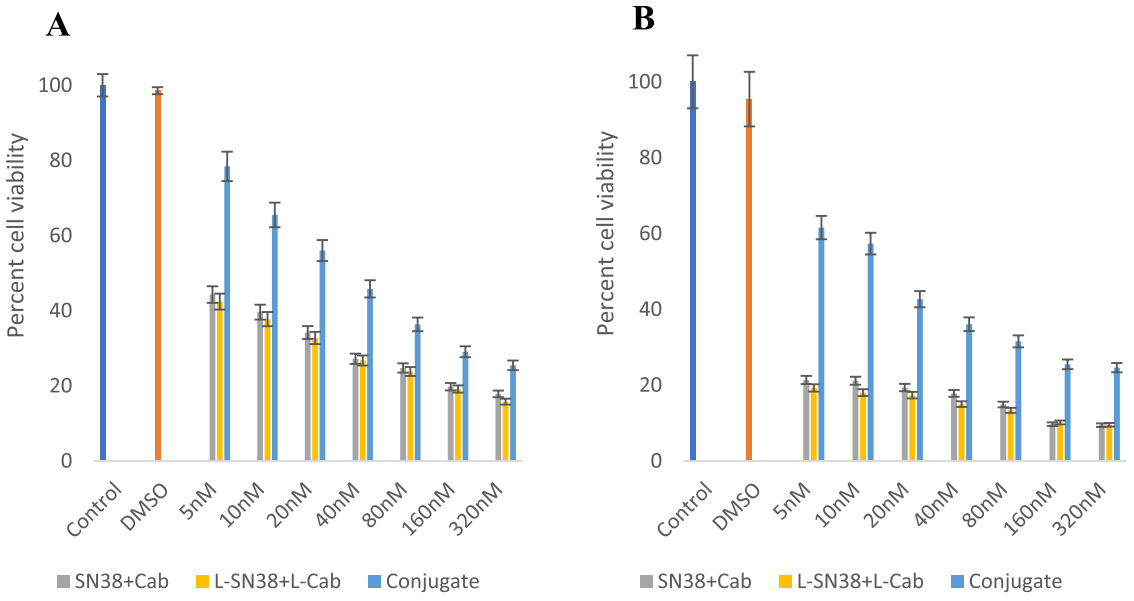

Figure 9. Cell viability data showing comparison of the effect of SN38 + cab, Leu-SN38 + Leu-cab, and the cabazitaxel−SN−38 conjugate on PC-3 cell line after (**A**) 48 h exposure to treatment and (B) 72 h exposure to treatment. Controls represent 0.05% DMSO in medium (which represents the highest concentration of DMSO used to dissolve the drugs) and growth medium only. Data represent mean ± SD (n = 4).

The cytotoxicity of SN-38-cabazitaxel conjugate in PC-3 cells over a period of 48 and 72 h is similarly reported (Figure 9). Even though PC-3 cells do not express PSA, the data show a concentration-dependent growth inhibition and time-dependent cytotoxicity on PC-3 cells by the conjugate (Figure 9a,b). However, the inhibition of growth observed in the dual drug conjugate-treated PC-3 cells was about 9-fold less when compared to LNCaP cells.

DISCUSSION

As a widely explored tumor biomarker, it has been reported that PSA expression is maintained even in androgen-resistant and metastatic tumors, indicating that PSA remains a viable target for cancer treatment.[13] While heterogeneous expression of PSA has been observed in mCRPC, it should be noted that extensive autopsy studies have shown that among several samples of metastatic PC sites that were examined, all of them had a detectable quantity of PSA. For example, a record of autopsy studies shows that within 265 samples of metastatic sites from 30 mCRPC patients, all of them have detectable expression of PSA.[47] These detectable amounts are high enough concentrations to initiate the cleavage of PSA activatable prodrugs. Thus, the design of a PSA-targeted drug delivery system holds the potential for site-specific delivery, thereby improving the efficiency of drug treatment and reduces the incidence of adverse effects.

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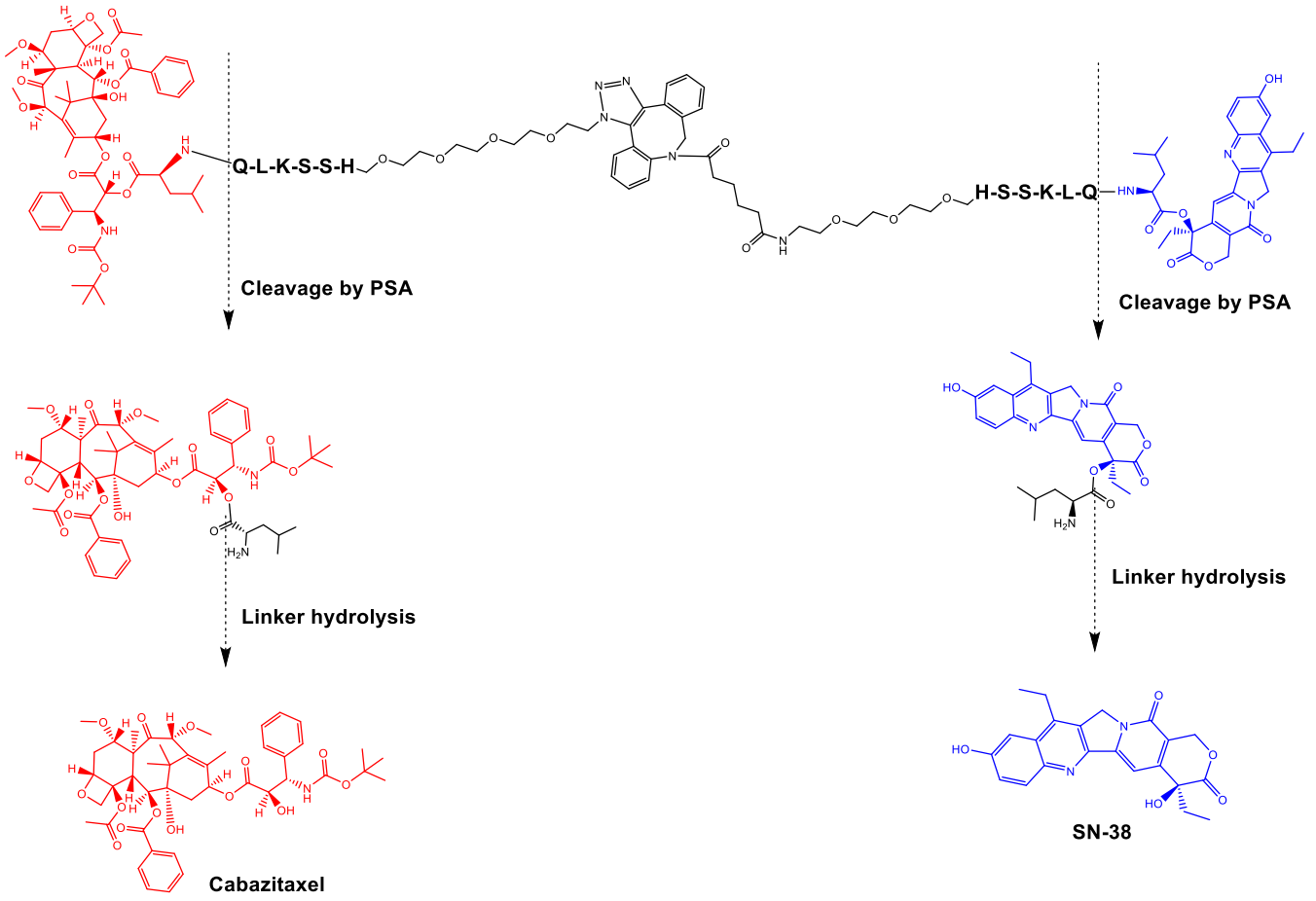
Scheme 5. Representation of the Proposed Cleavage Mechanism and Process of Release of Free Drugs from the Conjugate



The PSA substrate HSSKLQ has been used in the synthesis of several conjugates for targeted drug delivery for PC therapy using the prodrug approach.[9,48,49] HSSKLQ conjugates facilitate selective targeting of PSA-expressing tumors versus nonexpressing tumors. The conjugation of HSSKLQ via a leucine spacer or a self-immolative linker plays a crucial role in releasing the parent drug at the desired site of action and has been used to develop prodrugs of thapsigargin, doxorubicin, vinblastine, etc.[9,10,13,48−51] Various spacers or self-immolative linkers that have been employed in literature for conjugating HSSKLQ to chemotherapeutic agents include serine−leucine which enables drug release by self-immolation;[10] on the other hand, leucine,[49] *p*-aminobenzyl alcohol (PABA), and ethylene diamine[50] have been used as spacers to enhance enzyme cleavage efficiency by eliminating steric hindrance where cleavage inefficiency is a concern. These mechanisms can improve the specificity and selectivity of drug delivery, allowing for efficient release of the drug at the desired site of action.[52]

In this study, the addition of leucine to cabazitaxel and to the hydroxyl group on position 20 of SN-38 as a spacer is essential to remove steric hindrance and facilitates cleavage by PSA. Also, it has been reported that covalent binding at the 20-OH position stabilizes the lactone ring, which prevents ring opening to the inactive carboxylate form.[53,54]

The choice of the boc-protecting group for the selective protection of the 10-OH and the leucine at the 20-OH of SN-38 is justified because SN-38 is stable to acids.[40] Thus, boc- can be selectively cleaved from SN-38 by trifluoroacetic acid (TFA) treatment without degradation.

Following the synthesis of compounds **12** and **14**, the compounds were linked together using strain-promoted alkyne−azide cycloaddition (SPAAC) to afford the dual drug SN-38-cabazitaxel conjugate (**15**). This is a type of click chemistry reaction that involves the reaction between an azide group and an alkyne group to form a triazole.[55] The 1,2,3-triazole formed after conjugation of the azide and DBCO are stable and resistant to degradation.[56,57] Overall, this approach ensures that the chemotherapeutic agents will (i) be released in the tumor microenvironment where PSA is enzymatically active, hence avoiding nonselective cleavage and the attendant off-target toxicities, and (ii) have similar drug biodistribution in the tumor microenvironment.

Following the successful synthesis and characterization of the dual drug conjugate, the evaluation of conjugate stability at pH 7.4 as an index of stability in blood, cleavage studies in the presence of exogenous PSA, and biological characterization of the conjugate using PC cell lines were carried out to ensure that the conjugate meets its design attributes.

As shown in Scheme 5, in the presence of enzymatically active PSA within the tumor tissue, the SN-38-cabazitaxel conjugate is expected to cleave the peptide between glutamine and leucine residues (HSSKLQ//L-Drug; where // signifies the cleavage site), with linker hydrolysis or esterases trimming the leucine to release the free drugs.[10,45] The free drugs are then available in the tumor microenvironment to exert their cytotoxic effect. The hydrolysis of the ester bond between the drug and the PSA targeting peptide does not occur in PBS at pH 7.4, indicating that the conjugate is very stable to

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hydrolytic degradation. Also, we do not expect esterases to induce hydrolysis in blood because the ester bonds in the conjugate are sterically hindered and will not be accessible to these enzymes until cleavage by PSA. It is also expected that the conjugate will be stable in blood because PSA is enzymatically inactive as a result of complexation with α1-antichymotrypsin.[9]

For this study, LNCaP PC cells as a PSA-expressing cell line and PC-3 PC cells as a control cell line that does not express PSA were used. The diversity in the choice of cells is important to test the effect of PSA on drug release from the conjugate and to evaluate the relative cytotoxic efficacy of the conjugate in the presence and absence of PSA. Additionally, we examined whether released Leu−SN−38 + Leu-cabazitaxel was still active to induce cytotoxicity in PC cells. As demonstrated (Figures 6a,b and 7a,b), there was similarity in the cytotoxic effect of pure drug when compared to Leu-drugs. Furthermore, since combination drug delivery is the focus of this work, it is imperative that the interaction between SN-38 and cabazitaxel be evaluated. The Chou-Talalay method was used for evaluating the effect of the combination of the chemotherapeutic agents[43] cabazitaxel and SN-38. The combination index showed strong synergism, with values ranging from 0.04862 to 0.30784 (Table 1). This suggests that the combination of the two agents is suitable for synergistic combination therapy.

The cytotoxicity of the SN-38-cabazitaxel conjugate was examined in both LNCaP and PC-3 cells. Increased cytotoxicity was observed in both LNCaP, and PC-3 cell lines as the duration of treatment increased from 24 to 72 h. The calculated IC_{50} value for the SN-38-cabazitaxel conjugate was 0.71 nM in LNCaP cells and 6.35 nM in non-PSA expressing PC-3 cells (Table 1). The result obtained in this study mimics the findings of Bakthavatsalam et al. (2020).[10] They reported that their synthesized prodrug Ac-HSSKLQL-PAB-dithiocarbamate, containing a similar PSA peptide substrate, demonstrated cytotoxicity in PC-3 cells even though PC-3 cells do not express PSA.[10] Based on the determined IC_{50} values, the data from these studies indicate that the conjugate is about 9 times more cytotoxic to the PSA producing LNCaP cells than to the non-PSA producing PC-3 cells.

This difference in cytotoxicity could be linked to the differential expression of PSA in the cell lines as data from cytotoxicity studies using mixtures of solutions of the individual drugs, i.e., SN-38+Cabazitaxel at 72 h post-treatment, and mixtures of solutions of the leucine-coupled individual drugs, i.e., Leu−SN−38+Leu-cabazitaxel showed that PC-3 cells were more sensitive to the treatments (lower IC_{50} values) when compared to LNCaP cells (higher IC_{50} values) (Table 1). Thus, the reversal in potency to the cell lines after drug conjugation, and the 9-fold greater cytotoxicity to the PSA-producing LNCaP compared to the non-PSA-producing PC-3 cells confirm that targeted activation of the drug molecules is done mainly by the PSA.

CONCLUSIONS

The objective and motivation of this study is to develop a drug delivery platform for the site-specific delivery of potent combination cytotoxic drugs for PC. By covalently connecting small-molecule drugs with peptides via biocleavable linkers, drug conjugates present the drug in an inactive form, until it is processed proteolytically within the tumor microenvironment.

We successfully synthesized the dual drug SN-38-cabazitaxel conjugate, and the in vitro cleavage studies confirmed PSA-specific cleavage and release of Leu−SN−38 and Leu-cabazitaxel. The release of the active drug from Leu−SN−38 and Leu-cabazitaxel to SN-38 and cabazitaxel is proposed to occur by the action of esterases or by nonspecific hydrolysis of the ester bonds. Additionally, stability studies showed that the conjugate was stable in PBS for the duration of the time tested. The cytotoxicity of the conjugate was tested in vitro to evaluate the percent cell viability in LNCaP and PC-3 PC cell lines. The IC_{50} values obtained for the SN-38-cabazitaxel conjugate indicate that the conjugate is more cytotoxic against LNCaP cells than PC-3 cells.

From literature reviews, this report represents a first in the use of HSSKLQ conjugates for combination drug delivery. It also represents a first in the use of SN-38 in the development of peptide drug conjugates for the treatment of PC. The novel approach of PSA-targeted combination SN-38 and cabazitaxel delivery via conjugate design and development enables the targeted delivery of a combination of potent chemotherapeutic agents. Recently, sacituzumab-govitecan, an antibody drug conjugate with SN-38 as the cytotoxic agent, was approved by the FDA. Thus, our approach has great translational potential not just for targeting cancer cells, which is expected to improve therapeutic efficacy and reduce adverse effects but also to provide the "bystander effect" that is essential for heterogeneous PC.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c11483.

Additional experimental details, such as synthetic schemes of peptides via SPPS, ESI-MS spectra of all synthesized compounds, ^{1}H NMR spectra for select compounds, analytical HPLC spectra for synthesized peptides and conjugates, and FT-IR spectra of select compounds (PDF)

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■ REFERENCES

(1) Siegel, R. L.; Giaquinto, A. N.; Jemal, A. Cancer statistics, 2024. *CA Cancer J Clin.* **2024**, *74* (1), 12−49.
(2) Fontana, F.; Marzagalli, M.; Montagnani Marelli, M.; Raimondi, M.; Moretti, R. M.; Limonta, P. Gonadotropin-Releasing Hormone Receptors in Prostate Cancer: Molecular Aspects and Biological Functions. *Int. J. Mol. Sci.* **2020**, *21* (24), 9511.
(3) Karantanos, T.; Corn, P. G.; Thompson, T. C. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. *Oncogene* **2013**, *32* (49), 5501−5511.
(4) Chandrasekar, T.; Yang, J. C.; Gao, A. C.; Evans, C. P. Mechanisms of resistance in castration-resistant prostate cancer (CRPC). *Transl. Androl. Urol.* **2015**, *4* (3), 365−380.
(5) Wang, L.; Lu, B.; He, M.; Wang, Y.; Wang, Z.; Du, L. Prostate Cancer Incidence and Mortality: Global Status and Temporal Trends in 89 Countries From 2000 to 2019. *Front. Public Health* **2022**, *10*, 811044.
(6) Akinboye, E. S.; Brennen, W. N.; Denmeade, S. R.; Isaacs, J. T. Albumin-linked prostate-specific antigen-activated thapsigargin- and niclosamide-based molecular grenades targeting the microenvironment in metastatic castration-resistant prostate cancer. *Asian J. Urol.* **2019**, *6* (1), 99−108.
(7) DeFeo-Jones, D.; Garsky, V. M.; Wong, B. K.; Feng, D. M.; Bolyar, T.; Haskell, K.; Kiefer, D. M.; Leander, K.; McAvoy, E.; Lumma, P.; Wai, J.; Senderak, E. T.; Motzel, S. L.; Keenan, K.; Zwieten, M. V.; Lin, J. H.; Freidinger, R.; Huff, J.; Oliff, A.; Jones, R. E. A peptide-doxorubicin 'prodrug' activated by prostate-specific antigen selectively kills prostate tumor cells positive for prostate-specific antigen *in vivo*. *Nat. Med.* **2000**, *6* (11), 1248−1252.
(8) Kumar, S. K.; Roy, I.; Anchoori, R. K.; Fazli, S.; Maitra, A.; Beachy, P. A.; Khan, S. R. Targeted inhibition of hedgehog signaling by cyclopamine prodrugs for advanced prostate cancer. *Bioorg. Med. Chem.* **2008**, *16* (6), 2764−2768.
(9) Baiz, D.; Pinder, T. A.; Hassan, S.; Karpova, Y.; Salsbury, F.; Welker, M. E.; Kulik, G. Synthesis and Characterization of a Novel Prostate Cancer-Targeted Phosphatidylinositol-3-kinase Inhibitor Prodrug. *J. Med. Chem.* **2012**, *55* (18), 8038−8046.
(10) Bakthavatsalam, S.; Wiangnak, P.; George, D. J.; Zhang, T.; Franz, K. J. Dithiocarbamate prodrugs activated by prostate specific antigen to target prostate cancer. *Bioorg. Med. Chem. Lett.* **2020**, *30* (11), 127148.
(11) Descotes, J. L. Diagnosis of prostate cancer. *Asian J. Urol.* **2019**, *6* (2), 129−136.
(12) Kostova, M. B.; Brennen, W. N.; Lopez, D.; Anthony, L.; Wang, H.; Platz, E.; Denmeade, S. R. PSA-alpha-2-macroglobulin complex is enzymatically active in the serum of patients with advanced prostate cancer and can degrade circulating peptide hormones. *Prostate* **2018**, *78* (11), 819−829.
(13) Di Marco, L.; Zhang, J. Z.; Doan, J.; Kim, B. J.; Yamamoto, N.; Bryce, N. S.; Hambley, T. W. Modulating the Cellular Uptake of Fluorescently Tagged Substrates of Prostate-Specific Antigen before and after Enzymatic Activation. *Bioconjugate Chem.* **2019**, *30* (1), 124−133.
(14) Denmeade, S. R.; Lou, W.; Lövgren, J.; Malm, J.; Lilja, H.; Isaacs, J. T. Specific and efficient peptide substrates for assaying the proteolytic activity of prostate-specific antigen. *Cancer res.* **1997**, *57* (21), 4924−4930.
(15) Chandran, S. S.; Nan, A.; Rosen, D. M.; Ghandehari, H.; Denmeade, S. R. A prostate-specific antigen activated N-(2-hydroxypropyl) methacrylamide copolymer prodrug as dual-targeted therapy for prostate cancer. *Mol. Cancer Ther.* **2007**, *6* (11), 2928−2937.
(16) Dong, P.; Lv, H.; Jia, W.; Liu, J.; Wang, S.; Li, X.; Hu, J.; Zhao, L.; Shi, Y. Polysaccharide dextran-based conjugate for selective co-delivery of two synergistic drugs docetaxel and docosahexaenoic acid to tumor cells. *Drug delivery* **2023**, *30* (1), 40−50.
(17) Agarwal, N.; Sonpavde, G.; Sartor, O. Cabazitaxel for the treatment of castration-resistant prostate cancer. *Future Oncol.* **2011**, *7* (1), 15−24.
(18) Oudard, S.; Fizazi, K.; Sengeløv, L.; Daugaard, G.; Saad, F.; Hansen, S.; Hjälm-Eriksson, M.; Jassem, J.; Thiery-Vuillemin, A.; Caffo, O.; Castellano, D.; Mainwaring, P. N.; Bernard, J.; Shen, L.; Chadjaa, M.; Sartor, O. Cabazitaxel Versus Docetaxel As First-Line Therapy for Patients With Metastatic Castration-Resistant Prostate Cancer: A Randomized Phase III Trial-FIRSTANA. *J. Clin. Oncol.* **2017**, *35* (28), 3189−3197.
(19) Hwang, C. Overcoming docetaxel resistance in prostate cancer: a perspective review. *Ther. Adv. Med. Oncol.* **2012**, *4* (6), 329−340.
(20) Abidi, A. Cabazitaxel: A novel taxane for metastatic castration-resistant prostate cancer-current implications and future prospects. *J. Pharmacol. Pharmacother.* **2013**, *4* (4), 230−237.
(21) Sousa-Pimenta, M.; Estevinho, L. M.; Szopa, A.; Basit, M.; Khan, K.; Armaghan, M.; Ibrayeva, M.; Sönmez Gürer, E.; Calina, D.; Hano, C.; Sharifi-Rad, J. Chemotherapeutic properties and side-effects associated with the clinical practice of terpene alkaloids: paclitaxel, docetaxel, and cabazitaxel. *Front. Pharmacol.* **2023**, *14*, 1157306.
(22) Maloney, S. M.; Hoover, C. A.; Morejon-Lasso, L. V.; Prosperi, J. R. Mechanisms of Taxane Resistance. *Cancers* **2020**, *12* (11), 3323.
(23) Kunos, C. A.; Stefan, T.; Jacobberger, J. W. Cabazitaxel-induced stabilization of microtubules enhances radiosensitivity in ovarian cancer cells. *Front. Oncol.* **2013**, *3*, 226.
(24) Prota, A. E.; Lucena-Agell, D.; Ma, Y.; Estevez-Gallego, J.; Li, S.; Bargsten, K.; Josa-Prado, F.; Altmann, K. H.; Gaillard, N.; Kamimura, S.; Mühlethaler, T.; Gago, F.; Oliva, M. A.; Steinmetz, M. O.; Fang, W. S.; Díaz, J. F. Structural insight into the stabilization of microtubules by taxanes. *eLife* **2023**, *12*, No. e84791.
(25) Paller, C. J.; Antonarakis, E. S. Cabazitaxel: a novel second-line treatment for metastatic castration-resistant prostate cancer. *Drug Des., Dev. Ther.* **2011**, *5*, 117−124.
(26) Hoang, B.; Ernsting, M. J.; Tang, W. S.; Bteich, J.; Undzys, E.; Kiyota, T.; Li, S. D. Cabazitaxel-conjugated nanoparticles for docetaxel-resistant and bone metastatic prostate cancer. *Cancer letters* **2017**, *410*, 169−179.
(27) Zhang, S.; Wang, Y.; Chen, Z.; Kim, S.; Iqbal, S.; Chi, A.; Ritenour, C.; Wang, Y. A.; Kucuk, O.; Wu, D. Genistein enhances the efficacy of cabazitaxel chemotherapy in metastatic castration-resistant prostate cancer cells. *Prostate* **2013**, *73* (15), 1681−1689.
(28) Mohammadian, J.; Sabzichi, M.; Molavi, O.; Shanehbandi, D.; Samadi, N. Combined Treatment with Stattic and Docetaxel Alters the Bax/Bcl-2 Gene Expression Ratio in Human Prostate Cancer Cells. *Asian Pac J. Cancer Prev* **2016**, *17* (11), 5031−5035.
(29) Zhang, Q.; Lu, Q. B. New combination chemotherapy of cisplatin with an electron-donating compound for treatment of multiple cancers. *Sci. Rep.* **2021**, *11*, 788.
(30) Wang, H.; Chen, J.; Xu, C.; Shi, L.; Tayier, M.; Zhou, J.; Zhang, J.; Wu, J.; Ye, Z.; Fang, T.; Han, W. Cancer Nanomedicines Stabilized by π-π Stacking between Heterodimeric Prodrugs Enable Exceptionally High Drug Loading Capacity and Safer Delivery of Drug Combinations. *Theranostics* **2017**, *7* (15), 3638−3652.
(31) Roy, A.; Cunningham, D.; Hawkins, R.; Sörbye, H.; Adenis, A.; Barcelo, J. R.; Lopez-Vivanco, G.; Adler, G.; Canon, J. L.; Lofts, F.; Castanon, C.; Fonseca, E.; Rixe, O.; Aparicio, J.; Cassinello, J.; Nicolson, M.; Mousseau, M.; Schalhorn, A.; D'Hondt, L.; Kerger, J.; Hossfeld, D. K.; et al. Docetaxel combined with irinotecan or 5-fluorouracil in patients with advanced oesophago-gastric cancer: a randomised phase II study. *Br. J. Cancer* **2012**, *107* (3), 435−441.
(32) Wu, C.; Zhang, Y.; Yang, D.; Zhang, J.; Ma, J.; Cheng, D.; Chen, J.; Deng, L. Novel SN-38 derivative-based liposome as anticancer prodrug: an in vitro and in vivo study. *Int. J. Nanomed.* **2019**, *14*, 75−85.

https://doi.org/10.1021/acsomega.4c11483
ACS Omega 2025, 10, 17611−17625

(33) Bala, V.; Rao, S.; Li, P.; Wang, S.; Prestidge, C. A. Lipophilic Prodrugs of SN38: Synthesis and in Vitro Characterization toward Oral Chemotherapy. *Mol. Pharmaceutics* **2016**, *13* (1), 287−294.
(34) Afkham, A.; Aghebati-Maleki, L.; Siahmansouri, H.; Sadreddini, S.; Ahmadi, M.; Dolati, S.; Afkham, N. M.; Akbarzadeh, P.; Jadidi-Niaragh, F.; Younesi, V.; Yousefi, M. Chitosan (CMD)-mediated co-delivery of SN-38 and Snail-specific siRNA as a useful anticancer approach against prostate cancer. *Pharmacol. Rep.* **2018**, *70* (3), 418−425.
(35) Doi, H.; Kida, T.; Nishino, K.; Nakatsuji, M.; Sakamoto, S.; Shimizu, S.; Teraoka, Y.; Tamura, Y.; Kataoka, Y.; Inui, T. Solubility-Improved 10-O-Substituted SN-38 Derivatives with Antitumor Activity. *ChemMedChem* **2017**, *12* (20), 1715−1722.
(36) Akinboye, E. S.; Rosen, M. D.; Bakare, O.; Denmeade, S. R. Anticancer activities of emetine prodrugs that are proteolytically activated by the prostate specific antigen (PSA) and evaluation of *in vivo* toxicity of emetine derivatives. *Bioorg. Med. Chem.* **2017**, *25* (24), 6707−6717.
(37) Erickson, P. W.; Fulcher, J. M.; Spaltenstein, P.; Kay, M. S. Traceless Click-Assisted Native Chemical Ligation Enabled by Protecting Dibenzocyclooctyne from Acid-Mediated Rearrangement with Copper(I). *Bioconjugate Chem.* **2021**, *32* (10), 2233−2244.
(38) Wang, X.; Gobbo, P.; Suchy, M.; Workentin, M. S.; Hudson, R. H. E. Peptide-decorated gold nanoparticles via strain-promoted azide−alkyne cycloaddition and post assembly deprotection. *RSC Adv.* **2014**, *4* (81), 43087−43091.
(39) Zhao, H.; Rubio, B.; Sapra, P.; Wu, D.; Reddy, P.; Sai, P.; Martinez, A.; Gao, Y.; Lozanguiez, Y.; Longley, C.; Greenberger, L. M.; Horak, I. D. Novel prodrugs of SN-38 using multiarm poly(ethylene glycol) linkers. *Bioconjugate Chem.* **2008**, *19* (4), 849−859.
(40) Moon, S. J.; Govindan, S. V.; Cardillo, T. M.; D'Souza, C. A.; Hansen, H. J.; Goldenberg, D. M. Antibody conjugates of 7-ethyl-10-hydroxycamptothecin (SN-38) for targeted cancer chemotherapy. *J. Med. Chem.* **2008**, *51* (21), 6916−6926.
(41) Metcalf, C. A., III; Svenson, S.; Hwang, J.; Tripathi, S.; Gangal, G.; Kabir, S.; Lazarus, D.; Cole, R.; Sweryda-Krawiec, B.; Shum, P.; Brown, D.; Case, R. I.; van der Poll, D.; Rohde, E.; Harlfinger, S.; Teng, C. H.; Eliasof, S. Discovery of a Novel Cabazitaxel Nanoparticle-Drug Conjugate (CRLX522) with Improved Pharmacokinetic Properties and Anticancer Effects Using a β-Cyclodextrin-PEG Copolymer Based Delivery Platform. *J. Med. Chem.* **2019**, *62* (21), 9541−9559.
(42) Gutkin, S.; Green, O.; Raviv, G.; Shabat, D.; Portnoy, O. Powerful Chemiluminescence Probe for Rapid Detection of Prostate Specific Antigen Proteolytic Activity: Forensic Identification of Human Semen. *Bioconjugate Chem.* **2020**, *31* (11), 2488−2493.
(43) Chou, T. C. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer res.* **2010**, *70* (2), 440−446.
(44) AAT Bioquest, Inc. April 13. *Quest Graph IC_{50} Calculator*, AAT Bioquest. 2023. https://www.aatbio.com/tools/ic50-calculator.
(45) Aloysius, H.; Hu, L. Synthesis and evaluation of new peptide-linked doxorubicin conjugates as prodrugs activated by prostate-specific antigen. *Med. Chem. Res.* **2020**, *29*, 1280−1299.
(46) Chaudhary, M.; Tushir, R.; Chopra, N. Polymer−drug conjugation using ester and ortho-ester bond: Mechanism, protocols, and applications. *Polymer-Drug Conjugates* **2023**, *5*, p121−p145.
(47) Shah, R. B.; Mehra, R.; Chinnaiyan, A. M.; Shen, R.; Ghosh, D.; Zhou, M.; Macvicar, G. R.; Varambally, S.; Harwood, J.; Bismar, T. A.; Kim, R.; Rubin, M. A.; Pienta, K. J. Androgen-independent prostate cancer is a heterogeneous group of diseases: lessons from a rapid autopsy program. *Cancer Res.* **2004**, *64* (24), 9209−9216.
(48) Tomao, L.; Sbardella, D.; Gioia, M.; Di Masi, A.; Marini, S.; Ascenzi, P.; Coletta, M. Characterization of the prostate-specific antigen (PSA) catalytic mechanism: a pre-steady-state and steady-state study. *PloS one* **2014**, *9* (7), No. e102470.
(49) Denmeade, S. R.; Jakobsen, C. M.; Janssen, S.; Khan, S. R.; Garrett, E. S.; Lilja, H.; Christensen, S. B.; Isaacs, J. T. Prostate-specific antigen-activated thapsigargin prodrug as targeted therapy for prostate cancer. *J. Natl. Cancer Inst.* **2003**, *95* (13), 990−1000.
(50) Kumar, S. K.; Williams, S. A.; Isaacs, J. T.; Denmeade, S. R.; Khan, S. R. Modulating paclitaxel bioavailability for targeting prostate cancer. *Bioorg. Med. Chem.* **2007**, *15* (14), 4973−4984.
(51) Brady, S. F.; Pawluczyk, J. M.; Lumma, P. K.; Feng, D. M.; Wai, J. M.; Jones, R.; DeFeo-Jones, D.; Wong, B. K.; Miller-Stein, C.; Lin, J. H.; Oliff, A.; Freidinger, R. M.; Garsky, V. M. Design and synthesis of a pro-drug of vinblastine targeted at treatment of prostate cancer with enhanced efficacy and reduced systemic toxicity. *J. Med. Chem.* **2002**, *45* (21), 4706−4715.
(52) Gonzaga, R. V.; do Nascimento, L. A.; Santos, S. S.; Machado Sanches, B. A.; Giarolla, J.; Ferreira, E. I. Perspectives About Self-Immolative Drug Delivery Systems. *J. Pharm. Sci.* **2020**, *109* (11), 3262−3281.
(53) Sepehri, N.; Rouhani, H.; Tavassolian, F.; Montazeri, H.; Khoshayand, M. R.; Ghahremani, M. H.; Ostad, S. N.; Atyabi, F.; Dinarvand, R. SN-38 polymeric nanoparticles: in vitro cytotoxicity and in vivo antitumor efficacy in xenograft balb/c model with breast cancer versus irinotecan. *Int. J. Pharm. Drug* **2014**, *471* (1−2), 485−497.
(54) Edupuganti, V. V. S. R.; Tyndall, J. D. A. T. D. A.; Gamble, A. B. Self-immolative Linkers in Prodrugs and Antibody Drug Conjugates in Cancer Treatment. *Recent Pat. Anti-Cancer Drug Discovery* **2021**, *16* (4), 479−497.
(55) Jang, S.; Sachin, K.; Lee, H. J.; Kim, D. W.; Lee, H. S. Development of a simple method for protein conjugation by copper-free click reaction and its application to antibody-free Western blot analysis. *Bioconjugate Chem.* **2012**, *23* (11), 2256−2261.
(56) Derks, Y. H. W.; Rijpkema, M.; Amatdjais-Groenen, H. I. V.; Loeff, C. C.; de Roode, K. E.; Kip, A.; Laverman, P.; Lütje, S.; Heskamp, S.; Löwik, D. W. P. M. Strain-Promoted Azide-Alkyne Cycloaddition-Based PSMA-Targeting Ligands for Multimodal Intraoperative Tumor Detection of Prostate Cancer. *Bioconjugate Chem.* **2022**, *33* (1), 194−205.
(57) Tang, W.; Becker, M. L. Click″ reactions: a versatile toolbox for the synthesis of peptide-conjugates. *Chem. Soc. Rev.* **2014**, *43* (20), 7013−7039.

https://doi.org/10.1021/acsomega.4c11483
ACS Omega 2025, 10, 17611−17625