molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

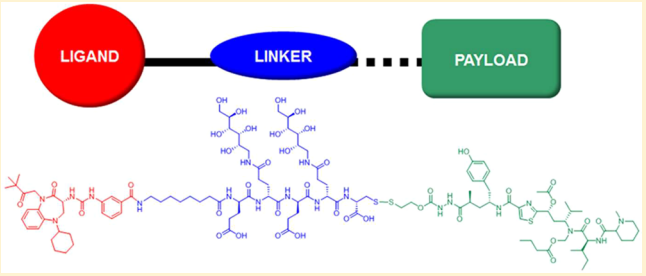
Selective Tumor Targeting of Desacetyl Vinblastine Hydrazide and Tubulysin B via Conjugation to a Cholecystokinin 2 Receptor (CCK2R) Ligand

Charity Wayua,[†,‡,§] Jyoti Roy,[†,‡,§] Karson S. Putt,[‡] and Philip S. Low*[,†,‡]

[†]Department of Chemistry and [‡]Center for Drug Discovery, Purdue University, West Lafayette, Indiana 47907, United States

Supporting Information

ABSTRACT: As the delivery of selectively targeted cytotoxic agents via antibodies or small molecule ligands to malignancies has begun to show promise in the clinic, the need to identify and validate additional cellular targets for specific therapeutic delivery is critical. Although a multitude of cancers have been targeted using the folate receptor, PSMA, bombesin receptor, somatostatin receptor, LHRH, and $\alpha_v\beta_3$, there is a notable lack of specific small molecule ligand/receptor pairs to cellular targets found within cancers of the GI tract. Because of the selective GI tract expression of the cholecystokinin 2 receptor (CCK2R), we undertook the creation of conjugates that would deliver microtubule-disrupting drugs to malignancies through the specific targeting of CCK2R via a high affinity small molecule ligand. The cytotoxic activity of these conjugates were shown to be receptor mediated in vitro and in vivo with xenograft mouse models exhibiting delayed growth or regression of tumors that expressed CCK2R. Overall, this work demonstrates that ligands to CCK2R can be used to create selectively targeted therapeutic conjugates.



KEYWORDS: *CCK2R, cholecystokinin 2 receptor, tubulysin, vinblastine, targeted therapy*

INTRODUCTION

Conventional nontargeted chemotherapeutics, such as antimetabolites,[1] microtubule inhibitors, and DNA intercalating[2]/alkylating agents,[3,4] are effective at killing cancer cells, but due to their indiscriminate penetration into nearly all cells, they can also damage healthy cells, causing such toxicities as myelosuppression, alopecia, mucositis, peripheral neuropathy, and cardiotoxicity.[5,6] To minimize such collateral damage to healthy tissues, physicians must often either reduce the dosage or decrease the frequency of drug administration, leading to incomplete elimination of diseased tissue. On the basis of these considerations, recent approaches to cancer therapy have focused on developing methods that specifically target chemotherapeutic agents to cancer cells, allowing for improved tumor suppression with fewer adverse events.

The most common approach to drug targeting has relied on the specificity of a monoclonal antibody for its tumor-specific antigen. Through the conjugation of a highly cytotoxic drug to a tumor-specific antibody, tumor-selective drug delivery can be achieved with little drug deposition in healthy tissues.[7,8] Examples of such tumor-targeted antibody−drug conjugates (ADCs) include trastuzumab emtansine[9,10] and brentuximab vedotin.[11] Although several ADCs have shown significant success in preclinical and clinical settings, some questions as to their ability to penetrate solid tumors have been raised.[12−16] A related strategy to achieve tumor-selective drug delivery involves the use of low molecular weight targeting ligands that can similarly deliver attached drugs specifically to cancer cells. Low molecular weight ligand−drug conjugates (Figure 1A) also target receptors that are overexpressed on malignant cells, and their much smaller sizes may permit more thorough tumor penetration.[17−20]

In this paper, Z-360,[21] a low molecular weight ligand of the cholecystokinin 2 receptor (CCK2R), is modified to deliver two of the more potent antimicrotubule agents currently available (desacetyl vinblastine monohydrazide and tubulysin B hydrazide). CCK2R is a transmembrane receptor primarily found in epithelial cells of the GI tract and brain, where it can bind Gastrin and other amidated peptides of the gastrin-cholecystokinin family.[22,23] CCK2R is overexpressed on many cancers of the lung, pancreas, liver, and GI tract (esophagus, colon, and gastrointestinal stromal tumors).[24−29] Because CCK2R in the brain is inaccessible to drugs that cannot pass the blood brain barrier, this restricted expression pattern renders CCK2R a possible candidate for ligand-targeted drug delivery. We report here the design, synthesis, and biological evaluation of two highly potent CCK2R-targeted chemotherapeutic agents and demonstrate their efficacy and

Received: March 18, 2015
Revised: May 19, 2015
Accepted: June 1, 2015
Published: June 4, 2015

DOI: 10.1021/acs.molpharmaceut.5b00218
Mol. Pharmaceutics 2015, 12, 2477−2483

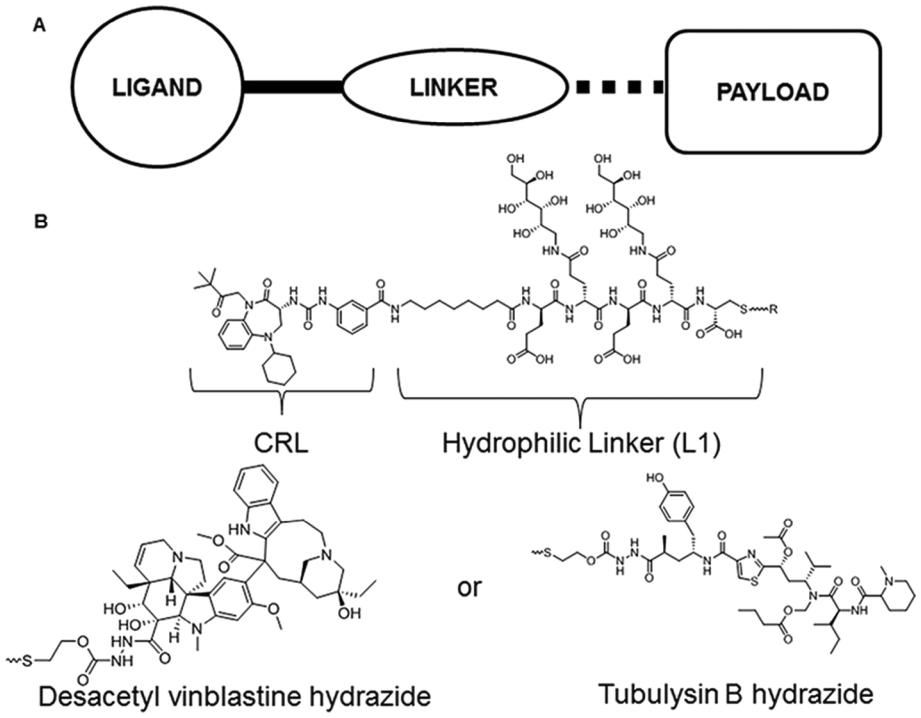


Figure 1. Ligand targeted drug conjugates. (A) General representation of ligand conjugated to cytotoxic payload via a peptide linker. The circle represents the cholecystokinin 2 receptor (CCK2R) binding ligand, whereas the linker is represented by an oval. The cytotoxic drug, or payload, is indicated by a rectangle. The solid black line represents a covalent bond between the ligand and the linker, and the dotted line symbolizes a cleavable self-immolative bond. (B) Chemical structures of the CCK2R ligand CRL conjugated to the cytotoxic antimicrotubule agents desacetyl vinblastine hydrazide and tubulysin B hydrazide via a hydrophilic peptide linker.

tolerability in treating CCK2R-expressing human tumor xenografts in *nu/nu* mice.

Materials. Protected amino acids were purchased from Chem-Impex Intl. (Chicago, IL). *H*-Cys (Trt)-2-Cl-Trt resin was obtained from Novabiochem (San Diego, CA). 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]-pyridinium 3-oxid hexafluorophosphate (HATU) was obtained from Genscript Inc. (Piscataway, NJ). Diisopropylethylamine (DIPEA), piperidine, dimethylformamide (DMF), isopropyl alcohol (*i*-PrOH), and all other reagents were purchased from Sigma-Aldrich. Tubulysin B and desacetyl vinblastine hydrazide and their activated derivatives were a kind gift from Endocyte Inc. (West Lafayette, IN). Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute medium (RPMI), fetal bovine serum (FBS), and G418 (Geneticin) were all purchased from GIBCO (Grand Island, NY). Glutamine and penicillin-streptomycin were obtained from Life Technologies. HC Matrigel was obtained from BD Biosciences (San Jose, CA).

Cell Culture. The HEK 293 cell line transfected with wild type CCK2R was a generous gift from Dr. Mark Hellmich (Galveston, Texas). Cells were cultured as monolayers in DMEM supplemented with 10% fetal bovine serum, G418 disulfate (400 μg/mL), 1% of 2 mM glutamine, and 1% of penicillin-streptomycin at 37 °C in a 5% CO_2:95% humidified-air atmosphere. KB cells were cultured from frozen stocks in RPMI and supplemented with the same concentrations of fetal bovine serum, penicillin-streptomycin, and glutamine as the HEK 293 cells.

Conjugate Synthesis. *Synthesis of Ligand–Linker Conjugate (CRL-L1).* The CCK2R ligand (Z-360) was synthesized as previously described[21] and abbreviated CRL to be consistent with previous publications.[30] The peptide spacer was prepared using Fmoc-protected solid phase peptide synthesis as outlined in Scheme 1 (Supporting Information (SI), Figure 1) and named L1. As shown in Scheme 1 (SI, Figure 1), CRL was coupled to the peptide spacer on the solid phase and cleaved from the resin using a standard cleavage cocktail solution. Crude CRL-L1 was purified by preparative RP-HPLC [A = 2 mM ammonium acetate buffer (pH 5.0), B = CH_3CN, solvent gradient: 5% B to 80% B in 25 min] to yield the requisite product. LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{72}H_{110}N_{12}O_{27}S$, 1607.79; found, 1608.

Synthesis of CRL-L1-desacetyl vinblastine hydrazide (CRL-L1-DAVBH). A solution of saturated sodium bicarbonate (2 mL) and HPLC grade water was bubbled with argon continuously for 10 min. CRL-L1 (36 mg, 0.0226 mmol) was dissolved in argon-purged HPLC grade water (2.0 mL), and the pH of the reaction mixture was increased to ~7 using argon-purged sodium bicarbonate. A solution of disulfide activated-DAVBH (11.10 mg, mmol) in THF (2.0 mL) was then added to the reaction mixture (SI, Figure 2). The progress of the reaction was monitored using analytical LRMS-LCMS, and after stirring for 20 min, the reaction was found to reach completion. Crude CRL-L1-DAVBH was purified by preparative RP-HPLC [A = 20 mM ammonium acetate (pH 7.2), B = CH_3CN, solvent gradient: 5% B to 80% B in 30 min], yielding the desired product. LRMS (LC/MS) (m/z): $[M + H]^+$ calcd for $C_{118}H_{168}N_{18}O_{36}S_2$, 2478; found, 2478.

Synthesis of CRL-L1 Tubulysin B Hydrazide (CRL-L1-TubBH). CRL-L1-TubBH was synthesized from activated tubulysin B hydrazide following the same procedure used for the synthesis of CRL-L1-DAVBH (SI, Figure 2). After removing the THF under reduced pressure, CRL-L1-TubBH

DOI: 10.1021/acs.molpharmaceut.5b00218
Mol. Pharmaceutics 2015, 12, 2477–2483

was purified by preparative RP-HPLC [A = 2 mM ammonium acetate buffer (pH 7.0), B = CH_3CN, solvent gradient: 5% B to 80% B in 25 min] to yield the requisite product. LRMS-LC/MS (m/z): $[M + H]^+$ calcd for $C_{117}H_{177}N_{19}O_{38}S_3$, 2553.96; found, 2554.

Synthesis of Nontargeted Desacetyl Vinblastine Hydrazide (L1-DAVBH) and Nontargeted Tubulysin Hydrazide (L1-TubBH). L1-DAVBH and L1-TubBH were synthesized from activated DAVBH and TubBH, respectively, following the procedure outlined for the synthesis of CRL-L1-DAVBH (SI, Figure 1 and 3). Each compound was then purified by reverse phase HPLC [A = 2 mM ammonium acetate buffer (pH 7.0), B = CH_3CN, solvent gradient: 5% B to 80% B in 25 min] to yield the requisite product. LRMS-LC/MS (m/z): $[M + H]^+$ calcd for L1-DAVBH, $C_{89}H_{134}N_{14}O_{32}S_2$, 1976.22; found, 1976; and (m/z): $[M + H]^+$ calcd for L1-TubBH $C_{88}H_{143}N_{15}O_{34}S_3$, 2051.35; found, 2051. All conjugates were found to be stable in saline for at least four weeks as monitored by LC-MS.

General Procedure for the in Vitro Determination of Cell Viability (IC_{50}). CCK2R-transfected HEK 293 cells (100,000 cells/well) were seeded on amine-coated 24-well plates and allowed to form monolayers. The spent medium in each well was replaced with fresh medium containing increasing concentrations of the test agents. After incubating for 2 h at 37 °C, cells were rinsed 3× with fresh medium and then incubated an additional 66 h at 37 °C in fresh medium. Spent medium in each well was again replaced with fresh medium (0.5 mL) containing ^{3}H-thymidine (1 μCi/ml), and the cells were incubated for an additional 4 h. After washing the cells 3× with medium, they were dissolved in 0.5 mL of 0.25 M NaOH. Thymidine incorporation was then determined by counting cell-associated radioactivity using a scintillation counter (Packard, Packard Instrument Company). The IC_{50} value was derived from a plot of the percent of ^{3}H-thymidine incorporation versus log concentration using Graph Pad Prism 4 and TableCurve 2D software.

Tumor Model and Therapy. HEK 293 cells expressing CCK2R (5.0×10^6 in 50% HC matrigel) were injected into the shoulders of 5−6 week old female *nu/nu* mice. An age-matched group of animals was similarly implanted with 1×10^6 KB cells in 100 μL of cell culture medium. Because KB cells do not overexpress CCK2R, the KB xenograft model served as a negative control. Tumors were measured in two perpendicular directions 3× per week with vernier calipers, and their volumes were calculated as 0.5 x L x W^2, where L is the longest axis (in millimeters), and W is the axis perpendicular to L (in millimeters). Dosing was initiated when the subcutaneous tumors reached ~100 mm^3 in volume. Dosing solutions were prepared in saline and filtered through a 0.22 μm filter. Solutions were administered either intraperitonealy (CRL-L1-DAVBH, L1-DAVBH) or intravenously (CRL-L1-TubBH, L1-TubBH, CRL-L1). Each mouse received 2 μmol/kg of the test or control agent in 100 μL of saline per injection. Injections were given 3× per week for 3 weeks, and the mice were weighed at each dosing as a measure of gross toxicity. All animal work was performed under the guidance of the Purdue Laboratory Animal Program and was reviewed by the Purdue Animal Care and Use Committee.

Histological Staining. Tumors were excised, fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Purdue Histology and Phenotyping Laboratory.

RESULTS

Proper design of a ligand-targeted chemotherapeutic agent requires (i) selection of a high affinity ligand with good selectivity for a cancer-enriched receptor, (ii) identification of a therapeutic agent with sufficient potency to kill cancer cells when captured by a cancer-specific receptor, and (iii) construction of a linker that will enable delivery and release of the attached drug preferentially within the targeted cells. Because cholecystokinin receptor ligand (CRL) has been shown to exhibit high affinity (0.47 nM) and strong selectivity for CCK2R (>600-fold specificity over CCK1R[29]), it was selected for exploration as a targeting ligand for drug delivery to CCK2R-expressing cancer cells (Figure 1B).[21,30]

To avoid nonspecific adsorption to CCK2R negative cells, we incorporated a water-soluble peptide spacer,[31] referred to as L1, between the ligand and its therapeutic payload (Figure 1B). Previous results from our lab have shown only a slight loss of affinity with no effect on specificity when CRL is conjugated to its payload via hydrophilic linkers.[30]

In the present study, two highly potent microtubule inhibitors, desacetyl vinblastine hydrazide (DAVBH) and tubulysin B hydrazide (TubBH), were attached to the CRL-L1 peptide spacer through a self-immolative disulfide linker.[31] This linker allows for selective release of the cytotoxic agent upon entry into the reducing environment of cancer cells. Detailed schemes for the synthesis of CRL conjugates of DAVBH and TubBH are described in SI Figures 2 and 3 and complete chemical structures for all conjugates can be found in SI Figures 4−7.

To determine the cytotoxicity and targeting specificity of the CRL-L1-DAVBH conjugate, we incubated CRL-L1-DAVBH, free DAVBH, and nontargeted L1-DAVBH with CCK2R-transfected HEK 293 cells for 2 h followed by incubation of the cells in drug-free medium for 66 h. Cell viability was then measured via incorporation of ^{3}H-thymidine. As shown in Figure 2, the potency of free DAVBH and CRL-L1-DAVBH was 9 and 29 nM, respectively, whereas the potency of the

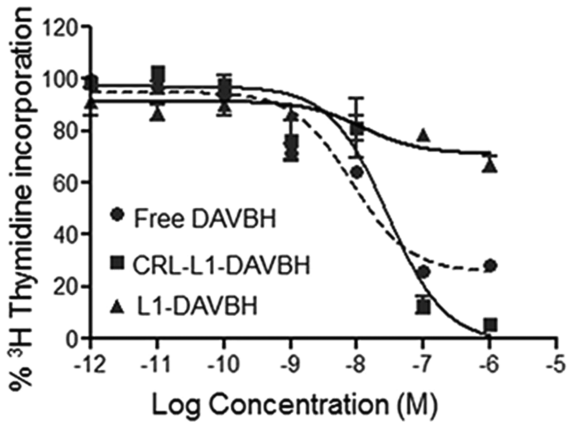


Figure 2. In vitro cytotoxicity of DAVBH derivatives. The cytotoxicity of free DAVBH (circles), the nontargeted L1-DAVBH (triangles), and the targeted CRL-L1-DAVBH (squares) conjugates in HEK 293 cells transfected with CCK2R when pulsed for 2 h at 37 °C, washed with culture medium three times, and then incubated for an additional 66 h. Cells were incubated with ^{3}H-thymidine for 4 h and washed three times. After the final wash, the cells were dissolved in 0.5 mL of 0.25 M NaOH. Viability of the cells was assessed by analyzing the incorporation of ^{3}H-thymidine. Error bars represent standard deviation.

DOI: 10.1021/acs.molpharmaceut.5b00218
Mol. Pharmaceutics 2015, 12, 2477−2483

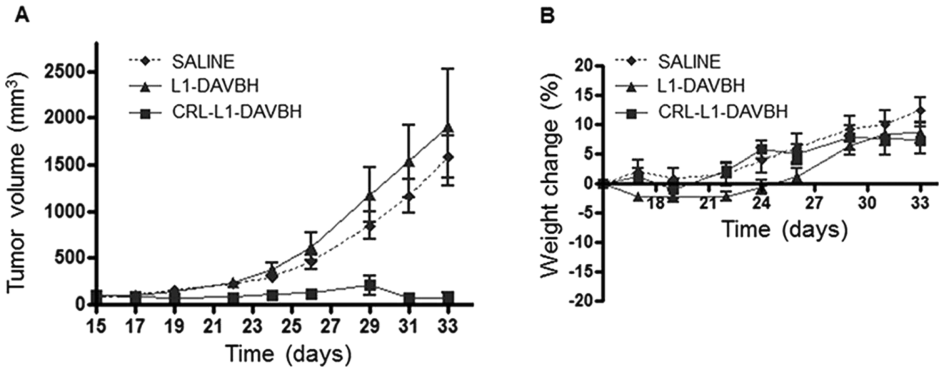


Figure 3. In vivo therapeutic efficacy and gross toxicity of DAVBH conjugates. (A) Volume of tumors and (B) percent weight change in *nu/nu* mice subcutaneously injected with CCK2R-transfected HEK 293 cells (5.0×10^6 in 50% HC matrigel) on day 0. Mice were randomized to different treatment groups ($n = 5$) and began treatment on day 15, when tumor volume was approximately 100 mm^3. Saline (diamonds), nontargeted L1-DAVBH (triangles), and targeted CRL-L1-DAVBH (squares) conjugates were administered intraperitoneally following a TIW schedule for ~3 weeks. Error bars represent standard deviation.

nontargeted conjugate L1-DAVBH was markedly reduced by a factor of >1000 (IC_{50} value >50 μM). Importantly, CRL by itself was found to show no cytotoxicity toward HEK 293-CCK2R cells (SI, Figure 8), demonstrating that the aforementioned cytotoxicity was due to the targeted therapeutic agent rather than obstruction of CCK2R by CRL.

To determine in vivo efficacy, we injected 2 μmol/kg CRL-L1-DAVBH intraperitoneally into mice bearing CCK2R-transfected HEK 293 xenografts at a frequency of 3x per week for ~3 weeks ($n = 5$). This dose was selected based on previous studies showing significant therapeutic benefit when similar folate- and DUPA-targeted chemotherapeutics were administered at the same concentration.[20,32,33] As shown in Figure 3A, CRL-L1-DAVBH was found to markedly delay tumor growth but not lead to complete tumor eradication. To determine whether the residual lesion was comprised predominately of cancer or stromal cells, H&E staining was performed on excised tumors (day 33). Unlike the saline control, which was comprised of almost entirely of cancer cells (Figure 4A), CRL-L1-DAVBH-treated tumors exhibited a greatly decreased ratio of cancer to stromal cells (Figure 4B). As expected, the nontargeted L1-DAVBH exerted little impact on tumor growth, demonstrating the critical role of ligand targeting on CRL-L1-DAVBH efficacy (Figure 3A). Importantly, body weights in the CRL-L1-DAVBH-treated group remained essentially constant over the course of the study, suggesting that the targeted therapy was not grossly toxic to the animals (Figure 3B).

Because complete tumor remission was not achieved at a dose of 2 μg/kg with CRL-L1-DAVBH, we elected to develop a more potent CCK2R-targeted conjugate. For this purpose, tubulysin B hydrazide (TubBH), a microtubule inhibitor with ~10× the potency of DAVBH, was conjugated to CRL via the same L1 linker (Figure 1, SI Figures 2 and 3). As shown in Figure 5A, free tubulysin B hydrazide was found to be very potent in vitro, exhibiting an IC_{50} of 2.7 nM on HEK 293-CCK2R cells. Similarly, the targeted CRL-L1-TubBH conjugate yielded an IC_{50} of 2 nM, whereas nontargeted L1-TubBH displayed the anticipated significant drop in potency (IC_{50} of ~310 nM) due to its membrane impermeability and lack of

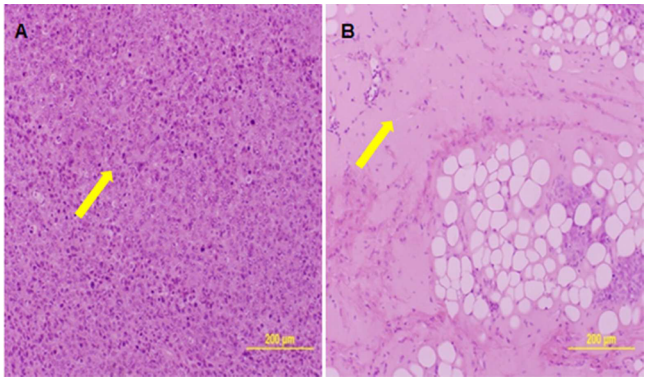


Figure 4. Histopathological staining of CCK2R-transfected HEK 293 tumor xenografts. Excised tumors from mice treated with (A) saline or (B) CRL-L1-DAVBH were sectioned and stained with hematoxylin and eosin. Arrows show the abundance of cancer cells in panel A and a reduced number of cancer cells in panel B.

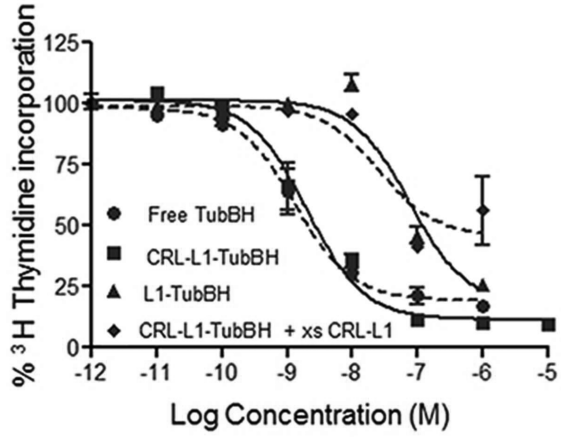


Figure 5. In vitro cytotoxicity of TubBH derivatives. The cytotoxicity of free TubBH (circles), the nontargeted L1-TubBH (triangles), the targeted CRL-L1-TubBH (squares), and the targeted CRL-L1-TubBH in the presence of 100-fold greater concentration competing CRL-L1 (diamonds) conjugates in HEK 293 cells transfected with CCK2R when pulsed for 2 h at 37 °C, washed with culture medium three times, and then incubated for an additional 66 h. Cells were incubated with ^{3}H-thymidine for 4 h and washed three times. After the final wash, the cells were dissolved in 0.5 mL of 0.25 M NaOH. Viability of the cells was assessed by analyzing the incorporation of ^{3}H-thymidine. Error bars represent standard deviation.

DOI: 10.1021/acs.molpharmaceut.5b00218
Mol. Pharmaceutics 2015, 12, 2477−2483

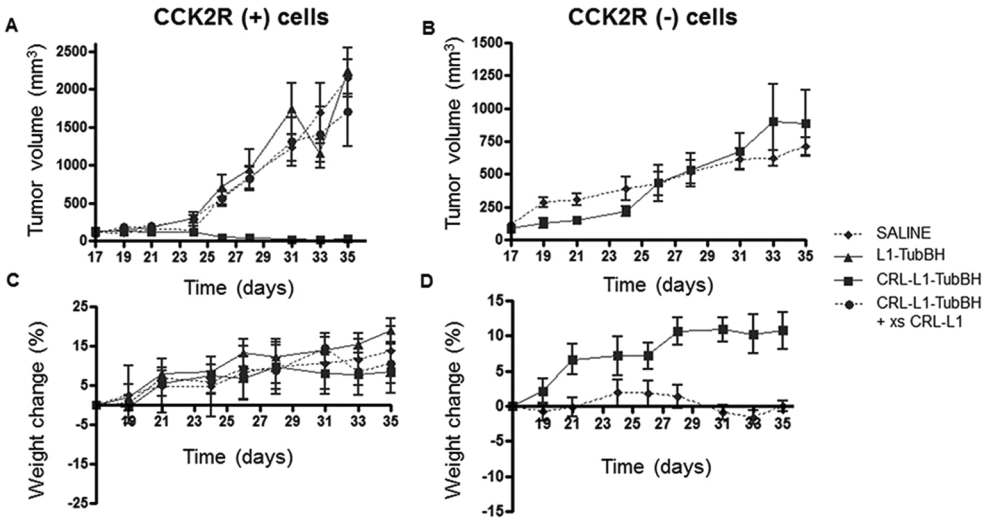


Figure 6. In vivo therapeutic efficacy and gross toxicity of TubBH conjugates. (A,B) Volume of tumors and (C,D) percent weight change in *nu/nu* mice subcutaneously injected with 5.0×10^6 CCK2R-transfected HEK 293 cells (CCK2R(+) cells) in 50% HC matrigel (A,C) or 1.0×10^6 KB cells (CCK2R(−) cells) in 50% HC matrigel on day 0. Mice were randomized to different treatment groups ($n = 5$) and began treatment on day 17, when the tumor volume was ∼100 mm^3. Saline (diamonds), nontargeted L1-TubBH (triangles), targeted CRL-L1-TubBH (squares), and targeted CRL-L1-TubBH in the presence of 100-fold greater concentration of competing CRL-L1 (circles) conjugates were administered via lateral vein injection following a TIW schedule for ∼3 weeks. Error bars represent standard deviation.

targeting. To ensure that the cytotoxicity of CRL-L1-TubBH was receptor mediated, cells were incubated with CRL-L1-TubBH in the presence of 100-fold excess CRL-L1. As expected, the IC_{50} value of the competed CRL-L1-TubBH was nearly identical to that of the nontargeted L1-TubBH conjugate (IC_{50} of ∼340 and 310 nM, respectively).

To determine the efficacy of CRL-L1-TubBH in vivo, we followed the same treatment regimen used for DAVBH conjugates except that TubBH conjugates were injected intravenously. CRL-L1-TubBH showed efficient antitumor activity, eliminating all detectable malignant lesions from all five mice (Figure 6A) and prolonged survival (SI, Figure 9). As expected, nontargeted L1-TubBH showed no difference from the saline-treated control group when an identical concentration as that of the CRL-L1-TubBH targeted conjugate was tested. Moreover, when CRL-L1-TubBH was competed with 100-fold excess of CRL-L1, the antitumor effect of the targeted TubBH was negated (Figure 6A). This indicates that the antitumor activity observed in the CRL-L1-TubBH group was entirely receptor mediated. Importantly, the CRL-L1-TubBH-treated mice displayed no visible signs of gross toxicity, and no weight loss was observed (Figure 6C).

Finally, to more thoroughly establish the necessity of receptor-mediated targeting for CRL-L1-TubBH efficacy, the targeted conjugate was tested using the same protocol in a CCK2R-negative KB xenograft model. In vitro CRL-L1-TubBH conjugate yielded an IC_{50} value of 269 nM (SI, Figure 9), similar to the IC_{50} values observed with the nontargeted and competed targeted conjugates in the CCK2R-expressing HEK cells. As shown in Figure 6B, CRL-L1-TubBH showed no difference in tumor growth from the vehicle control. Because free tubulyisn B hydrazide is known to be cytotoxic against KB cells,[34] the lack of reduction in tumor volume in the KB xenograft must derive from the absence of CCK2R and not from any intrinsic resistance to TubBH. Again, none of the mice in this study exhibited any signs of gross toxicity, and no substantial weight loss was observed (Figure 6D).

DISCUSSION

Conjugation of cytotoxic drugs to ligands that selectively target cancer cells has emerged as a promising method to both improve drug efficacy and reduce drug toxicity. Although only a few low molecular weight ligand-targeted cancer drugs have been tested in vivo to date, results suggest that the potential to design such targeted therapeutics for most human malignancies is promising. Folate receptor-targeted chemotherapeutic agents have demonstrated the capacity to treat malignancies of the ovary, lung, kidney, breast, and endometrium,[35,36] and PSMA-targeted drugs have shown promise for treating cancers of the prostate.[25] LHRH receptor- and $\alpha_v\beta_3$-targeted therapeutics can expand this list to include cancers of the ovary and breast,[37,38] but to date, few ligands are available to target cancers of the GI tract. In this study, we attempted to obtain a ligand that would deliver attached drugs to gastroinstestinal malignancies. On the basis of literature demonstrating overexpression of CCK2R in gastrointestinal stromal tumors and colon, stomach, esophageal, and pancreatic cancers,[24−29] the CRL conjugates described in this paper should have the potential to address this deficiency.

One of the more interesting results to emerge from this study was the high specificity displayed by both CRL-L1-DAVBH and CRL-L1-TubBH for CCK2R-expressing cancer cells. CRL, DAVBH, and TubBH were all found to be very hydrophobic, predicting that any conjugate of the ligand to a drug would be extremely lipophilic, resulting in a strong proclivity to associate nonspecifically with all cell membranes. Thus, to increase hydrophilicity, a water-soluble linker (L1) was utilized to conjugate CRL to the cytotoxic drugs. CCK2R-dependent binding of CRL-L1-DAVBH and CRL-L1-TubBH was established by the absence of toxicity when (i) CRL was deleted, (ii) conjugate binding was competed with excess ligand, or (iii) CCK2R was absent from the targeted cancer cell. These data suggest that, otherwise, highly hydrophobic ligand−drug conjugates can be rendered tumor specific by the judicious choice of a hydrophilic linker due to the linker impeding the diffusion of the hydrophobic drug through the cell membranes,[39] thus reducing nonspecific activity. However,

DOI: 10.1021/acs.molpharmaceut.5b00218
Mol. Pharmaceutics 2015, 12, 2477−2483

this effect is somewhat concentration dependent as some conjugates can diffuse into cells, and therefore at high concentrations, nonreceptor mediated activity can occur. In the case of linking CRL to DAVBH and TubBH, the insertion of a bulky water-soluble linker between the ligand and drug converted an otherwise nonspecific drug conjugate into a highly receptor-targeted drug conjugate at physiologically relevant concentrations.

Our CRL-TubBH conjugates were also tested against xenograft tumors similar to those exposed to the DAVBH conjugates, except an additional treatment group was included where CRL-L1-TubBH was competed with excess CRL-L1. Similar to the DAVBH conjugates, the nontargeted L1-TubBH conjugate did not appear to have any effect on tumor growth. The lack of efficacy of L1-conjugated cytotoxic compounds in vivo is most likely due to the hydrophilic linker, which does not allow for the cytotoxic compound to readily diffuse through the cell membrane, whereas the targeted CRL-L1-DAVBH and CRL-L1-TubBH both showed efficacy in vivo.

Somewhat surprisingly, the CRL-L1-DAVBH conjugate did not regress tumor volume even though similar concentrations of DAVBH conjugated to folate or DUPA did exhibit tumor regression.[20,32,33] This result is most likely due to a lower number of CCK2R receptors present on the cell surface as compared to folate or PSMA. This resulting lack of tumor regression in vivo encouraged the testing of a more potent cytotoxic agent with a similar mechanism of action. When the more potent CRL-L1-TubBH conjugate was tested, not only do it halt tumor growth, but it even regressed the tumor to an indistinguishable level. Importantly, the CRL-L1-TubBH treatment group that was competed with CRL-L1 showed no effect on inhibiting tumor growth, similar to both the vehicle control and the nontargeted conjugate. To further assess the necessity of receptor-mediated targeting, we tested xenografts of the receptor-negative KB cell line with the targeted CRL-L1-TubBH conjugate. Unlike the CCK2R-positive HEK 293 xenografts, the KB xenografts showed neither regression nor a delay of tumor growth when treated with the conjugate. Taken together, these results strongly support the necessity of receptor-mediated targeting with these conjugates for any in vivo efficacy.

In summary, the current study demonstrates that CCK2R-targeting ligands can be used to deliver drugs selectively to CCK2R-positive tumors to generate a dramatic receptor-specific antitumor effect without observable gross toxicity to healthy tissues. Because current treatments for colon, lung, pancreatic, and related cancers commonly cause hair loss, bone marrow suppression, weight loss, and so forth, the prospects for developing a less toxic treatment for these malignant diseases offer some optimism for the management of these cancers in the future.

■ ASSOCIATED CONTENT

Supporting Information

Conjugate syntheses, chemical structures, and cytotoxicity and survival curves. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00218.

■ AUTHOR INFORMATION

Corresponding Author

*Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907. E-mail: plow@purdue.edu. Phone: 765-494-5272. Fax: 765-496-2677.

Author Contributions

§C.W. and J.R. contributed equally to this article.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Endocyte for their kind gift of tubulysin B hydrazide and desacetyl vinblastine hydrazide. We are also thankful to Dr. Mark Hellmich (Galveston, Texas) for his generous gift of cholecystokinin 2 receptor-transfected HEK 293 cells. This work was supported by funds from Purdue University.

■ REFERENCES

(1) Scott, R. B. Cancer chemotherapy the first twenty-five years. *Br. Med. J.* **1970**, *4*, 259−265.

(2) Braña, M. F.; Cacho, M.; Gradillas, A.; de Pascual-Teresa, B.; Ramos, A. Intercalators as anticancer drugs. *Curr. Pharm. Des.* **2001**, *7*, 1745−1780.

(3) Kaestner, P.; Bastians, H. Mitotic Drug Targets. *J. Cell. Biochem.* **2010**, *111*, 258−265.

(4) Jordan, M. A.; Wilson, L. Microtubules as a target for anticancer drugs. *Nat. Rev. Cancer* **2004**, *4*, 253−65.

(5) Azim, H. A., Jr.; de Azambuja, E.; Colozza, M.; Bines, J.; Piccart, M. J. Long-term toxic effects of adjuvant chemotherapy in breast cancer. *Annals of Oncology* **2011**, *22*, 1939−1947.

(6) Allen, T. M. Ligand targeted therapeutics in anticancer therapy. *Nat. Rev. Cancer* **2002**, *2*, 750−763.

(7) Schrama, D.; Reisfeld, R. A.; Becker, J. C. Antibody targeted drugs as cancer chemotherapeutics. *Nat. Rev. Drug Discovery* **2006**, *5*, 147−159.

(8) Firer, M. A.; Gellerman, G. Targeted drug delivery for cancer therapy: the other side of antibodies. *J. Hematol. Oncol.* **2012**, *5*, 70.

(9) LoRusso, P. M.; Weiss, D.; Guardino, E.; Girish, S.; Sliwkowski, M. X. Trastuzumab Emtansine: a unique antibody-drug conjugate in development for human epidermal growth factor receptor 2−positive cancer. *Clin. Cancer Res.* **2011**, *17*, 6437−6447.

(10) Girish, S.; Gupta, M.; Wang, B.; Lu, D.; Krop, I. E.; Vogel, C. L.; Burris, H. A.; LoRusso, P. M.; Yi, J. H.; Saad, O.; Tong, B.; Chu, Y. W.; Holden, S.; Joshi, A. Clinical pharmacology of trastuzumab emtansine (T-DM1): an antibody−drug conjugate in development for the treatment of HER2-positive cancer. *Cancer Chemother. Pharmacol.* **2012**, *69* (5), 1229−1240.

(11) Vaklavas, C.; Forero-Torres, A. Safety and efficacy of brentuximab vedotin in patients with Hodgkin lymphoma or systemic anaplastic large cell lymphoma. *Ther. Adv. Hematol.* **2012**, *3*, 209−225.

(12) Teicher, B. A.; Chari, R. V. J. Antibody conjugate therapeutics: challenges and potential. *Clin. Cancer Res.* **2011**, *17*, 6389−6397.

(13) Ricart, A. D. Immunoconjugates against solid tumors: Mind the gap. *Clin. Pharmacol. Ther.* **2011**, *89*, 513−523.

(14) Salmon, D. J.; Leyland-Jones, B.; Shak, S.; Fuchs, H.; Paton, V.; Bajamonde, A.; Fleming, T.; Eiermann, W.; Wolter, J.; Pegram, M.; Baselga, J.; Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N. Engl. J. Med.* **2001**, *344*, 783−792.

(15) Firer, M. A.; Gellerman, G. Targeted drug delivery for cancer therapy: the other side of antibodies. *J. Hematol. Oncol.* **2012**, *5*, 70.

(16) Vasalou, C.; Helmlinger, G.; Gomes, B. A mechanistic tumor penetration model to guide antibody drug conjugate design. *PLoS One* **2015**, *10* (3), e0118977.

DOI: 10.1021/acs.molpharmaceut.5b00218
Mol. Pharmaceutics 2015, 12, 2477−2483

(17) Hanawa, M.; Suzuki, S.; Dobashi, Y.; Yamane, T.; Kono, K.; Enomoto, N.; Ooi, A. EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus. *Int. J. Cancer.* **2006**, *118* (5), 1173−1180.
(18) Hartmann, L. C.; Keeney, G. L.; Lingle, W. L.; Christianson, T. J.; Varghese, B.; Hillman, D.; Oberg, A. L.; Low, P. S. Folate receptor overexpression is associated with poor outcome in breast cancer. *Int. J. Cancer.* **2007**, *121*, 938−942.
(19) Schulz, S.; Pauli, S. U.; Schulz, S.; Handel, M.; Dietzmann, K.; Firsching, R.; Hollt, V. Immunohistochemical determination of five somatostatin receptors in meningioma reveals frequent overexpression of somatostatin receptor subtype sst2A. *Clin. Cancer Res.* **2000**, *6* (5), 1865−1874.
(20) Kularante, S. A.; Wang, K.; Santhapuram, H. K.; Low, P. S. Prostate-specific membrane antigen targeted imaging and therapy of prostate cancer using a PSMA Inhibitor as a homing ligand. *Mol. Pharmaceutics* **2009**, *6*, 780−789.
(21) Shinozaki, K.; Yoneta, T.; Murata, M.; Miura, N.; Maeda, K. 1,5-Benzodiazepine Derivatives. US patent 6,344,452, February 5, 2002.
(22) Dufresne, M.; Seva, C.; Fourmy, D. Cholecystokinin and gastrin receptors. *Physiol. Rev.* **2006**, *86* (3), 805−847.
(23) Ashurst, H. L.; Varro, A.; Dimaline, R. Regulation of mammalian gastrin/CCK receptor (CCK2R) expression in vitro and in vivo. *Exp. Physiol.* **2008**, *93* (2), 223−236.
(24) Reubi, J. C.; Waser, B. Unexpected high incidence of cholecystokinin B/gastrin receptors in human medullary thyroid carcinomas. *Int. J. Cancer.* **1996**, *67*, 644−647.
(25) Sethi, T.; Herget, T.; Wu, S. V.; Walsh, J. H.; Rozengurt, E. CCK-A and CCK-B receptors are expressed in small cell lung cancer lines and mediate Ca^{2+} mobilization and clonal growth. *Cancer Res.* **1993**, *53*, 5208−5213.
(26) Reubi, J. C.; Schaer, J. C.; Waser, B. Cholecystokinin(CCK)-A and CCK-B/gastrin receptors in human tumors. *Cancer Res.* **1997**, *57*, 1377−1386.
(27) Hur, K.; Kwak, M. K.; Lee, H. J.; Park, D. J.; Lee, H. K.; Lee, H. S.; Kim, W. H.; Michaeli, D.; Yang, H. K. Expression of gastrin and its receptor in human gastric cancer tissues. *J. Cancer Res. Clin. Oncol.* **2006**, *132*, 85−91.
(28) Körner, M.; Waser, B.; Reubi, J. C.; Miller, L. J. CCK(2) receptor splice variant with intron 4 retention in human gastrointestinal and lung tumours. *J. Cell. Mol. Med.* **2010**, *14*, 933−943.
(29) Kawasaki, D.; Emori, Y.; Eta, R.; Iino, Y.; Hamano, H.; Yoshinaga, K.; Tanaka, T.; Takei, M.; Watson, S. A. Effect of Z-360, a novel orally active CCK-2/gastrin receptor antagonist on tumor growth in human pancreatic adenocarcinoma cell lines in vivo and mode of action determinations in vitro. *Cancer Chemother. Pharmacol.* **2008**, *61*, 883−892.
(30) Wayua, C.; Low, P. S. Evaluation of a Cholecystokinin 2 Receptor-Targeted Near-Infrared Dye for Fluorescence-Guided Surgery of Cancer. *Mol. Pharmaceutics* **2014**, *11*, 468−476.
(31) Vlahov, I. R.; Santhapuram, H. K.; You, F.; Wang, Y.; Kleindl, P. J.; Hahn, S. J.; Vaughn, J. F.; Reno, D. S.; Leamon, C. P. Carbohydrate-based synthetic approach to control toxicity profiles of folate-drug conjugates. *J. Org. Chem.* **2010**, *11*, 3685−3691.
(32) Reddy, J. A.; Dorton, R.; Dawson, A.; Vetzel, M.; Parker, N.; Nicoson, J. S.; Westrick, E.; Klein, P. J.; Wang, Y.; Vlahov, I. R.; Leamon, C. P. In vivo structural activity and optimization studies of folate-tubulysin conjugates. *Mol. Pharmaceutics* **2009**, *6*, 1518−1525.
(33) Leamon, C. P.; Reddy, J. A.; Vlahov, I. R.; Westrick, E.; Parker, N.; Nicoson, J. S.; Vetzel, M. Comparative preclinical activity of the folate-targeted Vinca alkaloid conjugates EC140 and EC145. *Int. J. Cancer.* **2007**, *121*, 1585−1592.
(34) Leamon, C. P.; Reddy, J. A.; Vetzel, M.; Dorton, R.; Westrick, E.; Parker, N.; Wang, Y.; Vlahov, I. Folate Targeting Enables Durable and Specific Antitumor Responses from a Therapeutically Null Tubulysin B Analogue. *Cancer Res.* **2008**, *68*, 9839−9844.
(35) Kalli, K. R.; Oberg, A. L.; Keeney, G. L.; Christianson, T. J.; Low, P. S.; Knutson, K. L.; Hartmann, L. C. Folate receptor alpha as a tumor target in epithelial ovarian cancer. *Gynecol. Oncol.* **2008**, *3*, 619−626.
(36) Reddy, J. A.; Leamon, C. P.; Low, P. S. Folate-mediated delivery of protein and peptide drugs into tumors. In *Delivery of protein and peptide drugs in cancer*, 1st ed.; Torchilin, V, Ed.; World Scientific/Imperial College Press: London, 2006; pp 183−204.
(37) Kakar, S. S.; Jin, H.; Hong, B.; Eaton, J. W.; Kang, K. A. LHRH receptor targeted therapy for breast cancer. *Adv. Exp. Med. Biol.* **2008**, *614*, 285−296.
(38) Landen, C. N.; Kim, T. J.; Lin, Y. G.; Merritt, W. M.; Kamat, A. A.; Han, L. Y.; Spannuth, W. A.; Nick, A. M.; Jennings, N. B.; Kinch, M. S.; Tice, D.; Sood, A. K. Tumor-Selective Response to Antibody-Mediated Targeting of $\alpha v\beta 3$Integrin in Ovarian Cancer. *Neoplasia* **2008**, *10*, 1259−1267.
(39) Prokop, A.; Iwasaki, Y.; Harada, A. *Intracellular Delivery II Fundamentals and Applications*, 1st ed.; Springer Publishing Group: New York, 2014.

DOI: 10.1021/acs.molpharmaceut.5b00218
Mol. Pharmaceutics 2015, 12, 2477−2483