CLT1 Targets Bladder Cancer through Integrin α5β1 and CLIC3

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ABSTRACT

High grade non-muscle invasive bladder cancer is commonly treated with Bacillus Calmette-Guérin, an immunotherapeutic that depends on fibronectin and tumor cell integrin α5β1 for internalization into bladder cancer cells. We previously demonstrated that the anti-angiogenic peptide CLT1 forms cytotoxic complexes with fibronectin that are cooperatively internalized into proliferating endothelium through ligation of integrins and chloride intracellular channel 1. While CLT1 has no effect on mature, differentiated cells, we show here that CLT1 is highly cytotoxic for a panel of bladder tumor cell lines as well as a variety of cell lines derived from kidney, lung, breast and prostate cancer. Paralleling our previous results, we found CLT1-induced tumor cell death to be increased in the presence of fibronectin, which mediated CLT1 internalization and subsequent autophagic cell death in a mechanism that depends on tumor cell integrin α5β1 and chloride intracellular channel 3 (CLIC3). This mechanistic link was further supported by our results showing upregulation of α5β1 and CLIC3 in CLT1-responsive tumor cell lines and co-localization with CLT1 in tumor tissues. Incubating tumor tissue from bladder cancer patients with fluorescein-conjugated CLT1 resulted in a strong and specific fluorescence while normal bladder tissue remained negative. Based on its affinity for bladder tumor tissue and strong anti-tumor effects, we propose that CLT1 could be useful for targeting bladder cancer.
INTRODUCTION

Bladder cancer is the second most common and the third deadliest malignancy of the genitourinary tract, with a mortality of approximately 20 % (1). The majority of patients (75-85 %) have non-muscle invasive bladder cancer (NMIBC) at the time of diagnosis, which is typically treated with transurethral resection (TURBT) (2). While TURBT is effective in patients with low risk bladder cancer (i.e. small single tumors, non-invasive and low grade), the rate of recurrence and progression in patients with intermediate or high risk disease is considerable (3, 4). To reduce recurrence, TURBT is usually accompanied by an adjuvant intravesical treatment with chemo- or immunotherapy (5, 6). However, despite these therapies, the rate of recurrence and progression is up to 70 and 30 %, respectively (5). Other strategies to control bladder cancer include hyperthermia, photodynamic therapy and diverse imaging strategies that are intended to improve tumor visualization during cystoscopy and TURBT (2, 7, 8). While some of these approaches (e.g. hyperthermia) have shown benefit in the short term, their efficacy of controlling bladder cancer over a long period of time is not clear (7). Together, these data underscore the continued need to develop efficient and well-tolerated adjuvant treatments for high grade NMIBC.

The adjuvant treatment of choice for high risk NMIBC is the intravesical application of live bacteria, bacillus Calmette-Guérin (BCG), which is superior in reducing the risk of recurrence compared to intravesical chemotherapy with mitomycin (9-11). BCG has also been shown to reduce the risk of progression but it is not clear whether the
treatment yields a significant survival advantage (12, 13). The two major drawbacks of BCG are non-response and a high rate of side effects (up to 90 % of cases) ranging from irritative voiding, hematuria and cystitis to BCG-induced sepsis (13, 14). While the majority of BCG-induced side effects are considered as non-severe, it has been estimated that they cause nearly 30 % of the patients to discontinue therapy (14). The mechanism of action for BCG is to induce an immune reaction that orchestrates the eradication of bladder tumor cells and treatment complications are a direct result of this response (15). As such, side effects correlate positively with the efficacy of BCG while the lack of a sufficient immune response is predictive of treatment failure.

Using phage display, we previously identified a tumor homing peptide, CLT1 that associates with clotted plasma in tumor interstitial spaces (16). More recently, we found that CLT1 has strong anti-angiogenic activity in vivo and attributed this function to the capacity of CLT1 to induce an unfolded protein response and autophagic cell death in proliferating endothelial cells (17). Cytotoxicity towards endothelial cells was supported by fibronectin, which forms co-aggregates with CLT1 and as such, mediates binding to endothelial integrins and subsequent internalization through a novel CLT1 receptor, chloride intracellular channel 1 (CLIC1) (17). This mechanism is reminiscent of the role of fibronectin for BCG opsonization, which contributes to the integrin-mediated internalization of fibronectin-bound BCG into bladder tumor cells as a prerequisite of BCG anti-tumor activity (18, 19). Based on the role of fibronectin for BCG anti-tumor efficacy, we sought to determine if CLT1 has anti-tumor properties and if this function is pronounced in bladder cancer.
MATERIALS AND METHODS

Peptides. All peptides were purchased from Primm Biotech (Cambridge, MA). A lysine/alanine scan of CLT1 yielded CLT1 variant peptides GA (CALIIQKNEC), LK (CGKIIQKNEC), IK1 (CGLIIKQKNEC), IK2 (CGLIIQKNEC), QA (CGLIIAKNEC), KA (CGLIIQAEC), NA (CGLIIQKAEC), and EA (CGLIIQKNAC). CLT1 peptides were cyclized by exposure to air, which leads to the formation of intramolecular disulfide bonds between C- and N-terminal cysteines. Linear CLT1 (LCLT1, AGLIIQKNEA) was generated by replacing the cysteines with alanine. Carboxyfluorescein was conjugated to the peptides via a 2-aminoethoxy-2-ethoxycetic acid (AEEA)-linker for fluorescent studies.

Cell lines and treatments. J82, T24, UMUC3, TCCSUP, 5637 (bladder), DU145, PC-3 (prostate), RCC4, 786-0 (renal), MDA-MB-231, MCF-7 (breast), A549 (lung), PANC1 (pancreas) and RD (muscle) tumor cell lines were purchased from ATCC and cultured per manufacturer’s specifications. Human bladder epithelial cells were from Lifeline Cell Technology and cultured according to manufacturer recommendations in serum free Prostalife Basal Medium supplemented with L-glutamine, extract P, epinephrine, rh TGF-α, hydrocortisone hemisuccinate, rh insulin, apo-transferrin, and calcium chloride. Cells were cultured at 37°C under a humidified, 5% CO₂ atmosphere. All cells were treated at a plating density of ca. 50% to ensure a linear growth rate. Peptides were diluted in H₂O to 2 mg/ml and added to cells at concentrations ranging from 7.5-150 μg/ml in the presence of 2% fetal bovine serum (FBS). Human plasma fibronectin
(Sigma; 3-100 μg/ml), fibrinogen (Enzyme Research Laboratories; 30 μg/ml), nocodazole (Sigma; 10 μM), GRGDSP (RGD) or GRADSP (RAD) peptides (ea. 300 μM; EMD Chemicals) were added at the time of peptide treatment. Bafilomycin A1 (EMD Chemicals; 40-400 nM) was added 1 hour prior to CLT1 addition. Where indicated, FBS was depleted of fibronectin by passing the serum through a gelatin agarose column. For experiments utilizing serum free media, cells were serum starved for 8 hours prior to study onset.

Cytotoxicity assay. Cell death was assessed after 24 hours by measuring LDH release using the Cytotoxicity Detection Kit (Roche Applied Science). Results are normalized for background cell death observed in control cells treated in absence of CLT1 to the specified medium conditions. Maximal cell death was achieved by simultaneous treatment with camptothecin (1.4 μM; Sigma-Aldrich) and staurosporine (100 nM; Alexis Biochemicals).

Microscopy. To analyze internalization of CLT1 in tumor cell lines, cultured cells grown on coverslips coated with 10 μg/ml vitronectin (BD Biosciences) over night at 4°C were incubated with 25 μg/ml fluorescein-conjugated peptides for 24 hours in the presence of 30 μg/ml fibronectin or fibrinogen where indicated, fixed in 4% paraformaldehyde (PFA) and stained with DAPI-containing mounting media (Vectashield;Vector Laboratories). Cellular localization was analyzed at 40x magnification using a fluorescence microscope (Zeiss Axioplan 2) with image processing unit. Lysosomes were labeled with 100 nM Lysotracker Red DND-99 (Invitrogen) for 1 hour prior to fixation. For fibronectin and
CLIC3 staining, PFA-fixed cells were permeabilized with 0.5% triton X-100 and incubated with anti-fibronectin (Millipore), anti-CLIC3 (Abcam) or isotype control, followed by incubation with Alexa Fluor 546-conjugated secondary antibody (Invitrogen) and analyzed using a confocal microscope (Leica TCSSL). Cell morphology was imaged by reflection confocal microscopy. To visualize nuclei, cells were stained with Draq5 (eBioscience).

De-identified tissues from clinical bladder cancer as well as normal adjacent bladder tissue were inspected by an anatomical pathologist following resection and then immediately distributed through the tissue bank of the University of Pittsburgh Cancer Institute. Upon receipt in our laboratory, tissue was placed in Keratinocyte Growth Media containing bovine pituitary extract, hEGF, insulin, hydrocortisone and antibiotics with or without 25 μg/ml fluorescein-conjugated CLT1 or control peptides (IK1, LCLT1). Fibronectin and fibrinogen were added where indicated. After overnight incubation at 37°C and 5% CO2, tissue samples were washed in PBS and either placed on a microscopy slide for en-face confocal microscopy or frozen in OCT. Frozen tissues were sectioned, fixed in acetone and analyzed for peptide uptake by fluorescence microscopy. For immunohistochemistry, acetone fixed sections were incubated with anti-CLIC3 or anti-α5 integrin (BD Bioscience) and Alexa Fluor 546-conjugated secondary antibody prior to confocal microscopy. Digitized images were processed with Adobe Photoshop.

**Western blot analysis.** Cells were lysed using the Subcellular Protein Fractionation Kit (Thermo Fisher Scientific) or by the addition of 2x SDS sample buffer. Proteins were
separated by SDS-PAGE, transferred onto PVDF and stained with 0.05% Ponceau S (Sigma) to ensure equivalent protein loading. Immunoblots were blocked with 5% bovine serum albumin and probed overnight at 4°C with anti-Cathepsin D, anti-LC3B (Cell Signaling Technology) or anti-CLIC3. Immunoreactivity was detected using peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody and visualized by enhanced chemiluminescence.

**siRNA mediated gene silencing.** Integrin α5 (L-008003-00), integrin β3 (L-004124-00), CLIC1 (L-009530-00), CLIC3 (L-011805-00), CLIC4 (L-013553-00), CLIC5 (L-020570-00) and non-targeting control (D-001810-10) On-TARGETplus SMARTpool siRNAs were purchased from Dharmacon. Cells were transfected with 10 nM siRNA in Opti-MEM medium (Invitrogen) using LipofectAMINE 2000 reagent (Invitrogen) for 5 hours, then placed in normal culture medium and grown for an additional 43 hours prior to treatment with CLT1. Target knockdown was confirmed by RT-PCR and western blot analysis (Supplementary Fig. S1).

**Flow cytometry assay.** Cells were suspended in media supplemented with 2% FBS and incubated with integrin α5 or isotype control antibody (BD Biosciences) for 30 minutes at 4°C, washed with ice cold media, and incubated for 30 minutes on ice with Alexa Fluor 488 anti-mouse F(ab')2 (Invitrogen). To measure CLIC3 expression, suspended cells were incubated on a rotor for 45 minutes at room temperature prior to incubation with CLIC3 antibody for 30 minutes at 4°C. Cell viability was monitored by
staining cells with 5 μg/ml propidium iodide (Roche Applied Science). Fluorescence was examined on 10,000 viable cells per sample using a tabletop cytometer (Accuri C6).

**Statistical analysis.** Data were analyzed using unpaired two-tailed Student’s *t* test or one-way ANOVA followed by the posthoc Tukey’s multiple comparisons test (GraphPad Prism 5). Treatment differences with a two-sided *p* value < 0.05 were considered significantly different. Error bars show mean ± SEM.

**RESULTS**

*CLT1 induces tumor cell death in cooperation with fibronectin.* We previously showed that CLT1 has significant anti-angiogenic activity, which correlates with the ability of CLT1 to generate cytotoxic complexes with the adhesion protein fibronectin (17). Here we demonstrate that CLT1 is cytotoxic for a diverse panel of human tumor cell lines derived from the bladder, prostate, kidney, breast and lung (Fig. 1A). The cytotoxic activity of CLT1 was strongly augmented in the presence of fibronectin, which alone had no effect on the tumor cell fate (Fig. 1A-B, Supplementary Fig. S2A). Cytotoxicity in proliferating tumor cells occurred within 24 hours of treatment at a fibronectin concentration of 10 μg/ml and reached a plateau at a CLT1 concentration of 75 μg/ml (Fig. 1C-D). A second plasma adhesion protein, fibrinogen, had the opposite effect and effectively prevented CLT1-induced cytotoxicity (Fig. 1A). Among the different tumor types tested, CLT1 activity was pronounced in bladder cancer cells, which responded consistently with extensive cell death even in the presence of urine (Fig. 1A,
Supplementary Fig. S2B). CLT1 was significantly more effective in proliferating than in serum-starved bladder tumor cells and largely inactive in primary bladder epithelial cells (Fig. 1A-B). To further dissect the role of fibronectin for bladder tumor cell death, we treated TCCSUP, J82 and UMUC3 cells with fibronectin-depleted serum, which in comparison to plasma fibronectin-supplemented serum was significantly less effective in mediating CLT1-induced cell death (Fig. 1E-F). Tumor cell death in response to CLT1 was also reduced when we transfected bladder tumor cells with siRNA against fibronectin; however this was only the case when tumor cells were treated in absence of exogenous fibronectin, suggesting that CLT1 interacts with cellular fibronectin as well as plasma fibronectin (Fig. 1F). Disabling structural features of CLT1 that are relevant for fibronectin complex formation, such as the LIIQK motif or the C- and N-terminal cysteines that mediate cyclization (17), rendered CLT1 inactive (Fig. 1G). Together, our results show that CLT1 has substantial anti-tumor activity, which is pronounced in bladder tumor cell lines, and that this activity depends on an intact LIIQK motif as well as the presence of fibronectin.

Tumor cells undergo autophagic cell death in response to CLT1. To further delineate the mechanism of CLT1 cytotoxicity, we incubated tumor cells with fluorescein-conjugated CLT1 and analyzed its subcellular localization using fluorescence and confocal microscopy. These experiments demonstrated that CLT1 uptake was significantly higher in J82 and UMUC3 bladder cancer cells that are sensitive for CLT1-induced cell death than in 786-0 kidney cancer cells that are largely resistant towards CLT1 (Fig. 2A, Supplementary Fig. S3). Conversely, CLT1 cytotoxicity was significantly
reduced when we inhibited CLT1 uptake with nocodazole (Fig. 2B-C). Co-staining with lysotracker revealed that CLT1 was transported into lysosomes, which appeared enlarged and dysformed after treatment with CLT1 compared to untreated cells (Fig. 2D-E). To determine if CLT1 induces lysosome dysfunction, we analyzed the cytoplasmic fraction of CLT1-treated tumor cells and found increased concentrations of the lysosomal protease cathepsin D as an indicator for lysosome leakage (Fig. 3A). In addition, we found that CLT1 causes LC3 conversion, indicating that sorting of CLT1 into lysosomes is mediated in the context of autophagy (Fig. 3B). Moreover, inhibiting fusion of autophagosomes and lysosomes with the autophagy inhibitor bafilomycin A1 significantly reduced CLT1 cytotoxicity in a panel of bladder and prostate tumor cells (Fig. 3C). Together, our results show that tumor cells internalize CLT1 and that this process leads to lysosome dysfunction and autophagic cell death.

CLT1-induced cell death correlates with fibronectin-mediated internalization of CLT1. An important function of fibronectin is to form complexes with CLT1 that promote CLT1 uptake into the angiogenic endothelium (17). Paralleling these results, we show that fibronectin, which co-localized with CLT1 inside the tumor cells, supports CLT1 uptake into bladder and prostate tumor cells while fibrinogen largely prevents CLT1 internalization (Fig. 4A-B). Moreover, CLT1 internalization was significantly reduced in the presence of a peptide GRGDSP that blocks the RGD recognition motif of fibronectin-binding integrins but not in the presence of an inactive control peptide (GRADSP) (Fig. 4C). Blocking integrin function with the RGD peptide or knocking down integrin α5, which binds to fibronectin in an RGD-dependent manner, also inhibited the
cytotoxic function of CLT1 while knockdown of another fibronectin-binding integrin, αvβ3, had no effect on CLT1-mediated tumor cell death (Fig. 4D-E). Flow cytometry showed that CLT1-sensitive tumor cell lines generally expressed higher levels of integrin α5β1 on their cell surface than tumor cell lines that were resistant towards CLT1 treatment (Fig. 4F, Supplementary Fig. S3A). Together, these results indicate that CLT1 internalization and cytotoxicity is largely determined by interactions of CLT1-fibronectin complexes with tumor cell integrin α5β1.

*CLT1 cytotoxicity is mediated by CLIC3.* We previously demonstrated that CLT1-fibronectin complexes are taken up into angiogenic endothelium through interaction with integrin αvβ3 and CLIC1 and that this interaction induces autophagic cell death (17). To determine if CLIC proteins cooperate with fibronectin-binding integrins in mediating CLT1 cytotoxicity, we transfected tumor cells with siRNA against various CLIC family members prior to treatment with CLT1. While knocking down CLIC1, 4, and 5 had no effect, we found that siRNA against CLIC3 significantly reduced CLT1-mediated cell death in a panel of bladder cancer cell lines as well as DU145 cells (Fig. 5A-B). Subcellular fractionation and flow cytometry revealed that CLIC3 is expressed in the tumor cell membrane and extends from there to the cell surface (Fig. 5C-D). Importantly, CLIC3 co-localized with CLT1 inside tumor cells suggesting a mechanistic link between CLIC3 and CLT1 internalization (Fig. 5E). A connection between CLIC3 and CLT1 became further apparent by analyzing total CLIC3 protein expression levels, which were overall increased in CLT1-responsive when compared to non-responsive
cell lines (Fig. 5F). Together, these results suggest that CLIC3 plays an important role for mediating the cytotoxic activity of CLT1.

**CLT1 binds to tumor tissue from bladder cancer patients.** To determine if CLT1 interacts with clinical bladder cancer, we incubated tumor tissue from seven bladder cancer patients with fluorescein-conjugated CLT1 over night and subsequently analyzed the samples by fluorescence and confocal microscopy. Corresponding H&E stained sections were analyzed by a clinical pathologist for morphologic confirmation of tumor (Supplementary Fig. S4). A summary of patient characteristics is provided in Supplementary Table 1. Fluorescence microscopy revealed that CLT1 was strongly taken up in freshly resected, unfixed tumor tissues from both non-muscle as well as muscle invasive bladder cancer (Fig. 6A-B). Notably, there was no detectable fluorescence in normal bladder tissue treated with CLT1 or in tumor tissue treated with an inactive CLT1 variant as control peptide indicating that the interaction of CLT1 with bladder cancer is highly specific. Additional analysis with confocal microscopy showed staining of single cells indicating that CLT1 is internalized in a manner similar to what we observed in cultured tumor cells (Fig. 6C). CLT1 binding was more efficient when tumor tissues were cultured in the presence of plasma fibronectin compared to fibrinogen (Fig. 7A). The role of fibronectin for CLT1 binding was further supported by our finding that CLT1 co-localized with integrin α5 and CLIC3 in bladder tumor tissue sections (Fig. 7B). Together, our results show that CLT1 binds to clinical bladder tumor tissue and that CLT1 binding is pronounced in areas that are rich in integrin α5β1 and CLIC3.
DISCUSSION

We previously showed that CLT1 forms aggregates with fibronectin that are cytotoxic for angiogenic endothelium (17). Here we demonstrate that CLT1 is strongly cytotoxic for tumor cells and attribute this activity to interactions between CLT1-fibronectin complexes and the fibronectin receptor integrin α5β1, which mediates CLT1 internalization and autophagic cell death in cooperation with CLIC3. Interestingly, CLT1 cytotoxicity is particularly pronounced in bladder tumor cell lines that express large amounts of α5β1 and CLIC3. Paralleling this result, we demonstrate that CLT1 binds strongly and specifically to clinical bladder cancer tissues that express integrin α5β1 and CLIC3.

CLT1 (CGLIIQKNEC) contains a unique hydrophobic peptide sequence, LIIQK that is critical for its anti-tumor effects. LIIQK is necessary for the formation of CLT1-fibronectin co-aggregates and as such, is important for CLT1 internalization, which causes endoplasmic reticulum stress and autophagic cell death (17). As part of the CLT1 complex, fibronectin has been shown to mediate interactions with integrin αvβ3 on angiogenic endothelium that lead to upregulation of the CLT1 receptor CLIC1 and subsequent internalization of CLT1-fibronectin complexes (17, 20). The mechanism of CLT1 cytotoxicity is similar in tumor cells, which internalize CLT1 in a fibronectin-dependent manner; however, instead of utilizing CLIC1 and integrin αvβ3, uptake of CLT1-fibronectin complexes into tumor cells is mediated through CLIC3 and the
fibronectin receptor integrin α5β1, both of which are upregulated on CLT1-responsive tumor cells. This internalization mechanism also explains the selectivity of CLT1 for tumor cells over resting epithelial cells, which lack integrin α5β1 and express significantly less CLIC3 than malignant cells (21-24). However, even in the presence of fibronectin, CLT1 exhibits only reduced levels of cytotoxicity in serum-starved bladder tumor cells suggesting that CLT1-fibronectin complexes are most effective in fast-growing, invasive urothelial cancers that lack cell cycle control as a result of mutations in p53, RB or certain receptor tyrosine kinase pathways (25). These mutations are typically associated with significant changes to the tumor microenvironment, which in return lead to the generation of a plethora of factors that promote tumor cell proliferation, survival and invasion (26). Moreover, pro-invasive and anti-apoptotic factors alter the interaction of integrins with the extracellular matrix by increasing turnover and recycling of activated integrins that are subsequently available for binding and internalization of CLT1-fibronectin complexes (27, 28).

The interaction between integrin α5β1 and fibronectin appears to be a major determinant of CLT1 cytotoxicity, because eliminating exogenous fibronectin in prostate tumor cells or replacing fibronectin with fibrinogen effectively antagonized CLT1 internalization and subsequent cell death. While there was minimal cell death in prostate tumor cells in the absence of plasma fibronectin, we achieved only partial inhibition of cytotoxicity in bladder tumor cells treated with CLT1 in plasma fibronectin-depleted media. Moreover, transfection with fibronectin siRNA caused additional reduction of CLT1-induced cell death suggesting that bladder tumor cells generate
endogenous fibronectin to support CLT1 internalization. This is in line with previous results showing that fibronectin is a significant component of urothelial tumor stroma (29, 30). Notably, for the purpose of CLT1 internalization, the origin of fibronectin appears to be irrelevant, as both plasma fibronectin and cellular fibronectin are able to interact with integrin α5β1 through their RGD-containing integrin recognition motif located within the fibronectin repeat III_{10} (31). More importantly, the interaction of CLT1-fibronectin complexes with α5β1 is preserved in the presence of urine, which is an important consideration for the intravesical treatment of bladder cancer in vivo.

In addition to fibronectin, we identified CLIC3 as an important factor for CLT1 cytotoxicity. However, while involved in CLT1 internalization and cytotoxicity, CLIC3 was unable to compensate for the loss of fibronectin, indicating that CLIC3 function depends on RGD-dependent ligation of α5β1 with the fibronectin component of the CLT1-fibronectin complexes. This is in agreement with a recent report showing that CLIC3 mediates internalization and recycling of integrin α5β1 from lysosomes after ligation with fibronectin (21). Endocytosis of fibronectin is important for the turn-over of focal contacts and recycling of unligated integrin α5β1 that mediates adhesive interactions with fibronectin during cell migration and invasion (21, 32). As a consequence, factors that promote recycling of activated α5β1 such as CLIC3 are upregulated in invasive tumor cell phenotypes (21). Based on this, we propose a model where amyloidogenic CLT1-fibronectin co-aggregates co-opt a CLIC3-dependent recycling mechanism for integrin α5β1 in invasive tumor cells, thereby inducing lysosome dysfunction and autophagic cell death. Considering the high degree of homology between CLIC3 and the CLT1 receptor
CLIC1, this mechanism could also include direct interactions of CLT1 with cell surface
CLIC3 (33).

The mechanism of CLT1 uptake shares similarities with the integrin α5-dependent
internalization of BCG-fibronectin complexes into bladder tumor cells suggesting that
CLT1 could be useful for detection and treatment of clinical bladder cancer (18). This
concept is further supported by our results showing that CLT1 is particularly effective in
inducing cell death in bladder tumor cell lines that exhibit high levels of integrin α5β1. In
addition, we demonstrate that CLT1 binds to human bladder tumor tissue ex vivo in the
presence of integrin α5β1, which has been shown to be upregulated in high grade
bladder cancer (23). Based on these data, we conclude that CLT1 function depends on
receptors that are commonly expressed in bladder cancer. Both BCG as well as CLT1
induce autophagy (17, 34); however, while internalization of BCG has no direct effects
on the tumor cell fate (35), we show here that CLT1 causes autophagic cell death in
bladder cancer cells. The anti-tumor mechanism of CLT1 is also different from
apoptosis-inducing chemotherapeutics such as mitomycin C, suggesting that CLT1
could be effective as a second line treatment or in combination with BCG, which has
been shown to render mitomycin C inactive (35). Overall, considering the robust anti-
tumor activity of CLT1 in combination with its specific labeling of bladder tumor tissue, it
will be interesting to determine if CLT1 can make a significant contribution to the
diagnosis and treatment of bladder cancer in vivo.

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REFERENCES

**FIGURE LEGENDS**

**Fig. 1** *CLT1 is cytotoxic for tumor cells.* (A), a panel of human bladder (J82, T24, UMUC3, TCCSUP), prostate (DU145), kidney (RCC4), breast (MDA-MB-231) and lung (A549) tumor cell lines and primary human bladder epithelial cells (HBEC) were treated for 24 hours with CLT1 (75 μg/ml) in the presence of 2% serum supplemented with 30 μg/ml fibronectin (2%+FN), fibrinogen (2%+FG) or media + 2% serum alone (2%). Cell death is shown as percent LDH release of cells treated with camptothecin/staurosporine. (B), LDH released by bladder tumor cells treated with CLT1 (75 μg/ml) in the presence of 2%+FN, serum-free media supplemented with FN (SF+FN) or serum-free media alone (SF). LDH release is normalized to absorbance values of vehicle control cells treated in absence of CLT1. LDH released in response to CLT1 in the presence of 2%+FN is set to 1. *, *P*<0.05; ***, *P*<0.001 versus 2%+FN. (C), LDH released by DU145 tumor cells cultured in FN-depleted serum and treated with CLT1 (75 μg/ml) and increasing concentrations of FN (3-100 μg/ml). (D), LDH released by DU145 cells treated with increasing concentrations of CLT1 (7.5-150 μg/ml) in the presence of 2%+FN. (E), LDH released by bladder tumor cells treated with CLT1 (75 μg/ml) in the presence of 2%+FN, fibronectin-depleted serum (FN minus) and FN depleted serum supplemented with FN (FN minus +FN). LDH released in response to CLT1 by cells grown in the presence of 2%+FN is set to 1. **, *P*<0.01; ***, *P*<0.001 versus 2%+FN or FN minus +FN. (F), LDH released by UMUC3 cells transfected with FN or control siRNA and then treated with CLT1 (75 μg/ml) in the presence of 2%+FN, FN minus or FN minus +FN media. LDH released in response to CLT1 by control siRNA
cells grown in the presence of 2%+FN is set to 1. **, \( P < 0.01 \); NS, non-significant versus control siRNA. (G), a lysine/alanine scan of CLT1. LDH release was assessed after treating DU145 for 24 hours with CLT1 and the variant CLT1 peptides (ea. 150 μg/ml). ***, \( P < 0.001 \) versus CLT1.

**Fig. 2** *CLT1 internalization correlates with cytotoxicity.* (A), uptake of fluorescein-CLT1 (25 μg/ml) was analyzed in the presence of 2% serum +FN after 24 hours in UMUC3, J82 and 786-0 cells by fluorescence microscopy. Data are shown as percent CLT1-positive cells per optical field (x40). ***\( P < 0.001 \) versus UMUC3 and J82 cells. (B), DU145 cells were treated with fluorescein-CLT1 alone or in the presence of nocodazole (10 μM) for 24 hours and analyzed for peptide internalization as percent CLT1-positive cells per optical field (x40). \( P < 0.001 \) versus DMSO. (C), LDH released by DU145 cells after 24 hours of CLT1 treatment (75 μg/ml) with or without nocodazole. ***, \( P < 0.001 \) versus DMSO. (D), UMUC3 (upper panel) and J82 cells (middle and lower panel) were treated with fluorescein-conjugated CLT1 (green) for 24 hours and lysotracker (Lyso, red; upper and middle panel) or DMSO (lower panel) for 1 hour prior to confocal microscopy. Merged images are shown in yellow. Reflection depicts cell morphology. Scale bar, 10 μm. (E), DU145 cells were treated with fluorescein-CLT1 (green) or vehicle (Con) for 24 hours, stained with lysotracker (Lyso; red) and analyzed by fluorescence microscopy. Nuclei were stained with DAPI (blue). Merged images are shown in yellow. Scale bar, 10 μm.
Fig. 3 **CLT1 internalization induces lysosome dysfunction and autophagic cell death.** (A), cytosolic fractions from DU145, UMUC3 and J82 tumor cells were treated with vehicle, CLT1, the inactive CLT1 variant IK1 (ea. 75 μg/ml), FN (30 μg/ml) or CLT1+FN for 24 hours and immunoblotted for cathepsin D (CTSD). (B), lysates isolated from UMUC3 and J82 cells cultured in 2% serum + FN were immunoblotted for the autophagy marker LC3 after 24 hours of treatment with CLT1 (25 μg/ml). (A-B), ponceau S (PS) staining shows equal protein loading. (C), LDH release in DU145, UMUC3 and J82 cells treated with 40-400nM bafilomycin A1 (BAF) and CLT1 (25 μg/ml) +FN for 24 hours. BAF was added 1 hour prior to CLT1. LDH released in response to CLT1 is set to 1. ***, P<0.001 versus CLT1.

Fig. 4 **CLT1-induced cell death correlates with fibronectin-mediated internalization of CLT1.** (A), internalization of fluorescein-CLT1 in the presence of FN or FG (ea. 30 μg/ml) was analyzed by fluorescence microscopy and is shown as percent CLT1-positive cells per optical field (x40). *** P<0.001 versus FN. (B), DU145 cells treated with fluorescein-CLT1 (green) and FN for 7 hours, were stained with anti-fibronectin antibody (FN, red) and analyzed by confocal microscopy. Nuclei were stained with Draq5 (blue). Scale bar, 10 μm. (C), DU145 cells were measured by fluorescence microscopy for fluorescein-CLT1 uptake in the presence of either an integrin-blocking RGD peptide or control RAD peptide. Data are shown as percent CLT1-positive cells per optical field (x40). *** P<0.001 versus RAD. (D), LDH release in DU145 cells after treatment with 75 μg/ml CLT1 in the presence of RGD and RAD peptides or following transfection with integrin α5, integrin β3 or control siRNA. ***, P<0.001 versus vehicle.
and control siRNA. (E), LDH release in CLT1-treated UMUC3, J82 and TCCSUP cells transfected with integrin α5 or control siRNA. LDH released in response to CLT1 by control siRNA cells is set to 1. **, P<0.01; ***, P<0.001 versus control siRNA. (F), cell surface expression of integrin α5β1 assessed by flow cytometry (Mean Fluorescence Intensity, MFI) was compared to CLT1 reactivity (++, cell death >75%; +, cell death 75-50%; +/-, cell death ca. 25%; -, cell death <25%).

**Fig. 5** *CLT1-induced cell death is mediated by CLIC3.* (A), LDH release in DU145 cells transfected with siRNA against CLIC1, CLIC3, CLIC4, and CLIC5 after treatment with 75 μg/ml CLT1. ***, P<0.001 versus control siRNA. (B), LDH release in CLT1-treated UMUC3, J82 and TCCSUP cells transfected with CLIC3 or control siRNA. LDH released in response to CLT1 by control siRNA cells is set to 1. *, P<0.05; ***, P<0.001 versus control siRNA. (C), membrane fractions isolated from DU145, J82, TCCSUP and UMUC3 cells were immunoblotted for CLIC3. DU145 compared to J82 and TCCSUP (left panel). DU145 compared to UMUC3 at 10x higher protein concentration (right panel). Ponceau S (PS) staining shows equal protein loading. (D), cell surface expression of CLIC3 on DU145 and UMUC3 cells was assessed by flow cytometry. Percent cells gated in M1 was determined after incubation with anti-CLIC3 or control antibody (IgG) based on fluorescence in FL1. **, P<0.01; ***, P<0.001 versus control IgG. (E), J82, TCCSUP and DU145 cells incubated with fluorescein-CLT1 (green) for 24 hours were stained with anti-CLIC3 antibody (red) or control IgG (lower panel) and analyzed by confocal microscopy. Merged images are shown in yellow. Reflection depicts cell morphology. Scale bar, 10 μm. (F), whole cell lysates from CLT1 reactive...
and non-reactive cells were probed for CLIC3 expression. CLT1 reactivity (++, cell death >75%; +, cell death 75-50%; +/-, cell death ca. 25%; -, cell death <25%). (C, F), PS staining shows protein loading.

**Fig. 6** CLT1 binds to clinical bladder cancer ex vivo. (A), fluorescence microscopy of tissues from muscle invasive (INV) compared to normal adjacent bladder tissue (NAT) after incubation with fluorescein-conjugated CLT1 (green) or LCLT1 as a control peptide (CP). Numbers correspond to patients in Supplementary Table 1. Nuclei (blue) were stained with DAPI. (B), fluorescence microscopy of non-muscle invasive bladder cancer (SUP) after incubation with fluorescein-conjugated CLT1 or LCLT1 (green). (A-B), Scale bar, 50 μm. (C), en-face confocal microscopy of freshly resected bladder tumor tissues compared to NAT after incubation with CLT1 (green) or IK1 as a control peptide (CP). Scale bar, 10 μm. (C) Nuclei (blue) were stained with Draq5 for confocal.

**Fig. 7** CLT1 co-localizes with integrin α5β1 and CLIC3 in clinical bladder cancer. (A), fluorescence microscopy analysis of bladder tumor sections after incubation with fluorescein-conjugated CLT1 (green) or LCLT1 as a control peptide (CP) in the presence of FN or FG (ea. 30 μg/ml). Scale bar, 50 μm. (B), tissue sections from invasive bladder cancer treated with fluorescein-CLT1 (green), were stained with anti-α5 integrin, anti-CLIC3 antibody or control IgG (red) and analyzed by confocal microscopy. Merged images are shown in yellow. Scale bar, 10 μm.
A

DU145

UMUC3

J82

CTSD

PS

Veh CLT1 Veh CLT1+FN

Full length

Cleaved

Cleaved

B

UMUC3

J82

LC3

PS

Veh CLT1+FN

C

CLT1 CLT1+BAF

DU145 UMUC3 J82

LDH (A490)

0 40 0 40 0 40 400

BAF (nM)

Knowles et al. Fig.3
Figure 4

A) Internalization of CLT1+ cells in different cell lines: UMUC3 and DU145

B) CLT1 immunofluorescence images withMerge overlay

C) Internalization of CLT1+ cells in different ligands: RAD and RGD

D) LDH (A490) levels with different treatments: Vehicle, RGD, RAD, α5, β3

E) LDH (A490) levels in different cell lines: UMUC3, J82, TCCSUP

F) α5β1 MFI (x 10^5) in different cell lines:

Knowles et al. Fig. 4
Figure 5: Immunohistochemical analysis of CLIC3 and CLT1 expression in various cell lines.

A) LDH (A490) measurement showing increased levels in CLIC3-overexpressing cells compared to controls.

B) LDH (A490) measurement for different cell lines and treatments.

C) Western blot analysis showing increased m-CLIC3 and PS levels in J82 and TCCSUP compared to DU145 and UMUC3.

D) Flow cytometry analysis showing increased percentage of cells in M1 in DU145 and UMUC3 in CLIC3-overexpressing conditions.

E) Immunofluorescence images of CLT1 and CLIC3 expression in J82, TCCSUP, DU145, and control IgG conditions.

F) Western blot analysis of CLIC3 and PS expression across different cell lines with varying CLT1 reactivity.

Knowles et al. Fig.5
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## CLT1 Targets Bladder Cancer through Integrin α5β1 and CLIC3

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