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Photoactivated inhibition of cathepsin K in a 3D tumor model

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Abstract

Collagenolytic activity of cathepsin K is important for many physiological and pathological processes including osteoclast-mediated bone degradation, macrophage function and fibroblast-mediated matrix remodeling. Here, we report application of a light-activated inhibitor for controlling activity of cathepsin K in a 3D functional imaging assay. Using prostate carcinoma cell line engineered to overexpress cathepsin K, we demonstrate the utility of the proteolytic assay in living tumor spheroids for the evaluation and quantification of the inhibitor effects on cathepsin K-mediated collagen I degradation. Importantly, we also show that utilizing the ruthenium-caged version of a potent nitrile cathepsin K inhibitor (**4**), *cis*-[Ru(bpy)₂(**4**)₂](BF₄)₂ (**5**), offers significant advantage in terms of effective concentration of the inhibitor and especially its light-activated control in the 3D assay. Our results suggest that light activation provides a suitable, attractive approach for spatial and temporal control of proteolytic activity, which remains a critical, unmet need in treatment of human diseases, especially cancer.

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Keywords

cathepsin K; 3D tumor model; photoactivation; prostate cancer; proteolysis; ruthenium-caged inhibitors

Introduction

Cysteine cathepsins are highly overexpressed in cancer and are linked to tumor growth, invasion, angiogenesis and metastasis (Mohamed and Sloane, 2006; Gocheva and Joyce, 2007). Of the various subtypes, cathepsin K (CK) plays a critical role in metastatic breast and prostate cancer, specifically in the bone tumor microenvironment, where it catalyzes collagen I degradation and contributes to bone resorption and remodeling (Podgorski, 2009; Podgorski et al., 2009; Herroon et al., 2013).

Due to the pivotal role that cysteine cathepsins play in cancer and many other human disease states, the development of small molecule inhibitors has been aggressively pursued by the pharmaceutical industry and academia (Otto and Schirmeister, 1997; Leung et al., 2000; Powers et al., 2002; Turk, 2006; Vasiljeva et al., 2007). Through significant efforts, selective inhibitors for most of the human cysteine cathepsins have been developed. All but a few of the small molecule inhibitors rely on electrophilic groups or ‘warheads’ that react covalently with the cysteine thiolate present in the active site of all cysteine cathepsin enzymes. Nitrile-based inhibitors, which react with the cysteine thiolate to form thioimides, have shown particular promise in animal models and clinical studies (Greenspan et al., 2001; Loeser et al., 2005; Altmann et al., 2006; Boxer et al., 2010; Fleming et al., 2010; Frizler et al., 2010).

Although potent and selective analogs have been known for decades (Powers et al., 2002), even showing efficacy in clinical trials, small molecule inhibitors of cysteine cathepsins have yet to reach the market (Turk, 2006; Duong et al., 2015). Clinical trials of odanacatib, a nitrile-based inhibitor of CK, were abandoned after Phase II studies for metastatic bone disease (Jensen et al., 2010; Sturge et al., 2011). Even though odanacatib showed promising results early on in the treatment of osteoporosis, side effects from Phase III clinical trials forced a delay in FDA filing. Balicatib, a specific and potent nitrile-based inhibitor of CK, showed promise early on (Desmarais et al., 2008, 2009). However Phase II trials were discontinued due to numerous off-target effects (Ruenger et al., 2012). Because odanacatib and balicatib show potent and selective inhibition of CK, side effects may actually be the result of off-site rather than off-target effects. In general, the site-selective inhibition or proteolysis remains a major hurdle to overcome. In addition to tumors, cysteine cathepsins, including CK, are required for normal cell functions throughout the body. Aside from its role in tumor-induced bone resorption, CK is known to be present in lung and dermal fibroblasts, where it contributes to remodeling of the extracellular matrix in the lung and skin (Ruenger et al., 2012). This potent protease is also present in multi-nucleated giant cells and epithelioid cells in granulomas involved in foreign body responses (Buhling et al., 2001). Because conventional small molecules are unable to differentiate between aberrant enzyme activity such as that found at a tumor site vs. proteolytic activity present in normal

tissue, the issue of site specificity may prove challenging to overcome without causing side effects.

One potential solution to the problem of site specificity is to use light in combination with caged compounds. Light gives researchers extraordinary capabilities to control where and when biologically active compounds are released from photolabile protecting groups (Young and Deiters, 2007; Lee et al., 2009; Brieke et al., 2012). Caged molecules are well developed and have advanced to the point where many analogs are commercially available (Klan et al., 2013). Metal-based caging groups, a special class of caged compounds that are labile with visible light, have been the focus of more recent attention (Farrer and Sadler, 2008; Farrer et al., 2009; Haas and Franz, 2009; Ciesinski and Franz, 2011; Sgambellone et al., 2013). Caged metal complexes have demonstrated particular efficacy in cell-based assays (Nikolenko et al., 2005; Salierno et al., 2008; Filevich and Etchenique, 2011; Araya et al., 2013; Zayat et al., 2013), often without toxicity, and have recently proven effective for *in vivo* applications (Yang et al., 2012), including xenografted tumor models (Westendorf et al., 2012).

As a collaborative effort, our laboratories developed a method for caging nitrile-based protease inhibitors with ruthenium complexes (Respondek et al., 2011, 2014; Sharma et al., 2014). Data against purified enzymes and lysates proved that enzyme inhibition is enhanced up to 89 fold using visible light irradiation. This approach was validated recently in cell-based assay using murine osteoclasts that express high levels of CK (Respondek et al., 2014). While this study demonstrated that photoactivated enzyme inhibition could be achieved using our approach, the evaluation process was not appropriate for screening large numbers of new photoactivated compounds for several reasons. First, osteoclasts are terminal in nature, and must be obtained through differentiation of bone marrow macrophages harvested from live animals, making supply of cells a major issue. Studies using other cell types were not an option, because CK is only expressed in abundance in a few cell lines (Buhling et al., 1999; Husmann et al., 2007; Duong et al., 2014). Measuring the specific activity of CK was another challenge, because fluorogenic substrates that are described as selective for one protease are also cleaved by other proteases, making specific measurement of CK activity difficult (Falgueyret et al., 2004). Although the issue of specificity had been addressed using radiolabelled irreversible inhibitors and radioimaging (Falgueyret et al., 2004, 2005; Desmarais et al., 2008, 2009), we sought to develop an imaging approach that did not carry the hazards of synthesizing and working with radioactive materials. As a final note, our previous study was carried out using cells cultured in two-dimensional (2D) monolayers (Respondek et al., 2014), which do not accurately mimic many of the characteristics found in the tumor microenvironment *in vivo*. Indeed, recent studies have proven that compound performance against cells cultured in three-dimensional (3D) systems provides a better prediction for *in vivo* efficacy (Elliott and Yuan, 2011; Lovitt et al., 2013). Unlike 2D systems, 3D cultures contain interactions with the extra-cellular matrix, cell polarity and cell-cell contacts present *in vivo* (Lovitt et al., 2015).

In this study, we report a new method that can be used to evaluate photoactivated inhibitors of CK in 3D functional imaging assays that is representative of the tumor microenvironment *in vivo*. A new cell line (PC3-CK) was constructed from prostate carcinoma cells stably

transfected with a CK vector, which has clinical relevance due to well-established role for CK in prostate cancer metastasis to bone. Inhibitors of CK were screened for their ability to block proteolysis of dyequenched collagen I (DQ-collagen) by these cells. We chose collagen I because it is a major component of bone matrix and a well-recognized substrate of CK (Garnero et al., 1998; Podgorski, 2009). Focusing on DQ-collagen cleavage avoided measurement of specific inhibition of CK, offering a more global readout for inhibition for proteolysis, rather than cleavage of fluorogenic substrates that are selective but not fully specific for CK. A CK inhibitor was identified that provided potent inhibition of DQ-collagen cleavage. A photocaged version of this inhibitor of CK was shown to significantly inhibit proteolysis in a light activated fashion with this new assay, demonstrating its promise to identify new leads in an environment representative of the tumor microenvironment *in vivo*.

Results

Generation of CK-overexpressing prostate carcinoma cells

The present study aimed to examine efficacy of inhibitors against human CK in a live cell-based system. For this purpose, we utilized PC3 cells, a prostate carcinoma line, that we have previously shown not to express CK (Podgorski et al., 2009), and thus represent an excellent system for introducing the enzyme exogenously for functional studies. We confirmed minimal presence of *CK* mRNA in PC3 wild type cells (CT values > 30; data not shown) and utilized TrueORF® Gold CK cDNA plasmids tagged with myc-DDK (OriGene) to establish stable CK-expressing clones (PC3-CK). The myc-DDK-tagged entry vector plasmids were used to establish the control cell lines (PC3-EV). A total of 18 CK-expressing clones were established with overexpression levels ranging from 6- to 80-fold over empty vector controls. Two clones (PC3-CK1 and PC3-CK2) expressing approximately 12-fold higher levels of *CK* mRNA than EV controls (Figure 1A) were chosen for the inhibitor studies. The presence of CK protein in selected clones was confirmed by Western blotting (Figure 1B) and by immunostaining for DDK tag (Figure 1C). Importantly, PC3-CK cells were shown to exhibit significantly higher levels of CK activity against Z-Gly-Pro-Arg-AMC compared to PC3-EV cells (Figure 1D), confirming the expression of the functional enzyme.

Potent CK inhibitors show efficacy against CK-mediated DQ collagen I degradation in 2D proteolysis assay

With a stably transfected cell line in hand, we examined the ability of potent CK inhibitors from the literature (compounds **1–4**, Figure 2) to inhibit CK activity in living PC3-CK cells using an established confocal assay employing quenched fluorescent derivative of collagen I (DQ-I) (Sameni et al., 2003; Podgorski et al., 2005; Jedeszko et al., 2008). Compound **1** is the potent CK inhibitor L-873724 [IC_{50} = 0.2 nM, Merck-Frosst, Canada; (Li et al., 2006; Podgorski et al., 2009)] that was a lead compound in the development of odanacatib (Gauthier et al., 2008). Compound **2** is balicatib, another potent inhibitor of CK (IC_{50} = 1.4 nM) that advanced to Phase II clinical trials (Palmer et al., 2005). Compound **3** is a 1,3-diaminoacetone potent CK that is commercially available (K_i = 22 nM) (Yamashita et al., 1997). Compound **4** is a potent nitrile based inhibitor with reported IC_{50} values of 9 nM

against purified CK (Altmann et al., 2006). This serine-derived compound has enhanced plasma stability making it an excellent candidate for *in vivo* applications. PC3-CK cells were cultured on top of collagen I matrix containing DQ-collagen I substrate in the absence or presence compounds **1–4**. Cells were cultured atop collagen I for 48 h and in agreement with our previous studies, grew as monolayers without forming 3D structures (Podgorski et al., 2005). The green fluorescence indicative of the presence of collagen I degradation products was captured at the extended depth of focus (Figure 3A). Because PC3 cells do not form spheroids when grown on collagen I, the mean fluorescence based on four arbitrary squares of defined area (22 500 Px2) per field was measured, quantified and represented as % fluorescence measured for DMSO control (Figure 3B). Compound **4** efficiently inhibited collagen I proteolysis by PC3-CK cells and the level of inhibition was comparable to that obtained for a potent CK inhibitor L-873724 (**1**, Figure 3B). Compounds **2** and **3** showed no inhibition of proteolysis within error of the vehicle DMSO. This was a promising indication that **4** does exhibit inhibitory effects on CK-driven collagen I degradation by living PC3-CK cells.

Effects of nitrile inhibitor on DQ collagen I degradation in 3D assay are due to inhibition of activity of CK

To confirm these results in a more physiologically relevant 3D live cell system, we modified well-established protocols for 3D spheroid formation (Shaw et al., 2004; Jedezsko et al., 2008). Specifically, cells were plated on rBM matrix mixed with quenched DQ-I substrate, and 2% rBM was added to the culture media. Using the mixture of rBM and DQ-I permitted for the 3D structure formation by the tumor cells, while allowing for the measurements of DQ-collagen I degradation. Using this system we were able to quantify the fluorescence from the DQ-I degradation in entire tumor spheroid and confirm that **4** effectively inhibits DQ-I proteolysis at the levels comparable to inhibitor **1** (Figure 4).

To test the idea that inhibitory effects of **4** on DQ-I proteolysis by living cells are indeed due to inhibition of CK activity, we compared DQ-I degradation between CTSK-overexpressing PC3-CK cells and control PC3-EV cells, which do not express the enzyme and should not respond to the inhibitor. As anticipated, in comparison to PC3-CK-induced proteolysis, low levels of DQ-I degradation were observed for PC3-EV cells cultured on DQ-I matrix under control conditions (DMSO, Figure 5). Treatment with **4** resulted in approximately 50% reduction in DQ-I fluorescence by PC3-CK cells, but had no effect on collagen I degradation by PC3-EV cells. This suggests that PC3-CK-driven collagen I degradation that is successfully reduced by **4** is due to activity of CK. The remaining 50% fluorescence persisting after CK inhibition in PC3-CK cells indicates that enzymes other than CK might be involved in generation of fluorescent degradation products by these cells. This is in agreement with our previous report demonstrating the involvement of another cysteine protease, cathepsin B as well as matrix metalloproteases (MMPs) in DQ-I degradation by PC3 cells (Podgorski et al., 2005). These enzymes are also likely responsible for modest levels of proteolysis by PC3-EV cells.

Light-enhanced inhibition of DQ-I degradation with ruthenium-caged version of nitrile inhibitor

Next, we performed the DQ-I proteolysis assay with **5**, a caged version of **4** that shows excellent potency in CK inhibition (IC_{50} of 25 nM), with a dark to light ratio of 88:1 against the purified enzyme (Respondek et al., 2014). Inhibition of CK-mediated degradation of collagen I was measured after 48 h under light and dark conditions, using a 40 min irradiation time and two treatments with vehicle or **5** (100–500 nM) at 0 and 24 h. More than 50% inhibition of DQ-I degradation by PC3-CK cells was observed upon the treatment with 100 nM caged complex **5** under light exposure (Figure 6A, top panels and C), which represents a 10-fold less concentration of inhibitor needed to reach the same levels of inhibition that were observed for **4**. Treatment with **5** under dark conditions had a minimal effect on DQ-I proteolysis (Figure 6A, bottom panels and C), with data not significantly different from treatment with the vehicle DMSO. To probe for effects of the ruthenium-based caging group, control experiments with the complex *cis*-[Ru(bpy)₂(MeCN)₂](PF₆)₂ were carried out under light and dark conditions. This complex, which releases MeCN rather than a protease inhibitor (Liu et al., 2009), showed minimal inhibition of DQ-I proteolysis (Figure 6B, D), indicating release of the protease inhibitor **4**, rather than the ruthenium-based caging group, was responsible for observed inhibition with **5**. Taken together, these studies prove that inhibition of DQ-I collagen degradation can be activated with light with **5**, and provide support for further screening of photocaged inhibitors by our method.

Because inhibition of DQ-I collagen proteolysis could be the result of toxicity, we evaluated **4** and **5** (light and dark) for effects on cell viability. PC3 cells were treated with **4** (1 nM–100 μ M) or **5** (1 nM–100 μ M, + or – irradiation, $\lambda_{irr} > 395$ nm) and viability was determined after 48 h by the MTT assay. Our results revealed no observable cytotoxic effects for free inhibitor **4** on PC3 cells within the range of tested concentrations (1 nM–100 μ M; Figure 7A). The caged complex **5** had no effects on PC3 cell viability at concentrations up to 10 μ M under light and dark conditions (Figure 7B). Visible toxicity occurred with **5** at the highest concentration tested (100 μ M). Importantly, this toxicity was observed at a concentration three orders of magnitude higher than the effective dose for **4** under light conditions (100 nM), supporting the idea that inhibition of DQ-I collagen degradation was due to release of **4** from **5**, rather than toxicity from the ruthenium complex.

Discussion

Cysteine proteases play a major role in human disease states, including cardiovascular, infectious and inflammatory diseases, cancer and neurological disorders (Berdowska, 2004; Jedszko and Sloane, 2004). Due to this fact, the development of cysteine protease inhibitors has been aggressively pursued (Otto and Schirmeister, 1997; Leung et al., 2000; Powers et al., 2002; Turk, 2006; Vasiljeva et al., 2007). Despite decades of research, and the availability of potent and selective analogs for most human cysteine cathepsins, small molecule inhibitors have not yet reached the market. One challenge that remains to be solved is how to target aberrant activities of cysteine cathepsins that are associated with human disease states, while leaving normal cell function undisturbed. Given the fact the same enzymes are involved in aberrant, disease-associated behavior and healthy ‘housekeeping’

activities in normal cells, site specificity may prove to be a challenging problem to solve with conventional, small molecule inhibitors.

An important step in the development of new protease inhibitors is evaluation in live cell-based systems, which helps to bridge the gap between purified enzymes and animal models. Recent studies have shown that assays with cancer cells cultured in 3D provide a better prediction of *in vivo* performance than assays with 2D monolayers (Jedeszko et al., 2008; Mullins et al., 2012; Lovitt et al., 2013, 2015). The 3D functional imaging assays recapitulate many aspects found *in vivo*, including cell-cell contacts, cell polarity and interactions with the extracellular matrix. We report herein a new method that can be used to evaluate inhibitors of CK under 3D conditions, which are much more representative of the tumor microenvironment than 2D monolayers. Generation of CK-overexpressing PC3-CK cell line allowed us to overcome the critical issues in supply of cells that abundantly express CK, whose specific activity has been hard to measure in the presence of more abundant proteases like cathepsins B and L. Importantly, this robust living cell-based system represents a model with clinical relevance. CK is a critical player in tumor-induced bone disease associated with metastatic prostate and breast cancers where it is predominantly expressed by osteoclasts and macrophages in the bone tumor microenvironment (Littlewood-Evans et al., 1997; Brubaker et al., 2003; Le Gall et al., 2007). Of the inhibitors screened in this study, compounds L-873724 (**1**) and **4** showed the most potent inhibition of DQ-collagen I degradation, agreeing with previous investigations using radiolabeled inhibitors (Falgueyret et al., 2004, 2005; Desmarais et al., 2008) and supporting the development of caged versions of these inhibitors to gain light control. Failure of compounds **2** and **3** to reduce proteolysis of DQ collagen I is consistent with data in the literature, which showed that potent inhibition of purified CK is not always correlated with efficacy in cell-based models (Desmarais et al., 2009).

Light activation is an attractive method for achieving spatial and temporal control over biological activity. This report describes application of a light-activated inhibitor for controlling CK activity in a 3D functional imaging assay. The ruthenium-caged version of protease inhibitor **4**, *cis*-[Ru(bpy)₂(**4**)₂](BF₄)₂ (**5**), not only provided an advantage in terms of its effective concentration in the assay, but also provided significant levels of light-activated control. Under light conditions, complex **5** was active at a concentration roughly 10 fold lower than **4**, which may reflect the release of greater than 1 equivalent of **4** from **5**, and enhanced solubility of **5** relative to neutral **4** in aqueous solution. Complex **5** also proved to be more potent in the light than under dark conditions. While there was some level of inhibition by **5** in the dark, which was not statistically significant, DQ collagen-I degradation was reduced by over 50% under light conditions. Regarding inhibition in the dark, recent studies with **5** and other nitrile-based inhibitors caged with ruthenium complexes have shown that the caged complexes are less stable in cell growth media than in simple aqueous buffers or DMSO, where half-lives of > 1800 days have been observed, suggesting that some level of inhibitor release may occur under prolonged exposure to cell culture conditions (Respondek et al., 2014; Ramalho et al., 2015). The method used herein represented a significant challenge for our caged complexes, which need to be stable over the course of the assay (48 h) to provide high levels of light control. Future development of caged inhibitors

will focus on enhancing stability under prolonged assay conditions, in addition to tuning the wavelength for inhibitor release towards longer wavelengths for *in vivo* applications. Nonetheless, studies reported in this manuscript provide a straightforward approach for screening new, photocaged inhibitors of CK that could one day be used to gain spatial control over inhibition of this important enzyme *in vivo*.

In conclusion, this work demonstrates successful utilization of a 3D proteolysis assay employing CK-overexpressing cells to evaluate efficacy of light-activated CK inhibitors. Our method provides a useful, quantitative approach to measure CK-mediated collagen I degradation by living cells. Importantly, our results reveal that light activation can be efficiently utilized to achieve spatial control over proteolytic activity. This provides a strong basis for extended use of our approach towards the inhibition of other enzyme targets in living cells and for future tuning of the ruthenium-based caging group and shifting the wavelength of inhibitor release to ultimately target proteolytic activity using *in vivo* animal models.

Materials and methods

Materials

Dulbecco's modified Eagle's medium, sodium bicarbonate, antibiotics, dimethyl sulfoxide, and other chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO, USA). Fetal bovine serum and trypsin-EDTA were obtained from Fisher Scientific (Waltham, MA, USA). The fluorogenic substrate Z-Gly-Pro-Arg-AMC was purchased from Bachem (King of Prussia, PA, USA). CA074 was purchased from Peptides International (Louisville, KY, USA) and CK inhibitor L-873724 (Compound **1**) was obtained from (Merck-Frosst, Canada) (Gauthier et al., 2008) Compounds **2** (Palmer et al., 2005), **3** (Yamashita et al., 1997), **4** (Altmann et al., 2006) and **5** (Respondek et al., 2014) were synthesized using literature protocols. DQ- collagen I substrate was from Life Technologies (Carlsbad, CA, USA) and CultrexTM (reconstituted basement membrane; rBM) was from Trevigen (Gaithersburg, MD, USA). Rabbit anti-human CK antibodies were purchased from Abcam (Cambridge, MA, USA) and anti-DDK antibodies were from OriGene (Rockville, MD, USA). Horseradish peroxidase-labeled goat anti-rabbit IgG was from Pierce (Rockford, IL, USA).

Cell lines

PC3 cell line derived from a bone metastasis of a high-grade adenocarcinoma (Kaighn et al., 1979) was purchased from American Type Culture Collection (Manassas, VA, USA). Parental PC3 cells stably transfected with empty vector (pCMV6-Entry; EV) or CK (Myc-DDKtagged Human CK in pCMV6-Entry vector; CK) plasmids (OriGene, Rockville, MD, USA). Eighteen stable CK clones and six EV clones were established. CK overexpression was confirmed by RTPCR in all 18 clones and a pool of two CK clones (CK1 and CK2) was used for subsequent experiments.

Taqman RT-PCR analyses

The cDNA from cells was prepared from 2 µg of total RNA using High-Capacity cDNA Reverse Transcription kit (Life Technologies). The analyses of CK expression levels was performed using TaqMan® Individual Gene Expression assays for human CK (Hs00166156). Assays were done on three biological replicates using TaqMan® Fast Universal PCR Master Mix and 50 ng of cDNA/well and all reactions were run on an Applied Biosystems StepOnePlus™ system. Data were normalized to hypoxanthine phosphoribosyltransferase (HPRT1; Hs 99999909). DataAssist™ Software (Applied Biosystems) was used for all analyses.

Immunoblot analyses

For assessment of CK expression, cell lysate samples were loaded on 12% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted for CK (Abcam, 1:500) and DDK (OriGene, 1:1000). Tubulin (Hybridoma Bank, 1:1000) was used as a loading control. The horseradish peroxidase-labeled secondary antibodies (Pierce) were used at 1:10 000.

Immunofluorescence analyses

The efficiency of transfection was examined by immunofluorescence analysis of DDK in CK-transfected PC3 cells, using mouse anti-human DDK (OriGene; 1:100), and Alexa 488 secondary antibodies. Fluorescent images were captured with a Zeiss LSM510 META NLO confocal microscope using 40 × oil immersion lens. Controls were run in the absence of primary antibodies.

CK activity

Cell lysates collected in 250 mM sucrose, 25 mM MES, 1 mM EDTA, pH 6.5, and 0.1% Triton X-100 (SME) were lysed by sonication and diluted 8 times with assay buffer solution containing 400 mM sodium acetate, pH 5.5, 4 mM EDTA, and 8 mM DTT. Enzymatic activity of CK was measured against the fluorescent substrate Z-Glycine-prolinearginine-7-amido-4-methylcoumarin-HCl (Z-Gly-Pro-Arg-AMC) from Bachem Chemical (100 µM; Torrance, CA, USA). The reaction was performed in the presence of the selective inhibitor to cathepsin B, CA074 (2 µM) to eliminate the activity due to cathepsin B-mediated cleavage of Z-Gly-Pro-Arg-AMC (Podgorski et al., 2009). The progress of the reaction was monitored every minute for a period of 30 min on a Tecan Infinite 200 PRO Microplate Reader. Results of activity assays are expressed as maximum fluorescence units formed per minute. Equal amounts of cell lysate were used for each assay.

DQ-collagen I live cell proteolysis assay

Cleavage of DQ-collagen I substrate (Life Technologies, Carlsbad, CA, USA) by live PC3-EV and PC3-CK cells was assayed in real time and quantified based on published protocols (Sameni et al., 2003; Podgorski et al., 2005; Jedeszko et al., 2008) with some modifications. For 2D images PC3 cells were plated on top of collagen I matrix containing DQ-collagen I (1:40) and imaged after 48 h. Fluorescence images of DQ-collagen I degradation products (green fluorescence) were taken at an extended depth of focus (Zeiss LSM-510) and fluorescence intensities were measured using ImageJ Software (National Institutes of

Health, Bethesda, MD, USA). For 3D images, single cell suspensions of tumor cells were plated on top of coverslips coated with Cultrex™ (Trevigen) containing DQ-collagen I (1:40) and overlaid with 2% Cultrex™. Cells were treated with inhibitors or vehicle upon seeding and again at 24 h. For experiments with caged inhibitor, cells were exposed to inhibitor or vehicle, incubated under dark (no irradiation) and light (250 W, 395–750 nm) for 40 min as described previously (Respondek et al. 2014), incubated an additional 60 min at 37°C, then washed 3 times and overlaid with 2% Cultrex™. The inhibitor treatment, irradiation protocol and washes were repeated after 24 h. At 48 h, proteolysis of DQ-collagen I was imaged by capturing z-stacks through the depth of structures using a Zeiss LSM 510 confocal microscope with a 40× water immersion objective. Intensity of green fluorescence/tumor spheroid was quantified in each 3D reconstructed spheroid using Volocity Software (Perkin Elmer, Waltham, MA, USA).

Viability assays

The cell viability of PC3 cells in the presence of uncaged and caged compounds was measured using the MTT assay according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). Briefly, the cells were grown in a 96-well plate for 24 h. Cells were then treated with inhibitor or vehicle, and the 'dark' plate was wrapped in aluminum foil while the 'light' plate was exposed to light. The photolysis was conducted for 40 min (250 W, 395–750 nm) as described above and cells were placed in 37°C incubator under 5% CO₂ atmosphere. After 48 h, the MTT assay was performed as described previously (Respondek et al., 2014) and absorbance at 540 nm was measured using Tecan Infinite 200 Pro Microplate Reader. Cell viabilities were reported relative to the vehicle control.

Statistical analyses

All data analyses were performed using GraphPad Software version 6.05. Data are presented as mean $\pm$ SD and statistically analyzed using Student's *t*-test. For three or more groups, one-way analysis of variance was used.

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Figure 1. Generation of CK (CTSK) overexpressing human prostate tumor cells

(A) Taqman RT-PCR analysis of human *CK* gene expression in CK-1 and CK-2 clones. Data are normalized to HPRT1 and shown as average fold increase relative to EV (for 3 replicate experiments). (B) Western blot analysis of CK protein (37 kDa proenzyme and 28 kDa mature enzyme; top panel) and DDK (middle panel) expression in representative samples from PC3 prostate carcinoma cells stably transfected with empty vector (PC3-EV) or CTSK plasmids with DDK tag (PC3-CTSK); tubulin was used as loading control. (C) Immunofluorescent staining for DDK (green, left panel) indicative of CTSK expression; no primary antibody staining is shown as negative control (right panel); DAPI (blue) indicates nuclei; 40 × original magnification. (D) CK activity in CK clones. Assay was run against CK substrate Z-Gly-Pro-Arg-AMC the presence of cathepsin B inhibitor CA074. Data are shown as fold increase relative to CTSK activity in PC3-EV cells and a representative of three independent experiments. ** $p < 0.001$; *** $p < 0.0001$ (values considered statistically significant).

Figure 2.
Structures of CK inhibitors **1–4** and ruthenium-caged inhibitor $[\text{Ru}(\text{bpy})_2(\text{4})_2](\text{BF}_4)_2$ (**5**).

Figure 3. Degradation of DQ-collagen I by living human PC3-CK cells is effectively reduced in the presence of compounds 1 and 4 in 2D proteolysis assay

(A) Single cell suspensions of tumor cells were mixed with diluent (DMSO, 0.1%;), 1 μ M L-873724 (Merck; **1**), 1 μ M balicatib (**2**), 1 μ M 1,3-diaminoacetone inhibitor (**3**), or 1 μ M nitrile-based inhibitor (**4**). Cells were plated on top of DQ-collagen I-coated dishes and imaged at 48 h. Fluorescence images of DQ-collagen I degradation products (green fluorescence) were taken at an extended depth of focus (Zeiss LSM-510) and merged with Hoechst (nuclear stain, blue) and DIC images. (B) Fluorescence intensities in the absence and presence of **1–4** were quantified from confocal fluorescent images using ImageJ Software (National Institutes of Health, Bethesda, MD, USA). Mean fluorescence based on four arbitrary squares of defined area (262 144 Px²) per field was calculated and shown as % fluorescence for cells grown under control conditions (0.1% DMSO). * $p < 0.05$ is considered statistically significant.

Figure 4. Compound 4 shows significant efficacy in 3D DQ-I proteolysis assay

(A) Single cell suspensions of tumor cells were plated on top of coverslips coated with CultrexTM containing DQ-collagen I and overlaid with 2% CultrexTM. Cells were treated with inhibitors (compounds **1** and **4**; 1 μ M) or vehicle (1% DMSO) upon seeding and at 24 h. Proteolysis of DQ-collagen I (green fluorescence) was observed by capturing z-stacks through the depth of structures using a Zeiss LSM 510 confocal microscope with a 40 $\times$ water immersion objective; optical slice images through the middle of the spheroid are shown for Control cells (left panels), **1**-treated cells (middle panels) and **4**-treated cells (right panels). (B) Intensity of green fluorescence/tumor spheroid was quantified in each 3D reconstructed spheroid using Volocity Software. Fluorescent intensity per cell was quantified in the entire volume; nuclei were stained with Hoechst 33342 (blue) at the time of imaging and counted. Data are shown as average ($\pm$ SD) of three independent experiments with at least three independent spheroids measured/experiment; * $p < 0.05$.

Figure 5. DQ-I proteolysis by living PC3-CK cells is largely due to CTSK activity

3D proteolysis assay for PC3-CK (A) and PC3-EV (B) cells. Cells were plated on top of CultrexTM/DQ-1-coated coverslips with 2% CultrexTM overlay and treated with vehicle (1% DMSO) or **4** at 0 and 24 h. Proteolysis of DQ-collagen I (green fluorescence) was captured as described in Figure 3. Tumor spheroid structures were reconstructed in 3D using ImageJ software. (C) Intensity of green fluorescence/tumor spheroid was quantified in each 3D reconstructed spheroid using Volocity Software (PerkinElmer, Waltham, MA, USA). Fluorescent intensity per cell (C) was quantified in the entire volume; nuclei were stained with Hoechst 33342 (blue) at the time of imaging and counted. Data are shown as average (+/- SD) of 3 independent experiments with at least three independent spheroids measured/experiment; **p* < 0.05. ***p* < 0.01. Significant reduction of DQ-I fluorescence is observed in PC3-CK cultures treated with **4**. Baseline DQ-I fluorescence levels in PC3-EV cultures are comparable to DQ-I fluorescence in **4**-treated PC3-CK cells.

Figure 6. Light activation of caged complex 5 inhibitor has significant effects on DQ-I degradation by the living PC3-CK cells

3D cultures of PC3-CK cells were established as described in the legend to Figure 3. (A) Cultures were treated with caged complex **5** (100 nM and 500 nM) or vehicle (1% DMSO) and irradiated (250 W, 395–750 nm) for 40 min (Light) or kept in the dark (Dark). Following irradiation, cells were incubated for 60 min in the dark at 37°C, washed three times, and were overlaid with 2% Cultrex™, then allowed to grow for 48 h. The inhibitor/vehicle treatment with irradiation was repeated at 24 h. (B) Same culture and treatment conditions were applied to experiments with *cis*-[Ru(bpy)₂(MeCN)₂](PF₆)₂. Proteolysis of DQ-collagen I (green fluorescence) was captured as described in Figure 3. Tumor spheroid structures were reconstructed in 3D using ImageJ software. (C, D) Intensity of green fluorescence/tumor spheroid was quantified in each 3D reconstructed spheroid using Volocity Software. Fluorescent intensity per cell in the absence or presence of caged complex **5** (C) or *cis*-[Ru(bpy)₂(MeCN)₂](PF₆)₂ (D) under the light or dark conditions was quantified in the entire volume; nuclei were stained with Hoechst 33342 (blue) at the time of imaging and counted. Data are shown as average (+/- SD) of three independent experiments with at least 3 independent spheroids measured/experiment; **p* < 0.05. ***p* < 0.01 (values considered statistically significant).

Figure 7. Evaluation of cytotoxicity of complex 4 and caged complex 5 on PC3 cells

Using PC3-CTSK cells, cytotoxicity of complex 4 (A) and caged complex 5 (B) were evaluated. 24 h after plating, cells were treated with the representative complex, and either incubated in normal tissue culture conditions (A) or kept in the dark or irradiated with tungsten halogen lamp (250 W, $\lambda_{\text{irr}} > 395$ nm, H₂O filter, 40 min); 10 μ M Thapsigargin (TPG) was used as a positive control (B). Cell viability was determined by MTT assay after 48 h, and is reported relative to DMSO control. Error bars represent the standard deviations of triplicate wells ($\pm$ SD), and data are representative of three independent experiments.