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Cathepsin L targeting in cancer treatment

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Abstract

Proteolytic enzymes may serve as promising targets for novel therapeutic treatment strategies seeking to impede cancer progression and metastasis. One such enzyme is cathepsin L (CTSL), a lysosomal cysteine protease. CTSL upregulation, a common occurrence in a variety of human cancers, has been widely correlated with metastatic aggressiveness and poor patient prognosis. In addition, CTSL has been implicated to contribute to cancer associated osteolysis; a debilitating morbidity affecting both life expectancy and the quality of life. In this review we highlight the mechanisms by which CTSL contributes to tumor progression and dissemination and discuss the therapeutic utility of CTSL intervention strategies aimed at impeding metastatic progression and bone resorption.

Keywords

Cathepsin L; metastasis; bone resorption; cancer; protease targeting

1.0. Introduction: Proteases as targets in cancer treatment

Proteolytic enzymes are critical in several stages of malignant tumor progression (Lankelma, et al., 2010; Palermo & Joyce, 2008). Extracellular proteases can promote tumor growth and angiogenesis through proteolytic activation of latent growth factors and pro-angiogenic factors (Bogenrieder & Herlyn, 2002; DeClerck, et al., 2004; S. D. Mason & Joyce, 2011). In the metastatic cascade, proteolytic enzymes are actively involved in several key steps including, tumor cell detachment, degradation of extracellular and interstitial matrices and basement membranes, intravasation and extravasation across the capillary/lymphatic system (Gupta & Massague, 2006; Joyce & Pollard, 2009; Tan, Peng, Lu, & Tang, 2013). Consequently the identification of malignancy promoting proteases and the development of intervention strategies designed to interfere with their activities has gained increasing attention (Lah, Duran Alonso, & Van Noorden, 2006; Palermo & Joyce, 2008). Recognition

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of the importance of matrix metalloproteases (MMPs) in tumor progression and metastasis subsequently led to the development of a broad range of MMP inhibitors (B. Turk, 2006). However, numerous MMP inhibitor clinical trials had to be discontinued due to adverse side effects and/or lack of clinical benefit (Fingleton, 2007; Overall & Lopez-Otin, 2002; Zucker, Cao, & Chen, 2000). Failure of these early generation MMP inhibitors in patients can be ascribed to a variety of factors but the most critical reason was the lack of specificity of these agents (Kruger, Kates, & Edwards, 2010; Palermo & Joyce, 2008). Most MMP inhibitors tested in the clinic exhibited a broad spectrum activity against many members of the large MMP family which also includes anti-tumoral proteases and proteases that are critical to normal tissue functioning. In light of the clinical failure of these MMP inhibitors, protease targeting as a means of inhibiting tumor progression largely fell out of favor. More recently the concept of proteolytic enzyme targeting is being revived because research exploring other proteolytic targets has shown that if selective inhibitors of pericellular proteolysis can be developed, such agents would have the potential to significantly impair tumor progression and metastasis (Chavarria, et al., 2012; Fisher & Mobashery, 2006; Tomoo, 2010; Zucker & Cao, 2009). For example, several cysteine cathepsins of the papain superfamily of cysteine proteases have been widely implicated as facilitators of neoplastic progression (Gocheva & Joyce, 2007; Joyce, et al., 2004; Mohamed & Sloane, 2006; Palermo & Joyce, 2008). The focus of this review is on one of these, Cathepsin L (CTSL), a family member widely associated with malignant progression and metastatic disease.

2.0 Cathepsin L upregulation in human cancers

CTSL is a ubiquitously expressed lysosomal endopeptidase that is primarily involved in terminal degradation of intracellular and endocytosed proteins (Barrett & Kirschke, 1981; Dennemarker, et al., 2010). However, Gottesman and colleagues observed a strong association between CTSL synthesis and malignancy of Kirsten virus transformed NIH 3T3 cells (Gottesman & Sobel, 1980). Subsequent investigations performed in several different transformation models (different viral oncogenes and chemical carcinogens) reported consistent CTSL over-expression irrespective of the mode of transformation (Doherty, et al., 1985; Gottesman & Sobel, 1980; Lah, et al., 1996; Rabin, Doherty, & Gottesman, 1986). In addition, tumor secreted cytokines that have been widely implicated in malignant progression including VEGF, FGF, PDGF, EGF, NGF, $\text{INF}\gamma$ and IL-6 have also been shown to significantly enhance CTSL promoter activity and synthesis (Asanuma, Shirato, Ishidoh, Kominami, & Tomino, 2002; Frick, Doherty, Gottesman, & Scher, 1985; Gallardo, de Andres, & Illa, 2001; Gerber, Wille, Welte, Ansorge, & Buhling, 2001; Keerthivasan, Keerthivasan, Mittal, & Chauhan, 2007; Lah, Hawley, Rock, & Goldberg, 1995; Nilsen-Hamilton, Hamilton, Allen, & Massoglia, 1981; Scher, Dick, Whipple, & Locatell, 1983). Consistent with these in-vitro observations, CTSL upregulation has been reported in a wide range of human malignancies including ovarian, breast, prostate, lung, gastric, pancreatic and colon cancers (Chauhan, Goldstein, & Gottesman, 1991) (Table 1). Importantly, evidence indicates that CTSL expression may be linked to cancer grade and stage. For example, in endometrial cancer patients, CTSL levels increased 50-fold and positively correlated with tumor grade, growth regulatory genes such as Ki-67, cyclin B1 and p21, and HER2 receptor status (Skrzypczak, et al., 2012). Furthermore, Chauhan and colleagues

observed significantly elevated CTSL levels in pancreatic adenocarcinomas compared to benign endocrine tumors and islet cells obtained from adjacent normal tissue thereby suggesting a strong association between CTSL expression levels and pancreatic cancer aggressiveness (Chauhan, et al., 1991). In colorectal cancer, numerous clinical studies have reported upregulated CTSL levels and activities (Adenis, et al., 1995; Herszenyi, et al., 1999; Sheahan, Shuja, & Murnane, 1989). The concomitant upregulation of its downstream target urokinase plasminogen activator-1 in colorectal cancer tissues and the strong correlation with metastatic incidence and overall survival further substantiates the argument that CTSL upregulation is a key factor driving neoplastic progression (Herszenyi, et al., 1999).

Chronic myeloid leukemia (CML) patients from both chronic and blast crisis/accelerated disease phases exhibit significantly higher CTSL mRNA and enzymatic activity compared to healthy individuals (Samaiya, Bakhshi, Shukla, Kumar, & Chauhan, 2011). CTSL promoter sequence analysis in CML patients revealed that CTSL expression levels are determined by the methylation status of a CpG island flanking the major transcription initiation site (Jean, Guillaume, & Frade, 2002; Jean, Rousselet, & Frade, 2006). Bisulfite sequencing of the CTSL promoter revealed that promoter flanking CpG dinucleotides were significantly hypomethylated in CML patients compared to healthy individuals thus resulting in CTSL overexpression. Independent investigations performed in melanoma and glioblastoma multiforme further demonstrated that in addition to promoter hypomethylation, CTSL upregulation could also be achieved by other distinct mechanisms such as gene amplification and increased promoter activity (Jean, et al., 2006; Mao & Hamoudi, 2000).

Activity assessments have shown significantly higher CTSL proteolytic activity in neoplastic tissues compared to benign tumors or matched tumor adjacent normal tissues (Frohlich, et al., 2001; Kageshita, et al., 1995). In addition to its expression status, CTSL function is tightly regulated by the concentration of its endogenous inhibitors (V. Turk, et al., 1986). In normal tissues, the extracellular matrix is protected from undesirable proteolytic activities by endogenous cathepsin inhibitors such as cystatins and stefins. Studies in various tumor settings have shown a steep downregulation of these CTSL inhibitors with tumor progression (Chambers, Colella, Denhardt, & Wilson, 1992; Friedrich, et al., 1999; Lah, et al., 1997; Samaiya, et al., 2011; Zajc, Sever, Bervar, & Lah, 2002). Lah and colleagues noted elevated CTSL activity in breast cancer patients due to an imbalance between CTSL to cystatin and stefin ratio; with CTSL activity levels strongly correlating with tumor grade (Lah, et al., 1997). Similarly, significant downregulation of cystatin C levels has been observed in chronic myeloid leukemia patients leading to heightened CTSL proteolytic function (Samaiya, et al., 2011). Consistent with these clinical observations, independent experimental investigations have reported striking imbalance between CTSL and endogenous inhibitor level in highly invasive breast and prostate cancer cells compared to poorly invasive and weakly tumorigenic cells (Zajc, Sever, Bervar, & Lah, 2002; Sudhan & Siemann, 2013). Importantly, Chambers and colleagues demonstrated that this inequity in CTSL and endogenous inhibitor levels in transformed cells is mediated through oncogenic Ras activity (Chambers, Colella, Denhardt, & Wilson, 1992). Thus CTSL upregulation is not paralleled by a commensurate increase in the activity of its endogenous inhibitors and as a

result it evades regulation and could engage in unregulated activation of proteolytic cascades.

2.1 CTSL secretion by tumor cells

Under normal physiological conditions, CTSL is sequestered within the lysosomes. However, in tumor cells, alterations in expression levels and trafficking pathways result in secretion of CTSL into the extracellular milieu (Denhardt, Greenberg, Egan, Hamilton, & Wright, 1987; Doherty, et al., 1985; Gal, Willingham, & Gottesman, 1985; Gottesman & Sobel, 1980; Rabin, et al., 1986). Comparative analysis of the secretome of transformed cells with that of non-transformed cells revealed that the secretion of a glycoprotein was strikingly enhanced (~200-fold) in the conditioned media of transformed cells (Gottesman, 1978). This upregulation occurred consistently irrespective of the means of transformation (oncogenic v-ras, v-src or v-mos or chemical tumor promoters) and this mannose-6-phosphate containing glycoprotein was termed as the major excreted protein (MEP) of transformed cells (Doherty, et al., 1985; Gottesman & Sobel, 1980; Rabin, et al., 1986). MEP was subsequently identified as cathepsin L by several independent investigations based on cDNA characterization (Denhardt, et al., 1986; Troen, Gal, & Gottesman, 1987), amino acid sequence homology (Gal & Gottesman, 1988; Troen, et al., 1987), immunolocalization and electron microscopy (Gal, et al., 1985), activity profile (Gal & Gottesman, 1986; R. W. Mason, Gal, & Gottesman, 1987), substrate specificity and cleavage pattern (Gal & Gottesman, 1986).

In agreement with these in-vitro observations, elevated serum CTSL levels have been reported in patients with lung, pancreatic and ovarian cancer when compared to healthy donors (Chen, et al., 2011; Leto, et al., 1997; Nishida, et al., 1995; Siewinski, et al., 2004; Tumminello, et al., 1996; W. Zhang, et al., 2011). Unlike the clinically used CA-125 ovarian cancer biomarker which may also be upregulated in patients with benign tumors yielding false positive results; the selective upregulation of CTSL in aggressive ovarian cancers compared to benign tumors implies significant utility for CTSL as a diagnostic marker (Nishida, et al., 1995). Moreover, its close correlation with tumor grade and tumor invasion suggests that pre-operative serum CTSL levels could serve as indicators of the extent of tumor invasion at the time of surgical resection (W. Zhang, et al., 2011). Similarly, urinary CTSL levels have been shown to be predictive of metastatic spread and tumor recurrence in patients with bladder urothelial cell carcinoma (Svatek, et al., 2008).

Several studies have been undertaken to determine the mechanisms underlying the altered trafficking of this lysosomal enzyme into the extracellular space. During the process of protein translocation, mannose-6-phosphate receptors present within transport vesicles are responsible for accurate and efficient sorting of lysosomal enzymes to the lysosomes. However, (Dong, Prence, & Sahagian, 1989) demonstrated that compared to other lysosomal enzymes, CTSL intrinsically exhibits a low affinity for the mannose-6-phosphate receptor. Thus transformation dependent upregulation in CTSL synthesis combined with poor affinity for the lysosome targeting receptor ultimately results in default secretion of CTSL through the constitutive secretory pathway. Furthermore, growth stimulation limits mannose-6-phosphate receptor concentrations within the golgi through receptor re-distribution which

causes the few receptors that are available to be occupied by high affinity lysosomal enzymes whereas, the low affinity CTSL, failing to bind to these receptors is shunted into the secretory pathway (Prencipe, Dong, & Sahagian, 1990; Stearns, Dong, Pan, Brenner, & Sahagian, 1990). Subsequently, Barbarin and colleagues demonstrated that Rab4a protein is the key regulator of CTSL secretion in melanoma cells. Rab proteins are important regulators of vesicle transport between organelles of the endocytic and secretory system (Barbarin & Frade, 2011). Transfection of highly tumorigenic and metastatic melanoma cells with dominant negative Rab4a mutant, significantly hampered CTSL secretion from these cells and also dramatically impaired their tumorigenic and metastatic potential (Barbarin & Frade, 2011). Collectively these data suggest that upregulated CTSL secretion by tumor cells is achieved through several different mechanisms.

2.2 Nuclear CTSL

In addition to the secreted form, the presence of a truncated nuclear isoform of CTSL has been reported by numerous independent investigations. Nuclear CTSL is translated from a downstream AUG translation initiation site and is consequently devoid of the endoplasmic reticulum targeting signal peptide (Goulet, et al., 2004; Goulet, et al., 2007). However, it does contain a putative nuclear localization signal which might be responsible for its translocation to the nucleus (Hiwasa & Sakiyama, 1996). Nuclear CTSL proteolytically processes and activates the CCAAT-displacement protein/cut homeobox (CDP/Cux) transcription factor which has been strongly associated with poor disease prognosis (Goulet, et al., 2007; Goulet, Truscott, & Nepveu, 2006; Moon, et al., 2002). CTSL processed CDP/Cux exhibits enhanced DNA binding properties which in turn confers a replicative and metastatic advantage to tumor cells (Figure 1). CDP/Cux promotes tumor cell proliferation by accelerating entry into the S phase of cell cycle, and also augments tumor invasion by stimulating hallmark epithelial to mesenchymal transition features such as snail and slug upregulation and E-cadherin repression (Kedinger, et al., 2009).

Nuclear CTSL has been documented to promote tumor progression through other CDP/Cux independent mechanisms as well. Nuclear CTSL is frequently upregulated in triple negative breast cancer patients and patients with either a germline or somatic mutation in BRCA1 tumor suppressor gene (Grotsky, et al., 2013). In fact, increased nuclear CTSL levels serve as a predictive biomarker for treatment response in this subset of breast cancer patients. In the absence of BRCA1, 53BP1 suppresses homologous repair of DNA double strand breaks (DSBs) and instead activates the error prone non homologous end joining (NHEJ) substitute pathway which results in accumulation of unrepaired DNA DSBs, chromosomal translocations and consequently cell death and tumor suppression (Bouwman, et al., 2010; Snouwaert, et al., 1999; Ward, Minn, van Deursen, & Chen, 2003). BRCA1 deficient cells overcome this genomic instability and growth arrest by over-expressing nuclear CTSL which in turn proteolytically degrades 53BP1 (Gonzalez-Suarez, et al., 2011; Grotsky, et al., 2013). Thus nuclear CTSL not only confers a survival advantage on BRCA1 deficient tumor cells, but, by improving their DNA repair capacities it also renders them resistant to radiation and genotoxic chemotherapeutics such as cisplatin, PARP inhibitors and mitomycin C (Grotsky, et al., 2013).

3.1 Role of CTSL in tumor metastasis

In transformed cells extracellular CTSL levels increase up to 200-fold and comprise up to 40 % of total secreted proteins (Gottesman, 1978). Transformation dependent CTSL secretion augments the invasive/metastatic potential of cancer cells through direct degradative proteolysis of several components of the extracellular matrix (ECM) and basement membrane (Baricos, Zhou, Mason, & Barrett, 1988; Chambers, et al., 1992; Colella, Jackson, & Goodwyn, 2004) (Figure 1). In the presence of cell surface glycosaminoglycans, secreted CTSL degrades ECM components such as laminin, Type I and IV collagen, fibronectin and elastin (Gal, et al., 1985; Ishidoh & Kominami, 1995; R. W. Mason, Johnson, Barrett, & Chapman, 1986; Novinec, et al., 2007). Furthermore, it plays a critical role in amplification of the proteolytic cascade by activating latent pro-forms of other key metastasis associated proteases including urokinase plasminogen activator, pro-heparanase, other cathepsins, as well as certain MMPs (Everts, et al., 2006; Goretzki, et al., 1992; Laurent-Matha, Derocq, Prebois, Katunuma, & Liaudet-Coopman, 2006). Cell adhesion protein E-cadherin is significantly reduced during tumor invasion and thus loss of E-Cadherin is widely accepted as a marker of tumor invasiveness. CTSL proteolytically degrades the extracellular domain of E-cadherin and abolishes its adhesive property (Gocheva, et al., 2006). Consistent with these observations, tumor progression studies in a CTSL^{-/-} RIP1-Tag2 pancreatic carcinogenesis model revealed that CTSL deficiency significantly hampers the progression of benign encapsulated tumors into invasive carcinomas thus indicating that CTSL has a non-redundant consequential role in the process of tumor invasion (Gocheva, et al., 2006) (Figure 2). Likewise, numerous studies have reported that an increase in CTSL secretion is a key feature driving the switch of poorly tumorigenic and non-metastatic human melanoma cells to a highly tumorigenic and aggressively metastatic phenotype (Frade, et al., 1998; Jean, et al., 1996; Rozhin, Wade, Honn, & Sloane, 1989). In agreement, treatment with an anti-CTSL antibody resulted in significantly retarded tumor growth and decreased lung metastases incidence (Frade, Rousselet, & Jean, 2008; Jean, et al., 1996). CTSL has also been shown to promote melanoma cell survival and tumor growth by inhibiting complement mediated tumor cell death (Jean, et al., 1996; Jean, et al., 1995). The well-orchestrated modulation of CTSL expression as a metastatic tumor cell transitions through different phases of the metastatic cascade further highlights its significance in the metastatic dissemination process. Metastasizing tumor cells upregulate CTSL expression as they undergo epithelial to mesenchymal transition but once the cells become established at a distant site, CTSL expression is down-regulated as the cells revert back to their epithelial state to promote colonization (Chen, et al., 2013; Keding, et al., 2009).

In contrast to the well oxygenated and near neutral pH conditions observed within most normal tissues, the tumor microenvironment is frequently marked by hypoxia and acidosis (Jensen, et al., 2010; Lunt, Chaudary, & Hill, 2009; Siemann DW, 2015). It is now a well-recognized fact that these very aberrant microenvironmental conditions are responsible for aggressive tumor progression and metastatic occurrence which ultimately results in poor patient survival (Schwickert, Walenta, Sundfor, Rofstad, & Mueller-Klieser, 1995; Siemann DW, 2015; Walenta, et al., 2000). Hypoxic and acidic conditions are known to result in enhanced tumor cell secretion of CTSL by eliciting two distinct mechanisms, namely,

upregulated expression and lysosomal exocytosis (Cuvier, Jang, & Hill, 1997; Sudhan & Siemann, 2013). Lysosomes are normally peri-nuclear in localization. However, in response to hypoxic and acidic exposures, the lysosomes redistributed and undergo anterograde trafficking to the plasma membrane and released their contents (including CTSL) into the extracellular milieu (Sudhan & Siemann, 2013). Since cathepsins exhibit optimal activity under acidic conditions (Ishidoh & Kominami, 1995), CTSL released in an acidic milieu would engage in heightened proteolysis of extracellular matrix components and promote invasiveness. Indeed, hypoxia and acidosis triggered CTSL upregulation closely correlated with metastatic properties of tumor cells thereby suggesting that CTSL may be a key player in microenvironment triggered metastatic aggressiveness (Figure 3) (Sudhan & Siemann, 2013).

Supporting the observations made in experimental metastasis models, several clinical studies in cancer types including breast, pancreatic, colorectal and lung have shown that tumor CTSL level is a strong prognostic indicator of patient outcome (Table 2). Grotsky and colleagues demonstrated that CTSL along with 53BP1 and vitamin D receptor constitutes a triple biomarker signature for therapeutic resistance to radiation, DNA damaging chemotherapeutics and PARP inhibitors among triple negative breast cancer patients and patients with BRCA1 mutation (Grotsky, et al., 2013). In breast cancer patients, CTSL level inversely correlates with steroid hormone receptor status and is a strong predictor of tumor relapse and poor overall survival (Foekens, et al., 1998; Lah, Cercek, et al., 2000; Lah, Kalman, et al., 2000; Thomssen, et al., 1998; Thomssen, et al., 1995). In fact, Thomssen and colleagues have reported that the prognostic impact of CTSL was comparable to that of axillary lymph node status and tumor histological grading; and could be utilized for identification of node negative patients that are at a high risk of metastatic relapse (Thomssen, et al., 1998; Thomssen, et al., 1995).

3.2 Role of Cathepsin L in bone resorption

The involvement of CTSL in neoplastic bone diseases has been highlighted by numerous experimental and clinical reports of CTSL upregulation in primary and metastatic bone tumors. Several studies have reported elevated CTSL mRNA levels across various human osteosarcoma cell lines and tumor samples (Damiens, et al., 2000; Husmann, et al., 2008; Krueger, Kellner, Buehling, & Roessner, 2001; Park, et al., 1996). In comparison to normal tissue levels, CTSL levels were upregulated in ~50% of primary bone tumors, and in almost 100% of the bone metastases investigated (Park, et al., 1996). In agreement with these observations Husmann and colleagues have reported that highly metastatic SAOS-2 sublines LM5 and LM7 expressed significantly higher levels of CTSL mRNA compared to parental SAOS-2 osteosarcoma cells thereby highlighting the correlation between CTSL expression levels and metastatic aggressiveness of bone cancers (Husmann, et al., 2008). A serendipitous observation made in CTSL antibody producing hybridoma cells provided one of the first leads to the importance of CTSL in multiple myeloma. Highly tumorigenic and metastatic P3X63Ag8.653 murine myeloma cells secrete high levels of pro-cathepsin L (Weber, Gunther, Laube, Wiederanders, & Kirschke, 1994). Intriguingly, these authors observed that when these CTSL secreting myeloma cells were fused with spleen cells immunized with CTSL to generate CTSL antibody producing hybridoma cells, their

tumorigenic and metastatic capacity was remarkably diminished. Compared to the numerous peritoneal tumors and metastatic deposits observed in mice inoculated with either myeloma cells or control hybridoma cells, ~80% of the mice inoculated with CTSL antibody producing hybridoma cells failed to show signs of primary or metastatic tumor burden. Consistent with these findings, CTSL depletion has been reported to significantly retard the growth of SP and L myeloma tumors (Kirschke, Eerola, Hopsu-Havu, Bromme, & Vuorio, 2000).

In addition to these primary bone neoplasias, several human cancers such as lung, prostate and breast adenocarcinomas exhibit a marked proclivity to metastasize to the bone (Mundy, 2002; Roodman, 2004). Bone metastases are rarely indolent – they usually inflict devastating skeletal morbidities such as intractable bone pain, pathological fractures, paralysis due to nerve compression and hypercalcemia (Mundy, 2002; Roodman, 2004). These adverse skeletal complications are predominantly triggered by unrestrained osteoblastic and osteoclastic activities of bone metastases. Under normal physiological conditions, bone remodeling is tightly regulated through reciprocal interactions between bone resorbing osteoclasts and bone forming osteoblasts (Raggatt & Partridge, 2010). However, both primary and metastatic bone lesions perturb this equilibrium through sustained activation of osteoclasts or osteoblasts which ultimately leads to excessive osteolysis or bone formation. While it is well-recognized that osteolytic cancers perpetrate bone resorption through osteoclasts; even predominantly osteoblastic cancers such as prostate cancer, have been shown to depend on an initial osteoclastic trigger for subsequent osteoblastic events to occur (Inoue, et al., 2005; Keller & Brown, 2004; Roato, et al., 2008; J. Zhang, et al., 2003). Since inhibition of unrestrained osteoclastic activity can alleviate skeletal morbidities associated with both osteoclastic and osteoblastic metastases, significant efforts have focused on the identification of osteoclast targeting strategies and led to the development of agents including bisphosphonates, denosumab, and Cathepsin K inhibitors (Cleazardin, 2011; Neville-Webbe & Coleman, 2010; Onishi, Hayashi, Theriault, Hortobagyi, & Ueno, 2010; Rachner, Hadji, & Hofbauer, 2012).

Cathepsin K (CTSK) is the predominant protease in osteoclasts and is the prime executor of resorption during both normal and pathological bone turnover. Nonetheless, studies in knockout mouse models have shown other proteases; in particular CTSL to closely participate in conjunction with CTSK in the process of pathological bone resorption (Everts, et al., 2006; Potts, et al., 2004). Although osteoclastic CTSL levels are significantly lower than CTSK levels during normal bone remodeling, in the presence of bone resorption promoting inflammatory cytokines such as parathyroid hormone, IL-1 α , IL-6 and TNF- α , osteoclastic CTSL activity increases several fold and it plays a significant, non-redundant role in the process of pathological bone resorption (Cox, et al., 2006; Furuyama & Fujisawa, 2000a, 2000b; Kakegawa, et al., 1995; Tagami, et al., 1994). Using immunohistochemical procedures and electron microscopy, Goto and colleagues have demonstrated that in contrast to the low intracellular levels of CTSL observed within osteoclasts, the protease shows a strong extracellular presence and heavily co-localizes with collagen fibrils within resorption pits suggesting its involvement in degradation of the bone matrix (Goto, et al., 1994; Goto, et al., 1993). CTSL also has been widely implicated in the proteolytic degradation of bone and cartilage matrix components (Kakegawa, et al., 1995; Kirschke, Kambhavi, Bohley, &

Barrett, 1982; Leto, Sepporta, Crescimanno, Flandina, & Tumminello, 2010; Maciewicz, Wotton, Etherington, & Duance, 1990; Nakase, et al., 2000). In fact, serum CTSL levels were found to be significantly elevated in patients with low bone density conditions such as osteoporosis and osteopenia compared to individuals with normal bone mineral density (Lang, Willinger, & Holzer, 2004). Likewise, treatment with anti-resorptive agents such as bisphosphonates restored serum CTSL levels near normal levels. The close association of CTSL with various bone disorders involving destructive bone loss such as rheumatoid arthritis (Devauchelle, et al., 2004; Lemaire, et al., 1997; Schedel, et al., 2004), osteoporosis (Katunuma, Tsuge, Nukatsuka, & Fukushima, 2002; Lang, et al., 2004; Potts, et al., 2004), osteoarthritis (Cawston & Young, 2010; Solau-Gervais, et al., 2007) and periodontal disease (Cox, et al., 2006; Yamaguchi, Naruishi, Arai, Nishimura, & Takashiba, 2008) further suggests the likelihood of CTSL's participation in cancer associated bone resorption. Indeed, (Katunuma, Tsuge, Nukatsuka, Asao, & Fukushima, 2002; Katunuma, Tsuge, Nukatsuka, & Fukushima, 2002) showed that CTSL inhibition significantly mitigates osteolytic events and hypercalcemia in mice with bone metastases and importantly, this protective effect was shown to be superior to that of bisphosphonate.

In addition to cancer induced osteolysis, bone loss is also inflicted by several “standard of care” anti-cancer agents; a morbidity that severely affects the quality of life and life expectancy of cancer survivors (Holroyd, Cooper, & Dennison, 2008; Khan & Khan, 2008; VanderWalde & Hurria, 2011). Patients receiving chemotherapeutic agents such as methotrexate, cyclophosphamide, dexamethasone, prednisone and doxorubicin as post-operative adjuvant therapy, often suffer from hypogonadism triggered osteoporosis (Pfeilschifter & Diel, 2000; Reid, 1997; VanderWalde & Hurria, 2011). Similarly, surgical or medical hormonal ablation in patients with hormone responsive cancers such as breast, ovarian or prostate cancer can result in a drastic worsening of bone health (Fogelman, et al., 2003; Hadji, et al., 2011; Mincey, et al., 2006). Furthermore, steroidal hormones such as estrogen have been shown to negatively regulate osteoclastic synthesis of cathepsins L and K both directly by binding to osteoclastic estrogen receptor and indirectly by suppressing the expression of resorptive cytokines (Furuyama & Fujisawa, 2000a). Thus ovariectomized mice exhibit a significant upregulation of CTSK and particularly CTSL. In fact, CTSL deficient mice showed a marked resistance to osteoporosis upon ovariectomy (Potts, et al., 2004). Thus CTSL intervention strategies would not only serve to impede the metastatic dissemination of tumor cells but would also alleviate both treatment-induced and cancer-associated osteolysis.

4.0 Cathepsin L targeting

The role of CTSL in promoting tumor progression and metastatic aggressiveness, its contribution to cancer associated bone resorption and its strong association with disease relapse and patient mortality has raised significant interest in the development of CTSL intervention strategies. Since unrestrained CTSL activity stems from an imbalance between CTSL and endogenous inhibitor levels, several investigations have attempted to abolish CTSL proteolytic function through ectopic delivery/overexpression of endogenous inhibitors such as cystatins and stefins (Gianotti, Sommer, Carmona, & Henrique-Silva, 2008; Saleh, et al., 2003; Shridhar, et al., 2004). Notably, Gianotti and colleagues have reported that the

anti-invasive property of cystatin over-expression was mainly mediated through inactivation of extracellular but not intracellular CTSL; further validating the hypothesis that metastatic phenotype of tumor cells is primarily driven by secreted CTSL (Gianotti, et al., 2008). Likewise, CTSL downregulation through RNA interference in different tumor models including glioma, osteosarcoma, myeloma and melanoma resulted in consistent inhibition of tumorigenicity and invasiveness of neoplastic cells (Kirschke, et al., 2000; Krueger, et al., 2001; Levicar, et al., 2003; Yang & Cox, 2007). In addition to its anti-invasive effect, Levicar et al., reported that CTSL suppression in glioblastoma also enhanced the sensitivity of tumor cells to apoptotic agents such as staurosporine. Studies performed in the RIP1-Tag2 pancreatic carcinogenesis model comparing the effect of various cathepsin deficiencies on tumor progression showed that compared to other cathepsins, CTSL knockout led to the largest reduction in tumor burden and a significant impairment of the progression of benign encapsulated tumors to invasive carcinoma (Gocheva, et al., 2006).

Although anti-sense and genetic knockout strategies support the anti-metastatic significance of CTSL abrogation, the development of specific inhibitors of CTSL has been limited by the high degree of structural homology between different members of the cathepsin family (Sadaghiani, et al., 2007; V. Turk, Turk, & Turk, 2001). Indeed most currently studied cathepsin inhibitors have broad activity spectrums (Bell-McGuinn, Garfall, Bogyo, Hanahan, & Joyce, 2007; Elie, et al., 2010; Joyce, et al., 2004; Navab, Mort, & Brodt, 1997; Palermo & Joyce, 2008). While the anti-tumor effects exhibited by pan specific cysteine cathepsin inhibitors appear promising, the dismal outcomes of previous broad spectrum targeting approaches are disconcerting (Palermo & Joyce, 2008; B. Turk, 2006). Since the proteolytic function of all cathepsins has as yet not been deciphered, and given the critical normal tissue functions of some cathepsins, indiscriminate inhibition of all members of the cathepsin family could potentially entail the same negative consequences as reported for the application of broad spectrum MMP inhibitors (V. Turk, et al., 2001). A likely factor underlying the failure of MMP inhibitors in clinical trial was their lack of specificity. These broad spectrum inhibitors indiscriminately inhibited several members of the MMP family which also includes anti-cancer proteases and proteases that are crucial to normal tissue function (Kruger, et al., 2010; Lopez-Otin & Matrisian, 2007; Overall & Lopez-Otin, 2002). For example, owing to their undesired inhibitory activity against the closely related ADAM proteases, several patients experienced severe musculoskeletal toxicity, which necessitated a reduction in dosage to suboptimal concentrations that were ineffective at inhibiting MMPs (Coussens, Fingleton, & Matrisian, 2002; Fingleton, 2007; B. Turk, 2006; Zucker, et al., 2000).

Despite the challenges imposed by the high degree of homology between the S2 substrate recognition domains of cysteine cathepsins, a few CTSL specific inhibitors have been developed and tested in preclinical models (Katunuma, 2011; Katunuma, Tsuge, Nukatsuka, Asao, et al., 2002; Kishore Kumar, et al., 2010; Song, et al., 2013; Torkar, Lenarcic, Lah, Dive, & Devel, 2013). Kumar and colleagues generated and screened a library of functionalized benzophenone, thiophene, pyridine, and fluorene thiosemicarbazone derivatives for potent inhibitors of CTSL (Kishore Kumar, et al., 2010; Kumar, et al., 2010). The lead molecule, KGP94, significantly retarded the growth of both recently implanted and established tumors (Chavarria, et al., 2012). In addition, KGP94 treatment significantly

impaired various metastasis associated tumor cell functions such as migration, invasion and lung colonization (Figure 4) (Chavarria, et al., 2012; Sudhan & Siemann, 2013). Similarly, (Katunuma, Tsuge, Nukatsuka, Asao, et al., 2002; Katunuma, Tsuge, Nukatsuka, & Fukushima, 2002) showed that epoxysuccinate based CTSL inhibitors of the CLIK series, namely CLIK-148 and CLIK-195 not only attenuated cancer associated bone resorption and hypercalcemia but also led to a significant reduction in metastatic burden. Bone resorption not only provides room for the expansion of metastatic lesions but also leads to the release of several active growth factors from the bone matrix that supports aggressive neoplastic cell growth. Thus a 'vicious cycle' is created between the tumor and the bone: tumor secreted cytokines stimulate bone resorption by upregulating osteoclastic CTSL activity; and the resorbed bone in turn releases growth factors stimulating tumor-cell proliferation and further cytokine release (Leto, et al., 2010; Mundy, 2002). Conceivably, CTSL inactivation disengages this vicious cycle and thus not only alleviates bone resorption, but also decreases the tumor burden in the bone.

In addition to participating in bulk protein turnover within the lysosomes, CTSL is involved in numerous other activities critical to normal tissue functioning. CTSL is responsible for activation of several key pituitary neuropeptides including β -endorphin, enkephalin, α -melanocyte stimulating hormone and adrenocorticotrophic hormone present within zymogenic granules (Funkelstein, et al., 2008; Hwang, et al., 2007; Yasothornsrikul, et al., 2003). CTSL also plays a cardioprotective role through inhibition of apoptosis promoting Akt signaling pathway in cardiomyocytes (Tang, et al., 2009). The significance of CTSL in normal tissue functioning is reflected by numerous pathological conditions that stem from CTSL deficiency such as dilated cardiomyopathy, metabolic syndromes, brain atrophy, epithelial hyperplasia and hypotrichosis (Felbor, et al., 2002; Huang, et al., 2003; Petermann, et al., 2006; Potts, et al., 2004; Stypmann, et al., 2002). Thus inhibition of nuclear CTSL poses a significant risk of such undesirable side-effects due to inactivation of intracellular CTSL. One key difference between the normal tissue and neoplastic cell functions of CTSL is that while most normal tissue functions are furnished by intracellular CTSL, tumor and metastasis associated functions are mainly mediated through extracellular CTSL. Selective targeting of extracellular CTSL could therefore broaden the therapeutic window of CTSL inhibition strategies by obviating any toxicity resulting from inhibition of intracellular functions. In fact, the cell impermeable JPM-OEt inhibitor was able to achieve a significant inhibition of tumor growth, angiogenesis and invasion without exerting any overt normal tissue toxicity (Sadaghiani, et al., 2007).

Long term administration of selective inhibitors of CTSL poses a potential risk of therapeutic resistance through compensation by other members of the cathepsin family. Thus simultaneous inhibition of CTSL and other critical tumor promoting cathepsins could potentially yield a better outcome. Since CTSB and CTSK have been shown to significantly impact two distinct aspects of tumor progression, tumor metastasis and bone resorption (Jensen, et al., 2010; Roshy, Sloane, & Moin, 2003), respectively, it might be feasible to test the efficacy of CTS L+B or CTS L+K dual targeting strategies. Such protease inhibition approaches require careful deliberation supported by studies in conditional knockout models in order to achieve maximum anti-tumor effects while obviating normal tissue toxicity. Studies have shown that CTSL inhibition improves the therapeutic window of various

chemotherapeutic agents by lowering the effective concentrations at which they mediate cytotoxic effects (Zheng, Chou, Mirkin, & Rebbaa, 2004; Zheng, et al., 2009). These authors demonstrated that CTSL inhibition not only prevents the emergence of a drug resistant phenotype but also effectively reverses resistance to various cytotoxic and targeted agents including doxorubicin, etoposide, imatinib, trichostatin A and tamoxifen. While administration of highest tolerable dose of doxorubicin had no impact on the growth of doxorubicin resistant tumors, the CTSL inhibitor iCL yielded a 40 % reduction in tumor growth; combination treatment with doxorubicin restored the sensitivity to doxorubicin and led to a 90% reduction in tumor growth. Since the majority of anticancer drug therapy failures may be attributed to either inherent or acquired resistance, if drug sensitivity restoration by CTSL inhibition could be successfully translated to the clinic, such a strategy would have significant impact on patient treatment outcome.

Despite the promising results of CTSL inhibition in preclinical models, questions on protease targeting raised by the unanticipated outcomes of MMP inhibitor clinical trials need to be considered. One such issue is the development of reliable tools for the identification of patients who would best benefit from anti-CTSL therapy. While methodologies such as CTSL immunohistochemistry of tumor tissue and measurement of CTSL level/activity in serum samples have been proposed, it is critical to evaluate the reliability of these techniques. Recently, activity based probes (ABPs) have been developed as a non-invasive imaging tool that not only lends itself to patient identification but could also be used for monitoring treatment response (Blum, von Degenfeld, Merchant, Blau, & Bogoy, 2007; Paulick & Bogoy, 2008). ABPs are comprised of three distinct elements namely (i) a reactive site that covalently links to the active site of target enzyme based on its activity status, (ii) a target recognition motif that confers specificity towards its target enzyme and (iii) a reporter tag that enables direct visualization of probe labeled proteins. In contrast to the passive measurement of protein abundance offered by widely used proteomic tools that depend on antibody labeling, ABPs provide a direct readout of the activity status of its target enzyme both in-vivo as well as ex-vivo. These features of ABPs allow longitudinal in-vivo assessment of response to enzyme inhibitors using non-invasive imaging techniques (Paulick & Bogoy, 2008; Verdoes, et al., 2013). However, most ABPs that have been developed for the assessment of cysteine proteinases were designed based on broad spectrum reactive sites and are thus not amenable for detection of specific cathepsins such CTSL (Blum, et al., 2007; Paulick & Bogoy, 2008). Like CTSL inhibitors, the development of CTSL specific ABP has been hindered by the high degree of similarity between the substrate recognition pockets of different members of the cathepsin family. However, Torkar and colleagues recently developed a novel non-cell permeable CTSL specific photo-affinity based probe that could be utilized for the measurement of secreted CTSL activity ex-vivo (Torkar, et al., 2013).

Although issues such as patient identification, therapeutic response assessment and comparison of effectiveness of intracellular versus extracellular CTSL targeting need to be addressed, the abundance of favorable preclinical and clinical findings make CTSL targeting an attractive and promising approach to target tumor cell dissemination and associated skeletal morbidities.

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Abbreviations

MMPs	Matrix metalloproteases
CTSL	Cathepsin L
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
PDGF	Platelet derived growth factor
EGF	Epidermal growth factor
NGF	Neuronal growth factor
INFγ	Interferon gamma
IL	Interleukin
53BP1	p53 binding protein 1
TNF-α	Tumor necrosis factor- α

Figure 1. Distinct roles of secreted and nuclear CTSL

Secreted CTSL aids metastatic dissemination of tumor cells by degrading cell adhesion molecules and extracellular matrix proteins as well as activating other latent proteases. Nuclear CTSL activates the transcription of epithelial to mesenchymal transition genes and also confers therapeutic resistance.

Figure 2. CTSL deficiency impairs invasiveness of pancreatic islet tumors in RIP-1 Tag-2 mice
 Tumors in RT2 mice can be classified into three different grades **A**. Encapsulated tumor (Tum), **B**. microinvasive tumors (IC1) and **C**. invasive carcinomas (IC2). **D**. Representative image for the predominant grade in CTSL knockout mice is shown. **E**. Distribution of tumor types in control and CTSL knockout tumors. Immunofluorescence images indicating increased E-Cadherin levels in CTSL knockout (**H** and **I**) compared to control (**F** and **G**) tumors (Red, E-cadherin; blue, DAPI). Modified with permission from Gocheva et al (2006). Distinct roles for cysteine cathepsin genes in multistage tumorigenesis. *Genes & Development*, 20:543-556, under a Creative Commons License (Attribution-NonCommercial 4.0 International License).

Figure 3. Tumor microenvironmental conditions such as hypoxia and acidosis enhance CTSL secretion and tumor cell invasiveness

A. and **C**, CTSL secretion by prostate cancer PC-3ML cells upon exposure to hypoxic or acidic conditions. **B.** and **D**, PC-3ML invasiveness in response to such conditions. Modified with permission from Sudhan et al (2013). Cathepsin L inhibition by the small molecule KGP94 suppresses tumor microenvironment enhanced metastasis associated cell functions of prostate and breast cancer cells. *Clinical & Experimental Metastasis*, 30:891-902.

Figure 4. Effect of CTSL inhibition on metastasis

A. Effect of CTSL specific inhibitor KGP94 on migratory capacity of OS-156 osteosarcoma cells. **B.** Anti-invasive effect of KGP94 across various tumor cell types; osteosarcoma cells (OS-156), androgen dependent prostate cancer cells (LNCaP), androgen resistant bone metastatic prostate cancer cells (C42B), and highly lung metastatic breast cancer cells (LM2-4175). **C.** Effect of CTSLi KGP94 treatment on lung metastases forming capacity of SCCVII squamous carcinoma cells, mice ($N > 9$) treated with daily 20 mg/kg doses of the CTSL inhibitor KGP94. Line, median; bars 25th and 75th percentiles. **D.** Representative images of lungs from **C**.

Table 1
CTSL upregulation in human cancers

Cancer type	Observations	Citations
Colorectal cancer	Higher expression and activity in tumors, correlation with ras, association with uPA and PAI levels, CTSL levels could be of diagnostic significance	Adenis, et al., 1995; Anastakis Doxakis, 2013; Herszenyi, et al., 1999; Sheahan, et al., 1989; Troy, et al., 2004
Breast cancer	CTSL upregulation and increased activity in high grade tumors	Lah, et al., 1997
Chronic myeloid leukemia	Correlation with disease progression, metastatic incidence and overall survival, upregulation due to promoter hypomethylation	Samaiya, et al., 2011
Pediatric acute myeloid leukemia	CTSL upregulation, inverse correlation with endogenous inhibitor cystatin C, positive association with key AML angiogenesis factor VEGF	Jain, Bakhshi, Shukla, & Chauhan, 2010
Melanoma	Increased CTSL activity in melanocytic tumors compared to pigmented nevi and normal dermis	Frohlich, et al., 2001; Kageshita, et al., 1995
Gastrointestinal stromal tumors and gastric cancers	Overexpression and positive correlation with c-kit expression	Dohchin, et al., 2000; Farinati, et al., 1996; Miyamoto, et al., 2011; Watanabe, et al., 1987; Watanabe, et al., 1989
Endometrial cancer	CTSL upregulation and correlation with growth regulatory genes and HER2 receptor status	Skrzypczak, et al., 2012
Pancreatic cancer	CTSL upregulation in tumor as well as tumor associated macrophages	Niedergethmann, et al., 2004; Singh, et al., 2013; Sulpizio, et al., 2012
Glioma	Correlation with glioma progression with expression profile of low grade glioma < anaplastic astrocytoma < glioblastoma	Sivaparvathi, et al., 1996
Lung cancer	Higher CTSL activity in lung cancer compared to non-malignant tissue, association with tumor grade, upregulated serum levels	Chen, et al., 2011
Nasopharyngeal carcinoma	Overexpression in primary tumor and cervical lymph node metastases	Xu, Yuan, Liu, Zhang, & Chen, 2009
Oral squamous cell carcinoma	Elevated CTSL expression correlated with lymph node metastasis and poor survival, CTSL overexpression increases the likelihood of progression of oral dysplastic lesions into carcinomas	Macabeo-Ong, et al., 2003; Nakashima, Yasumatsu, Masuda, Clayman, & Komune, 2012

Table 2
CTSL in cancer prognosis

Cancer type	Observations	Citations
Breast cancer	Inverse correlation with steroid hormone receptor status and disease free survival and overall survival, prognostic value comparable to axillary lymph node status and tumor grading, biomarker for identification of node negative patients at a high risk of metastatic relapse, CTSL could predict response to surgical resection and adjuvant systemic therapy in patients with operable tumors.	Foekens, et al., 1998; Harbeck, et al., 2001; Jagodic, Vrhovec, Borstnar, & Cufer, 2005; Lah, Cercek, et al., 2000; Lah, Kalman, et al., 2000; Thomssen, et al., 1995
Gastric cancer	Upregulation in tumors locally invading the muscularis propria and tumors with venous invasion, upregulation in chronic atrophic gastritis with intestinal metaplasia and gastric tumors, may participate in gastritis to cancer progression	Dohchin, et al., 2000; Farinati, et al., 1996
Urothelial carcinoma of the bladder	Positive correlation with tumor stage and grade, local and metastatic recurrence and poor survival	Yan, et al., 2010
Pancreatic cancer	Association with tumor stage, lymph node invasion, disease recurrence and overall survival, predictor of disease relapse in patients undergoing curative resection	Niedergethmann, et al., 2004; Singh, et al., 2013
Glioma	correlation with glioma progression in the order of low grade glioma < anaplastic astrocytoma < glioblastoma	Sivaparthi, et al., 1996
Colorectal cancer	Higher expression and activity in tumors compared to matched normal mucosa, correlation with tumor progression, metastatic incidence and overall survival, high CTSL expression in patients with curative disease is a predictor of disease relapse and poor survival	Anestakis Doxakis, 2013; Herszenyi, et al., 1999; Troy, et al., 2004
Pediatric acute myeloid leukemia	CTSL upregulation exhibited inverse correlation with event free survival and overall survival	Jain, et al., 2010
Oral squamous cell carcinoma	Elevated CTSL expression correlated with lymph node metastasis and poor survival, expression of headpin - endogenous inhibitor to CTSL inversely correlated with tumor grade	Nakashima, et al., 2012
Nasopharyngeal carcinoma	Upregulation correlates with lymph node metastases and distant metastases, tumor stage and overall survival	Xu, et al., 2009