Cancer with low cathepsin D levels is susceptible to vacuolar (H\(^+\))-ATPase inhibition

Satoshi Kitazawa, Satoru Nishizawa, Hideyuki Nakagawa, Masaaki Funata, Kazuho Nishimura, Tomoyoshi Soga and Takahito Hara

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Correspondence
Takahito Hara, Oncology Drug Discovery Unit, Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, Fujisawa; *Biomolecular Research Laboratories, Takeda Pharmaceutical Company Limited, Fujisawa; †Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan

Vacuolar (H\(^+\))-ATPases (V-ATPases) have important roles in the supply of nutrients to tumors by mediating autophagy and the endocytic uptake of extracellular fluids. Accordingly, V-ATPases are attractive therapeutic targets for cancer. However, the clinical use of V-ATPase inhibitors as anticancer drugs has not been realized, possibly owing to their high toxicity in humans. Inhibition of V-ATPase may be an appropriate strategy in highly susceptible cancers. In this study, we explored markers of V-ATPase inhibitor sensitivity. V-ATPase inhibitors led to pH impairment in acidic intracellular compartments, suppression of macropinocytosis, and decreased intracellular amino acid levels. The sensitivity of cells to V-ATPase inhibitors was correlated with low cathepsin D expression, and cancer cells showed increased sensitivity to V-ATPase inhibitors after pretreatment with a cathepsin D inhibitor and siRNA targeting the cathepsin D gene (CTSD). In addition, V-ATPase inhibitor treatment led to the induction of the amino acid starvation response, upregulation of endoplasmic reticulum stress markers, and suppression of mammalian target of rapamycin (mTOR) signaling in cells expressing low levels of cathepsin D. Some colorectal cancer patients showed the downregulation of cathepsin D in tumor tissues compared with matched normal tissues. These findings indicate that V-ATPase inhibitors are promising therapeutic options for cancers with downregulated cathepsin D.
for 2 h, and culture medium was removed and replaced with PBS (Wako). Fluorescence (544/640 nm and 514/563 nm for the Acridine Orange and AcidFluor assays, respectively) was detected using the SpectraMax Paradigm (Molecular Devices, Sunnyvale, CA, USA).

**Macropinosome visualization and macropinocytosis assay.** T-24 cells were plated at 2500 cells/100 µL/well on collagen-coated 96-well plates (Corning) for macropinosome visualization and 2500 cells/25 µL/well on collagen-coated 384-well plates (Corning). Cells for the macropinosome assay in RPMI-1640 (Wako) with 10% FBS (35-076-CVR; Corning) and cultured overnight. Culture media were removed and replaced with serum-free RPMI-1640 (Wako) 24 h post-seeding. After 16 h, indicated concentrations of bafilomycin A1 (Calbiochem, Darmstadt, Germany) and EIPA (Toronto Research Chemicals, Toronto, Canada) were added to the wells. After 1 h, 0.4 mg/mL TMR-BSA (Life Technologies, Carlsbad, CA, USA) was added to the wells, and cells were incubated for 30 min. Cells were fixed with 4% paraformaldehyde (Wako). DNA in cell nuclei was stained with DAPI (Thermo Fisher Scientific, Waltham, MA, USA) for macropinosome visualization and Hoechst 33258 (Life Technologies) for the macropinocytosis assay, and cells were counted. Fluorescence was detected using the IN Cell Analyzer 6000 (GE Healthcare Life Sciences, Chicago, IL, USA). The TMR and DAPI signals were counted and normalized in each well using the cell number.

**Amino acid measurement by capillary electrophoresis time-of-flight mass spectrometry.** HCT116 human colon cancer cells were plated at 7.5 × 10^5 cells/10 mL in 10-cm dishes (Corning). Then 3 nM bafilomycin A1 was added 24 h post-seeding. After 8 h, the cells were washed with 5% d-mannitol (Wako). Samples were extracted with methanol and purified with chloroform (Wako). The concentrations of amino acids were analyzed by capillary electrophoresis time-of-flight mass spectrometry in the laboratory of Dr. Soga (Institute for Advanced Biosciences, Keio University, Tsuruoka, Japan), as previously described.\(^{(20)}\)

**Cell growth assay.** To test the effect of bafilomycin A1 on growth, cells were plated at either 2500 or 3000 cells/100 µL/well on 96-well plates (Corning) in RPMI-1640 (Wako) with 10% FBS and cultured overnight. Various concentrations of bafilomycin A1, concanamycin A (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and dyphylline (synthesized as described by Zhao et al.)\(^{(21)}\) were added 24 h post-seeding, and after 3 days, cell viability was assessed by a Cell Titer-Glo Luminescent Cell Viability Assay (Promega). For confirmation of knockdown efficiency of siCTSD, cells were plated at 6 × 10^4 cells/well and transfections with 10 nM siCTSD #1 and #2 or negative control siRNA in the same manner. After 48 and 72 h, cells were harvested for Western blotting.

**Generation of the anti-phospho-Thr899-GCN2 rabbit mAb.** The antibody was generated in collaboration with Epitomics (Cambridge, MA, USA). Rabbits were immunized by repeated injections of a phospho-GCN2 peptide (SDPSGHLpTGMTAC, where pT represents phosphorylated Thr) cross-linked to keyhole limpet hemocyanin. B cells were taken from the immunized rabbits and fused with a rabbit plasmacytoma cell line. The resulting hybridomas were selected and subcloned. Antibody screening was carried out by ELISA, Western blotting, and an immunofluorescence analysis.

**Western blot analysis.** Cells were washed with PBS at 4°C and lysed with cell lysis buffer containing 62.5 mM Tris-HCl (Wako), 10% glycerol (Wako), and 1% SDS (Wako). After heating at 100°C for 5 min, the protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific). Lysates were prepared with 3-mercapto-1,2-propandiol (Wako) and separated by SDS-PAGE using 7.5–15% or 5–20% SDS-PAGE gels (Perfect NT Gel W; DRC, Tokyo, Japan). Proteins were electroblotted onto a PVDF membrane (Wako) at 75 V for 120 min and blocked with Block Ace (DS Pharma Biomedical) in PBS containing 0.2% Tween-20 (PBS-T) or Starting Block T20 (PBS) Blocking Buffer (Thermo Fisher Scientific). Membranes were incubated with the specific primary antibody overnight at 4°C and washed three times with PBS-T. Membranes were incubated with an HRP-conjugated secondary antibody (Bioworld, San Diego, CA, USA) for 1 h at room temperature, and then washed three times with PBS-T. The immunoblots were visualized using ImmunoStar Zeta (Wako) or ImmunoStar LD (Wako). Signals were visualized using the LAS-3000 Image Analyzer (Fujifilm, Tokyo, Japan) and quantified using Multi Gauge version 3.1 (Fujifilm). The following antibodies were used: anti-phospho-Thr899-GCN2 (1:1000), anti-β-actin (conjugated with HRP, rabbit monoclonal, #5125, 1:5000), anti-GCN2 (rabbit polyclonal, #3302, 1:2000), anti-phospho-Ser51-Elf2α (119A1, rabbit monoclonal, #3597, 1:5000), anti-Elf2α (D7D3 XP, rabbit monoclonal, #5324, 1:5000), anti-phospho-Thr389-p70 S6K (108D2, rabbit monoclonal, #9234, 1:5000), anti-p70 S6K (rabbit polyclonal, #9202, 1:5000), anti-phospho-Thr235/244-S6 ribosomal protein (2F9, rabbit monoclonal, #4856, 1:5000), anti-S6 ribosomal protein (54D2, mouse monoclonal, #2317, 1:5000), anti-PEK (C33E10, rabbit monoclonal, #3192, 1:5000), anti-ATT4 (D48B, rabbit monoclonal, #11815, 1:5000), anti-LC3B (D11 XP, rabbit monoclonal, #3868, 1:5000), anti-cathepsin D (rabbit polyclonal, #2284, 1:5000), and anti-cleaved PARP (rabbit monoclonal, #9541, 1:5000), all supplied by Cell Signaling Technology (Danvers, MA, USA).

**Analysis of gene expression levels in cancer cell lines.** Log2-transformed gene expression levels of cathespins in cancer cell
lines were obtained from the Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle/search/geneInfoPage).

Transcriptomic analysis of colorectal tumors. Gene expression levels of CTSD in clinical colorectal tumors and their matched normal tissues were measured by the following transcriptome analysis. All samples were collected from patients with informed consent and ethics approval. Total RNA was purified from tissue derived from 39 colorectal cancer patients (41 tumor tissue and 39 normal tissue samples) using an RNeasy Mini Kit (Qiagen, Venlo, Netherlands). RNA samples were subjected to DNA microarray analysis according to a standard protocol. In brief, 100-ng aliquots of total RNA were used for the generation of Cy3-labeled complementary RNA, and the resulting probes were hybridized to the SurePrint G3 Human GE 8 × 60 K v2 microarray (Agilent Technologies, Santa Clara, CA, USA). The signal values were determined using Feature Extraction software (Agilent Technologies), and normalized by dividing by the trimmed mean calculated by excluding 2% of the highest and the lowest outliers of all signal values of each sample.

Statistical analyses. Statistical analyses were carried out using Student’s t-test or Williams’ test. A P-value of less than 0.05 for the Student’s t-test and 0.025 for Williams’ test was considered statistically significant. To analyze synergistic effects, the combination index, which indicates synergy for values lower than 1.0, was determined using the Bliss Independence model as described previously by Foucquier et al. (22)

Results

Vacuolar (H+)-ATPase inhibitor treatment led to pH impairment of acidic intracellular compartments, suppression of macropinocytosis, and decreased intracellular amino acid levels. Acridine Orange and AcidFluor staining, which were observed in untreated cells, disappeared in T-24 cells after treatment with bafilomycin A1 (Fig. 1a,b). Bafilomycin A1 also inhibited TMR-BSA uptake in T-24 cells as well as the macropinocytosis inhibitor EIPA (Fig. 1c). Bafilomycin A1 at concentrations greater than 10 nM suppressed Acridine Orange, AcidFluor staining, and TMR-BSA uptake in T-24 cells. Bafilomycin A1 also completely blocked the growth of T-24 cells at a concentration greater than 10 nM (Fig. 1d), suggesting that the V-ATPase inhibitor impairs the acidic compartment, which leads to the inhibition of T-24 cell growth. In addition, bafilomycin

![Fig. 1. Inhibition of intracellular pH regulation, macropinocytosis, intracellular amino acid levels, and cell growth by a vacuolar (H+)-ATPase (V-ATPase) inhibitor. (a,b) Acridine Orange and AcidFluor assays were undertaken in T-24 bladder cancer cells 2 h after treatment with the indicated concentrations (Conc.) of bafilomycin A1 (n = 4). Fluorescence (544/640 nm and 514/563 nm) was designated as the Y-axis. Data are presented as mean ± SD. (c) Macropinosome visualization was carried out with tetramethylrhodamine-conjugated BSA (TMR-BSA) for 30 min in T-24 cells after 1 h pretreatment with 100 nM bafilomycin A1 (Baf) and 30 μM 5-(N-ethyl-N-isopropyl) amiloride (EIPA). TMR dots (red) show levels of macropinocytosis. The macropinocytosis assay using TMR-BSA was carried out in the same manner with the indicated concentrations of bafilomycin A1 or EIPA (n = 4). TMR dots/cell are designated as the Y-axis. Data are presented as the mean ± SD. (d,e) T-24 cells and HCT116 colon cancer cells were treated with the indicated concentrations of bafilomycin A1. After 3 days, cell viability was assessed. Ordinate values were obtained by setting the control group value as 100%. Data are presented as mean ± SD (n = 3). (f) Amino acid levels were analyzed in HCT116 cells 8 h after treatment with 3 nM bafilomycin A1 using capillary electrophoresis time-of-flight mass spectrometry (n = 3). Ordinate values were obtained by setting the control group value as 100% and designated as the Y-axis. Data are presented as mean ± SD. *P < 0.05, Student’s t-test.]
Table 1. Log₂-transformed gene expression levels (Cancer Cell Line Encyclopedia; CCLE) and log₂ 50% growth inhibition values of vacuolar (H⁺)-ATPase (V-ATPase) inhibitors in various cell lines

| Cell lines | Tissue | Log₂-transformed gene expression levels (CCLE) | Log₂-GI₅₀ of V-ATPase inhibitors |
|------------|--------|-----------------------------------------------|----------------------------------|
|            |        | CTSA  | CTSB | CTSC | CTSD | CTSE | CTSF | CTSG | CTSH | CTSK | CTSL | CTSO | CTSS | CTSV | CTSW | CTSZ | Baf | Con | Dyp |
| RKO        | Colon  | 10.30 | 8.30 | 11.03 | 6.04 | 4.77 | 5.05 | 5.07 | 9.18 | 5.60 | 11.27 | 4.59 | 4.35 | 8.39 | 5.32 | 7.83 | -9.20 | -9.88 | -6.46 |
| HCT116     | Colon  | 10.33 | 9.29 | 9.42 | 6.89 | 4.32 | 4.78 | 4.25 | 8.13 | 5.18 | 9.71 | 4.43 | 4.27 | 9.18 | 4.98 | 7.85 | -8.83 | -9.61 | -6.19 |
| HCT15      | Colon  | 10.33 | 9.14 | 9.08 | 7.34 | 4.54 | 5.18 | 4.49 | 10.68 | 5.98 | 9.19 | 4.59 | 4.27 | 8.80 | 5.42 | 8.11 | -8.79 | -9.20 | -5.65 |
| ACHN       | Kidney | 10.79 | 9.04 | 11.61 | 7.51 | 4.63 | 5.01 | 4.43 | 8.84 | 6.30 | 9.76 | 7.25 | 5.72 | 7.89 | 5.04 | 6.47 | -8.72 | -9.63 | -5.70 |
| PATU8988T  | Pancreas | 10.51 | 9.27 | 10.23 | 9.03 | 11.21 | 6.81 | 4.66 | 7.59 | 5.65 | 10.43 | 6.39 | 4.43 | 9.79 | 5.26 | 9.10 | -8.92 | -9.87 | -5.13 |
| PATU8902   | Pancreas | 10.16 | 9.36 | 10.42 | 9.98 | 4.76 | 5.08 | 4.49 | 8.00 | 5.30 | 9.36 | 7.25 | 5.72 | 8.80 | 5.02 | 8.54 | -9.13 | -9.69 | -6.43 |
| MIAPACA2   | Pancreas | 11.08 | 9.79 | 11.82 | 8.92 | 4.28 | 5.05 | 4.60 | 10.22 | 7.39 | 10.26 | 4.82 | 4.22 | 9.08 | 5.03 | 7.12 | -8.10 | -8.51 | -5.93 |
| NCI-H661   | Lung   | 11.08 | 10.82 | 7.75 | 6.71 | 4.66 | 5.93 | 4.59 | 8.73 | 5.56 | 9.82 | 5.60 | 5.25 | 10.84 | 4.84 | 6.00 | -8.85 | -9.68 | -6.29 |
| A498       | Kidney | 12.53 | 10.48 | 10.89 | 10.21 | 4.49 | 6.56 | 4.42 | 11.36 | 5.62 | 12.24 | 8.49 | 7.01 | 8.92 | 5.44 | 8.27 | -8.90 | -9.56 | -6.33 |
| 786O       | Kidney | 11.21 | 9.12 | 9.71 | 8.38 | 4.43 | 4.92 | 4.29 | 7.16 | 5.84 | 10.70 | 5.52 | 7.30 | 9.15 | 5.40 | 10.38 | -8.96 | -9.96 | -5.53 |
| A549       | Lung   | 10.12 | 9.97 | 9.59 | 7.62 | 4.56 | 6.67 | 4.64 | 8.96 | 5.50 | 11.34 | 4.73 | 6.40 | 8.55 | 5.16 | 6.28 | -8.96 | -9.60 | -5.50 |
| NCI-H1734  | Lung   | 10.41 | 10.42 | 10.66 | 7.35 | 4.28 | 6.12 | 4.87 | 9.60 | 6.60 | 10.11 | 7.39 | 10.04 | 9.31 | 4.92 | 7.37 | -8.83 | -9.72 | -5.78 |
| NCI-H226   | Lung   | 10.13 | 10.10 | 9.70 | 7.10 | 4.54 | 5.30 | 4.53 | 9.47 | 5.88 | 10.49 | 6.87 | 4.14 | 6.77 | 5.19 | 8.36 | -8.68 | -9.70 | -5.91 |
| NCI-H1703  | Lung   | 10.50 | 8.78 | 8.81 | 6.83 | 4.19 | 5.66 | 4.57 | 5.92 | 7.13 | 10.45 | 6.54 | 4.14 | 9.51 | 4.87 | 4.28 | -8.88 | -9.52 | -5.59 |
| BXPC3      | Pancreas | 10.88 | 10.48 | 10.68 | 8.43 | 4.87 | 5.40 | 4.36 | 12.64 | 5.14 | 10.01 | 4.62 | 8.77 | 9.02 | 5.22 | 8.52 | -8.93 | -9.49 | >5.00 |
| NCI-H2228  | Lung   | 10.82 | 10.20 | 11.51 | 9.54 | 4.61 | 6.41 | 4.36 | 10.41 | 5.64 | 11.55 | 8.87 | 7.08 | 7.76 | 4.73 | 7.84 | -8.80 | -9.57 | -5.92 |
| HCC4006    | Lung   | 9.33 | 8.65 | 9.16 | 7.64 | 4.46 | 6.21 | 4.10 | 12.31 | 5.31 | 8.73 | 7.65 | 6.18 | 9.32 | 4.94 | 10.56 | -9.13 | -9.76 | -6.37 |
| Caki2      | Kidney | 11.72 | 9.57 | 11.90 | 10.63 | 4.51 | 4.91 | 4.45 | 9.76 | 5.26 | 10.58 | 8.87 | 4.24 | 7.55 | 5.22 | 10.40 | -8.73 | -9.21 | >5.00 |
| PAN1C      | Pancreas | 10.77 | 9.40 | 10.16 | 9.58 | 4.78 | 6.52 | 4.41 | 10.11 | 5.70 | 9.36 | 5.63 | 5.05 | 9.27 | 5.22 | 7.20 | -8.01 | -8.17 | -5.82 |
| RCC4       | Kidney | 11.66 | 11.01 | 10.51 | 11.13 | 4.53 | 7.60 | 4.56 | 9.00 | 5.57 | 10.68 | 7.58 | 4.63 | 9.33 | 5.41 | 8.82 | -8.15 | -8.95 | -5.06 |

Baf, bafilomycin A1; Con, concanamycin A; Dyp, dyphylline.
A1 strongly suppressed HCT116 cell growth at a concentration greater than 3 nM (Fig. 1e) and decreased amino acid levels in HCT116 cells with 3 nM bafilomycin A1 (Fig. 1f). These data suggest that the V-ATPase inhibitor suppressed macropinocytosis as well as the associated amino acid supply and pH maintenance in acidic intracellular compartments, and hence inhibited cancer cell growth.

Sensitivity to V-ATPase inhibitors is correlated with low cathepsin D expression. We next investigated sensitive and insensitive cancer cell lines to explore markers for patient selection for treatment with V-ATPase inhibitors. We examined whether lysosomal protease cathepsins are involved in sensitivity to lysosomal inhibitor. Most cathepsins are activated in low pH conditions in lysosomes and hence are known as lysosomal markers. Cathepsins participate in endolysosomal proteolysis as proteases involved in the degradation of protein waste and the recycling of proteins into amino acids; accordingly, these proteases may be affected by the inhibition of macropinocytosis and the maintenance of intracellular amino acids in response to V-ATPase inhibitors. We examined the gene expression levels of cathepsins in cancer cell lines from the public database Cancer Cell Line Encyclopedia (Table 1). We also tested the effects of several V-ATPase inhibitors (bafilomycin A1, concanamycin A, and dyphylline) on the growth of various cancer cell lines and calculated the 50% growth inhibition values (GI50) of these compounds (Table 1). We examined the correlations between the expression levels of cathepsin genes and the GI50 of V-ATPase inhibitors (Table 2). We found the highest correlation between

|   | Baf | Con | Dyp |
|---|-----|-----|-----|
| CTSD | 0.42 | 0.43 | 0.35 |
| CTSA | 0.36 | 0.28 | 0.28 |
| CTSB | 0.34 | 0.17 | 0.22 |
| CTSC | 0.25 | 0.24 | 0.23 |
| CTSD | 0.36 | 0.27 | 0.02 |
| CTSE | 0.26 | 0.17 | 0.19 |
| CTSF | 0.12 | 0.13 | 0.32 |
| CTSH | 0.08 | 0.25 | −0.08 |
| CTSS | −0.16 | −0.18 | 0.45 |
| CTSG | 0.04 | −0.08 | 0.11 |
| CTSZ | 0.00 | 0.05 | −0.02 |
| CTSE | −0.16 | −0.15 | 0.14 |
| CTSO | 0.02 | −0.05 | −0.08 |
| CTSL | −0.09 | −0.19 | 0.08 |
| CTSV | −0.19 | −0.25 | 0.10 |

Baf, bafilomycin A1; Con, concanamycin A; Dyp, dyphylline.
pepstatin A, an inhibitor of cathepsin D,(26) followed by treat-
pretreated V-ATPase inhibitor-insensitive RCC4-vec cells with
whether cathepsin D inhibitors affect cancer cell growth. We
sion and the sensitivity to V-ATPase inhibitors, we examined
on the observed correlation between lower cathepsin D expres-
down sensitized cancer cells to the V-ATPase inhibitor.

Fig. 2. Expression levels of CTSD in various cell lines. RCC4-vec and RCC4-VHL cells have lower levels of CTSD compared to RCC4-vec cells, while V-ATPase inhibitor-sensitive RKO cells have lower levels of CTSD compared to V-ATPase inhibitor-insensitive RKO cells.

Fig. 3. Enhanced growth inhibition effect of the vacuolar (H+)-ATPase inhibitor by pretreatment with a cathepsin D inhibitor or CTSD knockdown. (a, b) RCC4 renal cell carcinoma cells plus vector alone (RCC4-vec) and RKO colon cancer cells were treated with the indicated concentrations of pepstatin A. After 1 day, these cells were treated with the indicated concentrations of bafilomycin A1. After 3 days, cell viability was assessed. Ordinate values were obtained by setting the control group value as 100%. Data are presented as mean ± SD (n = 3). *P < 0.025; **P < 0.005; ***P < 0.0005, Williams' test. Each number indicates the combination index; synergistic effects of pepstatin A and bafilomycin A1 are indicated for values <1.0. (c) RCC4-vec cells were treated with siRNA targeting CTSD #1 and #2 or negative control siRNA (siControl). After 54 h, cells were treated with the indicated concentrations of bafilomycin A1. After 3 days, cell viability was assessed. Ordinate values were obtained by setting the control group value as 100%. Data are presented as mean ± SD (n = 3) (d) RCC4-vec cells transfected with siRNA targeting CTSD #1 and #2 or control siRNA were lysed after 48 and 72 h, and expression levels of CTSD and β-actin were determined by Western blot analysis.

We next examined the correlations between cathepsin D protein expression in various cell lines and the sensitivity of these cells to V-ATPase inhibitors (Fig. 2d). Cathepsin D protein expression levels were strongly negatively correlated with sensitivities to bafilomycin A1, concanamycin A, and dyphylline than RCC4-vec and RCC4-VHL cells, which show high CTSD expression levels (Fig. 2a–c).

We next examined the correlations between cathepsin D protein expression in various cell lines and the sensitivity of these cells to V-ATPase inhibitors (Fig. 2d). Cathepsin D protein expression levels were strongly negatively correlated with sensitivities to bafilomycin A1, concanamycin A, and dyphylline, with correlation coefficients of 0.64, 0.61, and 0.51, respectively (Fig. 2e–g). These results suggest that a low level of cathepsin D expression is a marker of V-ATPase inhibitor sensitivity.

Pretreatment with cathepsin D inhibitors or CTSD knock-
down sensitized cancer cells to the V-ATPase inhibitor. Based on the observed correlation between lower cathepsin D expression and the sensitivity to V-ATPase inhibitors, we examined whether cathepsin D inhibitors affect cancer cell growth. We pretreated V-ATPase inhibitor-insensitive RCC4-vec cells with pepstatin A, an inhibitor of cathepsin D,(26) followed by treatment with bafilomycin A1. Growth inhibition of RCC4-vec cells by bafilomycin A1 was significantly greater for cells pre-
treated with pepstatin A than for cells that were not pretreated (Fig. 3a). Based on analyses of bafilomycin A1 and pepstatin A at various concentrations, they had synergistic effects, as evidenced by the combination index <1.0 (Fig. 3a). Pretreat-
ment with pepstatin A also slightly enhanced the sensitivity of V-ATPase inhibitor-sensitive RKO cells to bafilomycin A1 (Fig. 3b). Furthermore, its effect was confirmed by the syner-
gistic cytotoxicity of CTSD knockdown and bafilomycin A1 in

V-ATPase inhibitor for low CTSD cancer

Vacuolar (H+)-ATPase inhibitors induce AAR, upregulate ER stress markers, and suppress mTOR signaling in cancer cells with low cathepsin D levels. Our data suggested that cancer cell sensitivity to V-ATPase inhibitors is dependent on the cathepsin D expression level. We next investigated the intracellular amino acid and protein degradation responses to V-ATPase inhibitors in sensitive and insensitive cancer cell lines. Several studies have reported that GCN2 and eIF2α are phosphorylated by the binding of uncharged tRNAs to GCN2 and stimulate its dimerization, referred to as the AAR.(27–29) We found increased phosphorylation of GCN2 (T899) and eIF2α (S51) in response to bafilomycin A1 in V-ATPase inhibitor-sensitive RKO cells than in V-ATPase inhibitor-insensitive RCC4-vec cells (Fig. 4). It is also well known that insufficient protein degradation leads to the accumulation of misfolded proteins at the ER, and this is called ER stress.(30) We detected a gel mobility shift of PERK, an ER stress marker, suggesting the activation of PERK due to the autophosphorylation and induction of the downstream factor ATF4 in response to bafi-

mTOR signaling is downregulated following amino acid deprivation, and V-ATPase is then necessary to activate mTOR complex 1.(11,31,32) Therefore, we investigated mTOR down-
stream molecules in cells treated with V-ATPase inhibitors. Bafilomycin A1 clearly impaired the phosphorylation of ribo-

somal protein kinase S6K (T389) and ribosomal protein S6 (S235/236) in V-ATPase inhibitor-sensitive RKO cells (Fig. 4).
levels of CTSD of cancer patients have tumors that harbor downregulated regulation in tumors. We next investigated whether a fraction V-ATPase inhibitors. Cathepsin D is important for cell growth inhibitory effects of bafilomycin A1 in RKO cells (Fig. 4). Interestingly, the levels than in RCC-vec cells, suggesting autophagy is inhibited by bafilomycin A1 more clearly in RKO cells at a lower concentration.

Somes for degradation, lysosomal inhibitors inhibit autophago-lysosome–lysosome dysfunction and the fusion of LC3-II, which results in autophagy inhibition and the accumulation of LC3-II. The autophagy marker LC3B-II was induced by bafilomycin A1 more clearly in RKO cells at a lower concentration than in RCC-vec cells, suggesting autophagy is inhibited by bafilomycin A1 in RKO cells (Fig. 4). Interestingly, the levels of the mature form of cathepsin D were attenuated by bafilomycin A1 treatment at the cell growth inhibitory concentration in both cells (Fig. 4), implying that the downregulation of cathepsin D is important for cell growth inhibitory effects of V-ATPase inhibitors.

Some colorectal cancer patients showed cathepsin D downregulation in tumors. We next investigated whether a fraction of cancer patients have tumors that harbor downregulated levels of CTSD. Overall, CTSD mRNA expression levels were significantly lower in colon tumor tissues than in normal tissues (Fig. 5a). Compared with matched paired samples, 34 of 39 tumors (87%) of colorectal cancer patients showed downregulated CTSD mRNA levels (Fig. 5b).

Discussion. We showed that Acridine Orange and AcidIfluor, which accumulate in acidic intracellular compartments, were inhibited by bafilomycin A1, in agreement with the inhibitory effects on macropinocytosis, intracellular amino acids, and cell growth. These results imply that the loss of activity in acidic intracellular compartments in response to bafilomycin A1 may impair the degradation of macropinocytosed BSA and further uptake of BSA, affecting nutrient supply, including the supply of intracellular amino acids, and leading to the inhibition of cancer cell growth.

We found that cancer cell lines with low cathepsin D expression were sensitive to several V-ATPase inhibitors. In addition, V-ATPase inhibitors led to the induction of AAR, elicitation of ER stress, and suppression of mTOR signaling more strongly in cells expressing low levels of cathepsin D than in those with high cathepsin D expression. Cathepsin D is a soluble lysosomal aspartic endopeptidase that functions in the clearance of proteins. The decreased levels of the mature form of cathepsin D in response to bafilomycin A1, shown in Figure 4, imply that the downregulation of cathepsin D is not only a potential selection marker, but also a mechanism of cell growth inhibition by V-ATPase inhibitors.

Cathepsin D is a multifunctional protein involved in cancer invasion and metastasis by mediating the intracellular degradation of matrix proteins and apoptosis, and it also functions to protect cells from apoptotic cell death induced by oxidative stress through autophagy activation or the degradation of damaged mitochondria when autophagy is impaired. V-ATPase inhibitors block the late stages of autophagy by interfering with the fusion of autophagosomes with endosomes and lysosomes. This is consistent with our results showing that LC3B-II is induced by bafilomycin A1 (Fig. 4). Our data also showed that V-ATPase inhibitors induce the dysfunction of intracellular compartments, and this may lead to the activation of AAR and induction of ER stress pathways in cells expressing low levels of cathepsin D. We hypothesized that when cathepsin D is highly active, cellular stress induced by V-ATPase inhibitors might be diminished by cathepsin D, including the activation of autophagy or clearance of damaged compartments. Our data showed that the cathepsin D inhibitor pepstatin A and CTSD knockdown increase the cell growth inhibition induced by bafilomycin A1, and this finding is supported by a previous report showing that pepstatin A inhibits cell growth when essential amino acids are depleted.

Cathepsin D is overexpressed in several cancer types and is considered to be involved in cancer progression and metastasis, especially in breast cancer because estrogen induces cathepsin D. However, patients with cathepsin D-negative colorectal adenocarcinoma show a worse prognosis, suggesting the need for therapeutic strategies for low cathepsin D-expressing tumors. This finding is reasonable because cathepsin D induces apoptosis as a tumor suppressor, either directly or in response to several stimuli. Our data indicated that cathepsin D is actually downregulated in colorectal tumors compared with matched normal tissues, and a previous report has shown that 58% of colorectal tumors are negative for cathepsin D suggesting that many patients with colorectal cancer can benefit from V-ATPase inhibitors using cathepsin D expression, as evaluated by immunohistochemistry, as a
marker for patient selection. In this study, we focused on the relationship between cathepsin D levels and susceptibility to V-ATPase inhibitors. However, other cathepsins may also be involved in V-ATPase inhibitor sensitivity, as we found that several cathepsin inhibitors, including the cathepsin B, H, and L inhibitor E-64-d, cathepsin B inhibitor CA-074, cathepsin K inhibitor II, cathepsin L inhibitor CAA0225, and cathepsin L inhibitor III, also enhanced the growth inhibition effect induced by the V-ATPase inhibitor bafilomycin A1 in RCC4-vec cells, and those effects were synergistic as evidenced by the combination index <1.0 (Fig. S1). It is necessary to elucidate which cathepsin is the most sensitive marker for treatment with V-ATPase inhibitors, and the underlying biological mechanism, in future studies.

In this study, we found that cancer cells with downregulated cathepsin D expression are highly susceptible to V-ATPase inhibitors, possibly owing to the low protein degradation activity and energy supply. We further showed that most clinical colorectal tumors have downregulated cathepsin D, suggesting that V-ATPase inhibitors are applicable for colorectal cancer patients. In conclusion, V-ATPase inhibitors are promising therapeutic options for cancer with downregulated cathepsin D.

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Disclosure Statement

S.K., S.N., H.N., M.F., K.N., and T.H. are employees of Takeda Pharmaceutical Company. T.S. has no conflict of interest.

Abbreviations

AAR amino acid starvation response
ATF4 activating transcription factor 4
CTSD cathepsin D gene
eIF2α eukaryotic initiation factor 2α
EIPA 5-(N-ethyl-N-isopropyl) amiloride
ER endoplasmic reticulum
GCN2 general control nonderepressible 2

gly

*** P < 0.00001, Student’s t-test. (b) CTSD mRNA expression levels of the clinical colorectal tumor relative to those of the matched normal tissue, determined by setting the normal tissue value as 100%, were divided into two groups, that is, low and high CTSD expression in tumor tissues.

Fig. 5. Downregulated cathepsin D gene expression levels in colorectal tumors compared with matched normal tissues. (a) CTSD mRNA expression levels (log2) of clinical colorectal tumor tissues (n = 41) and their matched normal tissues (n = 39) were quantified by a transcriptome analysis. Data are presented as mean ± SD. *** P < 0.00001, Student’s t-test. (b) CTSD mRNA expression levels of the clinical colorectal tumor relative to those of the matched normal tissue, determined by setting the normal tissue value as 100%, were divided into two groups, that is, low and high CTSD expression in tumor tissues.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** Enhancement of the growth inhibition effect of vascular (H+-) ATPase inhibitors by pretreatment with various cathepsin inhibitors. RCC4 renal cell carcinoma cells plus vector alone (RCC4-vec) cells were treated with 10 μM E-64-d, 10 μM CA-074, 10 μM cathepsin K inhibitor II, 30 μM CA0225, or 10 μM cathepsin L inhibitor III. After 1 day, these cells were treated with the indicated concentrations of bafilomycin A1. After 3 days, cell viability was assessed. Ordinate values were obtained by setting the control group value as 100%. Data are presented as mean ± SD (n = 3). Each number indicates the combination index; synergistic effects of cathepsin inhibitors and bafilomycin A1 are indicated for values <1.0.