**ORIGINAL ARTICLE**

**BCM-95 and (2-hydroxypropyl)-β-cyclodextrin reverse autophagy dysfunction and deplete stored lipids in Sap C-deficient fibroblasts**

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**Abstract**

Saposin (Sap) C deficiency is a rare variant form of Gaucher disease caused by impaired Sap C expression or accelerated degradation, and associated with accumulation of glucosylceramide and other lipids in the endo/lysosomal compartment. No effective therapies are currently available for the treatment of Sap C deficiency. We previously reported that a reduced amount and enzymatic activity of cathepsin (Cath) B and Cath D, and defective autophagy occur in Sap C-deficient fibroblasts. Here, we explored the use of two compounds, BCM-95, a curcumin derivative, and (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), to improve lysosomal function of Sap C-deficient fibroblasts. Immunofluorescence and biochemical studies documented that each compound promotes an increase of the expression levels and activities of Cath B and Cath D, and efficient clearance of cholesterol (Chol) and ceramide (Cer) in lysosomes. We provide evidence that BCM-95 and HP-β-CD enhance lysosomal function promoting autophagic clearance capacity and lysosome reformation. Our findings suggest a novel pharmacological approach to Sap C deficiency directed to treat major secondary pathological aspects in this disorder.

**Introduction**

Saposin (Sap) C deficiency (MIM#610539) is a rare variant form of Gaucher disease (GD) caused by mutations in the prosaposin gene (PSAP) affecting Sap C expression and/or function (1). Sap C acts as physiological activator of glucosylceramidase (GCase) in lysosomal breakdown of glucosylceramide (GC). Sap C deficiency results in GC accumulation followed by secondary storage of ceramide (Cer), unesterified cholesterol (Chol) and other lipids in the endo-lysosomal compartment, in spite of normal levels of GCase. Impaired GCase or Sap C function leads to a serious visceral pathology that can be associated with neurological manifestations in some instances. Based on the extent of neurological involvement, GD is clinically subdivided in non-neuronopathic (type 1, MIM#230800), acute neuronopathic (type 2, MIM#230900), and chronic neuronopathic (type 3, MIM#231000) variants (2). Six Sap C-deficient patients have been reported so far (3–8). Among them, two adult siblings exhibited a GD1 phenotype, while the other four subjects displayed a more severe condition (3–8). In the latter group, two died at young age.
Autophagy is a highly regulated and conserved catabolic process that, by cooperation of autophagosomes and lysosomes, provides to destroy and recycle cellular components, and contributes to the maintenance of cellular energy balance. Major steps of this pathway include autophagosome formation, autophagosome-lysosome fusion, substrate degradation and lysosome reformation (9,10). We recently documented defective autophagy in primary fibroblasts from Sap C-deficient patients. Specifically, we showed that PSAP mutations causing loss or substitution of Cys311, Cys346 or Cys382 result in almost complete lack of the protein, due to aberrant disulfide bridge arrangement and rapid degradation of the mature protein (11). Loss of Sap C function was shown to lead to aberrant lipid storage, reduced levels and catalytic activities of two lysosomal proteases, cathepsin (Cath) B and Cath D, lysosome dysfunction, delayed degradation of autolysosomes and relative altered autophagic pathway (12,13). Of note, such defective function in autophagy had not previously reported in fibroblasts for GD patients with deficit of GCase (13), indicating a possible more complex role of Sap C.

Treatments for Sap C deficiency are currently limited to substrate reduction therapy (SRT), which utilizes drugs reducing the accumulation of metabolites by inhibiting their synthesis (14). This therapy failed in two Sap C-deficient patients (15), highlighting the urgent need for more effective therapeutic strategies. To this goal, we investigated whether two small molecules, BCM-95, a novel bio-enhanced preparation of curcumin with addition of phosphatidylcholine and turmeric oil (16), and (2-hydroxypropyl)-ß-cyclodextrin (HP-ß-CD), could have beneficial effects in Sap C-deficient fibroblasts. Accumulating evidence suggests that curcumin, a hydrophobic polyphenol derived from the rhizome of Curcuma long, is characterized by an exceptional safety profile and a number of pleiotropic effects, including a positive modulatory role in autophagy (17–19). HP-ß-CD is a cyclic oligomer of seven glucose units that can be targeted to the late endosome/lysosome compartment via pinocytosis. In lysosomes, it depletes the stored unesterified Chol (20). Previous studies reported that HP-ß-CD led to a dramatic improvement in Chol homeostasis in Niemann–Pick type C (NPC) fibroblasts, and reduced inflammation and significant prolongation of lifespan in aNPC mouse model (21–24). This drug is currently under investigation in a clinical trial for NPC disease (http://clinicaltrials.gov/show/NCT01747135).

Here, we provide data indicating that BCM-95 and HP-ß-CD, individually, increase Cath B and Cath D expression and activity, rescue the impaired autophagic substrate degradation, and promote clearance of Chol and Cer deposits in Sap C-deficient fibroblasts. These results provide novel insights onto the different properties of BCM-95 and HP-ß-CD in improving lysosomal functionality, making them potential therapeutic agents in Sap C deficiency.

Results

Effects of BCM-95 and HP-ß-CD on cell viability, apoptosis and morphology of Sap C-deficient fibroblasts

Before exploring the pharmacological effects of BCM-95 and HP-ß-CD in Sap C-deficient fibroblasts, toxicity of these compounds on cell viability and apoptosis was examined. Sap C-deficient (P1 and P2), GCase-deficient GD and control (C) cells were treated with different concentrations (2.5–10 μM) of BCM-95 (referred to the curcumin content) for 24 h, and a single concentration (300 μM) of HP-ß-CD for 24, 48 and 72 h, as previously reported by Rosenbaum et al. (22), and cell viability was measured by MTS assay. BCM-95 treatment at high concentrations (10 μM) was demonstrated to affect viability of Sap C-deficient cell lines, whereas HP-ß-CD was not cytotoxic (Fig. 1A). The subG1 population of Sap C-deficient cells was not appreciably increased following BCM-95 or HP-ß-CD treatment (Fig. 1B), as shown by FACS quantification. These results indicated that apoptosis did not account for the decreased (35–40%) cell viability observed via MTS assay in 10 μM BCM-95 treated cells, suggesting a possible effect on suppression of cell proliferation. Prolonged exposure to BCM-95 at concentration range of 2.5–7.5 μM did not affect viability or induce apoptosis in control and patients fibroblasts (data not shown). At the same time, no difference in cellular morphology was observed by phase-contrast microscopy between cell lines left untreated or treated with the highest BCM-95 concentration (10 μM) and with the longest HP-ß-CD exposure time (72 h) (Fig. 1C).

BCM-95 treatment increases Cath B and Cath D expression level and enzymatic activity in Sap C-deficient fibroblasts

Recent studies indicated that the antifibrotic effects of curcumin are associated with overexpression of Cath K and Cath L in human lung fibroblasts (25). Based on these observations, the effect of BCM-95 treatment (2.5–10 μM, 24 h) on Cath B and Cath D expression was evaluated in control, GD and Sap C-deficient cell lines by immunofluorescence microscopy. As previously reported, the level of both proteases in untreated P1 and P2 cells resulted significantly reduced compared with what observed in control and GD fibroblasts. Such level, however, increased after treatment with BCM-95 proportionally to the dosage until the concentration of 5 μM, while it was less evident at higher doses. Addition of the compound did not produce significant increase of Cath B and Cath D expression in control and GD fibroblasts (Fig. 2A and B). The localization of these two proteases was visualized by double immunostaining utilizing Lamp 2, as a lysosomal marker, which confirmed colocalization of Cath B and Cath D with Lamp 2 before and after BCM-95 treatment. The comparison of the overlaid images also confirmed the increase of Cath B and Cath D levels after incubation with BCM-95 (Fig. 2C and D). Based on these findings, the concentration of 5 μM was considered for all the following experiments. The effect of BCM-95 on Cath B and Cath D level was also analyzed by western blot analysis. Both proteases showed a robust increase (∼50%, Cath B; ∼85%, Cath D) in P1 and P2 cells. No effect was observed in control and GD fibroblasts (Fig. 2E and F). Consistently, enhanced enzymatic activity, particularly of Cath B, was observed in P1 and P2 cells, while no significant change in Cath B and Cath D enzymatic activity was observed in control and GD cells (Fig. 2G and H). Cath B level and enzymatic activity were almost doubled in GD fibroblasts compared with control fibroblasts; these observations are consistent with our previously reported results (13). Overall, these data indicate that BCM-95 enhances both the expression level and enzymatic activity of the two lysosomal enzymes in Sap C-deficient cells.

HP-ß-CD treatment increases Cath B and Cath D expression level and enzymatic activity in Sap C-deficient fibroblasts

In our past work, we observed a secondary Chol accumulation in the endo/lysosomal compartment in Sap C-deficient cells (12). It has been reported that HP-ß-CD has the function to lower unesterified Chol stored in lysosomes (20), but there is no information
regards its possible modulatory role on lysosomal proteases function. Control, GD and Sap C-deficient fibroblasts were treated with HP-β-CD (300 μM, 24 h) to explore its effect on Cath B and Cath D expression and activity. Immunofluorescence analysis revealed that HP-β-CD-treated P1 and P2 fibroblasts displayed a higher level of both proteases as compared with untreated cells.
Figure 2. BCM-95 treatment increases Cath B and Cath D protein level, and enzymatic activity in Sap C-deficient fibroblasts. (A and B) Control, GD and Sap C-deficient P1 and P2 fibroblasts untreated and treated with different amounts of BCM-95 for 24 h were immunostained with Cath B antibody (green) (A) or Cath D antibody (red) (B). Nuclei were labeled with Hoechst. Scale bar, 10 μm. Quantification of Cath B (A) or Cath D (B) particles for cell was shown on the right. Graphical data are expressed as mean ± S.D. (**P < 0.01). (C and D) P1 and P2 cells were double-immunostained for Cath B (red) and Lamp 2 (green) (C) or for Cath D (green) and Lamp 2 (red) (D) before and after treatment with BCM-95 5 μM. (E and F) Representative western blots and densitometric analyses of Cath B (E) and Cath D (F) in untreated and treated (5 μM BCM-95 for 24 h) control, GD and Sap C-deficient cells, with actin as loading control. (G and H) Enzymatic activity of Cath B (G) and Cath D (H) in untreated and treated (5 μM BCM-95 for 24 h) control, GD and Sap C-deficient cells. Data refer to three independent experiments performed.
Figure 3. HP-β-CD treatment increases Cath B and Cath D protein level, and enzymatic activity in Sap C-deficient fibroblasts. (A and B) Control, GD and Sap C-deficient (P1 and P2) fibroblasts untreated and treated with 300 μM HP-β-CD for 24 h were immunostained with Cath B antibody (green) (A) and Cath D antibody (red) (B). Nuclei were labeled with Hoechst. Scale bars, 10 μm. Quantification of Cath B (A) or Cath D (B) particles for cell was shown on the right. Graphical data are expressed as mean ± S.D. (**P < 0.01). (C and D) P1 and P2 cells were double-immunostained for Cath B (red) and Lamp 2 (green) (C) or for Cath D (green) and Lamp 2 (red) (D) before and after treatment with 300 μM HP-β-CD. Scale bars, 10 μm. (E and F) Representative western blots and densitometric analyses of Cath B (E) and Cath D (F) in untreated and treated (300 μM HP-β-CD for 24 h) control, GD and Sap C-deficient cells, with actin as loading control. (G and H) Enzymatic activity of Cath B (G) and Cath D (H) in untreated and treated (300 μM HP-β-CD for 24 h) control, GD and Sap C-deficient cells. Data refer to three independent experiments performed.
(Fig. 3A and B). No effect was observed in control and GD fibroblasts. Immunostaining experiments performed to compare the intracellular distribution of Lamp 2 and Cath B (Fig. 3C) or Cath D (Fig. 3D) before and after HP-β-CD addition confirmed that both Caths almost completely colocalized with the lysosomal marker, and that the amount of Cath B and Cath D increased after treatment. To confirm these results, the level and enzymatic activity of both enzymes were also examined. Western blot analysis showed a significant increase of Cath B and Cath D in both cell lines (Fig. 3E and F). The enzymatic activity of Cath B increased about 55% in P1 and 71% in P2 fibroblasts, which were comparable values to those obtained after BCM-95 treatment (Fig. 3G). An increase of Cath D enzymatic activity in P1 (~44%) and more consistent (~130%) in P2 cell lines was also observed (Fig. 3H). There were no significant changes in HP-β-CD-treated control and GD fibroblasts. These findings provided first evidence that HP-β-CD has the capacity to enhance the level and enzymatic activity of Cath B and Cath D in Sap C-deficient fibroblasts. Remarkably, such an effect on Cath D activity was much more evident than that observed following BCM-95 treatment.

**BCM-95 or HP-β-CD treatment promotes autolysosome degradation and lysosome reformation in Sap C-deficient fibroblasts**

We previously showed that the reduction of the amount and enzymatic activity of Cath B and Cath D lead to defective autophagy in Sap C-deficient fibroblasts (13). To investigate whether the enhancement of Cath B and Cath D level and enzymatic activity, promoted by treatment with either BCM-95 or HP-β-CD, could restore the autophagic flux, the expression levels and turnover of microtubule-associated protein 1 light chain 3 (LC3) and p62, also called sequestosome 1 (SQSTM1), were monitored in Sap C-deficient cell lines. The assay is based on the observation that LC3-II, a specific marker of autophagosomes and p62, one of the best-known autophagic substrates, are degraded in autolysosomes. As expected, treatment with inhibitors of lysosomal proteases, such as leupeptin, blocked the degradation of LC3-II and p62, resulting in their accumulation (26,27). Untreated and drug-treated cells were incubated for 24 h with leupeptin (10 μg/ml) followed by short recovery time for LC3 and longer recovery for p62 in leupeptin-free medium. As shown in Figure 4A and B, LC3-II and p62 expression was elevated in all samples treated with leupeptin; after recovery, LC3-II and p62 levels were dramatically reduced in untreated and drug-treated GD and control fibroblasts, while their amount was aberrantly persistent in untreated Sap C-deficient cells. Remarkably, in these cells, LC3-II and p62 levels were significantly reduced after recovery in BCM-95 (24 h incubation) or HP-β-CD (72 h incubation), suggesting the capability of these molecules to rescue the autophagic flux. To further confirm these findings, we used a proteolytic assay directed to measure red Bodipy dye conjugated to bovine serum albumin (DQ-BSA) (28) as an LC3-independent readout for lysosome function. Control, GD, P1 and P2 cells incubated with DQ-BSA (10 μg/ml) in complete medium showed comparable lysosome proteolytic activity (Fig. 5, left panels). A different behavior was observed among cell lines upon 12 h of starvation in Earle’s Balanced Salt Solution (EBSS) medium. While control and GD cells remained still positive for DQ-BSA, showing an intense punctate pattern, Sap C-deficient cells exhibited a dramatically reduced positivity for DQ-BSA (Fig. 5, second row on the left), indicating inefficient lysosomal proteolytic activity (13). Treatment with BCM-95 (Fig. 5, third row) or HP-β-CD (Fig. 5, fourth row) rescued cell response to starvation. Specifically, cells incubated with DQ-BSA remained positive upon 12 h in EBSS medium, with an intense punctate staining filling the whole cytoplasm, providing evidence for a reactivated lysosomal degradation capacity in treated cells. Finally, to determine whether drug treatment could also restore lysosome reformation, which is a cellular event occurring during the terminal stage of autophagy, analysis of intracellular lysosomal positioning in response to starvation was performed. It has been reported that lysosomes are diffuse peripherally into the cytoplasm in basal conditions, whereas perinuclear clustering of these structures occurs during starvation, being this position of lysosomes optimal for fusion with incoming autophagosomes to generate autolysosomes. Their degradation leads to reformation of new lysosomes dispersed into the cytoplasm (29). Untreated and treated cells (24 h incubation for BCM-95 and 72 h for HP-β-CD) were starved for different times (4, 8, 16 h), and then immunostained with Lamp 2. Lamp 2 positive lysosomes appeared dispersed widely throughout the cytoplasm in basal conditions in all untreated and treated cell lines (Fig. 6, first row on the left), whereas lysosomes were aggregated in the perinuclear region after 4 h of starvation (Fig. 6, second row on the left). Restoration of peripheral lysosome localization began after 8 h, and was complete after 16 h of starvation in control and GD cells (Fig. 6, first and second lines). Conversely, the perinuclear position of lysosomes lasted until 16 h of starvation in both untreated Sap C-deficient cell lines (Fig. 6, third and sixth lines). Treatment of Sap C-deficient fibroblasts with BCM-95 (Fig. 6, fourth and seventh lines) or HP-β-CD (Fig. 6, fifth and eighth lines), resulted in a substantial relocalization of lysosomes that appeared distributed peripherally in the cytosol after 8 h of starvation, similarly to what observed in control cells. These observations suggest that both the utilized drugs rescue autolysosome degradation and lysosome reformation in P1 and P2 fibroblasts.

Similar observations were attained using higher concentrations of HP-β-CD (700, 1400 μM), whereas the duration of treatment makes the difference (72 h instead of 24 h).

**BCM-95 or HP-β-CD treatment depletes lysosomal Chol and Cer storage in Sap C-deficient fibroblasts**

Since addition of HP-β-CD has been shown to rescue unesterified Chol and glycosphingolipids accumulation in NPC fibroblasts (22,30), we examined whether this treatment would mediate depletion of Chol stored in GD and Sap C-deficient fibroblasts (12). Cells were cultured in basal medium with or without HP-β-CD for 24, 48 and 72 h, and Chol content was analyzed by filipin staining. As expected, untreated GD, P1 and P2 cells appeared positive (Fig. 7A, left panels), indicating aberrant Chol storage, while the same cells treated with HP-β-CD showed an almost complete reduction in staining intensity after 24 h (Fig. 7A, middle panels). Similar results were obtained for stored Cer, with a marked immunofluorescence observed in both untreated pathological cell lines (Fig. 7B, left panels), and no staining after 24 h of incubation with HP-β-CD (Fig. 7B, middle panels). The Chol and Cer level in the control fibroblasts and the Cer level in GD cells were below the detection limit.

It has been reported that curcumin has hypocholesterolemic effects by changing the expression of genes involved in Chol homeostasis (31). Moreover, elevated lipid levels in NPC1 mouse brain were observed to be reduced after curcumin treatment (32). Recently, it has also been demonstrated that curcumin ameliorates the U18666A-induced endo-lysosomal cholesterol accumulation by shuttling cholesterol and presumably other lipids out of the cell via exosomes/microvesicles secretion (33). These findings...
prompted us to investigate whether BCM-95 could have also the ability to reduce lysosomal Chol and Cer accumulation. GD, P1 and P2 fibroblasts were treated with this molecule for 24, 48 and 72 h, or left untreated. Remarkably, Chol (in GD, P1 and P2 cells) and Cer (in P1 and P2 cells) almost completely disappeared after 72 h of treatment (Fig. 7A and B, right panels, respectively). Of note, the effect of the two drugs exhibited a different kinetics since lipids decreased more rapidly following treatment with HP-β-CD compared with BCM-95. Moreover, HP-β-CD required a lower incubation time (24 h) and its action lasted for more than
three days in absence of further treatment. Overall, our findings provide evidence that both agents efficiently promote depletion of Chol and Cer deposits in Sap C-deficient cell lines.

Discussion

Since 1994 the effective treatment for GD patients is the enzyme replacement therapy (ERT), based on periodical infusion of recombinant GCase. This therapy reverses visceral disease progression, even though it is not efficacious in treating neuronopathic complications because the enzyme does not pass the blood–brain barrier. An alternative to ERT is SRT, which works by lowering the rate of synthesis of all GC-based glycosphingolipids. The goal of both treatments is to reduce GC storage. No efficient therapy is currently available for Sap C-deficiency. SRT treatment resulted unsuccessful in one treated patient (15). Two types of Sap C-deficient mice have been generated (34,35), but both models present a less severe phenotype compared with their human counterpart. This could be attributed to differences in lipid metabolism between species (35). The lack of bona fide animal models, and the few reported cases worldwide are significant factors that contribute to the dearth of treatment options available for this very rare disease.

Here, we evaluated the impact of pharmacological intervention with BCM-95, a novel bio-enhanced derivative of curcumin, and HP-β-CD in primary Sap C-deficient fibroblasts. Generally, the choice of a drug is based on its safety and potential efficacy that can vary depending on its concentration, period of exposure and targeted cell type. Curcumin has a safety profile but presents some drawbacks, including poor solubility, due to its lipophilic nature, and low bioavailability, due to its poor absorption, rapid metabolism (36). Instead, BCM-95 has a longer retention time, and its bioavailability is more than seven times higher than curcumin (16). Based on these considerations, the optimal concentration and exposure time of both compounds necessary to obtain a biochemical readout were analyzed. Preliminary experiments, indicated that BCM-95 is more stable than curcumin, as previously reported (16). This compound has been utilized as chemotherapeutic drug in human colon cancer cells (37) and in a clinical trial as antidepressant in patients with major depressive disorder (38). HP-β-CD has been reported to be neurotoxic at high doses (39), whereas has a safety profile at low doses. It has been demonstrated that treatment with low doses partially depletes unesterified Chol in NPC fibroblasts (40). Of note, lipid storage in Sap C-deficient cell lines is, usually, less marked than in NPC cells, and low doses of this compound were shown to be sufficient for complete Chol removal in Sap C-deficient fibroblasts. Recently, Song et al. have shown that low doses of HP-β-CD have also effect in lowering autofluorescent ceroid lipopigment in fibroblasts from a patient with late infantile neuronal lipofuscinosis (41).

We provided evidence for the first time that treatment with BCM-95 or HP-β-CD induces an enhancement of Cath B and Cath D expression and enzymatic activity in Sap C-deficient
The increase of both proteases was observed to lead to a normalization of autolysosome degradation and lysosome reformation, reversing dysfunctional autophagy in pathological cell lines. We also documented that both agents efficiently deplete lysosomal Chol and Cer deposits. Remarkably, both compounds exhibited the same effects in removal lipid storage, and rescuing autophagy impairment, even though they possibly operate on distinct processes. Based on experimental evidence, we hypothesize that BCM-95 is primarily implicated in promoting Cath B and Cath D upregulation, and indirectly in autophagic progression recovery and lipid storage clearance. This view is corroborated by previous data obtained after transient overexpression of Cath B and Cath D in Sap C-deficient fibroblasts (13). On the other hand, HP-β-CD is expected to promote lysosomal Chol and Cer clearance, making lysosomes more functional. This in turn possibly results in a more efficient autophagic flux. This picture is reinforced by the fact that BCM-95 normalizes the autophagic clearance after 24 h treatment, whereas HP-β-CD requires more time (72 h) despite being more effective in increasing Cath D activity in P2 cells. Consistently, HP-β-CD was observed to remove lipids quickly, with an effect visible after 24 h and lasting for up to three days, whereas BCM-95 depletes lipids...
only after 72 h, and must be added every day. Of note, no synergistic or additive effects of the combination of the two drugs were observed in pathological fibroblasts.

These findings suggest a novel role of BCM-95 and HP-β-CD in rescuing autophagy by increasing Cath B and Cath D and depleting Chol and Cer accumulated in lysosomal compartment (Fig. 8). The reported biochemical and cellular observations provide evidence that these two small molecules represent potentially effective drugs for treatment of Sap C deficiency. Taken together, the present data are consistent with the concept that BCM-95 or HP-β-CD therapy could be used to treat clinically relevant secondary pathological aspects in Sap C deficiency. The ability of these two agents to enhance lysosomal function could be used to achieve new therapies for the treatment of other lysosomal storage diseases.

Figure 7. HP-β-CD or BCM-95 treatment decreases stored lysosomal Chol and Cer in Sap C-deficient fibroblasts. GD, P1 and P2 fibroblasts, untreated or treated with 300 μM HP-β-CD (incubation for 24 h) or 5 μM BCM-95 (incubation for 72 h with the addition of drug every 24 h), were cytochemically stained with filipin for Chol (A); P1 and P2 cells were also immunostained for Cer (B). Nuclei were stained with propidium iodide (A) or Hoechst dye (B). Scale bars, 10 μm.
Materials and Methods

Reagents

Dulbecco Modified Eagle’s Medium (DMEM) (Gibco, 31966) and EBSS (Gibco, 24010–043) were purchased from Life Technologies. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS) (C5070), filipin (F9765), HP-β-CD (H107) and leupeptin (L2884) were obtained from Sigma. CellTiter 96® AQueous One Solution Cell Proliferation Assay (G3580) was purchased by Promega. BCM-95 (Eurochem Feinchemie GMBH, 340069) was a kind gift of Oliver Schultz, managing director of Eurochem Feinchemie GMBH.

Antibodies

Antibodies used in this study were against: actin (Sigma, A3853), Cath B (Santa Cruz, sc-6493), Cath D (Santa Cruz, sc-6487), ceramide (Glycobiotech, clone S58–9), Lamp 2 (Developmental Studies Hybridoma Bank, H4B4), LC3 (MBL, PM036) and p62/SQSTM1 (Santa Cruz, sc-28359). Goat anti-mouse (A21042 or A11005), goat anti-rabbit (A11008) and rabbit anti-goat (A11078 or A11080) secondary antibodies conjugated to Alexa Fluor 488 or 594 were purchased from Molecular Probes, while HRP-conjugated goat anti-rabbit (31460), rabbit anti-mouse (31450) and rabbit anti-goat (31402) antibodies were from Thermo Scientific.

Cell culture and treatment

Two variant GD fibroblast lines, from Sap C-deficient patient P1 (PSAPp.del.FDKMCSK342–348/del.FDKMCSK342–348) and P2 (PSAPp.M1V/C315S) were utilized in this study. Control fibroblasts were from apparently healthy age-matched subjects. Two GCase-deficient GD fibroblasts, one homozygous for the N370S mutation and one homozygous for L444P mutation, were used as controls for GD. All the experiments were performed in both the GD lines obtaining similar results, so we reported only those referred to the cell line L444P/L444P. Control, GD and Sap C-deficient cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml of penicillin and 100 µg/ml streptomycin, and maintained at 37°C in 5% CO2 incubator. The fibroblasts were used between passages 4 and 14. Experiments were carried out when cell monolayers were 80% confluent. BCM-95 (dissolved in DMSO as stock solution) was added at 2.5, 5.0, 7.5 or 10.0 µM, and HP-β-CD (dissolved in water and sterile-filtered) was added at 300 µM final concentration. The untreated cells were cultured in the same medium without the drug. To inhibit LC3-II degradation, cells were treated with leupeptin diluted in culture medium to a working concentration of 10 µg/ml for 24 h. To evaluate autophagy recovery, cells were washed twice with phosphate-buffered saline (PBS) after protease inhibitor treatment and leupeptin-free medium was added. To examine the reformation of lysosomes, cells were starved in EBSS for different times.

Cell death evaluation by MTS assay and by propidium iodide detection

Cells were seeded at a density of 5 x 10^4 cells per well in flat bottomed 96-well plates. After 24 h cells were treated as described in the text and in the figure legends. Finally, CellTiter 96® AQueous One Solution Reagent was added to each well according to the manufacturer’s instructions. After 2 h in culture, cell viability
was determined by measuring the absorbance at 490 nm using a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK).

Apoptosis was monitored by evaluating the hypodiploid cell population, after flow cytometric analysis. Cells were mechanically detached from the plates and centrifuged at 300 g for 5 min; pellets were washed with PBS, placed on ice, and overlaid with 0.5 ml of a solution containing 50 mg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate. After gentle resuspension in this solution, cells were left for 30 min at 4°C, in the absence of light, before analysis. Propidium iodide-stained cells were analyzed using a FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, USA); fluorescence was measured between 565 and 605 nm. The data were acquired and analyzed by the CELLQuest program (Becton Dickinson).

**Cell morphological examination**

Cell morphology of control and Sap C-deficient fibroblasts was analyzed by acquiring bright field images (20× objective) at the EVOS Fluid Cell Imaging Station (Life Technologies).

**Western blot analysis**

Cell samples were lysed with 50 mM Tris-base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, pH 7.4, containing one tablet of protease inhibitor mixture/10 ml (Complete™, Roche Diagnostics, 04693159001). Lysates were precleared by centrifugation at 10 000 g for 15 min at 4°C, and the supernatants used as fibroblast homogenates. Proteins (10–20 μg) were resuspended in Laemmli buffer, loaded on SDS–PAGE, and then transferred on polyvinylidene difluoride membranes (Bio-Rad, 170–4156). Membranes were probed with the indicated antibody. Target proteins were examined using enhanced chemiluminescence reagents (GE Healthcare, RPN 2232). Band intensity was determined by densitometry using Alphaview SA software (Protein Simple).

**Fluorescence and immunofluorescence analysis**

Cells were grown on Labtech chamber slides (Nunc, Naperville, IL, USA) and fixed with 4% paraformaldehyde in PBS. For intracellular free unesterified Chol staining, fixed cells were incubated 30 min with filipin solution (0.05% in PBS). Cells were observed with an UV 330–380 filter. For single immunofluorescence, cells were fixed with 4% paraformaldehyde, while for double immunostaining, were further post-fixed with absolute methanol. Fixed cells were rinsed with PBS, permeabilized 7 min with 0.05% saponin and incubated with 3% BSA for 2 h at room temperature. Cells were then incubated 1 h with a rabbit polyclonal (anti-Cath B), goat polyclonal (anti-Cath D) or mouse monoclonal (anti-ceramide, anti-Lamp 2) primary antibody, rinsed twice with PBS, and incubated 1 h with the secondary antibody (Alexa Fluor 488 or Alexa Fluor 594-conjugated goat anti-rabbit, goat antimouse or rabbit anti-goat). Finally, cells were rinsed twice with PBS, and the nuclear staining was performed with Hoechst 33342 (Sigma) 10 μg/ml in PBS for 5 min or with propidium iodide in presence of RNase I for 10 min at room temperature. After a final rinse with PBS, cells were mounted with ProLong antifade reagent and then analyzed by a fluorescence microscope (Olympus BX52) or a confocal microscope (Leica TCS SP2).

Expression of Cath B and Cath D before and after treatment was quantified using the Analyse Particle plugin in ImageJ (NIH). Quantification was carried out on at least 30 cells per condition from independent experiments. Lysosomal distribution was scored as described by Korolchuk et al. (29).

**Cath B and Cath D enzymatic activity**

Cell pellets were lysed in PBS containing 0.05% CHAPS (w/v) for 30 min on ice. After sonication for 20 s, lysates were centrifuged at 15 000 g for 15 min at 4°C. The resultant supernatants were used for enzymatic assays. The activity of Cath B was assayed using the fluorogenic substrate Z-Arg-Arg-AMC (Biomol International, BML-P137), as described by Barrett and Kirschke (42). The activity of Cath D was measured utilizing the fluorogenic substrate MCA-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(DNP)-D-Arg-NH2 (Calbiochem, 219360), as described by Yasuda et al. (43).

**Assay of lysosomal function by degradation of a chromogenic BSA**

A red BODIPY dye conjugated to bovine serum albumin (DQ-BSA) was used for the detection of the degradation capacity of lysosomes and autolysosomes (28). This BSA derivative is so heavily labeled that the fluorophore is self-quenched. Proteolysis of this compound results in dequenching and release of brightly fluorescent fragments. Control and pathological fibroblasts were grown on Labtech chamber slides, incubated with DQ-BSA (10 μg/ml) in complete culture medium for 1 h at 37°C. Cells were washed twice with PBS to remove excess probe and then incubated in starvation medium (EBSS) for 12 h at 37°C. After fixation in 4% paraformaldehyde, cells were immediately analyzed by a fluorescence microscope (Olympus BX52).

**Statistical analysis**

The data are expressed as mean ± standard deviation (S.D.) or standard error (s.e.m.). Statistical significance of differences was evaluated by Student's t test. Values of "P < 0.01 and "P < 0.05 were considered significant.

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**References**


