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Inhibitors of cathepsins B and L induce autophagy and cell death in neuroblastoma cells

Donna M. Cartledge, Rita Colella, Lisa Glazewski, Guizhen Lu, and Robert W. Mason
Nemours Biomedical Research, Alfred I. duPont Hospital for Children, 1600 Rockland Road,
Wilmington, Delaware 19803

Summary

This study was designed to test the hypothesis that specific inhibition of cathepsins B and L will cause death of neuroblastoma cells. Five compounds that differ in mode and rate of inhibition of these two enzymes were all shown to cause neuroblastoma cell death. Efficacy of the different compounds was related to their ability to inhibit the activity of the isolated enzymes. A dose- and time-response for induction of cell death was demonstrated for each compound. A proteomic study showed that inhibitor treatment caused an increase of markers of cell stress, including induction of levels of the autophagy marker, LC-3-II. Levels of this marker protein were highest at cytotoxic inhibitor concentrations, implicating autophagy in the cell death process. An in vivo mouse model showed that one of these inhibitors markedly impaired tumor growth. It is concluded that development of drugs to target these two proteases may provide a novel approach to treating neuroblastoma.

Keywords

Neuroblastoma; Cathepsin; Inhibitor; Autophagy

Introduction

Neuroblastoma is a unique cancer of childhood. Unlike most adult cancers that are caused by mutations that lead to de-differentiation and uncontrolled growth of cells, neuroblastoma is a developmental defect caused by failure of neural crest cells to differentiate into cells of the adrenal glands and peripheral nervous system[1,2]. Prognosis for infants is generally good and infants with a unique stage 4S neuroblastoma require little or no treatment and metastasized tumors spontaneously regress. It appears that although differentiation of neuroblasts in these patients is delayed, the tumors ultimately regress due to differentiation or apoptosis. The concept of inducing differentiation has led to the use of retinoic acid in treatment regimens for neuroblastoma[3]. Currently, the prognosis for patients who are diagnosed with metastatic neuroblastoma when they are two years or older is poor, with 5-year survival rates below 50%. In patients with poor prognosis, tumor cell proliferation continues and genetic instability leads to more aggressive disease. Subsets of neuroblastoma develop discrete genetic abnormalities such as N-myc amplification[4] and ALK activation mutations[5], some of which may lead to new treatments for sub-sets of patients[1].

Corresponding author: Robert W. Mason, 302-651-6885, Fax 302-651-6767, rmason@nemours.org.

Current addresses: RC, Department of Anatomical Sciences & Neurobiology, University of Louisville School of Medicine, Louisville, KY 40292.

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A significant barrier to developing new therapeutics for children with cancer is the low numbers of patients compared to the much larger incidence of cancers in adults. Consequently identification of common features of abnormally proliferating neuroblasts may provide better targets for development of treatments for neuroblastoma. In a major breakthrough, an immunotherapy that targets a neuroblast cell surface marker, ganglioside GD2, has shown a dramatic improvement in survival for advanced stage neuroblastoma patients [6]. There are significant side-effects due to expression of the marker on normal nerve cells, but the associated pain can be controlled by medication. This study clearly demonstrates the value and efficacy of targeting features that are common to all neuroblastoma tumors. We have developed a hypothesis that targeting two lysosomal proteases, cathepsins B and L, may offer a novel approach to treating neuroblastoma. Deletion of cathepsins B or L in mice causes no overt neurological defect whereas deletion of both enzymes results in rapid on-set neurodegeneration shortly after birth[7]. Induction of cell death is restricted to cells of the nervous system and proliferating granule cells within the cerebellum are particularly affected[7]. An inhibitor of cathepsins B and L, Z-Phe-Ala-CHN₂, specifically causes apoptosis of neuroepithelial and neural crest cells in developing embryos[8]. Lack of embryonic defects in cathepsin B and L knockout animals may be due to compensatory effects of one or more of the 8 additional cathepsins that are uniquely expressed in the rodent placenta[9,10]. After the proliferative stage of neuronal development, cathepsin inhibition is well tolerated. Inhibitors of cathepsins B and L have no significant toxicity to mature rodent cells and tissues[11,12], and actually protect mature brain tissue from ischemia[13]. For neuronal tissue development, the neonatal stage in mice corresponds to the third trimester fetal stage in humans [14,15] so specific inhibition of cathepsins B and L may selectively cause death of abnormally proliferating neuronal cells, sparing maturing neuronal cells and non-neuronal proliferating cells. We have shown that the cathepsin B and L inhibitor Fmoc-Tyr-Ala-CHN₂ (FYAD) specifically causes apoptosis of neuroblastoma cells[16]. In this study we show that a range of cathepsin inhibitors cause death of neuroblastoma cells and that ability to induce cell death is directly related to ability of each compound to inhibit cathepsins B and L and induce accumulation of the marker of autophagy, LC3-II. The potential value of targeting cathepsins B and L to treat neuroblastoma is supported by a preclinical *in vivo* model.

Materials and Methods

Neuroblastoma cell lines

Neuroblastoma cell lines SK-N-SH and IMR-32 were maintained in Minimum Essential Medium (MEM) supplemented with 1% final concentrations of non-essential amino acids and sodium pyruvate, and contained a 10% final concentration of fetal bovine serum (FBS).

Cathepsin inhibitors

FYAD is a specific irreversible inhibitor of cathepsins B and L developed in the Mason lab[17,18] and now available from Bachem (Torrance, CA). (3R,6S,8R)-8-(4-Bromophenyl)-6-(2-fluoro-2-methylpropyl)-5-oxo-8-(trifluoromethyl)-1-thia-4,7-diazacycloundec-9-yne-3-carbonitrile (U.S. patent application 12/532,652, L-264); N-(1-(((cyanomethyl)amino)carbonyl)cyclohexyl)-4-(2-(4-methyl-piperazin-1-yl)-1,3-thiazol-4-yl)benzamide (L-006235)[11]; and N-(1-(((cyanomethyl)amino)carbonyl)-2-ethyl-(3,5dimethylbenzyl))-4-(2-(4-methyl-piperazin-1-yl)-1,3-thiazol-4-yl)benzamide (L-625) were a gift from M. David Percival (Merck-Frosst, Canada). N-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl (K11777) was a gift from James McKerrow (University of California, San Francisco). Chemical structures of the inhibitors are shown (Fig 1).

Quantitative assessments of cell viability

Cathepsin inhibitor-induced cytotoxicity was measured using the cell titer blue viability assay (Promega, Madison, WI). Neuroblastoma cells were cultured in 24-well or 96-well plates. Cells seeded at 50% confluence were incubated at 37°C with 5% CO₂ for 24 h to allow cell attachment to plates. Inhibitors or vehicle controls were then added and cells were cultured for up to 8 more days. Media was changed every 3 days. At each time point, cell titer blue (5 µl of 1:5 PBS-diluted reagent per 100 µl media, equivalent to 1% final concentration) was added to each well and incubated for 4 h at 37°C. Fluorescence intensity was then measured (535/595 nm, excitation/emission). Data shown are representative of the mean $\pm$ standard deviation (SD) for multiple samples with statistical significance calculated using two-tailed type-two Student's t-test.

Western blotting

Total cellular proteins were dissolved in 7 M urea, 2 M thiourea, 1% chaps lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and/or protease and phosphatase inhibitor cocktails diluted to 1X (Sigma-Aldrich, Saint Louis, MO). Equal amounts of protein (20–30 µg/lane) were separated by SDS/PAGE electrophoresis and were transferred onto Immobilon-P PVDF membranes (Millipore, Bedford, MA). Proteins were identified by immunoblotting with the following antibodies: β -actin (A5441, Sigma, St Louis, MO), calreticulin (56259, QED Biosciences, San Diego, CA), Gp-96 (36–2600, Invitrogen, S. San Francisco, CA) and LC-3 (3868, Cell Signaling, Danvers, MA). Western blot membranes were probed with anti- β -actin antibodies as a control for protein loading. A solution consisting of 200 mM glycine, 0.1% SDS and 1% Tween-20 at pH 2.2 was used to strip membranes prior to re-probing with different primary antibodies.

Cell Fractionation

Cells were broken by homogenization in 250 mM sucrose, 5 mM Tris, 1 mM MgCl₂, pH 7.2 in a glass Potter-type homogenizer. The homogenate was centrifuged at 1500 g and 4°C for 2 min. The pellet was washed in fresh sucrose solution to improve purity of the nuclear pellet. The supernatant was re-centrifuged to pellet contaminating nuclear components and then centrifuged at 3000 g and 4°C for 15 min. The pellet from this centrifugation was washed with sucrose to obtain dense granules. The supernatant was re-centrifuged to pellet contaminating dense granules to obtain a cytosol fraction with low density endosomes.

DIGE analysis of proteins

SK-N-SH cells were treated with 5 µM FYAD for 2 days. Treated and control cells from either whole cell lysates and dense granules from treated and control cells were dissolved in DIGE lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5) and labeled with dyes as described previously[19]. Control and FYAD treated samples labeled with different dyes from either whole cells or dense granules were combined and then separated by 2D gel electrophoresis. Protein spots were quantified by scanning on a Typhoon Trio Scanner and by use of DeCyder 2-D Differential Analysis Software 6.5 to determine changes in levels of proteins.

Trypsin Digestion and Mass Spectrometry

Spots of interest were removed from the gel with a OneTouch Plus Spotpicker. Excised spots were digested with trypsin as described previously[19]. Tryptic peptides were isolated and concentrated using C₁₈ ZipTips (Millipore, Billerica, MA) prior to spotting onto MALDI plates in matrix solution (5 mg/ml α -cyano-4-hydroxycinnamic acid, 70% ACN, 0.1% TFA). Mass analysis was determined on ABI 4700 MALDI/TOF-TOF Proteomics

Analyzer and proteins identified using Mascot software. Only proteins identified with a minimum Mascot score of 100 and 95% confidence are listed.

Xenograft model of neuroblastoma

SCID hairless sho strain code 474 mice were injected s.c. with 10^7 SK-N-SH cells. After 35 days, tumors were injected twice daily with FYAD (25 mg/kg/day) in 100 μ l hydroxypropyl beta cyclodextrin (HPD) solution (45% HPD, 10% DMSO in PBS) or vehicle control for 10 consecutive days. This dose was at the limit of solubility of FYAD for a 100 μ l injection. Tumors were measured with calipers and tumor volume calculated using a modified ellipsoid formula $1/2(\text{Length} \times \text{Width}^2)$. The experiment was terminated 20 days after initiation of treatment when tumors in control animals approached a volume of 1000 mm³. In separate experiments, balb/c mice were treated with FYAD or vehicle control (25 mg/kg) and then tissues extracted 6 h later to measure residual activity of cathepsins B and L using Z-Phe-Arg-NHMec substrate [20]. All experiments were approved under IACUC protocol # NBR-2005-006.

Results

Both reversible and irreversible inhibitors of cathepsins B and L decrease neuroblastoma cell viability

Reversible nitrile inhibitors originally developed in a program to target cathepsin K as a potential treatment for osteoporosis show a concentration and time dependent effect on viability of neuroblastoma cells (Fig 2). We determined the dose of inhibitors required to cause 50% death of SK-N-SH cells after 72 h of exposure for L-006235, L-625, L-264, K11777 and FYAD to be 180, 15, 16, 22 and 10 μ M, respectively. L-006235, which is a tight binding inhibitor of cathepsin K (IC₅₀ 0.2 nM) that binds weakly to cathepsins B and L (IC₅₀ 1.1 and 6.3 μ M, respectively)[12], only caused cell death at high concentrations (over 100 μ M). Furthermore, after 8 days of treatment with 180 μ M L-006235, viable SK-N-SH cells still remained (Fig 2C). L-625, a tighter binding inhibitor of cathepsins B and L (IC₅₀ 5.1 and 32 nM, respectively, M. David Percival, unpublished results) was much more effective against SK-N-SH cells, with the majority of cells dying after 3 days treatment with 50 μ M inhibitor (Fig 2B). L-264, a non-basic inhibitor of cathepsins B and L (IC₅₀ 10 and 1.3 nM, respectively) was even more effective against SK-N-SH cells (Fig 2A). K11777, an irreversible inhibitor of cathepsins B and L that is currently being pursued as a potential treatment for Chagas disease by targeting structurally related parasitic proteases[21,22], also caused time- and concentration-dependent death of SK-N-SH cells (Fig 2D). All five inhibitors also caused cell death of the N-myc amplified neuroblastoma cell line, IMR-32 (Fig 2F and data not shown).

Proteomic analysis of proteins accumulating in cells and dense granules after inhibition of cathepsins B and L

In our original study showing that FYAD specifically caused apoptosis of neuroblastoma cells, electron microscopy showed accumulation of dense organelles[16]. FYAD treatment resulted in accumulation of several markers of cell stress, including heat shock proteins and chaperones (Table 1). These proteins were elevated within 48 h of inhibitor treatment, prior to induction of apoptosis of cells[16]. Cytoskeletal proteins such as actin, vimentin, tropomyosin and tubulin were less affected. Similar proteins were elevated in the dense organelles isolated from FYAD treated cells (Table 2). Two of these proteins, calreticulin and GP-96, are endoplasmic reticulum proteins that are primarily found in the cytosol/low density organelle fraction from untreated SK-N-SH cells (Fig 3). Levels of both proteins are enriched in FYAD-treated cells and a significant portion of the proteins are found in the dense organelle fraction.

Induction of autophagy by five different cathepsin inhibitors

Overall levels of cathepsin B are not significantly altered, although levels of the single chain form of the enzyme are increased relative to the heavy chain of the cleaved two-chain form of the enzyme as a result of FYAD treatment (Fig 3). Levels and cellular distribution of β -actin do not change upon FYAD treatment (Fig 3). Electron microscopic analysis of inhibitor treated cells had previously shown accumulation of double membrane compartments resembling autophagosomes[16] so we also probed the western blots for LC-3. On induction of autophagy, the C-terminus of LC-3 is lipidated, resulting in a product that migrates faster than the unprocessed protein in SDS/PAGE[23]. Control cells contained low levels of the unprocessed protein that was isolated in the cytosol/low density organelle fraction (Fig 3). FYAD treatment caused accumulation of the processed protein, most of which was found in the dense organelle or crude nuclear fractions. Increased levels of lipidated LC-3 were dependent on concentrations of inhibitors used in both SK-N-SH and IMR32 cells (Fig 4). All five inhibitors caused accumulation of modified LC-3 in both cell lines (Fig 4). Thus five different compounds that inhibit cathepsins B and L by three different mechanisms all have the same effect on neuroblastoma cells, causing cell death and accumulation of markers of autophagy.

Cathepsin inhibition and arrest of tumor growth *in vivo*

Treatment of sub-cutaneous tumors with FYAD twice daily for 10 days arrested tumor growth (Fig 5). During time of injections, measurement of tumor volume by calipers was complicated by the effect of injection volume at the tumor site and significant differences in volume were not detected in control and treated animals. However, after cessation of treatment, tumor volumes were significantly lower in treated animals than controls at all time-points. In separate experiments, tissues from treated and control animals were excised 6 h after administration of inhibitor to determine the effect of treatment on enzyme activity. In liver, kidney and brain tissue, cathepsin activity was inhibited by 72 $\pm$ 12%, 72 $\pm$ 7%, and 5 $\pm$ 3%, respectively. This indicates that *in vivo*, FYAD inhibits cathepsin activity by over 70% but is not effective against proteases in brain.

Discussion

Although neuroblastoma is a unique pediatric cancer, current therapies were originally developed for more common adult cancers and adapted for use in children. Unfortunately the major cytotoxic chemotherapies have severe side-effects in developing children and the response of children with advanced neuroblastoma to current therapies is poor. We found that an inhibitor of both cathepsins B and L, FYAD, caused selective cell death of neuroblastoma cells[16]. The primary goal of this study was to more rigorously test the hypothesis that inhibition of cathepsins B and L causes cell death in neuroblastoma cells using compounds that are already being developed in programs targeting similar enzymes as new therapeutic approaches to treat other ailments.

Efficacy of compounds in causing cell death is related to efficiency of cathepsin inhibition

L-006235 was the least effective inhibitor in causing death of SK-N-SH cells. This might be expected because although the IC_{50} for L-006235 for cathepsin K is 0.2 nM, it is a much weaker inhibitor of cathepsins B and L with IC_{50} of 1 and 6 μ M, respectively[24]. L-625 and L-264, are much tighter binding inhibitors of cathepsins B and L, with IC_{50} s in the nanomolar range. These two compounds caused neuroblastoma cell death at lower concentrations than needed for L-006235. K11777, an irreversible inhibitor that reacts with mammalian cathepsins B and L, was similar to FYAD in its ability to cause death of neuroblastoma cells. Thus the comparative sensitivity of neuroblastoma cells to these

compounds corresponded to the relative ability of each to hinder the activity of cathepsins B and L *in vitro*.

We have shown that complete inhibition (i.e. 100%) of both cathepsins B and L is achieved within 2–3 h after addition of the irreversible inhibitor, FYAD (10 μ M) to cultured cells[25]. For irreversible inhibitors, even very low concentrations of inhibitors should eventually block activity of cathepsins B and L, with steady-state levels of activity depending upon rate of inactivation and rate of synthesis of new enzyme. By reducing the concentration of FYAD and measuring residual cathepsin activity, it was shown that at least 90% inhibition of cathepsins must be achieved to cause SK-N-SH cell death[16]. The effectiveness of enzyme inhibition *in vivo* by reversible inhibitors is more difficult to determine. Cathepsin concentrations in lysosomes can be in the millimolar range[25], so mechanisms for increasing compound concentration in lysosomes are required to ensure effectiveness of reversible inhibitors. It has been shown that L-006235 competes with binding of cathepsins B and L to an irreversible inhibitor *in vivo* even though it binds weakly to these enzymes *in vitro*[12]. It was proposed that protonation of this compound could cause its accumulation in lysosomes, thereby increasing lysosomal concentration of inhibitor and subsequent inhibition of enzyme. However L-625, a tighter binding analog of L-006235 that can also be protonated, was no more effective in cell death induction than L-264 that is a similar tight-binding inhibitor that cannot be protonated in acidic conditions.

For tight binding reversible inhibitors, if diffusion equilibration across the cell into the lysosome results in a lysosomal inhibitor concentration that is significantly higher than its K_i , retention by protonation may not be as significant as retention by tight binding to enzymes. Binding to enzymes will reduce the concentration of free inhibitor and consequently diffusion equilibration will result in a net influx of inhibitor into the lysosome. If steady-equilibrium is achieved, 90% of the activity of cathepsins B and L will be inhibited by 60 μ M L-006235, which is similar to the concentration required to cause death of SK-N-SH cells experimentally. The calculated concentrations of L-625 and L-264 needed to cause 90% enzyme inhibition are 300 and 100 nM, respectively. Significantly higher concentrations were required to cause cell death by these two inhibitors. For these tight binding reversible inhibitors, the rate at which inhibitor diffuses into the lysosome may be the limiting step that prevents efficient enzyme inhibition. At lower concentrations, efficiency of inhibition of enzymes in lysosomes is probably regulated by a balance between slow rates of entry into lysosomes and synthesis of new enzyme, as proposed to explain incomplete inhibition of cathepsin B by low concentrations of the irreversible inhibitor FYAD[16]. The complex process of partitioning of inhibitors between extracellular compartments and sites of enzyme function will play a critical role in bioavailability and efficacy of inhibitors for treatment of neuroblastoma.

Efficacy of compounds in causing cell death is related to effect on autophagy

Proteomic studies showed that cell death was preceded by induction of markers of cell stress and autophagy. Efficacy of each inhibitor of cathepsins B and L in causing cell death corresponded with appearance of LC3-II, the lipidated form of LC-3, a key step in the formation of autophagosomes[26,27]. Treatment of cells with chloroquine and related compounds also results in up-regulation of markers of autophagy but these weak bases increase lysosomal pH and are proposed to result in leakage of lysosomal membranes, enabling lysosomal enzyme activity in the cytosol or nucleus to lead to cell death [28,29]. We did not previously find any evidence of lysosomal leakage by protease inhibition [16] but rather see accumulation of dense organelles that contain markers of cell stress and autophagy. Autophagy was originally discovered as a mechanism of cell survival during periods of nutrient deprivation but has more recently been recognized to be involved in cell death [30]. Although inhibition of cathepsins results in accumulation of markers of

autophagy, the cells are not under starvation conditions and cathepsin inhibition prevents degradation of proteins to provide amino acid nutrients. Consequently the initial effect of inhibition of cathepsins B and L will be to prevent turnover of proteins that are sequestered during constitutive autophagy. Accumulation of un-degraded proteins precedes induction of apoptosis [16], indicating that this perturbation of autophagy may be an early effect that ultimately leads to death. We conclude that the mechanism of selective neuroblastoma cell death by cathepsin inhibition is more likely due to perturbation of processing of as yet unidentified proteins in the lysosomal/autophagy pathway rather than a direct action of cathepsins released into the cytosol or a direct effect of cathepsin inhibition on apoptosis or induction of autophagy. Identification of the critical protein targets that accumulate and cause selective death of neuroblastoma cells would help understand the relationship between autophagy and cell death.

Cathepsin inhibition reduces neuroblastoma cell growth *in vivo*

FYAD was shown to inhibit growth of neuroblastoma tumors in a xenograft mouse model. FYAD inhibited activity of cathepsins B and L in mouse liver and kidney but does not appear to readily cross the blood brain barrier to inhibit cathepsins in the central nervous system. Neuroblastoma is derived from cells that normally develop into the peripheral nervous system and tumors rarely migrate into the CNS. Although tumor growth was impaired, the treatment protocol did not result in eradication of tumors. Cathepsin activity was inhibited by approximately 70% *in vivo* but our *in vitro* studies indicated that more than 90% inhibition is required to induce cell death [16]. Thus although a level of inhibition sufficient to reduce cell growth could be achieved, compounds that have better pharmacokinetic properties than FYAD are probably needed to eradicate neuroblastoma tumors.

The mechanism by which FYAD inhibited tumor growth in these *in vivo* experiments is not clear. Post-mortem examination of tissues failed to show accumulation of markers of autophagy (LC3-II) or apoptosis (cleaved PARP), supporting our *in vitro* observations that a higher degree of cathepsin inhibition is required to induce these effects [16]. The primary effect of FYAD *in vivo* appears to be slow tumor growth. Cathepsin inhibition restricts growth of many cancer cell types both *in vitro* and *in vivo* and may be caused by effects on cell proliferation, tumor cell invasion or angiogenesis [31–34]. While targeting these processes may provide a therapeutic approach to control tumor progression, there remains a need to develop therapies that cause selective cell death of cancer cells.

Inhibitors for cathepsins B and L for potential clinical use

The effect of FYAD on neuroblastoma cells both *in vivo* and *in vitro* provides proof of concept that inhibition of cathepsins B and L offers a potential novel therapeutic approach to treat neuroblastoma. A major limitation of this compound is its limited solubility that prevents complete enzyme inhibition *in vivo*. The panel of inhibitors used in this study was chosen because their bioavailability and efficacy have already been demonstrated in other clinical and preclinical programs that target similar proteases. The reversible nitrile inhibitors have been developed in a program to target cathepsin K to treat osteoporosis. A related specific inhibitor of cathepsin K is currently in phase 3 clinical trials[35]. K11777, an irreversible peptidyl vinyl sulfone inhibitor, is being developed to target cathepsin-like proteases of *Trypanosoma cruzi* as a novel treatment for Chagas disease [22]. Unlike standard chemotherapeutic approaches, these cathepsin inhibitors show remarkably low toxicity in pre-clinical animal studies and clinical trials [36,12]. Adoption of compounds from therapeutic studies that are already in clinical trials may accelerate the development of a new treatment for neuroblastoma. Further development of compounds such as K11777 or

the cathepsin K inhibitor analogs may provide drugs with better pharmacokinetics and become effective treatments for neuroblastoma.

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Fig. 1. Structures of cathepsin-inhibitory compounds. Fmoc-Tyr-Ala-diazomethane (FYAD) is a specific irreversible inhibitor of cathepsins B and L. L-006235, L-625 and L-264 are reversible inhibitors of cathepsins K, B and L. Each has a –CN group that binds tightly and reversibly to the active site cysteine of the enzymes. L-264 is a macrocyclic compound that was designed to improve stability and bioavailability of a cathepsin K inhibitor, but the modification reduced selectivity for cathepsin K over cathepsins B and L. K11777 a vinyl sulfone that, like FYAD, reacts covalently with the active site cysteine of cathepsins B and L.

Fig. 2.

Concentration and time-dependent effect of cathepsin inhibitors on neuroblastoma cell survival. **A–E, Left:** SK-N-SH cells were treated with L-264, L-625, K11777 or FYAD at 10–50 μM , with L-006235 at 100–300 μM or with DMSO vehicle control at 0.5%. **A–E, Right:** SK-N-SH cells were treated with L-264, L-625, L-006235, K11777, FYAD, or with DMSO vehicle control (0.22%) at approximated 72 h LD₅₀ doses (16, 15, 180, 22 and 10 μM , respectively). **F:** IMR-32 cells were treated with L-264 or L-625 (12.5–50 μM) or with DMSO vehicle control (0.5%). Media supplemented with individual inhibitors or with vehicle control was replaced every 3 days. At the end of incubation, viability was measured using cell titer blue. Graphical analysis represent mean $\pm$ standard deviation (SD) of quintuplicate (A–E, Left), octuplicate (A–E, Right) or quadruplicate (F) samples with readings normalized to vehicle control values. All inhibitors induced significant cellular death compared to cells treated with vehicle control at all concentrations ($p < 0.001$).

Fig. 3.

Expression of proteins in cells and dense fractions after FYAD treatment. Dense organelles were prepared from control and FYAD treated cells for western blot analysis. Relative levels of expression of Cathepsin B, two of the proteins found to increase after FYAD treatment in the proteomic study (calreticulin and GP96), LC-3 and β -actin were determined in treated and control cells. 50 μ g protein was loaded for each lane. Control and treated samples were separated on the same gel and transferred to the same blot to ensure that intensity of images reflects relative protein levels in the different samples. W represents whole cell protein; N, nuclear fraction; D, dense organelle fraction; and C, cytosol/light organelle fraction.

Fig. 4.

Expression of LC-3 in inhibitor treated neuroblastoma cells. A–B: Cells were treated with L-264 or L-625 (12.5–50 μ M), FYAD (10 μ M), or with DMSO vehicle control (0.5%). C–D: Cells were treated with L-264, L-625, L-006235, K11777, FYAD, or with DMSO vehicle control (0.22%) at approximated 72 h LD₅₀ doses (16, 15, 180, 22 and 10 μ M, respectively). Media supplemented with individual inhibitors or with vehicle control was replaced every 3 days. E: Cells were treated as indicated (L-264 at 25 μ M, L-625 at 35 μ M, L-006235 at 180 μ M, K11777 at 22 μ M, FYAD at 10 μ M or with DMSO at 0.35%). After 3 (A,B,E) or 6 (C,D) days total cellular lysates were collected and steady-state levels of LC-3 were determined by western blotting. β -actin was used as a loading control. Levels of lipidated LC-3 (LC-3-II) were augmented by all inhibitors in both neuroblastoma cell types.

Fig. 5.

Effect of FYAD on tumor growth *in vivo*: 10^7 SK-N-SH cells were injected subcutaneously into two groups of 10 hairless/SCID mice. When the first tumors were visible, vehicle or FYAD was injected at the tumor site and tumors measured daily with calipers. Tumors were significantly smaller in treated animals (* $p < 0.05$, ** $p < 0.01$).

Table 1

Changes in levels of proteins from whole cells. Proteins from DIGE gels were picked, digested with trypsin and identified by MALDI MS/MS. Fold change in proteins from FYAD treated cells compared to untreated cells are shown. For proteins represented by more than one spot, a range is shown.

Protein Name	Fold Change
BIP protein	4.4
GP-96	3.6
HSP 70	1.5–3.4
HSP 60	3.4
protein disulphide isomerase	2.9
calreticulin	2.7
prohibitin	2.7
ubiquitin carboxyl terminal esterase L1	2.4
calumenin	2.4
prolyl 4-hydroxylase	2.4
lactate dehydrogenase	2.0
nucleoside diphosphate kinase 1	1.9
triose phosphate isomerase	1.8
annexin V	1.7
tropomyosin	1.1–1.4
gamma actin	1.3–1.4
tyrosine 3-monooxygenase	1.3
tubulin	1.1
transitional ER ATPase	–1.1
HSP 90	–1.3
vimentin	–1.3–1.8

Table 2

Changes in levels of proteins in dense granules. Dense granules were isolated from FYAD treated and control cells and proteins compared by DIGE. Spots were picked, digested with trypsin and identified by MALDI MS/MS. Fold change in proteins from FYAD treated cells compared to untreated cells are shown. For proteins represented by more than one spot, a range is shown.

Protein Name	Fold Change
HSP 70	3.5
protein disulphide isomerase	3.4
HSP 60	1–3.4
BIP protein	3.3
GP-96	3.3
glucosidase II	2.2
lactate dehydrogenase	2.1
ATP synthase	2.0
protein disulphide isomerase	1.9
tubulin	1.8
tropomyosin	1.5
eef-2	1.4
gamma actin	–1.5–1.4
vimentin	–1.4
eif-4a1	–1.8