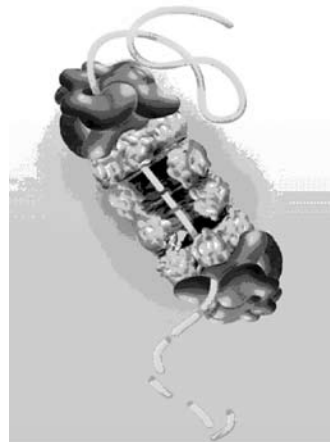


Augmentation and Reversal of the Apoptotic Effects of Proteasome Inhibition using RNAi

Esther Dubrovsky & Jennifer Fishe

Abstract

The 26S proteasome, a protease responsible for all non-lysosomal protein degradation, is vital for the cell cycle and cell survival. The proteasome inhibitor, lactacystin, is a common tool used to study proteasomal function. We used lactacystin and RNA interference (RNAi) methods to confirm the proteasome's function in the apoptotic pathway in *Drosophila* S2 cells. Proteasome inhibition induced apoptosis, as evidenced by a caspase activity assay and a subsequent rescue of cells transfected with the fly caspase DRONC. This observation has implications for new drug development, because manipulating apoptosis can be used to treat cancer or degenerative diseases such as Alzheimer's. In addition, proteasome inhibition in conjunction with a knockdown in DIAP1 showed an additive affect, suggesting a combination of both methods in cancer drug therapies.



Introduction

Apoptosis, or programmed cell death, occurs when the caspases, enzymes that break down peptide bonds, become active and dismantle vital proteins (Martin, 2002). Seven *Drosophila* caspases, DRONC, DCP-1, DRICE, DREDD, DECAP, DAMM, and STRICA have been identified, but only the first four play a demonstrable role in apoptosis (Kumar & Doumanis, 2000). It is possible to prevent apoptosis with inhibitor of apoptosis proteins (IAPs). DIAP1 is an IAP in *Drosophila* that marks caspases for destruction via ubiquitination. The ubiquitinated caspases are then drawn into the proteasome for degradation (Palaga & Osborne, 2002). Apoptosis can be activated by knocking down DIAP1 using RNAi, which initiates a signaling cascade (Martin, 2002). First, an initiator caspase such as DRONC is cleaved into its active form, which activates the other caspases. Because the proteasome also functions in the apoptotic pathway, it is believed that proteasome inhibition also leads to caspase accumulation and cell death (Daniel et al., 2005).

The *Drosophila* 26S proteasome is a protease consisting of two complexes, 20S and 19S. The 20S complex forms the catalytic core, which is insulated from the cytosol, and is capped by the 19S complex (Figure 1). The proteasome functions in two main steps: it recognizes proteins and then indiscriminately degrades them (Myung et al., 2001). Recognition is usually achieved through ubiquitination. Enzymes E1, E2, and E3 activate ubiquitin, determine the substrate protein, and attach the ubiquitin (Palaga & Osborne, 2002). The proteasome recognizes the polyubiquitin chain, unfolds the protein, degrades it into individual amino acids and ubiquitins, and releases everything back into the cytosol. This pathway (Figure 2) is known as the ubiquitin-proteasome-pathway (UPP) and also helps coordinate cell division (through destruction of cyclins), nuclear factor- α B activation, antigen presentation, cell differentiation, apoptosis, and organelle biogenesis (Adrain et al., 2004; Orłowski, 1999). Many proteasomal functions and target proteins were discovered using inhibitors such as lactacystin. Lactacystin is a very selective inhibitor that blocks chymotrypsin-like activity of subunit β 5 (Figure 1) and proteolysis (Fenteany et al., 1995), preventing caspase degradation. However, results vary with cell type, cell proliferation status, the amount of lactacystin, and the duration

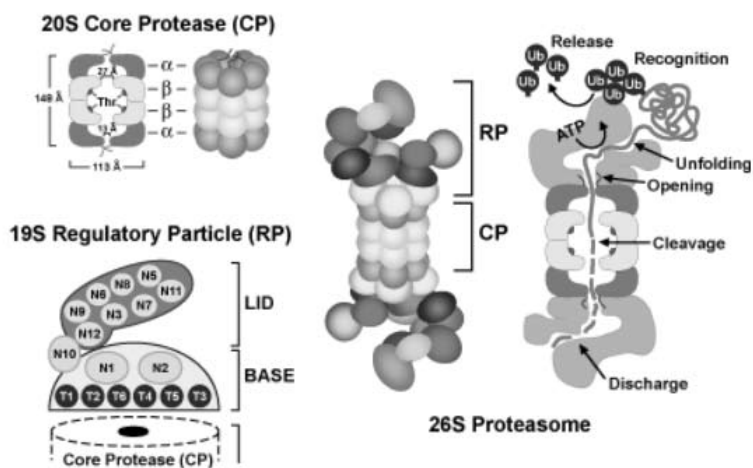


Figure 1. Drosophila 26S Proteasome
The 26S proteasome consists of the 20S catalytic core which is capped by the 19S complex. The 19S is necessary for the 20S to achieve the proper conformation for polyubiquitinated proteins to enter the proteasome. The 20S core is composed of 28 α and β subunits arranged in four stacked heptameric rings. The subunits form a hollow cylindrical structure that separates the catalytic inside from the cytosol (Groll & Huber, 2004).

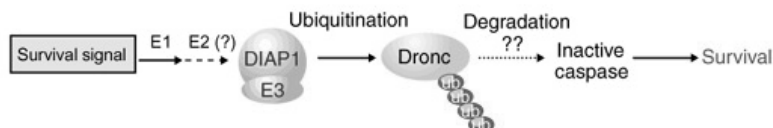


Figure 2. Ubiquitin Proteasome Pathway
When a cell receives a signal to survive, it activates DIAP1. DIAP1 allows the ubiquitinating enzymes to ubiquitinate caspases. The polyubiquitinated enzymes are recognized by the proteasome and degraded into individual amino acids and ubiquitins leading to cell survival.

of treatment (Groll & Huber, 2004). In this study, we used lactacystin to inhibit the proteasome and investigate its role in the apoptotic pathway.

Many cancers, such as multiple myeloma, exhibit a lack of apoptosis causing uncontrolled cell proliferation. Researchers have induced cell death and prevented malignant growth with proteasome inhibitors such as Bortezomib (Voorhees et al., 2003). However, treatment with proteasome inhibitors is limited since they are toxic at high levels. For example, if the apoptotic pathway were disrupted in multiple places, the degree of apoptosis could be enhanced. We used RNA interference (RNAi) to knockdown gene expression. RNAi uses double stranded RNA (dsRNA) to mediate mRNA degradation and silence gene expression at the post-transcriptional level (Downward, 2004). RNAi mediated knockdown of DIAP1 is known to induce apoptosis (Martin, 2002), thus we sought to enhance the apoptotic affect of proteasome inhibition with RNAi knockdown of DIAP1. Studies have found a combination of RNAi and proteasome inhibition effective on tumor cells (Gabai et al., 2005).

Proteasome inhibition might also be involved in degenerative diseases. A recent study (Song & Jung, 2004) proposed that amyloid beta ($A\beta$) induced proteasome inhibition causes cell death in Alzheimer's disease, highlighting the potential value of saving cells that are experiencing proteasome inhibition and caspase accumulation. Our approach was to knockdown the caspases by using RNAi in order to prevent caspase accumulation and cell death in lactacystin treated

cells. In addition, if caspase knockdown improves cell survival, it would show that the proteasome causes apoptosis via the caspase cascade.

Methods

Cell Culture

Drosophila S2 cells were cultured in liquid Schneider's medium containing 10% inactivated fetal calf serum and penicillin streptomycin. The cells were maintained at 25°C and serum starved at the beginning of each experiment. Serum starvation followed the 7.16 protocol (dsRNA Soaking) and included 24 hours in 2% serum medium before transfection, and then one hour soaking in serum free medium. The cells were placed in six-well plates immediately after being transferred to serum-free medium at a volume of 1mL per well and a concentration of 2×10^6 cells/mL.

In vitro Transcription

The double stranded RNA (dsRNA) used in the RNAi transfections was generated by using templates from Open Biosystems. In Vitro transcription was used to generate

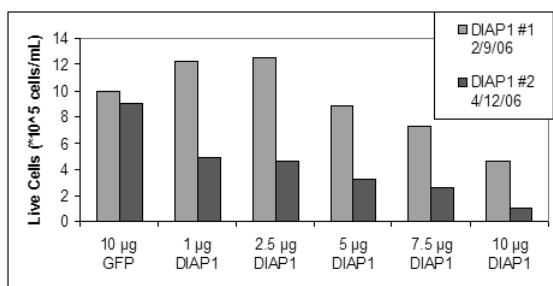


Figure 3. Half-maximal death curve for DIAP1
Cell count was conducted 24 hours after transfection with DIAP1. The half-max death concentration for the DIAP1 was 7.5µg and 3.7µg respectively.

dsRNA for transfections by using the Ambion Megascript kit. The concentration of dsRNA was determined with a spectrophotometer and 1% agarose gel electrophoresis helped detect contamination.

Establishing half-maximal cell death concentration of DIAP1

A half-maximal cell death curve was established to determine the concentration of DIAP1 necessary for these experiments. The curve indicates the amount of cell death caused by various concentrations of dsRNA. DIAP1 dsRNA was added to 2×10^6 cells in 1 mL of serum free medium and incubated for one hour as described above (Cell culture). After incubation, 2 mL of 15% serum medium was added to each well. Cell counts the following day generated two curves for two batches of DIAP1 that were made at different times (Figure 3).

Cell Assays

Three methods were attempted to quantify apoptosis generated by DIAP1 RNAi transfections and lactacystin treatment. Cell counts were completed for every experiment as a quantitative measure of cell death. Because cell counts do

not distinguish between apoptosis and necrosis, we attempted a quantitative measure of caspase activity after the initial experiments. Unfortunately, the assay was difficult to perform and rarely generated reliable results. Therefore we attempted to evaluate apoptosis by quantifying DNA fragmentation on an agarose gel, but the negative controls showed fragmentation similar to the positive controls.

Cell Count

Cells were stained with Trypan Blue (a dye that exclusively stains dead cells), and counted with a hemacytometer and light microscopy according to the 7.16 protocol (Counting Cells and assaying cell survival by Trypan Blue staining).

Caspase Activity Assay

A colorimetric assay of caspase activity was performed using the Caspase Colorimetric Substrate/Inhibitor QuantiPak™ AK-004. The assay was intended to show whether proteasome inhibition causes caspase activity, thus causing death through the apoptotic pathway. Treatment with DIAP1 was used as a control because it is known to cause apoptosis. The peptide substrate DEVD was also used because it is cleaved by caspases homologous to caspase-3: DRONC, DRICE, and DCP1. The absorbance at 405nm was measured and plotted over time to calculate the amount of caspase activity.

Genomic DNA Analysis

Genomic DNA was extracted from cells with the Qiagen DNeasy kit according to the DNeasy Tissue Handbook protocol. The DNA was inspected with a 1% agarose gel for apoptotic DNA fragmentation pattern.

RNAi and Lactacystin Treatment

Lactacystin Treatment

Lactacystin, obtained from CalBiochem, was dissolved in DMSO and added to cells in concentrations ranging from 1µM to 10µM lactacystin. To achieve these concentrations, only 5µL of lactacystin/DMSO was added to each well. Thus, a well with only 5µL of DMSO served as a control. Cells were treated with lactacystin/DMSO after the one hour incubation in serum free medium as described above (Cell Culture). Afterwards, 2mL of 15% serum media was added to each well. After 24 hours, cell count was conducted, cell extract was obtained for caspase activity assay, and genomic DNA was extracted.

DIAP1 Transfection and Lactacystin Treatment

Cells that were transfected with DIAP1 and treated with lactacystin were transfected at the beginning of the one hour incubation period in serum free medium and treated at the end of the one hour period (see Cell Culture). Afterwards, 2mL of 15% serum medium was added to each well. The amount of DIAP1 ranged from 1µg to 3µg of DIAP1 dsRNA and the concentration of lactacystin was 0.5µM and 1µM. The concentration of DIAP1 and lactacystin were below the concentration for half-maximal cell death because higher concentrations caused too much death. Thus, GFP dsRNA and DMSO served as a negative control whereas DIAP1 alone and

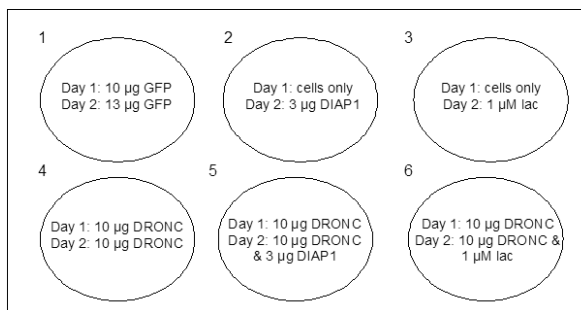


Figure 4. Example of DRONC transfection and lactacystin treatment. Cells were transfected with DRONC 24 hours prior to and again on the same day as lactacystin treatment in order to ensure caspase knockdown. The other caspases (DRICE, DREDD, and DCP1) were used in similar experiments.

lactacystin alone served as positive controls. Cell counts were conducted after 24 hours.

Caspase knockdown and Lactacystin Treatment

Cells were transfected over a two day period in order to knockdown the caspases before the proteasome was inhibited (Figure 4). After the first day of serum starvation, cells were transfected with 10µg of caspase (DRONC, DRICE, DREDD, or DCP1). On the second day, the cells were counted to make sure the number of cells in each well of a given plate was about the same. The cells were then placed in serum free medium and transfected with another 10µg of the same caspase. After one hour, the cells were treated with either 0.5µM or 1µM lactacystin. Various controls included GFP dsRNA and DMSO, caspase alone, DIAP1 alone, and lactacystin alone. Cells were counted and genomic DNA was extracted after 24 hours.

Results

Results of our initial experiments confirm that proteasome inhibition by lactacystin leads to cell death. A knockdown of DIAP1 in conjunction with lactacystin treatment led to more death than either manipulation alone. Finally, DRONC knockdown led to cell survival in cells treated with lactacystin, indicating that proteasome inhibition leads to cell death via the apoptotic pathway.

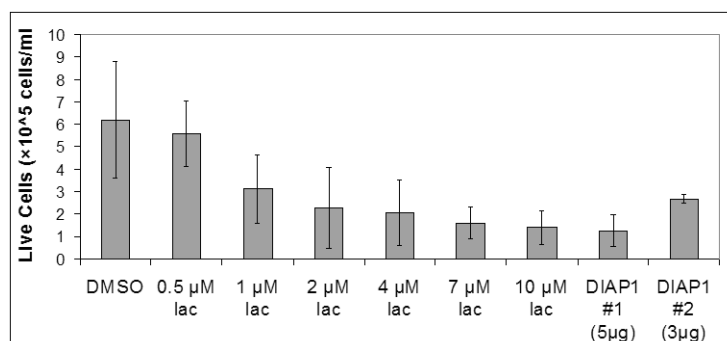


Figure 5. Lactacystin Treatment

Cells treated with varying concentrations of lactacystin displayed increased death correlating to an increase in lactacystin. The negative control, DMSO treated cells, showed a low amount of death since DMSO decreases cell viability. The positive controls, DIAP1 generated on different dates, displayed the expected apoptosis at values just below half-maximal death concentration. The half-maximal death concentration of lactacystin is just below 1µM.

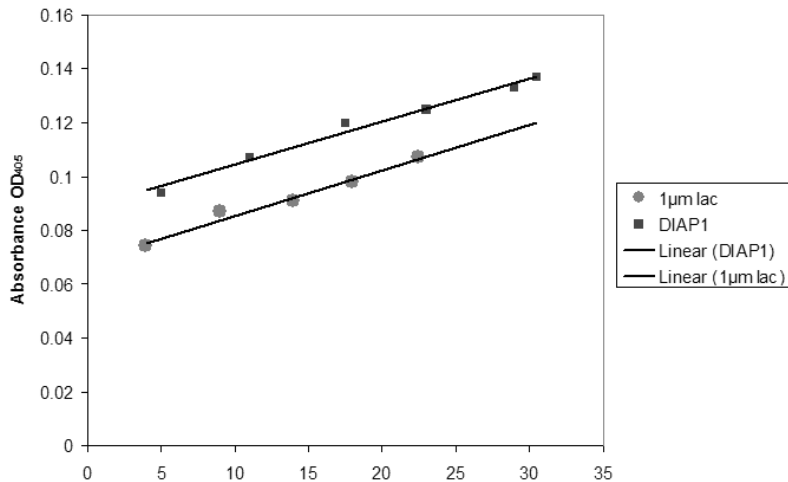


Figure 6. Lactacystin generated caspase activity
The absorbance readings of cell extract mixed with assay buffer and DEVD substrate generated a linear trend over time. The slope of the trendline multiplied by assay volume and conversion factor (provided by the manufacturer) revealed a caspase activity of 19.99 pmol/min for lactacystin treated cells and 22.42 pmol/min for DIAP1 transfected cells. The R2 values for both lines were above 0.97.

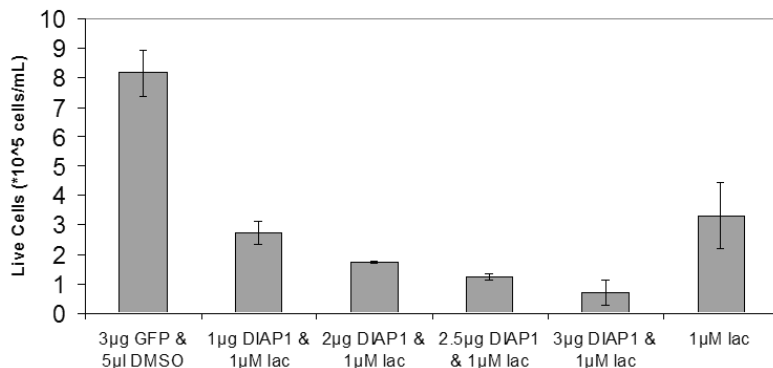


Figure 7. DIAP1 transfection in conjunction with 1µM lactacystin treatment
Cells treated with 1µM lactacystin displayed more death when transfected with DIAP1 dsRNA. Cell death increased with higher concentrations of DIAP1. The DIAP1 used for the experiment had a half-maximal cell death concentration of 3.7µg.

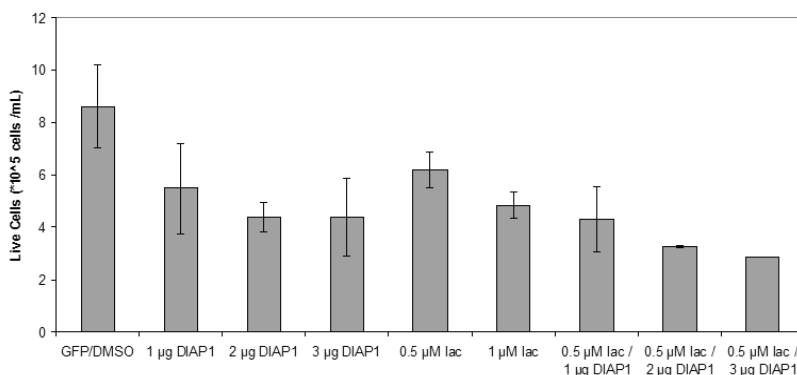


Figure 8. DIAP1 transfection in conjunction with 0.5µM lactacystin treatment
The six controls show the amount of death caused by 1µg, 2µg, and 3µg of DIAP1, and 0.5µM and 1µM lactacystin. Cells that were transfected with the varying concentrations of DIAP1 and also treated with 0.5µM lactacystin died more than cells subjected to either treatment alone.

Lactacystin Treatment

Cell death increased with higher lactacystin concentrations (Figure 5). The half-maximal cell death concentration was just below 1µM. Cells treated with concentrations greater than 4µM displayed indistinguishably high levels of death. The few surviving cells in the 10µM well appeared healthy, and were possibly resistant. Cells treated with DIAP1, the positive control, looked like fragments or appeared as though they were about to burst, whereas cells treated with lactacystin were blue and fragmented but did not look as though they would burst. Finally, the concentrations of DIAP1 were just below half-maximal for cell death and paralleled (when averaged) the level of cell death caused by 2µM concentration of lactacystin.

The caspase activity assay generated linear optical density (OD405) readings on the spectrophotometer for only two samples of cells. Cell extracts made from cells treated with 1µM lactacystin and 5µg DIAP1 #1 displayed caspase activity. The assay did not provide viable results for the other samples. The linear OD405 readings (Figure 6) indicate caspase activities of 19.99 pmol/min for lactacystin treated cells and 22.42 pmol/min for DIAP1 transfected cells.

DIAP1 Transfection and Lactacystin Treatment

Cells transfected with DIAP1 and treated with lactacystin displayed an additive effect. Cells treated with a range of DIAP1 concentrations and treated with 0.5µM lactacystin (Figure 8) or 1µM lactacystin (Figure 7) showed more cell death than cells transfected only with DIAP1 and cells treated only with lactacystin. Also, at a constant concentration of lactacystin, an increase in DIAP1 caused more cell death.

The genomic DNA extracted from cells transfected with DIAP1 and treated with lactacystin displayed DNA fragments indicative of apoptosis on a 1% agarose gel (Figure 9). Lanes with the negative control, however, were not loaded with enough dye. Thus, bands did not appear in the negative control lanes because the DNA dissolved in the gel buffer before gel electrophoresis began. A similar problem was encountered in Lane 4.

Caspase knockdown and Lactacystin Treatment

Cells transfected with DRONC dsRNA were rescued from cell death induced by lactacystin treatment (Figure 10). Cells treated with lactacystin only (negative control) demonstrated the same level of cell death as shown previously, and many cell fragments were apparent with light microscopy. A knockdown of DRONC and DIAP1 also rescued cells, as expected. Finally, knockdown of the caspases DRICE, DREDD, and DCP1 rescued cells from cell death.

Discussion

Our results confirm that 26S proteasome inhibition causes apoptosis in Drosophila S2 cells. The level of apoptosis is comparable with apoptosis induced by RNAi-mediated knockdown of DIAP1 measured through cell counts. The caspase activity further supports this comparison since the measured activity is very similar between the two samples. Proteasome inhibition combined with RNAi against DIAP1 increases the

amount of observed cell death, suggesting that these two treatments have an additive effect. Addition of lactacystin prevents the proteasome from degrading caspases, and the loss of DIAP1's inhibitory regulation of caspases appears to magnify this effect. Other factors that may affect the observed increase in apoptosis stem from the proteasome's role in many other cell pathways such as regulation of the cell cycle and the NF- κ B pathway (see Voorhees & Orlowski, 2006 for a review of the 26S proteasome's function in the cell). Loss of proteasomal function in these other pathways may also trigger apoptosis, which in turn could be accelerated due to loss of DIAP1. Measurement of the relative amount of apoptosis induced by loss of proteasome function in other pathways through RNAi knock-down of other vital proteins in each of the pathways would help clarify the apoptotic implications of each of the proteasome's different roles in the cell.

Blocking the apoptotic consequences of loss of proteasome function presented the other extreme in manipulating apoptosis. RNAi knock-down of the DRONC caspase was sufficient to rescue the cells from apoptosis triggered by lactacystin treatment. These results support the hypothesis that DRONC functions as a main regulatory caspase by confirming that DRONC knock-down effectively halts the caspase cascade. Furthermore, our results show that caspases function downstream of the proteasome in apoptosis, and that proteasome inhibition does not result in activation of other caspases such as DRICE, DCP-1, or DREDD in a DRONC-independent manner. Knock-down of these three caspases separately did not protect cells from apoptosis induced by proteasome inhibition, an observation consistent with the current assumption that these caspases function downstream of DRONC and do not have the regulatory power to singularly switch the caspase cascade "on" or "off."

Cancer therapy seeks to increase apoptosis; therefore, our demonstration that the combination of proteasome inhibition and RNAi increases cell death may be valuable in designing future clinical trials. If short interfering RNA (siRNA) proves to be a viable therapeutic option for humans, the combination of proteasome inhibitors with siRNA could represent an emerging paradigm in cancer therapy. Research on treatments for degenerative diseases such as Alzheimer's focuses on blocking apoptosis; we blocked apoptosis *in vitro* by RNAi-mediated silencing of the DRONC caspase. If apoptosis is implicated in the pathogenesis of degenerative diseases, this *in vitro* finding may translate into a novel clinical therapy.

The precise molecular mechanism by which proteasome inhibition triggers apoptosis remains unclear. This mystery is not easily resolved because proteasomal degradation of DIAP1 leads to apoptosis, but recent studies have shown that the proteasome also can prevent programmed cell death by degrading ubiquitinated DRONC (Martin, 2002). Because the phenotype of a non-functional proteasome is apoptosis, this suggests that the more important cellular role of the proteasome in the programmed cell death pathway is the degradation of caspases. Blocking apoptosis with RNAi silencing of DRONC in the context of a non-functional proteasome further supports this assumption. Biochemical measurements of different caspase activity under a variety of apoptotic stimuli would further support this hypothesis. Another open issue

is whether other caspases besides DRONC can be polyubiquitinated and targeted for destruction by the proteasome. Further investigation into potential substrates of the proteasome may benefit from the use of a proteomics-based screen, as utilized by Adrain et al. (2004).

The endpoint of apoptosis is by definition messy and convoluted: further experiments with the 26S proteasome and caspases will help clarify our knowledge of this molecular chain of events. In the more immediate future, the demonstration that the apoptotic effects of proteasome inhibition can be manipulated may have pharmaceutical applications in the treatment of cancer and Alzheimer's disease.

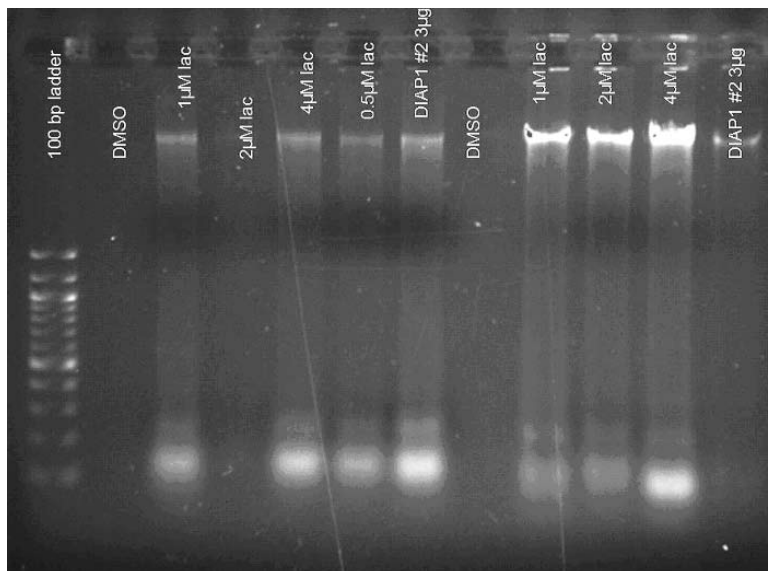


Figure 9. Genomic DNA analysis
The ladder indicates two bright bands in all the lanes except those with DMSO (negative control). Cells treated with DMSO should not undergo apoptosis, unlike cells transfected with DIAP1 (positive control). Lanes with genomic DNA from cells treated with lactacystin looked like lanes with the positive control.

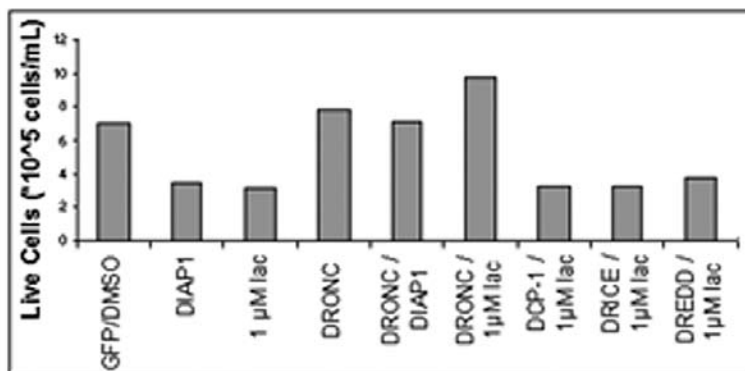
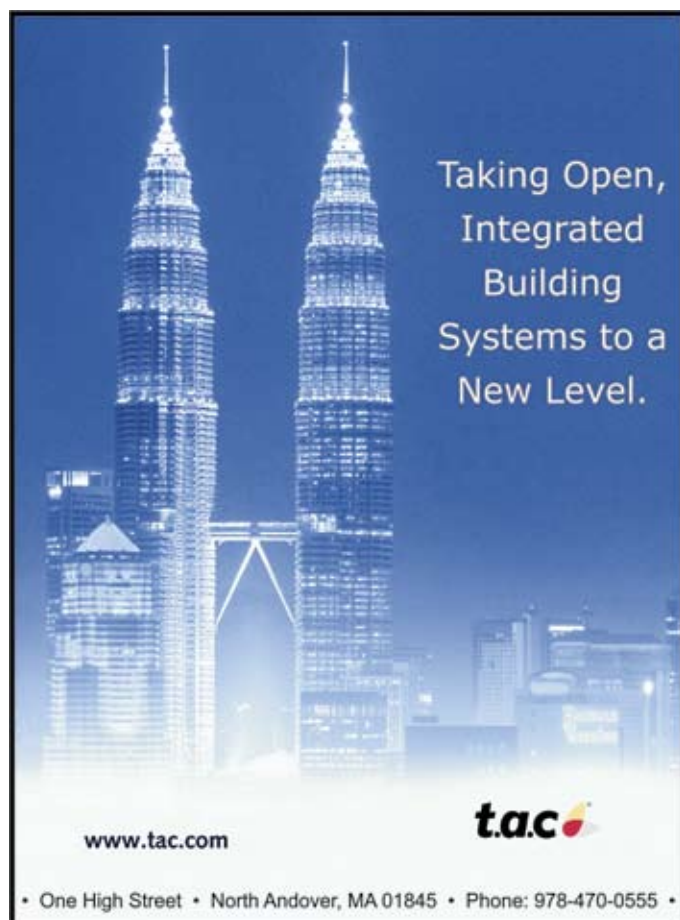


Figure 10. Rescue of lactacystin treated cells via RNAi transfection of DRONC Cells transfected with DIAP1 or treated with lactacystin experienced a high level of death. However, cells with a knockdown of DRONC exposed to similar conditions were saved from death. The cells were also unaffected by transfection with DRONC dsRNA.

References

- Adrain C, Creagh EM, Cullen SP, and Martin SJ. (2004). Caspase-dependent Inactivation of Proteasome Function during Programmed Cell Death in *Drosophila* and Man. *Biol. Chem.* 279(35), 36923-36930.
- Daniel KG, Kuhn DJ, Kazi A, and Dou QP. (2005). Anti-Angiogenic and Anti-Tumor Properties of Proteasome Inhibitors. *Current Cancer Drug Targets* 5(7), 529-541.
- Ditzel L, Huber R, Mann K, Heinemeyer W, Wolf DH, and Groll M. (1998). Conformational constraints for protein self-cleavage in the proteasome. *J. Mol. Biol.* 279, 1187-1191.
- Downard J. (2004). RNA interference. *BMJ* 328, 1245-1248.
- Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, and Schreiber SL. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* 268, 726-731.
- Gabai VL, Budagova KR, Sherman MY. (2005). Increased expression of the major heat shock protein Hsp72 in human prostate carcinoma cells is dispensable for their viability but confers resistance to a variety of anticancer agents. *Oncogene* 24, 3328-3338.
- Groll M, and Huber R. (2004) Inhibitors of the eukaryotic 20S proteasome core particle: a structural approach. *BBA Mol Cell Research* 1695(1-3), 33-44. Kumar S, and Doumanis J. (2000). The fly caspases. *Cell Death and Differentiation* 7, 1039-1044.
- Martin SJ. (2002). Destabilizing influences in apoptosis: sowing the seeds of IAP destruction. *Cell* 109, 793-796.
- Myung J, Kim KB, and Crews CM. (2001). The ubiquitin-proteasome pathway and proteasome inhibitors. *Med Res Rev.* 21(4), 235-73.
- Orlowski RZ. (1999) The role of the ubiquitin-proteasome pathway in apoptosis. *Cell Death Differ.* 6, 303-313.
- Palaga T, and Osborne B. (2002). The 3D's of apoptosis: death, degradation and DIAPs. *Nature Cell Biol.* 4, E149-151.
- Song S, and Jung YK. (2004). Alzheimer's disease meets the ubiquitin-proteasome system. *Elsevier* 10(11), 565-570.
- Voorhees PM, Dees EC, O'Neil B, and Orlowski RZ. (2003). The Proteasome as a Target for Cancer Therapy. *Clinical Cancer Research* 9, 6316-6325.



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