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Evidence that aberrant protein metabolism contributes to chemoresistance in multiple myeloma cells

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Abstract. Multiple myeloma (MM) is an incurable B lymphocyte cancer. To date, a comparative analysis of global protein metabolism for the MM cell line CCL-155 (RPMI-8226) and the non-cancerous B lymphocyte cell line CCL-156 (RPMI-1788) has not been published. Here, we report that both global protein synthesis and degradation occur at a higher rate in MM cells and demonstrate that alkylating agents can reduce global protein degradation in both cell lines, but the effect is greater in CCL-156 cells. Treatment with melphalan plus the proteasome inhibitor MG132 reduced global protein degradation for MM cells to roughly 60% of that seen without drugs, but the reduction was approximately three times greater for CCL-156 cells. This drug combination was growth inhibitory for both cell lines, but CCL-156 inhibition was 2-fold greater than that of the MM cell line. Additionally, treatment with melphalan plus the lysosomal inhibitor chloroquine did not affect growth of MM cells more than melphalan alone, whereas this combination drastically inhibited growth of CCL-156 cells despite protein degradation being maintained at 60% level for both cell lines. This suggests that a lysosomal function other than protein degradation is required for recovery from alkylation damage in CCL-156 cells. In general, CCL-156 cells were affected to a greater extent for both protein degradation and growth inhibition with most drug combinations tested. Statistical analysis of our data ($P=0.066$) provides evidence that aberrant proteasome-mediated protein degradation correlates with chemoresistance in MM cells, but that lysosome-mediated protein degradation does not.

Introduction

In the last few years proteasome inhibitors such as bortezomib (PS-341 or Velcade) have emerged as important chemothera-

peutic tools to treat multiple myeloma (MM) due to their ability to modulate the ubiquitin-proteasome system (1-5). Recent, clinical trials have resulted in very high complete recovery rates and increased rates of remission and longevity for a cohort of elderly MM patients when the proteasome inhibitor bortezomib was added to the classical regimen of melphalan (Alkeran) plus prednisone (6). The FDA approved the use of Velcade in the treatment of MM in 2003 (7), although the molecular mechanism for the enhanced anticancer activity has not been defined (2,3,8).

Bortezomib is a potent and selective inhibitor of the proteasome, the major site of protein degradation in human cells (9). The proteasome is a large multi-subunit protein complex that is localized in the cytoplasm and nucleus and has multiple sites to rapidly degrade ubiquitinated proteins in a highly regulated and selective manner. The proteasome has many disparate roles in cell metabolism including cell signaling, DNA repair, transcription, cell adhesion and angiogenesis (1,3,10,11). Bortezomib has been shown to induce apoptosis by inhibiting transcription factor NF- κ B activation. However, this activity alone cannot fully explain the anticancer activity of bortezomib (2,12).

Alkylation damage to DNA by melphalan can block DNA replication (13). Numerous studies have established a strong link between DNA repair and the proteasome and it can be surmised that a fully functional proteasome is essential for recovery after exposure to DNA damaging agents (14-19). In fact, proteome protection has been shown to be crucial for cell survival after exposure to radiation (20). Additionally, protein adduct formation after melphalan exposure has been observed and quantification of protein adducts has been proposed as a method to monitor chemotherapeutic toxicity and mutagenicity in patients (21,22).

The proteasome and the vacuole (an organelle analogous to the lysosome in mammals) are the two major sites of protein degradation in yeast (23). We have recently demonstrated in yeast that global protein degradation is modulated after exposure to a broad range of DNA damaging agents including the S_N2 alkylating agent methyl methanesulfonate (MMS) (17). Our results have established a role for both the proteasome and vacuole in recovery after exposure to MMS. These experiments identified the regulation of protein degradation as a critical factor for determining cell fate after exposure to DNA damaging agents. Thus, the molecular mechanism for the anticancer activity in MM patients treated with melphalan

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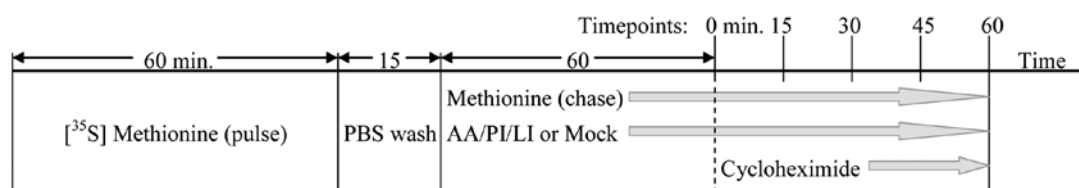


Figure 1. Protein degradation assay time-course outline. AA, alkylating agent; PI, proteasome inhibitor; LI, lysosome inhibitor.

and bortezomib may involve perturbation of global protein metabolism.

Like the proteasome, the lysosome is responsible for degrading proteins in the cell. However, the lysosome is responsible for bulk turnover of proteins by the non-selective process of autophagic uptake (23-26). The lysosome is also known to be involved in the activation of apoptotic pathways (27-29), causing cell death by necrosis and the upregulation of the autophagic pathway can lead to autophagic cell death (30,31). Therefore, drugs such as chloroquine, which inhibits lysosome-mediated protein degradation, have become of interest in cancer research (32-36).

In this study, we investigate the differences in protein metabolism between a MM cell line (CCL-155) and a non-cancerous B lymphocyte cell line (CCL-156). These two cell lines were derived from the peripheral blood of male patients by the same researcher during the same time period (37,38). We observed that both protein synthesis and degradation occurred at a higher rate in CCL-155 cells. We demonstrated that alkylating agents can reduce the level of global protein degradation in both cell lines, but that CCL-156 cells are affected to a greater extent. Our data establish that growth inhibition of MM cells from the combined exposure to melphalan and proteasome inhibitors does not result from a gross reduction in global protein degradation. Using a drug cocktail containing both chloroquine and proteasome inhibitor we present evidence that protein metabolism in MM cells is aberrant and propose that this metabolic state contributes to chemoresistance.

Materials and methods

Cell lines, culture media and materials. Melphalan (MP Biomedicals, Solon, OH) and MG-132 (EMD Chemicals, Gibbstown, NJ) were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) at a stock concentration of 10 mM. Chloroquine (MP Biomedicals) was dissolved in H₂O to the same concentration and all solutions were stored at -20°C. Methyl methanesulfonate (MMS) was purchased from Acros Organics (Geel, Belgium). CCL-155 (RPMI-8226) and CCL-156 (RPMI-1788) cell lines (ATCC, Manassas, VA) were maintained and assayed in untreated sterile tissue culture flasks or plates containing RPMI-1640 media supplemented with 20% FBS (HyClone, Logan, UT) and 100 U/ml penicillin and 100 µg/ml streptomycin (MP Biomedicals) in a humidified incubator at 37°C with 5% CO₂ (standard conditions). Both cell lines were maintained at a density of 0.50-2.0x10⁶ cells/ml under sterile conditions. Prior to each assay, cell density and viability was determined using Trypan Blue (Alfa Aesar, Ward

Hill, MA) dye exclusion with triplicate measurements. Dead cells were excluded from the investigation. Viabilities between 80-95% were typical for both cell lines.

Measurement of global protein synthesis. Our protocol for measuring global protein synthesis was adapted from the protocols of Pong *et al* (39) and Mans and Novelli (40). Cultures of CCL-155 and CCL-156 cells were incubated under standard conditions until the cell density reached ~1x10⁶ cells/ml. Both cell lines were processed simultaneously in the following manner. Cultures (5 ml) were resuspended in an equal volume of methionine-free RPMI media supplemented with 10 µCi/ml L-[³⁵S]-methionine (>1,000 Ci/mmol) (Perkin-Elmer, Boston, MA) and quickly mixed by pipetting. Aliquots (60 µl) were immediately removed in duplicate and added to a Whatman No. 3MM chromatography 2.3 cm filter disk (zero time point) which was briefly dried with lightly compressed air for 15 sec. Next, the filter disks were placed in an ice-cold 10% trichloroacetic acid (TCA) (Sigma-Aldrich) bath for 60 min to allow intact proteins to precipitate onto the filter disk followed by a 30-min wash in an ice-cold 5% TCA bath. The above steps were repeated for the 30 and 60-min time points. Two untreated filter disks were placed in the 10% TCA bath prior to the experiment and carried through the entire procedure to determine background counts per minute (CPM). Filter disks were collected, dried overnight, added to 5-ml scintillation fluid and subjected to scintillation counting with a Beckman 6500 scintillation counter. Relative CPM incorporated were calculated by averaging the duplicate CPM readings and subtracting both the background CPM and the zero time point CPM from each timepoint.

Measurement of global protein degradation. Our protocol for measuring global protein degradation was adapted from the protocols of Rock *et al* (41) and Meriin *et al* (42) (Fig. 1). Cultures of either CCL-155 or CCL-156 cells at ~1x10⁶ cells/ml were resuspended in methionine-free RPMI media supplemented with 10 µCi/ml L-[³⁵S]-methionine and incubated under standard conditions for 1 h. Next, cells were washed with phosphate-buffered saline (PBS) (MP Biomedicals) and resuspended in the same media with 0.2 mg/ml DL-methionine (Calbiochem, Los Angeles, CA). The culture was then split into separate 5 or 10-ml cultures and preincubated with appropriate drugs or vehicle for 1 h. Next, cycloheximide (MP Biomedicals) was added to each culture at a final concentration of 100 µg/ml, and 450 µl aliquots were immediately removed in duplicate and added to TCA (10% final concentration) at appropriate time intervals. Samples were mixed by brief vortexing, placed on ice for 1 h

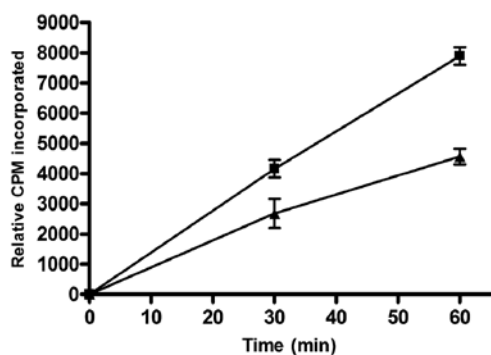


Figure 2. Protein synthesis profile for CCL-155 and CCL-156 cells. Actively growing cultures were incubated under standard conditions in methionine depleted RPMI media supplemented with L-[³⁵S]-methionine. Aliquots were removed in duplicate every 30 min, processed as outlined in Materials and methods and relative CPM incorporated was calculated. Data are plotted as the mean \pm SE of three independent experiments. ■, CCL-155; ▲, CCL-156.

and centrifuged in a microfuge at maximum speed for 5 min. Aliquots of supernatant were removed and subjected to scintillation counting with a Beckman 6500 scintillation counter. Percent degradation was calculated by averaging the duplicate CPM readings and using the following equation: % degradation = [(Avg CPM time point X/Avg CPM time point zero) - 1] x 100%. The increase in CPM detected in the supernatant corresponds to the release of free amino acids over time.

Cell proliferation test. Media was delivered to a 96-well plate so that the final volume, with drugs and cells included, would be 150 μ l/well. The drugs at 10-mM stock concentrations were serially diluted with media so that delivering 15 μ l of drugs to a well would yield the appropriate working concentrations. Cells were diluted in media so that delivering 90 μ l would seed 7,500 cells/well. Media-only wells were included to determine background. The assay was incubated for 72 h under standard conditions in triplicate, and viability was determined using the CellTiter 96[®] AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. Absorbance (abs) was read using a Spectramax 250 Plate Reader (Molecular Devices, Sunnyvale, CA) at 490 nm to determine viability and 600 nm to determine non-specific abs. The 600 nm abs was subtracted from the 490 nm abs to obtain the adjusted abs. The adjusted abs for each treatment was averaged, and the average adjusted abs of media-only wells was deducted from the average adjusted abs of each treatment to obtain the final abs.

Statistical analysis. Statistical significance was determined using unpaired two-tailed Student's t-tests with a 95% confidence interval using the built-in statistical analysis tool in the GraphPad Prism software program. Linear regression analysis was performed using Microsoft Office Excel 2007.

Results

Rates of global protein synthesis and degradation for the CCL-155 and CCL-156 cell lines. In general, we observed that protein metabolism occurred at a faster rate for the MM cell line, CCL-155, than the immortalized B-cell line, CCL-156

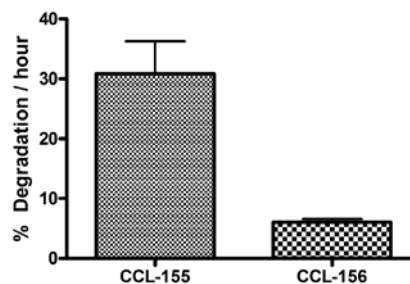


Figure 3. Percent protein degradation per hour for CCL-155 and CCL-156 cells. Actively growing cultures were incubated under standard conditions in methionine depleted RPMI media supplemented with L-[³⁵S]-methionine for 1 h. Cells were washed and resuspended in the same media with excess DL-methionine. Next, cells were incubated for 1 h and cycloheximide was added to each culture. Duplicate aliquots were removed immediately and 60 min later, processed as outlined in Materials and methods and percent degradation was calculated. Data are plotted as the mean \pm SE of three independent experiments.

(Figs. 2 and 3). To measure global protein synthesis, actively growing cultures were incubated with L-[³⁵S]-methionine. The level of protein synthesis was determined by measuring the amount of radioactivity incorporated into intact cellular proteins (Materials and methods). Fig. 2 indicates that protein synthesis for both cell lines is linear, and that the level of radioactivity incorporated into intact proteins reaches 7892 CPM on average for CCL-155 cells after 1-h incubation with [³⁵S]-methionine, while the CCL-156 cells reach an average of 4563 CPM under the same conditions. At the 60-min time point the rate of protein synthesis for CCL-155 is about 1.7-fold greater than the rate for the CCL-156 cell line (P=0.001). This is consistent with transcriptional profiling data which showed that the genes involved in protein synthesis are upregulated in MM cells compared to normal plasma cells (43).

To measure global protein degradation, actively growing cultures were incubated (pulse) with L-[³⁵S]-methionine for 1 h followed by incubation (chase) with unlabelled methionine for 1 h (Fig. 1). Next, cycloheximide was added to stop protein synthesis, and duplicate aliquots were immediately removed and processed to determine the background level of radioactivity (time zero). After 1-h incubation in cycloheximide and unlabelled methionine duplicate aliquots were removed and processed. This methodology provided consistency with measurements made in the presence of drugs (see below).

Percent protein degradation was calculated relative to the level of radioactivity at time zero. For CCL-155 cells, we observed that about 30% of the cellular proteins had been degraded by 1 h (Fig. 3). This value is in good agreement with previous protein degradation measurements in human cell cultures (41,44). Fig. 3 shows that the average rate of protein degradation was 5 times greater for CCL-155 cells (P<0.05), with CCL-156 cells degrading about 6% of the cellular proteins in 1 h. It should be noted that the CCL-155 cells consistently grow at a slower rate than the immortalized CCL-156 cells (Fig. 4). Therefore, the differences we observed in protein metabolism cannot be attributed to a gross difference in intrinsic growth rates for these cell lines in culture.

Effect of an alkylating agent on global protein degradation. The above protein degradation protocol was followed, but

Table I. Percent difference in the average relative levels of protein degradation or cell proliferation between the CCL-155 and CCL-156 cell lines.

| | Treatment with melphalan/MG132/chloroquine | | | | | | |
|---------------------|--|--------------|-------|-------------|-------|-------------|-------------|
| | +/-/- | -/+/- | -/-/+ | +/+/- | +/-/+ | +/+/+ | -/+/+ |
| Protein degradation | 54.0 | 19.7 | 46.1 | 42.6 | -7.4 | 34.4 | 46.1 |
| Cell proliferation | 32.3 | -15.0 | 9.3 | 19.2 | 60.5 | 28.2 | 41.3 |

Bold indicates correlation between protein degradation and cell proliferation (P=0.066).

Collectively, we observed that global protein degradation in CCL-155 cells is less affected by addition of drugs to the media. Fig. 6 indicates that addition of 500 μ M melphalan to the media results in a slight but insignificant decrease in the level of protein degradation for CCL-155 cells, but that the level of protein degradation for CCL-156 cells is reduced over 50% (P<0.05). In general, treatment of CCL-155 with any drug combination, other than melphalan alone, resulted in a 30-50% decrease in the level of protein degradation. Treatment with 50 μ M MG132 resulted in a roughly 50% reduction in the level of protein degradation for both cell lines. Treatment with 1 mM chloroquine or melphalan plus MG132 caused the level of protein degradation for CCL-156 to be reduced at least twice as much as CCL-155 (P<0.05). Treatment with melphalan plus chloroquine had a similar effect on the level of protein degradation for both cell lines. Treatment with all three drugs reduced the level of protein degradation in CCL-156 about twice as much as the levels in CCL-155 (P<0.05). Treatment of cells with MG132 plus chloroquine reduced protein degradation in both cell lines, but the level of protein degradation for CCL-156 cells was drastically reduced to about 5% of the level for untreated CCL-156 cells. This level of protein degradation is approximately 10-fold less than that observed for similarly treated CCL-155 cells (P<0.05). For most drug treatments, we observed a statistically significant difference in the level of protein degradation between CCL-155 and CCL-156 cells. This indicates that global protein degradation is grossly aberrant in CCL-155 cells.

CCL-155 cells are less sensitive to the growth inhibition effects of various drug cocktails. The effects of different combinations of melphalan, MG132 and chloroquine on cell proliferation were investigated. Drug concentrations were chosen which caused no greater than 30% average growth inhibition for CCL-155 cells when administered alone.

In general, CCL-155 cells were less sensitive to growth inhibition by these drug combinations than CCL-156 cells (Fig. 7). For instance, treatment with melphalan alone, melphalan plus MG132 or melphalan plus chloroquine resulted in at least a 2-fold difference between cell lines for average cell proliferation with these drug combinations exerting more growth inhibition in CCL-156 cells. No discernable differences were observed with either MG132 or chloroquine alone, and growth inhibition with these treatments was minimal in both cell lines. However, combination of MG132 plus chloroquine proved to be growth inhibitory only for CCL-156 (P<0.05).

Finally, treatment with all three drugs greatly inhibited cell proliferation in both cell lines with CCL156 cells exhibiting a 3-fold lower average cell proliferation than CCL-155 cells. It should be noted that the highest degree of growth inhibition for CCL-155 cells was achieved only with combined exposure to melphalan plus MG132, consistent with clinical use of this drug combination to treat MM (6).

Statistical support for a correlation between proteasome-mediated protein degradation and cell proliferation. For several drug treatments, we noticed that differences in the relative protein degradation between the cell lines often corresponded to similar differences in relative cell proliferation (comparing Figs. 6 and 7). Therefore, we sought to determine if there was a correlation between the level of relative global protein degradation and the relative level of cell proliferation for CCL-155 and CCL-156 cells. Our analysis included data points derived from treatments which included melphalan alone and MG132 in any combination. For this analysis, we took the average values for the relative level of protein degradation or cell proliferation and calculated the difference between CCL-155 and CCL-156 (CCL-155 minus CCL-156) for each treatment (Table I). We next plotted the differences for protein degradation versus the differences for cell proliferation. Using linear regression analysis with these five data points we obtained an r^2 value of 0.7278 ($r=0.8531$). Next, we performed a Student's t-test assuming three degrees of freedom and obtained a P-value of 0.066. This correlation was abolished when we considered treatment with chloroquine in the absence of MG132.

Discussion

Protein synthesis and degradation are thought to be ongoing processes in mammalian systems, and proteins are hypothesized to exist in a dynamic equilibrium within the cell (51). Our results show that the intrinsic rate of protein metabolism for the MM cell line CCL-155 is elevated compared to the non-cancerous B lymphocyte cell line CCL-156. Significantly higher rates were observed for both global protein synthesis and global protein degradation in CCL-155 cells (Figs. 2 and 3). It is well known that protein metabolism is elevated in a typical cancer cell (52-54); however, relatively speaking, MM is a slow growing cancer and this is reflected in cultured cells (Fig. 4). Therefore, considering cell doubling time, it can be extrapolated that global protein synthesis and degradation

in CCL-155 is elevated roughly 4-fold and 12-fold per cell generation, respectively. The deficit in the rate of synthesis vs. degradation may in part explain the development of uremia in some MM patients (55).

Our measurements of global protein degradation revealed that CCL-155 did not have a significant change in the level of protein degradation after exposure to melphalan, whereas MMS caused a 2-fold reduction in protein degradation. The discrepancy between our results with melphalan and MMS can be explained by the fact that the melphalan concentration in our assays is roughly 10-fold less than the MMS concentration. Furthermore, Lawley and Thatcher have determined that *in vivo* MMS preferentially alkylates proteins (56) whereas melphalan has equivalent reactivity with nucleic acids and proteins (57).

In contrast, exposure of CCL-156 cells to the same concentration of melphalan resulted in a greater than 2-fold reduction of global protein degradation compared to untreated CCL-156 cells (Fig. 6). Additionally, melphalan was observed to be more toxic to CCL-156 cells (Fig. 7). Indicators of differences in protein metabolism and stress response phenotypes between MM cells and B cells can be found in the literature. For instance, transcriptional profiling experiments have shown that genes involved in the unfolded protein response, protein synthesis and the stress response are upregulated in patient-derived MM cells compared to plasma cells derived from an unaffected twin (43). Additional experiments showed that *XBPI*, which mediates the unfolded protein response, as well as a group of disulfide isomerase genes, is expressed at a higher level in CCL-155 than in mature B cells (58). Considering that global protein synthesis and degradation is increased in MM cells (Figs. 2 and 3), the data suggests that MM cells are better prepared to process and replace alkylation damaged proteins.

We primarily observed that cell line CCL-155 showed significantly higher levels of global protein degradation for varying combinations of melphalan, MG132 and chloroquine than cell line CCL-156. The most notable difference between CCL-155 and CCL-156 was observed when both the proteasome and lysosome were inhibited. The greatest growth inhibition for both cell lines was observed when melphalan plus MG132 were included in the drug cocktail even though the protein degradation level for CCL-155 remained at about 60% of the untreated level. The growth inhibition result with CCL-155 is consistent with a previous published study (4); however, it is surprising to observe that the non-cancerous cell line actually appears more sensitive to this drug combination. This observation may help explain the hematological toxicities and compromised immune responses observed in clinical trials (6). Importantly, our results establish that the sensitivity of CCL-155 to melphalan plus MG132 cannot be attributed to a gross reduction in protein degradation. Overall, we surmise that the increased rate of protein metabolism in CCL-155 cells renders this cell line less prone to modulation of protein degradation by proteasome inhibitors, lysosomal inhibitors and alkylating agents.

Several studies have identified both the proteasome and lysosome (or counterpart) to be important for recovery from DNA damaging agents (14-19). Our previous results in yeast showed that inhibition of proteasomal and vacuolar proteases inhibited growth in cultures treated with MMS (17). Here,

we observe similar results with proteasome and lysosome inhibitors. One interesting result is observed when we treat cells with melphalan plus chloroquine. For this drug cocktail, protein degradation was reduced to about 60% of the untreated level for both cell lines, but this treatment proved much more effective at inhibiting growth for CCL-156. Chloroquine-induced destabilization of the lysosomal membrane has been shown to cause release of pro-death signals into the cytosol (29,34,35). Differential destabilization of the lysosomal membrane between these cell lines may explain this observation. On the other hand, this result may indicate that a lysosomal function other than protein degradation is required for recovery from alkylation damage in CCL-156 cells such as the metabolism of damaged carbohydrates and/or lipids. Another interesting observation is that treatment of CCL-155 with melphalan plus chloroquine results in the same degree of growth inhibition as melphalan alone while treatment with melphalan plus MG132 is the most growth inhibitory treatment for CCL-155. Furthermore, exposure of either cell line to melphalan plus MG132 plus chloroquine does not increase growth inhibition compared to treatment with melphalan plus MG132 for either cell line. These data are consistent with studies in yeast where the proteasome proved to be the protein degradation machinery most important for recovery from alkylating agents (17).

Upon further analysis of our protein degradation and cell proliferation data, we find fairly good evidence for a correlation between the degree of protein degradation reduction and the degree of growth inhibition for treatments which include melphalan alone and MG132 in any combination (comparing Figs. 6 and 7, summarized in Table I). Statistical analysis of the data resulted in a P-value of 0.066, which suggests that drug treatments that result in a greater reduction in protein degradation levels for CCL-156 compared to CCL-155 also result in greater growth inhibition for CCL-156 compared to CCL-155. This correlation was not observed when we also considered treatment with chloroquine in the absence of MG132, further indicating that the proteasome has a more prominent role in recovery from alkylating agents. Assuming the data obtained with the non-cancerous CCL-156 cell line to represent a normal response, these data infer that CCL-155 cells are resistant to pharmaceutical modulation of global protein degradation rates and that this phenotype contributes to the chemoresistance of MM cells. Our data provides new insight into the metabolic state of MM cells and is important to consider as new therapies are developed to treat MM.

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