

# Bortezomib inhibits the proteasome, leading to cell death via apoptosis in feline injection site sarcoma cells in vitro

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## OBJECTIVE

To determine the in vitro effects of the proteasome inhibitor bortezomib in feline injection site sarcoma (FISS) cell lines.

## SAMPLE

In vitro cultures of the FISS cell lines Ela-1, Hamilton, and Kaiser.

## PROCEDURES

Cells were treated with increasing doses of bortezomib or vehicle alone (dimethyl sulfoxide) and evaluated for cell viability via an adenosine triphosphate concentration assay, proteasome activity via a commercially available proteasome assay, accumulation of ubiquitinated proteins via Western blot, and apoptosis via flow cytometry.

## RESULTS

All 3 cell lines were sensitive to bortezomib with a 50% inhibitory concentration after 48 hours of treatment at 17.46 nM (95% CI, 15.47 to 19.72 nM) for Ela-1, 19.48 nM (95% CI, 16.52 to 23.00 nM) for Hamilton, and 21.38 nM (95% CI, 19.24 to 23.78 nM) for Kaiser. In the Ela-1 cell line, 20 nM bortezomib inhibited 20S proteasome activity by 90.9% compared with the vehicle-only control. In the Kaiser cell line, 20 nM bortezomib decreased 20S proteasome activity by 70%, compared with the untreated vehicle-only control. Last, treatment with bortezomib (25 and 40 nM) resulted in statistically significant decreases in viable cells accompanied by a statistically significant increase in apoptotic cells.

## CLINICAL RELEVANCE

Treatment options for FISS, especially nonresectable FISS, are currently very limited. These results support further investigation of bortezomib either alone or in combination with other treatments in such cases.

**F**eline injection site sarcomas (FISS) are believed to occur as a result of malignant transformation secondary to chronic inflammation at the site of previous injections, most commonly with vaccinations.<sup>1</sup> The prevalence of FISS has been reported to be between 4 and 16 cases/10,000 vaccinations.<sup>2</sup> FISS are locally invasive with relatively low rates of distant metastasis. However, because of their high degree of local invasion, local recurrence is common.

Radical surgery, defined as 5-cm lateral margins with a depth of 2 fascial planes, has demonstrated the lowest rate of local recurrence (14%).<sup>2</sup> Unfortunately, radical margins are not achievable in many cats. Surgery combined with radiation is recommended for tumors not amenable to radical excision, but local recurrence rates are still between 28% and 48%.<sup>3</sup> Even in the face of frequent local recurrence, in one study, surgery combined with radiation therapy resulted in

a median survival time of 520 days for 79 cats.<sup>4</sup> Neoadjuvant chemotherapy and immunomodulation with interleukin 2 have also led to reduced local recurrence rates, as seen in two preliminary studies.<sup>5,6</sup>

Despite modest advances in local control by combining other treatment modalities with surgery, treatment options for nonsurgical FISS are much more limited. Treatment of gross-disease FISS with radiation therapy has not led to durable control. The best result for gross-disease FISS was reported in a study examining stereotactic body radiation therapy, which found an objective response rate of 72.7%, with a median progression-free interval of 242 days in 11 cats.<sup>7</sup> However, cats with advanced, nonsurgical or metastatic FISS often have no durable treatment options.

Bortezomib is a first-generation proteasome inhibitor that works by binding reversibly to and thereby inhibiting the catalytic subunit (20S) of the

26S proteasome.<sup>8</sup> Inhibition of the proteasome has multiple effects on cancer cells, including accumulation of proapoptotic proteins leading to cancer cell death via apoptosis.<sup>9</sup> Bortezomib is licensed for use in humans with multiple myeloma and mantle cell lymphoma. It has also shown efficacy in other hematologic cancers such as non-Hodgkin lymphoma and solid malignancies such as cancers of the pancreas, lung, colon, breast, and head or neck.<sup>10,11</sup> In veterinary medicine, bortezomib has shown in vitro efficacy against canine malignant melanoma, lymphoma, and osteosarcoma.<sup>12-15</sup> In the study reported here, we sought to understand the effects of bortezomib on FISS cells, focusing initially on whether bortezomib was able to inhibit the feline proteasome. Furthermore, we sought to define the effect on cell viability and induction of apoptosis.

## Materials and Methods

### Reagents

Bortezomib (Selleck Chemicals) was purchased as a 5-mg powder and was reconstituted in 1 mL dimethyl sulfoxide (DMSO) to make a 5-mg/mL (13.0127-mM) stock, which was stored in 50- $\mu$ L aliquots at  $-80^{\circ}\text{C}$ .

### FISS cell lines

Three immortalized cell lines (Ela-1, Hamilton, and Kaiser), all derived from histologically confirmed FISS tissue samples, were used in this study. Cells were cultured in a humidified incubator at  $37^{\circ}\text{C}$  with 5% carbon dioxide in Dulbecco's modified Eagle medium (Gibco, Life Technologies Corp) supplemented with 10% fetal bovine serum (Corning Life Sciences), penicillin (100 U/mL), and streptomycin (100  $\mu\text{g}/\text{mL}$ ; Gibco, Life Technologies Corp).

### Adenosine triphosphate cell viability assay

FISS cell lines were seeded in quadruplicate in 96-well plates at 5,000 cells/well (Ela-1 and Kaiser) or 3,000 cells/well (Hamilton). These concentrations were previously determined to be optimal for preventing cell overcrowding after 48 to 72 hours in culture. Once plated, cells were allowed to adhere and grow under normal conditions overnight (at least 12 hours). After incubation overnight, cells were washed once with sterile phosphate-buffered saline (PBS) solution, and then complete media containing either DMSO (vehicle control) or increasing concentrations of bortezomib (0 to 1,000 nM) was added. Cells were incubated for 48 hours in the presence of bortezomib, and cell viability was assessed using an adenosine triphosphate (ATP)-based luminescent cell viability assay according to the manufacturer's protocol (CellTiter-Glo Luminescent Cell Viability Assay; Promega Corp). Briefly, the wells were washed once with PBS solution, after which the ATP substrate was added. After incubation at  $23^{\circ}\text{C}$  for 15 minutes, the contents of each well were transferred to a white-walled 96-well plate, and luminescence was measured on a microplate reader (Synergy 4 microplate

reader; Bio-Tek Instruments). A blank well containing the greatest concentration of bortezomib (1,000 nM) without cells was measured and subtracted from each experimental well to account for bortezomib auto-luminescence. The percentage of luminescence in each treatment condition versus untreated (DMSO alone) was calculated. The 50% growth inhibitory concentration ( $\text{IC}_{50}$ ) was determined via nonlinear regression analysis using commercially available statistical software (Prism v9.0.1; GraphPad Software).

### Bortezomib protein time course

FISS cells were grown in the presence or absence (DMSO only) of bortezomib for 6 to 36 hours. After the indicated time, whole-cell lysates were collected via lysis of cells in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease/phosphatase inhibitor cocktail (Pierce Biotechnology Co). The concentration of total protein in each sample was measured via a bicinchoninic acid protein assay (Pierce Biotechnology). Protein samples were then subdivided into 20- $\mu\text{g}$  aliquots and stored at  $-80^{\circ}\text{C}$  until analysis via Western blot.

### Western blots

Protein samples (20  $\mu\text{g}/\text{lane}$ ) were mixed with sodium dodecyl sulfate loading buffer and boiled for 5 minutes at  $95^{\circ}\text{C}$  and then separated by size on 4% to 15% polyacrylamide gels (Mini-Protean TGX gels; Bio-Rad Laboratories). The proteins were then transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories). The membranes were incubated in blocking buffer (Thermo Fisher Scientific) under constant agitation for 1 hour at  $23^{\circ}\text{C}$  and then overnight (14 to 18 hours) under constant agitation at  $4^{\circ}\text{C}$  in primary antibody diluted as indicated in the same blocking buffer. Primary antibodies used in this study were  $\beta$ -actin (1:4,000 dilution) and ubiquitin (1:1,000 dilution; Cell Signaling Technology).

Following overnight incubation in primary antibody, blots were washed 3X for 10 minutes each in Tris-buffered saline solution with 0.1% Tween-20 under constant agitation and then incubated at  $23^{\circ}\text{C}$  for 1 hour in a species-appropriate horseradish-peroxidase conjugated secondary antibody (Amersham Biosciences). Blots were then washed 3X for 10 minutes each in Tris-buffered saline solution with 0.1% Tween-20 and developed using a commercial horseradish-peroxidase substrate (Thermo Fisher Scientific) according to the manufacturer's instructions. Images were obtained with an imaging system (Azure 300; Azure Biosystems). After imaging, antibodies bound to the membrane were disrupted by incubation with a commercially available stripping buffer (Life Technologies Corporation) for 5 minutes at  $23^{\circ}\text{C}$  under constant agitation. After stripping, blots were reblocked with blocking buffer and probed with a second primary antibody as described earlier.

### Proteasome assay

Proteasome activity was measured using a commercially available 20S proteasome assay (Sigma-Aldrich Inc) according to the manufacturer's

instructions. Briefly, two FISS cell lines (ELA-1 and Kaiser) were seeded in triplicate at 5,000 cells/well in black-walled 96-well plates. Cells were allowed to adhere and grow under normal conditions overnight (at least 12 hours). After incubation overnight, the cells were washed once with PBS solution, and then media containing increasing concentrations of bortezomib (0 to 20 nM) were added. Cells were incubated for 15 hours, at which time a substrate (LLVY-RR110) of the 20S proteasome was added and the cells were incubated an additional 3 hours at 37 °C. After 3 hours of incubation, fluorescence was measured with excitation at 480 to 500 nM, and emission at 520 to 530 nM. A proteolytically active proteasome will cleave the substrate releasing a green fluorescent peptide (R110). Therefore, the degree of fluorescence measured in a sample correlates directly with the activity of the 20S proteasome.

### Flow cytometry for evaluation of apoptosis and necrosis

FISS cells were plated at 150,000 cells/well in 6-well plates and allowed to grow overnight (at least 12 hours) under standard conditions as described earlier. After 12 hours of culture, the media were changed to complete media containing increasing concentrations of bortezomib or DMSO alone (vehicle control). Cells were allowed to grow under standard conditions for 24 to 72 hours, collected via trypsinization, washed once with PBS solution, and resuspended in 1 mL PBS solution. Total cell recovery was enumerated via trypan blue exclusion using an automated cell counter (Cellometer Auto-T4; Nexcelom Biosciences). Cell suspensions were subsequently diluted to  $1 \times 10^6$  cells/mL. A total of 100  $\mu$ L ( $1 \times 10^5$  cells) of each treatment sample and a positive control (boiled sample) were transferred to a 96-well plate, collected by centrifugation, washed once with PBS, and resuspended in binding buffer. Fifty microliters of annexin V-FITC was added to each well and allowed to incubate on a shaker in the dark for 10 minutes according to the manufacturer's protocol (Abnova Corp). Fifty microliters of 7 amino-actinomycin D (7-AAD) was then added to each well and allowed to incubate an additional 10 minutes on a shaker in the dark. The cells were then washed

once with PBS solution, resuspended in PBS solution, read on a flow cytometer (BD Accuri C6 Plus flow cytometer; BD Biosciences), and analyzed using the associated software.

### Statistical analysis

For cell viability, proteasome inhibition, and apoptosis studies, three independent experiments with 3 replicates each were performed, and differences among groups were analyzed using statistical software. Comparisons were made using nonparametric ANOVA with the post hoc Tukey test. Significant was set at  $P \leq 0.05$ .

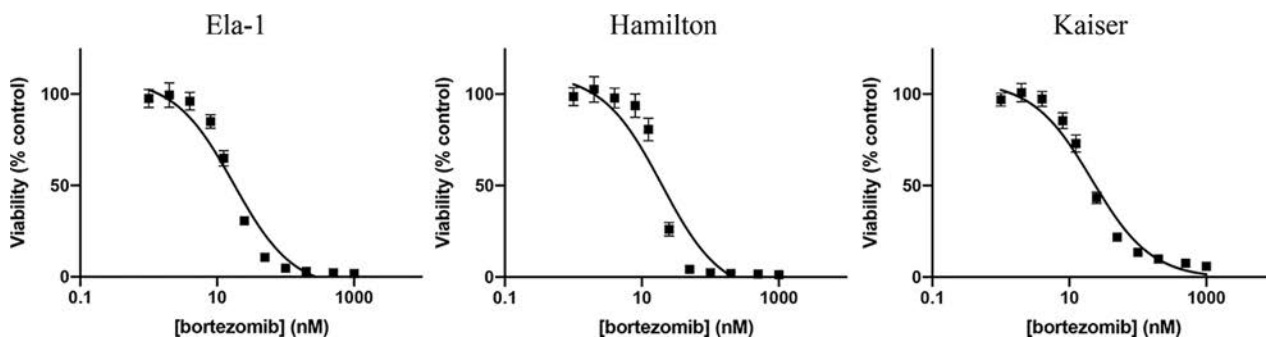
## Results

### FISS cells are sensitive to bortezomib

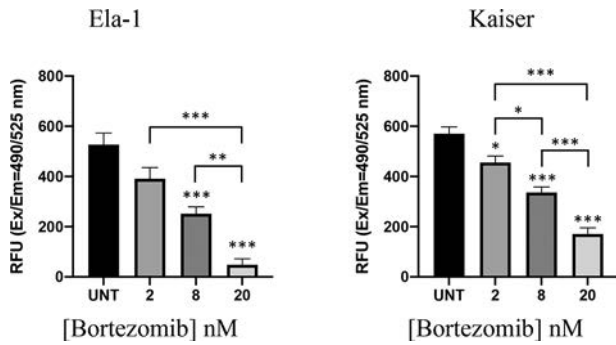
To assess the effects of bortezomib on cell viability in FISS cells, 3 FISS cell lines (ELA-1, Hamilton, and Kaiser) were treated with increasing concentrations of bortezomib (0 to 1,000 nM) for 48 hours. After treatment, total cellular ATP was measured as a correlate for cell viability. Increasing concentrations of bortezomib resulted in a dose-dependent decrease in cell viability in all 3 cell lines (**Figure 1**). The resulting curves were used to determine the  $IC_{50}$  for bortezomib in each cell line. The  $IC_{50}$  for the Ela-1 cell line was calculated to be 17.46 nM (95% CI, 15.47 to 19.72 nM) at 48 hours. The  $IC_{50}$  for the Hamilton cell line was calculated to be 19.48 nM (95% CI, 16.52 to 23.00 nM) at 48 hours. The  $IC_{50}$  for the Kaiser cell line was calculated to be 21.38 nM (95% CI, 19.24 to 23.78 nM) at 48 hours. These data indicate that FISS cells are sensitive to bortezomib in a dose-dependent manner, with  $IC_{50}$  concentrations in the low nanomolar range.

### Bortezomib inhibits 26S proteasome activity in FISS cells

To confirm that bortezomib inhibits the feline proteasome, we chose two cell lines (Ela-1 and Kaiser) and assessed proteasome activity in response to bortezomib treatment. In both the Ela-1 and Kaiser cell lines, bortezomib treatment resulted in concentration-dependent decrease in 20S proteasome activity.



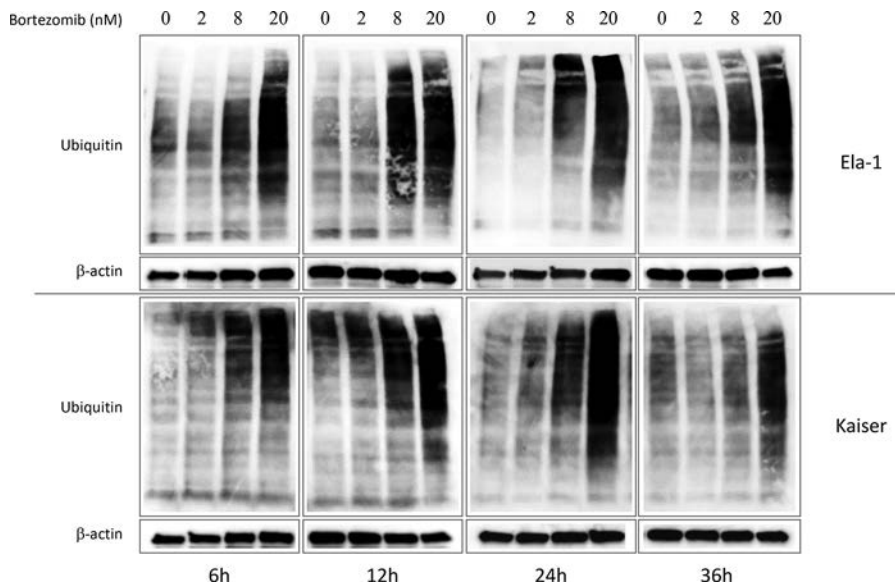
**Figure 1**—Mean  $\pm$  SEM viability of feline injection site sarcoma cells exposed to increasing concentrations of bortezomib. Data are presented as percentage viability versus the untreated (dimethyl sulfoxide only) condition. Nonlinear regression was applied to determine the 50% growth inhibitory concentration for each cell line. Data were derived from 3 independent experiments.



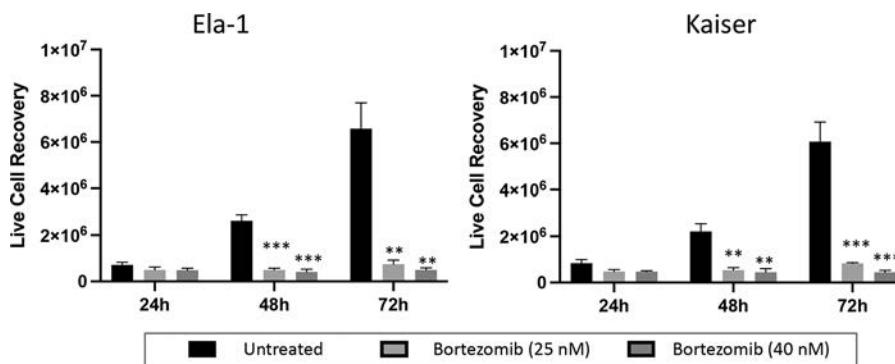
**Figure 2**—Mean  $\pm$  SEM proteasome activity (relative fluorescent units [RFU]) for feline injection site sarcoma cells exposed to increasing concentrations of bortezomib. Data were derived from 3 independent experiments. Ex/Em = Excitation/emission. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , 1-way ANOVA.

In the Ela-1 cell line (**Figure 2**), 2-, 8-, and 20-nM concentrations of bortezomib resulted in approximately 25.8%, 52.3%, and 90.9% reductions in proteasome activity, respectively. In the Kaiser cell line (**Figure 2**), the 2-, 8-, and 20-nM concentrations of bortezomib resulted in approximately 20.1%, 41.0%, and 70% reductions in proteasome activity, respectively. In both cell lines, there was a statistically significant ( $P < 0.001$ ) reduction in the 8- and 20-nM concentration groups when compared to the untreated cells (DMSO alone). In the Kaiser cell line, bortezomib treatment resulted in significant ( $P < 0.05$ ) proteasome inhibition even in the lowest dose (2 nM) when compared to the untreated cells (DMSO alone). Furthermore, a concentration response was observed in both cell lines in which a significant ( $P < 0.01$ ) decrease in proteasome activity was observed between the 2- and 20-nM doses and the 8- and 20-nM doses. The Kaiser cell line also showed a significant ( $P < 0.05$ ) reduction in proteasome activity between the 2- and 8-nM doses. These findings provided evidence that bortezomib inhibits the feline 26S proteasome in a concentration-dependent manner.

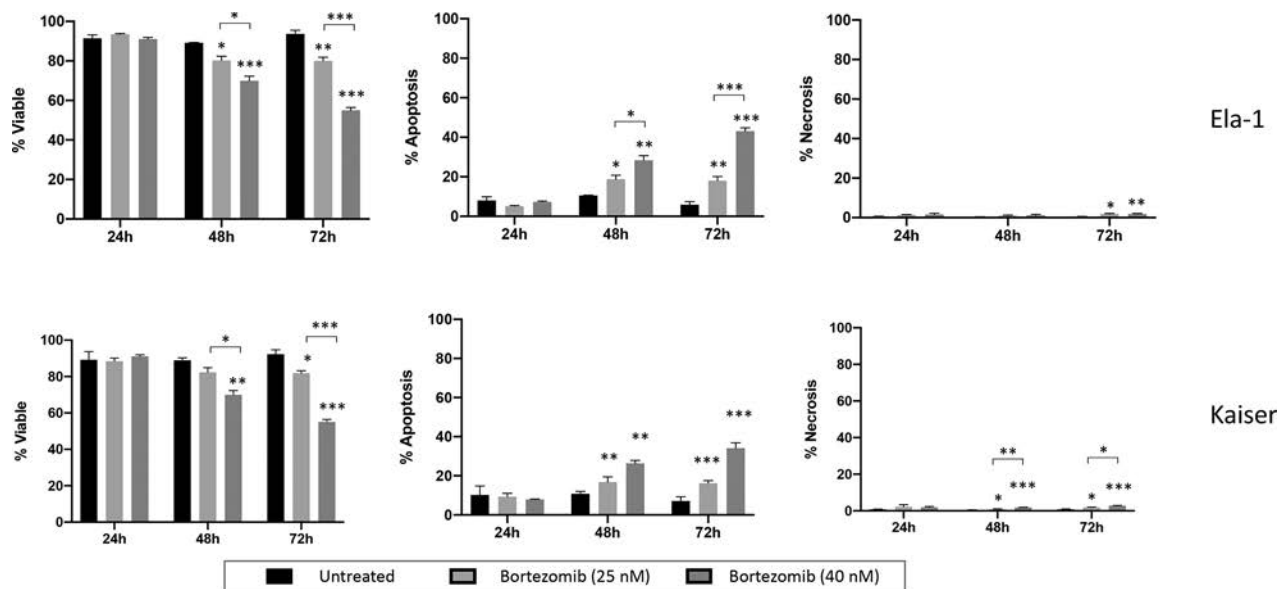
To investigate the impact of bortezomib on FISS cells further, cells were treated with the same concentrations of bortezomib as in the proteasome assay just described (2, 8, and 20 nM) for 6, 12, 24, and 36 hours. At each time point, cells were collected and lysed in RIPA buffer, and total protein was isolated. This protein was analyzed via Western blot for polyubiquitinated protein accumulation as described earlier (**Figure 3**).  $\beta$ -Actin was included as a loading control. Western blot analysis revealed a dose-dependent accumulation of polyubiquitinated proteins after treatment with bortezomib. This accumulation of polyubiquitinated proteins is visualized as a smear on the blot. In both cell lines, this accumulation was present as early as 6 hours post-treatment at the 8- and 20-nM concentrations. In the Kaiser cell line, this accumulation appeared to peak at 12 to 24 hours and began to recede by 36 hours. These findings supported the proteasome assay, showing that proteasome inhibition via



**Figure 3**—Immunoblot of total protein isolated from cells exposed to increasing concentrations of bortezomib.



**Figure 4**—Mean  $\pm$  SEM total live feline injection site sarcoma cells recovered following exposure to increasing concentrations of bortezomib. Data were derived from 3 independent experiments with 3 technical replicates each. \* $P < 0.01$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , 1-way ANOVA.



**Figure 5**—Mean  $\pm$  SEM percentage versus control of viable, apoptotic, and necrotic feline injection site sarcoma cells after exposure to increasing concentrations of bortezomib. Data were derived from 3 independent experiments with 3 technical replicates each. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , 1-way ANOVA.

bortezomib leads to accumulation of polyubiquitinated proteins that would otherwise be degraded.

### Bortezomib treatment results in FISS cell death via apoptosis

Induction of apoptosis is an important mechanism of bortezomib-induced cell death reported in other cancer types.<sup>16</sup> To assess the effect of bortezomib on apoptosis, FISS cells were treated with 2 doses of bortezomib (20 and 40 nM) for 24, 48, and 72 hours. At 24 hours, the number of total cells recovered was similar between treated and untreated groups in both the Ela-1 and Kaiser cell lines (**Figure 4**). After 48 hours, the total cell recovery in the Ela-1 cell line was reduced by 82.6% and 79.6% at the 25- and 40-nM doses, respectively, compared to the control. After 72 hours, the total cell recovery in the Ela-1 cell line was reduced further by 87.5% and 92.4% at the 25- and 40-nM concentrations, respectively, when compared to the control. After 48 hours, the total cell recovery in the Kaiser cell line was reduced by 74.0% and 73.5% at the 25- and 40-nM concentrations, respectively, when compared to the control. After 72 hours, the total cell recovery in the Kaiser cell line was reduced further by 88.8% and 92% at the 25- and 40-nM doses, respectively, when compared to the control. These decreases were significant ( $P < 0.01$ ) at 48 and 72 hours. No statistical difference was seen in total cell recovery between the 25- and 40-nM concentrations at any time point in either cell line.

Recovered cells were stained with annexin V-FITC and 7-AAD and evaluated via flow cytometry. Like the previous findings, the number of viable cells was largely unchanged at 24 hours in both cell lines (**Figure 5**). However, at 48 and 72 hours, the number of viable cells was statistically ( $P < 0.05$ ) decreased

in both cell lines at the 20- and 45-nM concentrations when compared to the untreated condition. Furthermore, a concentration response was seen with significantly ( $P < 0.05$ ) less viable cells recovered between the 25- and 40-nM concentrations in both cell lines. Likewise, differences in total apoptosis were not observed at 24 hours but were elevated significantly in the 25 and 40-nM conditions in both cell lines at 48 and 72 hours compared to the untreated condition. In the Ela-1 cell line, a concentration response was seen as there were significantly ( $P < 0.05$ ) more apoptotic cells in the 40-nM dose compared to the 25-nM concentration.

### Discussion

Our study identified bortezomib as a potent inhibitor of the 26S proteasome in FISS cells. We demonstrated that FISS cells are sensitive to bortezomib and that treatment with bortezomib leads to proteasome inhibition and accumulation of ubiquitinated proteins. Furthermore, FISS cell treatment with bortezomib led to induction of apoptosis.

Bortezomib is licensed for use in multiple myeloma and mantle cell lymphoma in humans. To our knowledge, there are no published pharmacokinetic or pharmacodynamic data in veterinary species, including cats or dogs. A small study examining bortezomib use in Golden Retrievers with Golden Retriever muscular dystrophy has been published.<sup>17</sup> In that study, dogs were treated with intravenous bortezomib (1.3 to 1.6 mg/m<sup>2</sup>) on a schedule that consisted of twice weekly infusions (Monday and Thursday) for 2 weeks followed by 10 days off. The study found a significant decrease in proteasome activity in the blood of dogs treated with bortezomib when compared to untreated dogs. This was

not a full-dose study and no other pharmacokinetic or pharmacodynamic data were presented. To our knowledge, no studies have been reported that examined bortezomib use in cats.

The low-nanomolar IC<sub>50</sub> doses identified in our study are likely to be achievable in vivo based on human studies.<sup>18</sup> In humans, pharmacokinetic research has demonstrated similar exposure between subcutaneous and intravenous routes, with reduced incidence of adverse effects including peripheral neuropathy.<sup>19</sup> Subcutaneous dosing may be attractive for some cats that are either fractious or present difficulties with regard to venous access. However, in the population of cats diagnosed with FISS, repeated subcutaneous injections will need further consideration. Bortezomib has also been shown to lead to cell cycle arrest at the G2/M checkpoint, which leads to radiation sensitization.<sup>20</sup> This raises the question of whether bortezomib combined with radiation therapy could be a viable strategy in FISS.

Limitations of our study included those commonly associated with in vitro cell culture studies, including the question of how representative responses in cell lines are to an actual tumor response in vivo. Nevertheless, our results provided early evidence that bortezomib may be a viable treatment option for cats with nonresectable FISS. This is important because treatment options are currently severely lacking for nonresectable FISS. Furthermore, a better understanding of the role of proteasome inhibitors in the treatment of sarcomas in cats may lay the groundwork for exploration of their use in human sarcomas.

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The authors declare that there were no conflicts of interest.

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