Biologic Activity of the Novel SINE Compound KPT-335 Against Canine Melanoma Cell Lines

THESIS

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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The Ohio State University
2014

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Abstract

**Background:** Exportin 1 (XPO1, also known as CRM1), is a protein responsible for the export of over 200 target proteins out of the nucleus. XPO1 is upregulated in several human cancers and its expression is also linked to the development of chemotherapy resistance. Recent studies using both human and murine cancer cell lines have demonstrated that XPO1 is a relevant target for therapeutic intervention. The present study sought to characterize the biologic activity of an orally bioavailable selective inhibitor of nuclear export (SINE), KPT-335, against canine melanoma cell lines as a prelude to future clinical trials in dogs with melanoma.

**Results:** We evaluated the effects of KPT-335 on 4 canine malignant melanoma cell lines and found that KPT-335 inhibited proliferation and induced apoptosis of treated cells. Additionally, KPT-335 downregulated XPO1 protein while inducing a concomitant increase in XPO1 messenger RNA. Lastly, KPT-335 treatment of cell lines induced the expression of the tumor suppressors p53 and p21, with enhanced nuclear localization.

**Conclusion:** KPT-335 demonstrates biologic activity against canine melanoma cell lines at physiologically relevant doses, suggesting that KPT-335 may represent a viable treatment option for dogs with malignant melanoma.
Acknowledgments

I would like to thank my advisors, Cheryl London, William Kisseberth, Sandra Barnard and Emma Warry for their support and guidance during my residency program. I would also like to thank Michael S. Kent for providing the melanoma cell lines. Karyopharm INC. provided the drug for this experiment and also providing experimental support. In particular Dr. Yosef Landesman provided guidance on experimental design and his lab technician Trinayan Kashyap performed the real time for p21 and MIC1. None of the experiments would have been possible without the guidance and patient teaching of Misty D. Bear. I am grateful to Joelle Fenger for assistance with real time PCR as well as other aspects of the study design and research methods. Thank you to Timothy Vojt for his hard work preparing the figures. Also thank you to Jerry R. Harvey in the College of Veterinary Medicine Biomedical Media and Instructional Technologies department for the images of the clonogenic assays. I am grateful for the training and technical support provided by The Ohio State University campus microscopy and imaging facility.
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Chapter 1. Literature Review

1.1 XPO1 and Cancer

The nuclear export protein exportin 1 (XPO1, also called Chromosome Region Maintenance protein 1 [CRM1]) was first identified in yeast.\[1\] The function of this protein was initially delineated by showing that the antifungal/antibiotic drug leptomycin B (LMB), caused the nuclear accumulation of multiple proteins and was covalently bound to XPO1.\[2, 3\] XPO1 is one of seven known nuclear export proteins responsible for shuttling cargo proteins from the nucleus to the cytoplasm \[4-6\]. It is a member of the karyopherin β family of transport receptors that binds over 200 target proteins through a hydrophobic leucine-rich nuclear export signal (NES) present in the cargo \[7\]. XPO1 has many important roles including directing embryogenic development through the neurula stage, progression through the cell cycle, regulation of centrosome duplication, and directing cellular apoptosis.\[1, 8, 9\]

Recently, XPO1 has been identified as a promising target for therapeutic intervention in cancer. XPO1 is the sole nuclear exporter of several major tumor suppressor and growth regulatory proteins (TSPs and GRPs, including p53, p75, Rb, p21, p27, STAT3, FOXO and IκB among others) \[10, 11\]. There is now substantial data demonstrating that XPO1 is upregulated in both hematologic malignancies and solid tumors such as multiple myeloma and cervical cancer \[4-6, 12, 13\]. Furthermore, overexpression of XPO1 correlates with a poor prognosis in many human cancers such as
pancreatic cancer, osteosarcoma, glioma and ovarian cancer.[14-17] The upregulation of XPO1 has also been associated with chemotherapy drug resistance.[5, 6]

One way that XPO1 can affect carcinogenesis is by altering nuclear-cytoplasmic protein trafficking which can result in aberrant localization of key proteins. Tumor suppressor protein p53 is altered in 50% of human malignancies, including HPV positive cervical cancer and esophageal squamous cell carcinoma.[18, 19] The subcellular localization of p53 in the nucleus of the cell is critical for it to be able to exert its activity as a transcription factor and induce cell cycle arrest. Degradation of p53 occurs in the cytoplasm and requires MDM2 being transported from the nucleus.[20] Treatment with leptomycin B has been shown to increase nuclear localization of p53 in cancer cells.[21] The localization of other proteins such as survivin also affects their function. Survivin has anti-apoptotic activity in the cytoplasm and is transported by XPO1 out of the nucleus.[4, 22, 23] A decrease in transcription by survivin when XPO1 is blocked has been shown to increase apoptosis in triple negative breast cancer cells.[24] Finally, p27 protein is an important regulator of the cell cycle. When it is located in the nucleus it inhibits proliferation and when it is localized in the cytoplasm it promotes cytoskeleton remodeling. Downregulation of p27 or increased levels in the cytoplasm are correlated with a poor prognosis. [25] XPO1 is important in transporting p27 out of the nucleus into the cytoplasm where p27 facilitate the G1/S transition.[26] The decrease of nuclear localization of p27 in breast tumors in people has been shown to be associated with progression to an invasive phenotype and is associated with prognosis.[27-29] The expression of p27 and XPO1 has also been shown to be important in cervical cancer,
ovarian cancer lymphoma and glioma.\cite{13, 16, 30, 31} Clearly, protein localization is critical to and helps define many normal and dysregulated protein functions, thus making it a potential target for therapeutic intervention.

1.1.2 XPO1 Inhibitors

LMB had good in vitro success in treating cancers such as leukemia, cervical cancer, esophageal squamous cell carcinoma, lung carcinoma and prostatic carcinoma.\cite{21, 32-39} Unfortunately, LMB failed a phase I clinical trial due to dose limiting toxicity.\cite{40} Several LMB analogues were developed to target XPO1 but lessen the toxicity. One way of accomplishing this was to have a reversible binding affinity. The molecule PKF050-638 is an analog of a class of drugs called N-azolylacrylates and it is a specific inhibitor of XPO1 with reversible binding that was shown to prevent export of the HIV-1 Rev protein.\cite{41} Other drugs that have been developed to prevent the transport of viral proteins out of the nucleus are valtrate and acetoxychavicol acetate. They have been shown to competitively bind to XPO1 at the reactive cysteine site. They have been shown to prevent export of HIV1 virus and influenza virus RNP without damaging the host cells.\cite{42} XPO1 inhibitors that can effect cancer cells and spare normal cells are of particular interest. One group of investigators screened for nuclear export inhibitors of the FOXO family of transcription factors and found 11 small molecule inhibitors that bound covalently to XPO1 at cysteine 528, similar to LMB.\cite{43} Further work has not been done as of yet to show the efficacy of these compounds. The small molecule inhibitor CBS9106 is a reversible inhibitor of
XPO1 that binds at cysteine 528. It has been shown to induce cell cycle arrest and induce apoptosis in 60 different cancer cell lines. It also has been shown to reduce tumor growth and increase survival in mouse xenograft models.[44] The most active current research of inhibitors of XPO1 is with selective inhibitors of nuclear export or SINE compounds which are discussed below.

1.1.3 SINE Compounds

Recently, novel orally bioavailable, small-molecule selective inhibitor of nuclear export (SINE) compounds that specifically bind to XPO1 at the reactive site Cys 528 residue have been developed and tested both in vitro and in vivo [45-51]. SINE compounds induce apoptosis and block proliferation in different cancer cell types, including cell lines derived from colon [10], pancreas [45], and breast carcinomas [49] as well as chronic lymphocytic leukemia (CLL) [48], while sparing normal cells [52]. SINE inhibitors have been shown to directly bind XPO1 and decrease its protein expression while increasing mRNA transcription.[53] Apoptosis of treated cancer cells occurs by several mechanisms, including: forcing nuclear localization of tumor suppressor proteins (FOX03a, p53, IĸB), induction of autophagy, and inhibition of ribosomal biogenesis and translational flux.[54] Further studies have shown potent anti-cancer activity and good tolerability of SINE in vivo using mouse human xenograft (subcutaneous, orthotopic, or leukemograft) models of pancreatic cancer [45], renal cancer [55], CLL [48], mantle cell lymphoma (MCL) [51], multiple myeloma [53] and acute myelogenous leukemia (AML)
Early clinical trials of the SINE KPT-330 (selinexor) have demonstrated biologic activity of XPO1 inhibition in lymphoid malignancies.

The SINE compound KPT-335 (verdinexor, structurally similar to selinexor) previously has been evaluated in canine lymphoma cell lines and found to have good activity in the low nanomolar range.[56] Additionally, a phase I clinical trial of KPT-335 in dogs with primarily lymphoma demonstrated evidence of single agent activity consisting of both partial responses and stable disease for over 4 weeks with excellent tolerability over long-term dosing.[56] Lastly, KPT-335 exhibits good oral bioavailability with an average Cmax of approximately 250 ng/ml and an average AUC of 1800 ng/ml. The purpose of this study was to evaluate the activity of KPT-335 against established canine malignant melanoma cell lines as a prelude to future testing in dogs with metastatic melanoma.

1.2 Human Melanoma

Melanoma in people represents 5% of skin cancers but is responsible for greater than 80% of skin cancer deaths.[57] Dermal melanoma in people is associated with ultraviolet (UV) light exposure as a risk factor.[57] This is in contrast to melanoma in dogs which is not thought to be UV induced. Mucosal melanomas in people, similar to oral melanoma in dogs, are not UV induced and are highly metastatic. However, mucosal melanoma represents a small subset of human melanoma cases (1.3%).[58]

Standard, cytotoxic, chemotherapy has been generally ineffective for treating metastatic melanoma in people, as has been the case in dogs. Melanoma is known to be
an immunogenic tumor and this is thought to be the reason why there can be a long latency period from the time of initial diagnosis to development of widespread metastasis in some patients and spontaneous regression of metastases in others. [59, 60] For this reason, much of the work to develop immunologic approaches to cancer treatment has focused on melanoma. Some of the first immunologic treatments investigated for metastatic melanoma were with the type 1 cytokines: IFN-α-2B and interleukin-2 (IL-2). Treatment with high dose IFN-α-2B was associated with an increase in relapse free survival but not overall survival.[61, 62] High dose IL-2 has a reported 16-23% response rate, with 5-10% of patients experiencing long term responses.[63, 64] Patients can experience significant toxicities including hypotensive shock and renal failure.[59] Since these therapies have low response rates and significant toxicity profiles, more recent research has focused on immunologic approaches with more targeted treatment strategies. BRAF inhibitors were developed to target the 40-50% of patients with a mutation in the gene that encodes the serine/threonine protein kinase BRAF. Treatment with the BRAF inhibitor Vemurafenib resulted in increased survival in patients with tumors harboring BRAF mutations.[65] These patients had an overall response rate of 48% and a median progression free interval of 5.3 months, compared to the control group receiving dacarbazine (MPI 5% and PFI 1.6 months). [66] These results were encouraging, but resistance was a problem and patients who do not have BRAF mutations are unlikely to experience a benefit from such inhibitors.

Some of the newest therapies are monoclonal antibodies that target specific proteins controlling T-cell inactivation. These include ipilimumab which inhibits CTLA4
function (a surface protein on T cells that negatively regulates activation) and nivolumab/lambrozolizumab which target PD1 (inhibitory receptor on T cells).[59] Ipilimumab demonstrated efficacy in patients with metastatic cutaneous and mucosal melanomas.[67-69] In a phase III clinical trial patients with metastatic melanoma were treated with dacarbazine, ipilimumab, or a placebo. The patients treated with ipilimumab experienced a survival benefit of 11.2 months vs. 9.1 months for dacarbazine alone. Patients receiving ipilimumab did suffer from a greater number of toxicities in the form of elevated liver enzymes, endocrine disorders and gastrointestinal toxicity (56.3% vs. 27.5% with dacarbazine alone), which were alleviated with discontinuation of therapy and treatment with glucocorticoids.[69, 70] Treatment with nivolumab also has shown a survival benefit in patients with melanoma.[71-73] Patients with advanced melanoma who receive nivolumab had a median overall survival of 16.8 months and a 1 and 2 year survival of 62% and 43% respectively.[71] Nivolumab and ipilimumab are also being investigated for combination with each other and with other immune modulators such as vaccines.[72, 73]

1.3 Canine Malignant Melanoma

Canine oral malignant melanoma is a biologically aggressive tumor with a reported metastatic rate of up to 90%. [74] Local disease can be treated with surgery and coarse-fractionated radiation therapy, with success dependent on location and stage. [74] Despite options for local control most dogs will eventually die of metastatic disease.[75] Similar to the case with human malignant melanoma, the use of traditional chemotherapy
agents, such carboplatin and cisplatin, has little impact on both the primary tumor and metastatic disease, resulting in no substantial survival benefit.[76-80] In one study, dogs with primary oral tumors had a 28% overall response rate but treatment with carboplatin after local control was achieved showed no survival benefit in a retrospective study.[76, 77, 80] No case controlled prospective clinical trials with chemotherapy have been performed in veterinary medicine to date. Other treatment strategies, such as immunotherapy using a variety of vaccine based approaches, including the commercially available xenogeneic melanoma vaccine (Oncept), have been explored as options for the management of canine malignant melanoma.[81-83] The Oncept vaccine consists of transdermal delivery of a plasmid vector expressing human tyrosinase. While the Oncept vaccine was proven to be safe and preliminary data indicated that it significantly enhanced survival when used in the setting of loco-regional control; [84] a recent retrospective clinical study failed to replicate these findings.[83-87] There is limited data with respect to the use of small molecule inhibitors such as toceranib phosphate (Palladia) with this disease in dogs; however, data regarding efficacy is lacking. [88, 89] Clearly, just as in human medicine new therapeutic approaches to treatment are needed. Furthermore dogs may also serve as a model of natural disease for humans with mucosal melanomas since in both species cases appear to be unrelated to UV exposure, are highly metastatic, and share histopathologic characteristics.[90]
1.4 Molecular Abnormalities in Melanoma

1.4.1 Human Melanoma

There are multiple molecular pathways that are altered in human melanomas: tyrosine kinase receptors pathways (e.g. VEGFR, HER, TGFB), Ras / Raf / MEK / ERK pathway, PI3K / Akt / PTEN / mTOR pathway, cell cycle regulation pathways (Rb / p53 / p16INKA / p14ARF / HDM2), epigenetic gene expression regulation (DNA methylation, histone acetylation and microRNA), programmed cell death (apoptosis) pathways (e.g. death receptor pathway: FAS, TRAILR, TNFR; mitochondrial pathway: Bcl2 family), common apoptosis effectors (e.g. caspases), protein chaperoning and degradation (HSP, proteasome).[91-111] The most common pathway altered is the MAP kinase pathway with the most common alteration in this pathway being in BRAF at the 600 position. The BRAF mutation is present in about 50-60% of melanomas.[92, 112-114] The next most common mutation is in the N-isoform of RAS and this occurs in about 17-25% of cases.[92, 93, 115] These two mutations occur together <1% of the time.[115, 116] Both mutations result in hyperactivation of the MAPK pathway. Mucosal melanomas show less frequent alteration in BRAF and NRAS but have an increased number of C-kit mutations.[58, 117, 118] Another mutation occurring in about 10-15% of cases is a loss-of-function mutation in the tumor suppressor gene neurofibromatosis (NF1). [119] This too can alter MAPK signaling and also leads to constitutive activation of RAS. Other less common gene mutations that can lead to upregulation of the MAPK pathway are KIT, GNAQ and GNA11.[120-122]
In addition to these oncogenic mutations there are other genes that can be mutated that are important for the cell cycle. Cyclin dependent kinases (CDK), CDK inhibitors (i.e. p16) and cyclin D are mutated in 70% of cases. Alterations in \( PTEN \) are seen in 30% of cases. Mutations in p53 have been observed in 20% of the time and its downstream target p21 has deletions in 8-12% of familial melanomas. Downregulation of these cell cycle modulators, as well as Bcl-2 proteins, have been shown to be prognostic and are related to the induction of apoptosis in melanoma cells.

1.4.2 *Canine Melanoma*

There are no appreciable \( BRAF \) mutation as in canine melanomas but there may be alterations more similar to mucosal melanomas in people. There have been alterations seen in N-ras and p-ERK which may alter the MAP kinase pathway which is also commonly deregulated in human melanomas. The PI3K/AKT/MTOR pathway is also suspected to be altered. Total as well as phosphorylated AKT has been shown to be increased in more than one study of canine melanoma cell lines.

Cell cycle regulators such as CDKs have also been shown to be altered in canine melanoma. In particular mutations of the \( CDKN2A \) gene have been shown to occur along with inactivation of \( TP53, RB, CDKN1A \) and \( PTEN \) genes. The most frequent abnormality found in canine cell lines is a reduction in p16 and PTEN expression and the exclusion of retinoblastoma protein and p53 from the nucleus. Similar to human melanomas p53 may be important for apoptosis and increased nuclear localization.
and expression of the downstream target p21 is appreciated after treatment with cytotoxic drugs.[133] In one study looking at p53 expression in canine and feline tumors using immunohistochemistry, only 1 in 20 tumors expressed p53. However, interestingly, the feline samples included metastatic lymph nodes from two cats that had an increase in p53 expression. [134] This could support the idea the p53 is not a primary driving mutation in primary melanoma tumors but could be altered in the metastatic phenotype.

Other stimulators of proliferation in cells have been found to be altered in canine melanoma. Oncogenes erb-b2 and c-myc have also been shown to be activated. [135] Expression of c-kit, an important driver of cellular proliferation, has also been observed.[136] Initial studies of c-kit expression in canine oral melanomas were unable to show differences in survival associated with KIT mutations, however recent work has shown an association with tumor recurrence.[136, 137]

1.5 Summary

There is a need for novel therapeutics in melanoma in dogs and people. There appears to be molecular similarities between canine oral melanoma and mucosal melanomas in people. The ability to affect the transport of key regulatory proteins between the nucleus and cytoplasm is a possible way to induce cell cycle arrest and apoptosis in cancer cells. There have been several drugs developed and currently the SINE inhibitors show promise in several types of cancers. The purpose of this study was to evaluate the activity of KPT-335 against established canine malignant melanoma cell lines as a prelude to future testing in dogs with metastatic melanoma.
Chapter 2. Materials and Methods

2.1 Cell Lines and Reagents

Canine melanoma cell lines Mel 23, Mel 36, Mel 69 and Mel 83 were generously provided by Michael S. Kent (University of California-Davis School of Veterinary Medicine, Davis, CA).[130, 133, 138] Three of the lines (Mel 23, 69 and 83) were derived from primary oral tumors and Mel 36 was generated from a metastatic lymph node. The cell lines were maintained in RPMI 1640 supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, penicillin, streptomycin, L-glutamine, and Hepes (4-(2-hydroxyethyl)-1-piperazineethanesolfonic acid) at 35 °C, supplemented with 5% CO₂. KPT-335 (provided by Karyopharm Therapeutics, Inc, Natick, MA) was dissolved in DMSO to generate stock solutions for use in vitro. The following antibodies were used for Western blotting and immunofluorescence experiments: anti-p53, anti-p21, anti-XPO1, and anti- β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA).

2.2 Cell Proliferation Assay

Melanoma cells (1.5x10³) were seeded in triplicate in 96-well plates overnight in 10% FBS supplemented media and incubated with DMSO or increasing concentrations of KPT-335. After 72 or 96 hours, the medium was removed and the plates were frozen at -80 °C overnight before processing using the CyQuant® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Cell
proliferation was calculated as a percentage of the DMSO-treated control wells and IC$_{50}$ values were derived after plotting proliferation values on a logarithmic curve. Each experiment was repeated 3 times.

2.3 Assessment of Apoptosis

The ability of KPT-335 to induce apoptosis in treated cells was assessed using annexin V/propidium iodide (PI) staining as previously described [139]. Briefly, 1.0 $\times$ 10$^6$ canine melanoma cells were treated with 0.1% DMSO, 0.1μM KPT-335, or 1μM KPT-335 for 96 hours at 37°C. Cells were collected, washed, and stained with annexin V–fluorescein isothiocyanate and PI for 15 minutes before evaluation by flow cytometry on a Becton Dickinson FACS Caliber flow cytometer, BD Biosciences, San Jose, California. Each experiment was repeated 3 times.

2.4 Immunoblotting

Melanoma cells (2x10$^6$) in 10% FBS medium were treated with DMSO, 0.1μM or 1μM KPT-335 for 4 or 24 hours. Protein lysates were prepared and quantified, separated by SDS-PAGE, and Western blotting was performed using previously described methods.[140] The membranes were incubated overnight with anti-XPO1, anti-p53 or anti-p21 antibodies, then incubated with the appropriate horseradish peroxidase linked secondary antibodies, washed, and exposed to substrate (SuperSignal West Dura Extended Duration Substrate, Pierce, Rockford, IL). Blots were stripped, washed, and reprobed for β-actin.
2.5 Quantitative RT-PCR

Total RNA was extracted from canine melanoma cells that were cultured in 10% FBS supplemented medium for 4 or 24 hours with DMSO, 0.1μM or 1μM KPT-335, using TRIzol (Invitrogen, Carlsbad, CA). cDNA was made from 2 μg of total RNA using Superscript III (Invitrogen), followed by real-time PCR with TaqMan-specific probes (Applied Biosystem, Rockford, IL) according to the manufacturer’s protocol. Real-time PCR for XPO1 was performed using the Applied Biosystems StepOne Plus Detection System and MIC-1 and p21 expression was detected using the ViiA™ 7 Real-Time PCR System (Life Technologies, Rockford, IL). Normalization was performed relative to 18S rRNA. All reactions were performed in triplicate and included no-template controls for each gene. Relative gene expression for all real-time PCR data was calculated using the comparative threshold cycle method.[141]

2.6 Immunofluorescence

Cells were plated in a 24-well plate with poly-lysine coated coverslips (35,000-50,000 cells per well) then treated with DMSO or 1μM KPT-335. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X. Next, the cells were blocked at room temperature in blocking buffer (1x PBS/5% bovine albumin/ 0.3% Triton-x) for 30 minutes and then were incubated with anti-p53 or anti-p21 for 1 hour at room temperature. A secondary FITC labeled anti-rabbit or anti-goat antibody was applied for 30 minutes, as appropriate [Alexa Fluor® 488 goat anti-rabbit IgG
(Invitrogen) or Alexa Fluor® 488 donkey anti-goat IgG (Invitrogen)]. Cells were also
stained with DAPI to visualize the nucleus (ProLong® Gold antifade reagent with DAPI,
Invitrogen). Intracellular localization of proteins was analyzed by immunofluorescence
microscopy using an Olympus FV1000 Spectral confocal microscope, Olympus America,
Center Valley, Pennsylvania.

2.7 Clonogenic Assay

Melanoma cell lines were grown in flasks until 80% confluent, then collected,
washed and plated at 2,000 cells per well in six-well plates. After 24 hours the cells were
treated with DMSO, 1nm, 10nM, 0.1 μM, 1 μM or 10μM KPT 335 and incubated at 35°C
and 5% CO₂, in RPMI 1640 supplemented with 10% FBS, non-essential amino acids,
sodium pyruvate, penicillin, streptomycin, L-glutamine, and Hepes (4-(2-hydroxyethyl)-1-
piperazineethanesulfonic acid for 7 days. Cells were then fixed with methanol/ acetic
acid (3:1), washed with PBS and stained with crystal violet (0.5%). The surviving cell
fraction was defined as the number of colonies counted divided by the number of cells
that were plated in the treated groups and then normalized to the plating efficiency.
Plating efficiency was defined as the number of colonies divided by the number of cells
plated in the untreated group. [130, 133] Vehicle control treated Mel 23 and Mel 36 cells
served as the plating efficiency control group. Experiments were performed in triplicate.
2.8 Statistics

All experiments were performed 3 times and/or performed in triplicate. A mean and standard deviation was derived from all repeated experiments. The apoptosis assays and clonogenic assays were evaluated using a Student’s t-test to compare treated groups to vehicle control. A one-way ANOVA comparison test was used to evaluate differences in gene expression among the KPT-335 treated and vehicle control treated groups for the quantitative RT-PCR assays and to compare multiple treatment groups in the cell proliferation assays. Values of p < 0.05 were considered statistically significant.
Chapter 3. Results

3.1 KPT-335 inhibits the proliferation of canine melanoma cell lines.

To assess whether KPT-335 was capable of inhibiting the proliferation of canine melanoma cells, canine melanoma cell lines were treated with increasing concentrations of KPT-335 and the CyQUANT assay was used to assess relative cell numbers after 96 hours of culture. As demonstrated in Figure 1, cell proliferation in all cell lines was significantly inhibited at nanomolar concentrations. The IC$_{50}$ values for the various cell lines ranged from 71 to 330 nM.
Figure 1. KPT-335 inhibits proliferation of canine melanoma cells. Canine melanoma cell lines were treated with DMSO or KPT-335 at increasing concentrations for 96 hours then analyzed using CyQUANT® cell proliferation assay kit. Proliferation values are listed as a percentage of DMSO control. Experiments were performed in triplicate and repeated three times. For each cell line there was a significant decreasing trend in cell proliferation with dose of KPT-335 (p <0.0001).

3.2 XPO1 inhibition induces apoptosis of canine melanoma cell lines.

To determine if inhibition of XPO1 resulted in apoptosis of canine melanoma cell lines, cells were treated with DMSO, 0.1μM KPT-335 or 1μM KPT-335 for 96 hours. Following collection, they were stained with markers of early (AnnexinV) and late (propidium iodide) apoptosis and then analyzed by flow cytometry. Figure 2 demonstrates that treated cells exhibited significant increases in early and late apoptosis when compared to the vehicle control in the 0.1 μM and 1μM treatment group, with the
exception of Mel 69 which only had a significant increase in late apoptosis in the 1 μM treatment group.
Figure 2. **KPT-335 induces apoptosis of canine melanoma cells.** Canine melanoma cell lines were treated with DMSO, 0.1μM KPT-335 or 1μM KPT-335 for 96 hours. Cells were collected and stained with Annexin V FITC/propidium iodide and analyzed by flow cytometry to quantitate early and late apoptosis. Cells treated with KPT-335 at 0.1 μM and 1μM demonstrated a substantial increase in early and late apoptosis compared to vehicle treated cells (p <0.05; with the exception of the Mel 69 in which only the 1 μM late apoptosis group showed statistical significance).

### 3.3 Colony formation is inhibited by KPT-335.

Mel 36 and Mel 23 previously have been demonstrated to exhibit colony formation in culture and inhibition of colony growth has been used as a tool to assess the effects of various treatments including rapamycin, nutilin-3 and DNA damaging agents on these canine melanoma cell lines.[130, 133] To determine whether blocking XPO1 function would impair the formation of colonies, Mel 23 and Mel 36 cells were seeded in 6-well plates and treated with DMSO or KPT-335 and evaluated daily. After 7 days of culture the plates were collected and colonies were counted following staining with
crystal violet. A significant inhibition of colony formation occurred in cells exposed to 
>10 nM of drug when compared to vehicle control (Figure 3).
Figure 3. Effect of KPT-335 on colony formation. Canine Mel 23 and Mel 36 cells were seeded at 2,000 cells per well in a 6-well plate for 24 hours, followed by treatment with DMSO, 0.001, 0.01, 0.1, 1 or 10 μM KPT-335 for 7 days. Cells were then fixed and stained with crystal violet and colonies with greater than 50 cells were counted. The surviving cell fraction was defined as the number of colonies counted divided by the number of cells that were plated in the treated groups and then normalized to the plating efficiency. Plating efficiency was defined as the number of colonies divided by the number of cells plated in the untreated group. Vehicle control treated Mel 23 and Mel 36 cells served as the plating efficiency control group. The surviving fraction was significantly decreased in cells treated with >10nM concentration of KPT-335, p<0.05.
3.4 KPT-335 downregulates XPO1 protein expression while inducing a concomitant increase in XPO1 mRNA.

Previous work has shown that XPO1 inhibition by SINE compounds results in loss of target protein, while simultaneously inducing upregulation of XPO1 gene expression.[53] Furthermore, increased expression of XPO1 mRNA has been used as a pharmacodynamic marker in people treated with the closely related SINE compound KPT-330 (selinexor).[54] To determine whether KPT-335 has a similar effect on XPO1 in canine melanoma cell lines, cells were incubated with DMSO or KPT-335 at 0.1μM or 1μM for 4 and 24 hours. Figure 4A demonstrates a time- and dosedependent loss of protein expression in treated cells. Concordant with results in human cell lines, XPO1 mRNA was similarly upregulated in the melanoma cells following incubation with KPT-335 for 24 hours (Figure 4b), demonstrating a compensatory response to loss of XPO1 protein.
**Figure 4 Impact of KPT-335 on CRM1/XPO1 message and protein expression in melanoma cell lines**

A. Canine melanoma cell lines were treated with DMSO or KPT-335 at 0.1 μM or 1 μM for 4 or 24 hours prior to collection. Protein lysates were generated, separated by SDS-PAGE and Western blotting for XPO1 and β-actin were performed.

B. Canine melanoma cell lines were treated with DMSO or KPT-335 1μM for 24 hours and RNA was collected. Quantitative RT-PCR was performed for XPO1. Relative expression was found to be increased in treated cell lines with at a p <0.05.

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<th>KPT-335 (μM)</th>
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**3.5 KPT-335 modulates the expression of p53 and its downstream targets and localization of p53 and p21 in canine melanoma cell lines.**
Consistent with other previous reports, only 3 of the 4 lines expressed p53 protein. KPT-335 treatment enhanced p53 expression in two of these lines after 24 hours of culture (Figure 5A). Real time PCR showed that expression of the down-stream targets of p53 (p21 and MIC-1), were significantly upregulated after canine melanoma cell lines were treated with DMSO or KPT-335 at 0.1 μM or 1 μM for 4 hours (Mel 69) or 24 hours (Mel 23 and Mel 83) (Figure 5B). Mel 69 was collected at an earlier time point because this cell line grew at a much faster rate than the other cell lines so over confluence in the vehicle control group was a problem at later time points of collection. Mic-1 was only significantly increased in Mel 23 and Mel 69. Similarly, p21 was expressed in 3 of the 4 lines and exposure to KPT-335 increased its expression.

Evaluation of nuclear versus cytoplasmic protein in treated cells revealed that with drug treatment both p53 and p21 expression were increased primarily in the nucleus, although levels were somewhat enhanced in the cytoplasm as well (Figure 5C). Lastly, confocal microscopy confirmed that p53 and p21 did demonstrate enhanced nuclear localization after KPT-335 treatment in Mel 23 and Mel 69 (Figure 6).
Figure 5 Evaluation of p53 and p21 expression and localization in canine melanoma cell lines after KPT-335 treatment. A. Canine melanoma cell lines were treated with DMSO or 1μM KPT-335 for 24 hours prior to collection. Protein lysates were generated, separated by SDS-PAGE and Western blotting for p53, p21 and β-actin were performed. B. Canine melanoma cell lines were treated with DMSO or KPT-335 at 0.1 μM or 1 μM for 4 hours (Mel 69) or 24 hours (Mel 23 and Mel 83). RNA was collected and quantitative RT-PCR was performed for MIC-1 and p21. Relative expression of p21 was found to be significantly increased in Mel 23, Mel 69 and Mel 83 cells treated with 0.1 μM and 1 μM KPT-335 and MIC-1 was significantly increase in Mel 23 and Mel 69 cells treated with 0.1 μM and 1 μM (p <0.05). C. Canine melanoma cell lines were treated with DMSO or 1 μM KPT-335 for 24 hours prior to collection. Nuclear and cytoplasmic protein lysates were generated and separated by SDS-PAGE and Western blotting for p53, p21 and β-actin was performed.
Figure 6 Confocal microscopy to detect p21 and p53 cellular localization following KPT-335 treatment of melanoma cell lines. The canine melanoma cell lines Mel 23 and Mel 69 were treated with DMSO or 1μM KPT-335 for 24 hours. Cells were fixed and stained with anti-p21 A. or anti-p53 B. and then a secondary FITC conjugated antibody. Cells were then counterstained with DAPI to identify the nucleus. Images were obtained using an Olympus FV1000 Spectral confocal microscope and overlayed to assess protein localization.
Chapter 4. Discussion

Unlike malignant melanoma in humans which occurs primarily in the skin and is typically associated with exposure to UV irradiation, malignant melanoma in dogs occurs primarily in the oral cavity and nail beds.[74] Approximately 60% of human cutaneous melanomas possess activating mutations in the cytoplasmic kinase BRAF whereas these mutations are not found in the canine disease.[90, 142] In both dogs and people, standard chemotherapy agents do not have substantial activity in either the primary disease or in the setting of metastasis.[74, 76-80, 143] While there are significant differences that exist between the clinical presentation and molecular biology of human and canine malignant melanoma, two recent review articles demonstrated that the diseases do share similar histopathologic features as well as alterations in both AKT and MAPK signaling pathways, with \textit{RAS} and \textit{PTEN} mutations present in tumors from both species.[90, 129]

In human patients with melanomas that carry \textit{BRAF} mutation the small molecule BRAF inhibitor vemurafenib (Zorafenib) has shown significant activity with response rates of approximately 50%. Unfortunately, these responses tend to be short-lived, and most patients relapse within 8-12 months.[144, 145] More recently, monoclonal antibodies that block signaling of CTLA4 (ipilimumab, Yervoy) and PD1 (nivolumab) have induced durable clinical remissions in human patients with widely disseminated
metastatic melanoma; when the two antibodies were combined, response rates were over 40% and in many instances, were durable.[72, 73]

There are currently no monoclonal antibodies that block CTLA4 or PD1 available for use in veterinary medicine, and while the chemotherapy agent carboplatin has demonstrated some activity against primary oral malignant melanoma in dogs, there is no definitive evidence that any chemotherapy agents work in the microscopic disease setting. Recently, the Oncept xenogeneic melanoma vaccine was approved for use in dogs with oral malignant melanoma in the adjuvant setting following control of the primary tumor/local disease.[146] However, many dogs still develop widespread metastatic disease necessitating the development of new therapeutic approaches.

Selective inhibitor of nuclear export (SINE) compounds that target the nuclear export protein XPO1 represent a potential target for therapeutic intervention in canine and human melanoma. Several studies have demonstrated that treatment of tumor cell lines with SINE compounds results in enhanced expression of p21 and p53, and redistribution of these proteins into the nucleus. [48, 50, 51, 53, 55, 144] With respect to melanoma, both p53 and retinoblastoma (rb) protein undergo increased nuclear export preventing them from engaging in active tumor suppression.[132] In canine melanoma p53 was shown to be excluded from the nucleus in 7 cell lines and 18 of 25 tumor samples.[131] The importance of p53 activity independent of mutation status has been demonstrated in human melanoma and therapies that increase the activity of this protein have shown promise.[124, 147, 148] Following treatment of human melanoma lines with a variety of SINE compounds (KPT-185, -251, -276, 330) in combination with a
small molecule BRAF inhibitor, stabilization of p53 was shown to be at least partly responsible for induction of cell cycle arrest and apoptosis. [144] Furthermore, the combination of XPO1 and BRAF inhibition was found to be synergistic, also altering the expression of rb protein and survivin. [149]

Given the similarity of canine and human melanoma with respect to tumor suppressor dysregulation, we were interested in determining whether SINE compounds would exhibit biologic activity against canine melanoma cells. Several SINE compounds have been tested in vitro and found to have good activity at nanomolar concentrations against human prostate, renal, pancreatic, and breast cancer cell lines, as well as against a variety of cell lines representing hematopoietic malignancies (CLL, mantle cell lymphoma, NHL, AML). [47-50, 52, 55]. Currently, the SINE compound KPT-330 (selinexor) is undergoing evaluation in human clinical trials, while the SINE compound KPT-335 (verdinexor) has completed both phase 1 and 2 canine clinical trials. Significant activity against canine NHL was observed in vitro in the low nanomolar range against canine diffuse large B cell lymphoma (DLBCL) samples, as well as in a canine DLBCL cell line. [56] In the phase 1 setting, partial response to KPT-335 was observed in dogs with NHL, and unlike previously tested XPO1 inhibitors such as leptomycin B [40], KPT-335 exhibited good tolerability with anorexia and weight loss as the main clinical effects noted. [56] Based on these findings, we elected to evaluate the potential utility of this SINE compound in canine melanoma lines prior to clinical testing in dogs with melanoma.
In the current study we found that KPT-335 inhibited the proliferation of canine melanoma cell lines with IC\textsubscript{50} concentrations ranging from 71-330 nM. These doses are biologically relevant and achievable based on pharmacokinetic data derived from studies in both healthy dogs and dogs enrolled in the phase 1 and 2 studies.\textsuperscript{[56]} Furthermore, we demonstrated that KPT-335 induced apoptosis of the melanoma cell lines, although these effects were somewhat delayed, occurring at 72-96 hours after drug exposure. This delay in effect is concordant with prior data generated in human melanoma lines in which BCL-2 was not down regulated until 32 hours following exposure to SINE compounds.\textsuperscript{[150]} Additionally, in human leukemia cell lines treated with SINE compounds, cycle arrest was noted at 24 hours but IC\textsubscript{50} values were best at 48 and 72 hours.\textsuperscript{[144]}

Although significant changes in proliferation or apoptosis were not found until 72-96 hours after drug exposure, the melanoma cell lines demonstrated changes in gene and protein expression at much earlier time points. This is likely a consequence of the manner in which tumor cells are disrupted. Inhibition of XPO1 results in a redistribution of nuclear proteins and consequently, an impact on gene transcription, largely due to restoration of tumor suppressor protein localization. This initiates a genome survey to determine whether sufficient critical non-repairable errors exist that necessitate induction of apoptosis. During this time, the cells may be able to compensate for changes in nuclear and cytoplasmic localization of key proteins for a period of time through such mechanisms as autophagy, until a critical point is reached and the process of apoptosis is initiated.\textsuperscript{[151]} The mechanism of action of XPO1 inhibitors is thus substantially different from that associated with small molecules that block known “driver” proteins.
often constitutively active through mutation, translocation or overexpression. In these cases, blocking the function of the driver protein typically results in rapid cell death as the tumor cell has become reliant on that particular protein for survival.

As expected, treatment of melanoma lines with KPT-335 increased the protein expression of p53 and p21 and the mRNA of p21 and Mic-1. There also was enhanced nuclear translocation of p21 and p53. Interestingly, the Mel 36 line, previously reported to be p53 null [133], was found to have basal p53 protein expression that increased after drug exposure. The reason for this discrepancy is not known, although it is possible there have been alterations in gene expression profiles over time. The effects of XPO1 inhibition on expression of these tumor suppressor proteins have been well-documented in a variety of human tumor cell lines treated with SINE compounds and this is likely a significant contributor to loss of cell viability. [48, 50, 51, 53, 55, 144] Prior studies with leptomycin B showed that prostate and neuroblastoma human tumor cell lines exhibited upregulation and activation of p53 that directly contributed to growth arrest and apoptosis.[39, 152] In contrast, apoptosis after inhibition of XPO1 can occur regardless of p53 status in some tumor lines indicating that this is not the sole mechanism for cell death.[150] Human melanoma cell lines were shown to undergo apoptosis after SINE compound exposure regardless of p53 status other mechanisms such as enhanced PUMA expression and decreased activation of NF-κB were found to be responsible.[53] These data are concordant with our studies as all melanoma lines treated underwent apoptosis independent of p53 status.
In addition to effects on transcription factors, our data demonstrate a direct effect of KPT-335 on its target protein, XPO1. Downregulation of XPO1 protein was observed in melanoma cell lines as early as 4 hours after drug exposure and expression was nearly completely eliminated by 24 hours of treatment. These findings are concordant with those generated in human myeloma cells treated with the KPT-185 and KPT-330.[53] Similarly, human myeloma cells treated with the reversible XPO1 inhibitor CBS9106 exhibited downregulation of protein that was dependent on the ubiquitin/proteasome pathway.[44] While protein was decreased, messenger RNA for XPO1 was increased in the canine melanoma lines following KPT-335 exposure, a finding also observed in human cell lines treated with the SINE compounds [53]. This likely represents a compensatory mechanism associated with loss of functional XPO1 protein. In fact, upregulation of XPO1 message is now being used as a biomarker of target inhibition in ongoing human clinical trials of the SINE compound KPT-330 (selinexor). [54]

4.1 Limitations

This study was limited to in vitro work with cell lines which may not translate into in vivo efficacy.

4.2 Future Directions

We would like to perform a phase II clinical trial in dogs with oral melanoma using KPT-335. A phase I trial has already been completed in dogs with lymphoma.[56] Additionally, we would like to further investigate the mechanism of delayed apoptosis
and perform real time PCR to look at mRNA important in autophagy to see if these are up-regulated.

4.3 Conclusions

In summary, KPT-335 (verdinexor), a novel orally bioavailable XPO1 inhibitor exhibited good in vitro single agent activity against canine malignant melanoma cell lines as evidenced by inhibition of proliferation and induction of apoptosis. KPT-335 downregulated XPO1 protein while upregulating XPO1 message, indicative of direct effects on the intended target. These biologic effects were achieved at nanomolar concentrations of drug. These concentrations have been shown to be biologically achievable in dogs following oral administration.
5. References


