IN VITRO ANTI-CANCER EFFECTS OF BENZIMIDAZOLES ON THE CANINE OSTEOSARCOMA D17 CELL LINE

BY

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THESIS

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ABSTRACT

The high morbidity and mortality of canine osteosarcoma (OS) despite standard therapy warrants the need to investigate new treatment options. One avenue in exploring novel therapies is drug repurposing – using drugs with known dosing, toxicity profiles, and pharmacokinetics, and re-purposing them "off-label" for their pharmacologic effects for other diseases. In the search for novel therapies for canine osteosarcoma (OS), the benzimidazole (BZ) drugs, a class of safe and inexpensive anti-parasitics, were identified as potential novel therapeutics.

Benzimidazole (BZ) drugs are used routinely as effective anti-parasitics in both human and veterinary medicine. Their safety is well-established and side effects are minimal in most veterinary species including dogs. Safety has been described both with short-term high doses as well as long-term chronic dosing in dogs and other species with minimal adverse effects.

BZs have demonstrated *in vitro* and *in vivo* anti-cancer effects in both people and animal tumor models. The mechanism of BZs is thought to be similar to the microtubule inhibitory actions of traditional chemotherapeutic drug classes such as taxanes and vinca alkaloids, leading to metaphase arrest (G2/M phase) and tumor cell apoptosis. BZs also demonstrate indirect anti-cancer activity by vascular disruption of endothelial cells and reduction in cancer cell secretion of the angiogenic cytokine vascular endothelial growth factor (VEGF).

In human OS, mitotic spindle inhibitors are routinely used as an adjuvant chemotherapy agent, and similarly mitotic spindle inhibitors demonstrate effect for canine OS. Given the proposed activity at the mitotic apparatus, BZs may have similar activity in canine osteosarcoma. In addition to direct cytotoxic effects, BZs may possess indirect anti-angiogenic effects in canine OS, including modulation of VEGF. In human OS, increased VEGF expression is a negative prognostic factor and a strong predictor of metastasis and poor survival. Similarly serum VEGF is elevated in many canine cancers including OS and correlates with poor disease free interval. This supports a role for VEGF-induced angiogenesis in the development and progression of metastatic disease in dogs with OS and provides another potential anti-neoplastic mechanism for BZs.

We hypothesize that BZs have direct and indirect anti-neoplastic effects *in vitro* for canine OS. The aims of this study were to assess the *in vitro* effects of the clinically-used veterinary benzimidazoles [mebendazole (MBZ), fenbendazole (FBZ), and albendazole (ABZ)]

on a canine OS cell line. Cell lines were evaluated for dose-dependent anti-neoplastic effects on the functions of cell proliferation, cell-cycle phase distribution, and cell death. Soluble VEGF secretion and the effect on tubulin polymerization were also evaluated *in vitro*. Specifically, the *in vitro* effects of ABZ, FBZ and MBZ on D17 canine OS cells were investigated by characterizing 1) cell proliferation with an MTS assay, 2) apoptosis via flow cytometry, 3) VEGF secretion via ELISA and 4) tubulin polymerization and 5) cell cycle distribution via flow cytometry.

The results of this study demonstrate that treatment with BZs inhibits cell proliferation in a dose and time dependent fashion. Flow cytometry demonstrates that BZ treatment induces cells arrest in G2/M and subsequently apoptosis. Mechanistically, the BZs affect microtubules by inhibition of polymerization. Additionally, exposure to the BZs results in decreased secretion of VEGF from D17 OS cells.

Our findings demonstrate that the clinically used veterinary BZs (ABZ, FBZ, and MBZ) possess anti-neoplastic activity in an OS cell line. In addition to direct effects on tubulin polymerization, cell cycle, proliferation, and cytotoxicity, BZs demonstrate indirect activity through modulation of a key pro-angiogenic cytokine. These findings are similar to what we would expect with a traditional mitotic spindle inhibitor such as a vinca alkaloid. *In vitro* effects are apparent at drug doses achievable *in vivo* with minimal expected adverse effects. This data supports the continued investigation into the use of BZs as an adjunctive therapy for canine osteosarcoma.

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This manuscript is dedicated to all the people in my life, who have stood by me through it all, lived with integrity over impropriety and because of their strength have made this world a better place. You know who you are and I love you for it.

Be who you are and say what you feel because those who mind don't matter and those who matter don't mind.

-Dr. Seuss

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CHAPTER 1
INTRODUCTION

Osteosarcoma (OS) is the most common skeletal tumor in dogs and is associated with high morbidity (severe bone pain) and mortality. The addition of adjunctive chemotherapy to local surgery (amputation or limb-spare) has led to prolonged survival time yet this tumor is highly metastatic and is considered terminal in most patients at diagnosis. Doxorubicin and/or platinum agents are considered standard of care adjuvant chemotherapeutics; however other drug classes such as microtubule inhibitors (vinca alkaloids and taxanes) have shown promise in both canine[1-3] and human OS.[4-6] The *in vivo* effects of systemic chemotherapy are thought to induce cytotoxicity of OS cells that have spread microscopically to the lungs. At both the primary (bone) and metastatic (lung) sites, tumors must promote malignant angiogenesis, or new blood vessel formation, to provide nutrients and oxygen for sustained growth and survival. Vascular endothelial growth factor (VEGF) is a key cytokine regulating malignant angiogenesis and its expression in human OS is a negative prognostic factor[7]and strong predictor of metastasis and poor survival.[8] Similarly, serum VEGF is elevated in many canine cancers including OS[9]and correlates with poor disease free interval,[10] demonstrating the role of VEGF-induced angiogenesis in the progression of metastatic disease in dogs with OS. Despite advances in veterinary oncology, the standard of care treatment and survival time for dogs with OS has remained fundamentally unchanged for twenty years, emphasizing the need for novel therapies. One avenue in exploring novel therapies is drug repurposing – using drugs with known dosing, toxicity profiles, and pharmacokinetics, and re-purposing them "off-label" for their pharmacologic effects for other diseases.

Benzimidazole (BZ) drugs have been used for decades in human and veterinary medicine primarily as effective anti-parasitics.[11, 12] Benzimidazoles have well-established safety and induce minimal adverse side effects in many veterinary species including dogs. Although usually prescribed for short-term usage for parasitic diseases, long-term safety has been reported in dogs at doses from 4mg/kg/day for 180 days to 250 mg/kg/day for 30 days for various BZs with no adverse effects. Recently, BZs have demonstrated additional pharmacologic effects, including *in vitro* and *in vivo* anti-cancer activity in both people and animal tumor models.[13-15]The mechanism of BZs is thought to be similar to the microtubule (MT) inhibitory actions of

clinically used anti-cancer drugs such as taxanes and vinca alkaloids.[16] These drugs inhibit cancer via interaction with tubulin and disruption of MT function, particularly of MTs comprising the mitotic spindle apparatus, leading to metaphase arrest (G2/M phase) and tumor cell apoptosis. [4-6, 17] BZs exhibit direct effects (inhibition of cell proliferation, promotion of cytotoxicity) [13-15] but also demonstrate indirect anti-cancer activity via modulation of angiogenesis, including vascular disrupting effects on endothelial cells and reduction in cancer cell secretion of the angiogenic cytokine VEGF.[18-21] Therefore, BZ anti-cancer activity can be attributed to MT inhibition resulting in cancer cell death as well as modulation of VEGF-related tumor angiogenesis.[15, 18, 19, 21, 22]

Based on clinical usefulness in dogs as well as reported anti-cancer activity in other species, the BZs fenbendazole (FBZ), mebendazole (MBZ), and albendazole (ABZ) appear the most promising of the BZ drug class for further study in pet dogs with cancer. Canine OS is an attractive *in vitro* tumor model based on reported sensitivity to MT inhibitors [1-3]and the *in vivo* role of VEGF in angiogenesis and tumor progression. [9, 10] We hypothesize that in canine OS cell lines, BZs inhibit MT formation resulting in cell-cycle arrest and apoptosis and may induce anti-angiogenic effects by reducing secretion of vascular endothelial growth factor (VEGF).

CHAPTER 2
LITERATURE REVIEW

2.1 INTRODUCTION

Osteosarcoma (OS) is the most common skeletal tumor in dogs as well as in children.[23] Osteosarcoma causes malignant osteolysis resulting in severe pain. Affected dogs are presented by their owners for acute or chronic lameness with inadequate response to oral analgesics. In human and veterinary medicine, OS is both locally aggressive and highly metastatic, resulting in high morbidity and mortality. Treatment with surgical removal (amputation or limb-spare surgery) in conjunction with chemotherapy (carboplatin, cisplatin, or doxorubicin) provide the longest median survival times in dogs.[24] Despite aggressive therapy, most dogs will die of metastatic disease approximately 1 year after diagnosis.[25-27] In people, the overall prognosis for localized disease is 65% survival at 5 years.[28] Given this poor survival, current treatment is aimed at alleviation of pain and reduction of tumor progression. The pain associated with OS is a result of malignant osteolysis and bone remodeling incited by the increased neoplastic osteoblastic as well as imbalanced osteoclastic activity. Osteolysis is also necessary in the establishment of bone metastasis.[29]

Investigation of novel therapies for the adjuvant treatment of OS is important to improve the survival and quality of life for these patients. Chemotherapy drugs with alternative mechanisms of action may help slow local tumor progression as well as delay micrometastatic disease which is thus far nearly inevitable in canine OS. Osteosarcoma cell lines and naturally occurring tumors have shown sensitivity to non-standard chemotherapy drugs including the mitotic spindle inhibitors vinca alkaloids and taxanes.[1, 4, 6, 30] However, these chemotherapy drugs retain similar adverse side effect profiles to clinically used drugs effective for OS and thus are unlikely to be superior in clinical use. Novel mitotic spindle inhibitors such as BZs that have limited toxicity profiles yet possess anti-cancer activity could be a viable addition to the current standard of care protocols.

2.2 BENZIMIDAZOLES

2.2.1 Use in Veterinary Medicine

A benzimidazole (BZ) is a heterocyclic aromatic organic compound that is formed by the fusion of an imidazole and benzene.[31] Structurally, the benzene is fused to the 4 and 5 position of the heterocyclic imidazole. (Figure 1) Chemical modifications in the 2 and 5 positions can also increase the activity of the drugs.[32] In 1961, 2-(4'-thiazolyl) benzimidazole or thiabendazole was found to have broad spectrum activity against gastrointestinal parasites. Following this discovery, additional compounds; parabendazole, cambendazole, mebendazole, and oxidbendazole were manufactured and intensely evaluated for anthelmintic use.[33] Today, BZs are used against helminths, fungi and peptic ulcers in people.[34] The BZ family of drugs is most frequently used in veterinary medicine for the treatment of nematode and trematode infections. They are poorly water soluble leading to limited absorption. The formulation of pro-drugs has helped to increase absorption however the BZs are most often given as an oral powder or paste for gastrointestinal parasites and as such intestinal absorption is not important for their primary use. BZs are routinely prescribed for many veterinary species including dogs.

2.2.2 Mechanism of action, toxicity, metabolism and efficacy

As an antiparasitic, a BZ acts by binding to the tubulin within the parasite resulting in inhibition of cell division and polymerization of microtubules via disruption of the mitotic spindle. *In vitro*, BZs prevent polymerization of tubulin into microtubules via suppression of the mitotic spindle and disruption of the microtubule-kinetochore interaction. The loss of tension leads to poor chromosomal alignment.[35] This causes the cells to arrest in G2/M and the cell cannot enter mitosis. Cell death ensues via apoptosis. BZs can also uncouple oxidative phosphorylation in mitochondria. This disruption causes altered metabolism and inhibition of cellular transport in the cell.[33,36] In a time dependent manner, energy reserves become depleted and waste excretion becomes inhibited leading to cell death.[37] Possible additional mechanisms of action have been reported with other BZs. The BZ carbendazim appears to directly target DNA; it can induce oxidative stress and epigenetic regulation through hypomethylation. Global changes in methylation patterns are a characteristic trait of many tumors. Benzimidazole derivatives have also been used as anti-cancer inhibitors of PgP (a drug efflux pump important in chemo-resistance) and topoisomerase I (an enzyme that that relaxes DNA supercoiling during

replication and transcription).[38] It has also been proposed that BZs can inhibit histone deacetylase resulting in hyperacetylation of histones and subsequently affect gene expression.[34]Mebendazole (MBZ) has been shown to induce apoptosis through inactivation of Bcl-2, a protein which suppresses apoptosis by preventing the activation of the caspase pathway. In addition, some BZ derivatives have demonstrated inhibition of EGFR, VEGFR-2 and PDGFR kinase activity, which are commonly upregulated in many cancers.[38, 39] Thus, although microtubule inhibition resulting in cell cycle arrest and apoptosis is likely to be the main anti-cancer mechanism of BZs, other intracellular mechanisms may play a role.

2.2.3 Safety, toxicity and metabolism

Hundreds of BZ derivative drugs exist, though few of the compounds have [40]been thoroughly evaluated. The overall safety of the BZs has been well documented in human and veterinary medicine. In general this class of drugs is well tolerated and has minimal toxicity. The largest volume of information is available for albendazole (ABZ). Bone marrow toxicity resulting in leukopenia and anemia has been reported in dogs, cats and people.[41] Reversible bone marrow hypoplasia secondary to ABZ administration has been noted in a dog, though this has never been reported in people. Bone marrow toxicity is less frequent with fenbendazole (FBZ) administration, possibly due to decreased gastrointestinal absorption compared to ABZ. [36]ABZ undergoes 1st pass metabolism in the liver (people, dogs and rats) into its active form albendazole sulphoxide which is 70% protein bound. Clearance of the parent drug is rapid.[41, 42] No comprehensive pharmacokinetic study has been conducted. Fatty meals enhance oral absorption up to 5 times in people and animals. Multiple studies examining doses in rats ranging from 0-168mg/kg daily (for 1-6 months) showed good tolerance at doses less than 30mg/kg. At doses higher than 30mg/kg/day for 6 months, reported toxicities included anemia, leukopenia, decreased body weight, relative liver enlargement and hypocellular bone marrow.[41]These findings are similar between dogs and rats. ABZ has been associated with teratogenic and embryogenic effects in rats. At doses of 50mg/kg twice daily anorexia has been noted. The oral LD50 of ABZ is >1320 mg/kg (rat) and >3000 mg/kg (mouse). No maximum toxic dose has been established in the dog.[43,44]

Fenbendazole is more than 50% metabolized in the liver in rats; no canine metabolic data is available. It has a greater affinity for helminth tubulin than mammalian tubulin.[13] The major

metabolites of FBZ are oxfendazole, sulfoxide and sulfone.[45] Absorption is quite variable and contradictory reports exist regarding the addition of a fatty meal to increase gastrointestinal absorption.[12] In toxicity studies, no adverse effects were noted at FBZ doses of 250mg/kg/day for 30 days or 125mg/kg for 90 days.[36] The oral LD50 of FBZ is >500mg/kg in rats.[46]

Mebendazole has limited solubility in water affecting its behavior and absorption in the body though uptake is increased with fatty meal. It undergoes 1st pass metabolism in the intestinal wall and liver in people and animals and primarily excreted in the bile and urine. The metabolites have not been individually identified, and are reported to lack anti-parasitic activity.[12, 41]Acute oral toxicity has been investigated in twelve animal species and mebendazole was well tolerated by all species. Noted side effects were diarrhea and weight loss in pigs and horses. The acute oral LD50 >640 mg/kg in dogs. Chronic oral toxicity has been investigated in four animal species including dogs. Oral administration of mebendazole to dogs at doses up to 40 mg/kg once daily for two years did not cause any significant abnormality.[47, 48] Overall, the BZ drugs have minimal toxicity and are well tolerated with variable absorption. (Table 1)

2.2.4 Benzimidazoles as anti-cancer agents

In the early 1950s the anti-cancer potential of the BZs were first discovered when they were added to other compounds such as nitrogen mustard and showed inhibition of carcinoma, mammary adenocarcinoma and sarcoma in mice.[49] In the 1980s further work with various benzimidazole alkylating agents in combination with nitrogen mustard derivatives and benzothiazole alkylating agents showed efficacy against lymphocytic leukemias.[50, 51]Over the next 30 years BZs were more thoroughly evaluated for their anticancer effects. Multiple BZ analogs have been evaluated in people and rodent tumor models. The results are extremely variable, dependent not only on species and tumor type but also dose rate and time. Early work with seven BZ analogs revealed significant inhibition of growth to normal lymphocytes via mitotic arrest by MBZ, parabendazole, cambendazole and FBZ. Other analogs, thiabendazole and oxfendazole (OFZ) showed no activity.[52] Limited information regarding the use of FBZ in both people and animals as a specific anticancer therapy exists. It was noted incidentally that FBZ routinely administered in rat food inhibited tumor growth of human xenograft lymphoma when combined with dietary vitamin supplementation (vitamin B, D, K, E, and A).[13]

2.2.5 *In vitro* and *in vivo* evidence of anti-cancer effects

Many BZ derivatives have been evaluated *in vitro* and *in vivo* over the last 20 years. ABZ has previously been shown to have strong antiproliferative *in vivo* and *in vitro* effects against colorectal cancer and hepatocellular carcinoma (HT-29) in people.[14, 53-55] MBZ induces mitotic arrest in non-small cell lung cancer in mice xenografts.[56] In another study, MBZ had antitumor effects on human lung cancer cell lines *in vitro* and *in vivo.* Flubendazole has demonstrated clinical activity against leukemia and myeloma xenografts especially in combination with vinca alkaloids.[57] A larger study demonstrated cytotoxic effects of carbendazim and benomyl on immortalized human cell lines and primary cell cultures from cancer patients, including leukemia, myeloma, lymphoma, small cell lung cancer, renal and cervical adenocarcinoma.[34] Benomyl appears more potent than the metabolite carbendazim (noted with many drugs) and is more effective against hematologic malignancies while the metabolite carbendazim is more effective against solid tumors. [34] Carbendazim also demonstrated potent antitumor activity against murine B16 melanoma and human HT-29 colon carcinoma cell lines.[58]

More recently, a new generation of synthesized BZ-based agents has also shown anticancer effects against murine melanoma models via PARP-1, which has a role in repair of single-stranded DNA (ssDNA) breaks.[59] The addition of BZ ligands to existing drugs such as cisplatin analogs has *in vitro* anti-proliferative effects on human MC7-breast and HeLa cervical cancer cell lines.[60] Derived compounds, such as 2-substituted BZs also have anticancer effects. Examples of this include bis-bezimidazole derivatives that interfere with DNA topoisomerase 1 and are cytotoxic against breast adenocarcinoma and skin epidermoid carcinoma. Another example is methyl-2-benzimidazole carbamate; this agent induces apoptosis in cancer cells.[55] A novel derivative, MPTB, can also induce apoptosis in human chondrosarcoma cells.[61] Various derivatives of 2-mercaptobenzimidazoles in one study showed antiproliferative activity with notable activity in G2/M phase arrest with time-dependent induction of apoptosis. Thiazolobenzoimidazole derivatives also activate apoptosis. [62] Thus, many BZ drugs exhibit anti-cancer properties, with demonstrated efficacy against a broad spectrum of tumor types and several potential mechanisms of action.

2.2.6 Benzimidazoles as possible therapy for resistant tumors

Acquired tumor resistance to chemotherapy necessitates novel therapies for successful cancer treatment. The drugs most often associated with acquired resistance are paclitaxel, docetaxel, vinca alkaloids, doxorubicin, daunorubicin, epirubicin, etoposide, dactinomycin, and mitomycin C.[63] Many mechanisms of tumor resistance exist. A frequently encountered mechanism involves cells with the MDR phenotype that utilize alterations in the Golgi apparatus, lysosomes and other organelles that affect post-translation pathways. The Golgi apparatus is central to maintaining growth and survival of cancer cells and may be an additional therapeutic target site. A BZ based chemical compound, 2-(substituted phenyl)-benzimidazole or 2 PB can displace the resident Golgi proteins by inhibiting its ability to recycle these proteins leading to inhibition of cell proliferation. [64] Another resistance mechanism involves PARP-1 (Poly (ADP-ribose) polymerase-1). PARP-1 is activated by DNA damage, causing it to cleave NAD+ and transfer ADP-ribose units which aid DNA repair, allowing cancer cells to evade apoptosis following DNA damage. Some cancer cells overexpress PARP-1, making it an attractive target. Various formulation and chemical alterations of BZ cores such the addition of a piperidyl or pyrrolidinyl +/- an alkyl group on the nitrogen at the 2 position demonstrate positive enzymatic and cellular assay results for PARP-1 inhibition. Continued modifications demonstrate improved pharmacokinetic properties and potent oral efficacy, making BZs yet more attractive as novel anticancer agents.[59]

2.3 TUBULIN AND MICROTUBULES

2.3.1 Structure and function

Tubulin is a heterodimer, made up of α and β subunits. The α and β tubulin subunits have masses of 55 kDa and both have approximately 450 amino acid residues.[65, 66] They are highly conserved across species (60%) and vertebrates have at least 6 genes responsible for tubulin formation. Tubulin is subject to multiple post translational modifications which are evolutionarily conserved. These include: acetylation of a lysine near the NH2-terminus of a tubulin by acetyltransferase, detyronization which removes the C terminal tyrosine residue of the α tubulin, and polyglutamylation, a modification that adds a glutamate side chain to the C terminal tail of both α and β tubulin.[66] The tubulin subunits interact in a covalent manner, forming a stable cylindrical structure that is 24nm in diameter. The tubulin dimers polymerize to

form a protofilament. This 3-D structure of the microtubule (MT) is composed of 13 protofilaments.[67] (Figure 2) Microtubules are an important component of the cytoskeleton and play a critical role in many processes such as motility, organelle function and distribution during interphase, vesicle trafficking, morphogenesis and chromosome segregation during mitotic cell division. As important parts of the cell scaffold, they help to localize and regulate cell signaling proteins.[17, 65] Many compounds isolated from plants influence MT assembly and stability. These include vincas, colchicine, taxol, and epothilone.[66] The two key binding sites for anti-neoplastic drugs are the colchicine and vinblastine binding sites. The colchicine binding site is located at the interface between α and β subunits of the same heterodimer. Some BZs including MBZ and FBZ are reported to bind to the colchicine binding site of mammalian tubulin. The vinblastine binding site lies between two tubulin heterodimers and includes tubulin residues that are located on the inner lumen of the MT. [68] (Figure 3) The combination of the crucial function in cell division plus multiple potential drug binding sites make MTs an attractive drug target with microtubule inhibitors (MTIs).[69-72]

2.3.2 β Tubulin

There are eight mammalian α tubulin and seven mammalian β tubulin isotypes. The β tubulin isotypes (I, II, III, IVa, IVb, V and VI) are highly conserved and are distinguished by their C-terminal sequence. [73] Aberrant expression of β tubulin has been reported in multiple cancers and this phenotype may be more aggressive and exhibit drug resistance. βIII tubulin is overexpressed in lung, prostate, ovarian and breast cancer cell lines and is associated with taxol drug resistance.[74-76] Increased expression of βIV tubulin and taxol resistance has also been demonstrated in OS cell lines.[77] Altered expression of β-tubulin isotypes in a taxol-resistant canine tumor-derived osteosarcoma-like cell line is reported. [78]As is typical in cancer therapy, there is variability in isotype expression based on tissue type and tumor type in people. Investigations into specific β tubulin isotype and effects of cancer therapies may represent another avenue for drug therapy.

2.3.3 Microtubule assembly and regulation in cells

Microtubules are assembled by stacking of the αβ-tubulin in a head to tail orientation forming the dynamic polarity. When MTs assemble, the plus end grows more rapidly than the minus end. MTs grow in an outward fashion, with the plus end leading, from the microtubule organizing center (MTOC) to the cell membrane. The minus ends remains attached to the MTOC. [68]This assembly is accomplished by the hydrolysis of GTP that is associated with β tubulin, resulting in MTs that consist primarily of GDP-tubulin stabilized at the plus end by a cap. [66] MTs assemble at temp >30 degrees C in the presence of GTP and magnesium ions and dis-assemble at lower temperatures.[79] MT plus end assembly occurs by the addition of tubulin dimers to a sheet-like lattice that then rolls up into a cylinder as the MT grows. The growth at both ends is interrupted at random times by shrinkage and growth, called dynamic instability.[66, 68, 80]

2.3.4 Microtubule Dynamics: Instability and Treadmilling

The dynamic nature of how tubulin polymerization results in spindle MTs can be summarized by the models of dynamic instability and treadmilling. Dynamic instability (DI) is the condition in which MT ends switch between growth and shortening. There are four parameters within DI which include: catastrophe (transition from growth or pause to shortening), rescue (transition from shortening to growth or pause), pause (time in which change is undetectable via microscopy), and dynamicity (overall detectable rate of change via light microscopy). Catastrophe and rescue occur when MTs grow out from the spindle to attach to the chromosomes at the kinetochore. When they attach, the bipolar spindle is formed. Dynamic instability is an important part of the MTs function during interphase for the establishment of interactions between the MT spindle and the chromosomes during mitosis. During cell division MTs attach chromatid pairs at the kinetochore and align the chromosomes to the mid plane at metaphase. Kinetochores selectively bind GTP over GDP, favoring the plus end over the minus. Treadmilling is the continued addition and removal of tubulin, causing a net lengthening on one end and a net shortening on another which results in tension on the spindle. Tension is necessary for the cell to progress from metaphase to anaphase. Suppression of dynamics prevents normal mitotic assembly and decreases tension at the kinetochore. Downstream, this leads to prolonged

mitotic arrest or apoptosis. The cells arrest in G2/M phase.[67] Thus, both inhibition of dynamic instability and treadmilling may be therapeutic targets of mitotic spindle inhibitors.

2.3.5 G2/M Mitotic Checkpoint

Chromosome alignment and segregation during cell division relies primarily on the interaction between the kinetochore (positive end embeds here) and the mitotic spindle. To avoid errors in chromosome segregation the kinetochore must secure the spindle MTs and connect the sister chromatids to the opposite spindle. MTs growing from the spindle pole search seek out the chromosomes. During these movements, MTs will undergo complete catastrophe, shortening almost completely and then rescue and re-grow. Kinetochore movement occurs at rate of approximately 10-50μm/min and utilizes MT depolymerization to move toward the spindle poles and MT assembly to move away from the poles. Cell cycle DNA damage check points occur in late G1, preventing entry into S and in late G2, preventing entry into mitosis. Regulation is via the Cdk protein kinases.[81] The late G2 check point, also referred to the spindle check point ensures accurate chromosomal segregation. If MT dynamics are perturbed chromosomes may not attached or lack the appropriate tension, resulting in check point blockage of progression to mitosis and eventually apoptosis.[67] Of the currently utilized chemotherapeutics, the vinca alkaloids and taxanes function to inhibit the G2/M transition.[62] More than 50% of human cancers have dysfunctional G1 check points and rely on the G2 check point to overcome the DNA damage caused by most cytotoxic cancer agents. The G2 cell cycle check point is infrequently utilized by normal cells, making the G2 check point an attractive target for anti-neoplastic drugs.[67]

2.4 MICROTUBULE INHIBITORS

As previously mentioned many naturally occurring agents inhibit MT function and have been used as anti-neoplastic agents for decades. In veterinary medicine, vinca alkaloids originally isolated from the periwinkle *Catharanthus roseus* are therapeutically important for multiple tumor types include lymphoma, leukemia and mast cell tumors. Broadly speaking, microtubule inhibitors (MTIs) are categorized as stabilizers or destabilizers. Stabilizers such as taxol, docetaxol, and epithilone enhance microtubule polymerization by suppressing MT growth and shortening at high concentrations. These agents bind on the inner surface of the MT. The

result is complete suppression of DI in the MT.[66, 67] The destabilizers include vinca alkaloids (vincristine, vinblastine, vindesine and vinflunine), dolastatins and colchicine; these function to inhibit MT polymerization at high concentrations, leaving cells in mitosis with condensed chromatin. Binding of the vincristine or colchicine domain occurs on the tubulin ends at binding sites named eponymously. (Figure 3) Interestingly, at low concentrations both groups are capable of acting in the opposite manner. With either mechanism, there is a decrease in dynamic movement. The attachment to the kinetochore can be blocked and the centromere stretching and relaxation is decreased which will stop the cell from progressing into anaphase.[66]

2.4.1 Mechanism of action: Taxanes and Vinca Alkaloids

The taxane taxol binds poorly to soluble tubulin but binds with high affinity to tubulin along the interior of the MT. This binding leads to stabilization of the MT, increasing polymerization while decreasing the number of protofilaments. It is assumed that there is a resultant conformational change that increases the affinity of the neighboring tubulin.[67] There is a single taxol binding site on every molecule of tubulin. In addition to MT stabilization, taxol increases cyclin B1 and stimulation of cdc2-B1 kinase demonstrating the multi-functional nature of this drug.[81] Epithilones influence MT architecture by altering the number of protofilaments and influencing the lateral interactions between tubulin subunits in the neighboring protofilament.[68] Taxane drug resistance occurs via the PgP drug transporter. Epithilones have the advantage over taxanes in that they are not transported by a drug transporter. [67] The vinca alkaloids' destabilizing effect results from the poisoning of the tubulin heterodimer. It is believed to prevent polymerization from occurring by blocking the region involved in heterodimer attachment. This has earned the vinca alkaloids the name "mitotic spindle poisons". Vinblastine binds to tubulin and directly to the MT at β tubulin via the vinca domain. Binding is rapid and reversible and induces a conformational change that leads to tubulin self-association. Drug resistance with vincristine and vinblastine is primarily due to PgP protein and MDR gene.[82] All MT targeted drugs have the ability to suppress MT growth resulting in decreased dynamicity and suppression which is adequate to prevent progression of mitosis. Both taxol and the vincas inhibit the stretch and relaxation that must occur between centrosomes of the paired chromatids to allow for separation and progression through cell division.

Vinca alkaloids and colchicine also have anti-proliferative effects on tumor vasculature. This effect has been seen a doses much lower than the MTI effects. Microtubule targeted vascular disruption has been under heavy investigation and colchicine-like drugs are in clinical trials (Auristatin PE and Trisenox).[67] This additional mechanism may allow MTIs to be used as anti-angiogenic and/or vascular-disrupting agents as well, allowing for additional mechanisms of action. (Table 2) Therapies that utilize multiple targets to promote cytotoxic effects may provide improved efficacy.

2.5 ANGIOGENESIS

Angiogenesis is the formation of new blood vessels from existing vascular structures. It is a key step in both tumor growth and the development of metastasis and plays a vital role in embryonic and post-natal development, menstruation and wound healing. Primary tumors as well as metastatic foci require oxygen and nutrient transport necessitating sustained angiogenesis. Capillaries must be within a distance of 1-2mm to ensure that cells receive appropriate oxygen and nutrients.[83] In malignant angiogenesis, continuous stimulation by the tumor and stromal cells is required to support new growth.[84] If new vessel growth cannot keep up with the rapid growth rate of the tumor, hypoxia ensues and the tumor can become necrotic. This condition of oxygen deprivation is an important trigger for tumor vessel growth. There are more than a dozen endogenous proteins that can act as positive regulators or activators of tumor angiogenesis. A hypoxic environment stabilizes hypoxia-inducible factor-1α (HIF-1 α).[85] HIF-1 α is the primary transcriptional regulator of VEGF. HIF-1 α also regulates other target genes: pro-angiogenic cytokines such as fibroblast growth factor-3 (FGF-3) and hepatocyte growth factor (HGF), transcription factors such as annexin V, insulin-like growth factor binding proteins 1,2 and 3 and heat shock factor, and various integrins and matrix metalloproteinases.[86] Angiogenesis can be promoted both by pro-angiogenic cytokines such as VEGF secreted from neoplastic and inflammatory cells as well as upregulation of pro-angiogenic receptors such as VEGFR. Additional important regulators of angiogenesis include tumor necrosis factor α (TNF-α), basic fibroblast growth factor (bFGF), angiopoietin-1 and angiopoietin-2, interleukin-8 (IL-8), and platelet-derived growth factor β (PDGF-β).[85, 87, 88] Pro-angiogenic factors are balanced by the major anti-angiogenic/inhibitory factors including angiostatin, endostatin, interferon-α, and interferon-β.[88]

For new vessels to develop, vasodilation and increased vascular permeability must occur. Endothelial cells become activated and migrate through the basement membrane. The milieu of activated cytokines promotes signaling and survival of these endothelial cells. The cells invade the extracellular matrix (ECM) and begin to form tubular structures. Subsequent re-organization of the ECM helps to support the newly formed tubular structures.[88]

The single most important factor in angiogenesis is vascular endothelial growth factor (VEGF-A/VEGF). VEGF, a selective mitogen for endothelial cells, increases microvascular permeability and leakage. Although necessary for normal development and wound healing, VEGF overexpression has been demonstrated in tumors across species and is associated with malignant angiogenesis. VEGF also increases malignant cell trans-endothelial migration *in vitro*.[86] Serum VEGF levels are elevated in dogs with osteosarcoma and pretreatment elevation is correlated with disease free interval. [10]In people, overexpression of VEGF is linked to disease progression and poor prognosis in various carcinomas as well as osteosarcoma. Thus, VEGF may be useful both a surrogate biomarker of drug biologic activity as well as a target for therapy. Another potential surrogate biomarker of anti-angiogenic therapy is tumor hypoxia. Direct tumor hypoxia can be measured by the use of a probe to measure oxygen concentration within the tumor, or in a surrogate manner using endogenous markers such as HIF-1 α or exogenous probes (pimonidazole and EF5). In human cancer patients, tumor hypoxia may have prognostic and predictive significance.[89]

2.5.1 Assessment of angiogenesis

Microvessel density (MVD) has historically been the standard method used to quantifying tumor vascularity in tumor tissue. Immunohistochemistry with CD31 or von Willebrands factor can be performed to detect endothelial cells. Based on the number of blood vessels in a unit of area, called the vascular hotspot, a MVD score can be determined.[90] In people a strong correlation between MVD and outcome has been identified in carcinomas such as breast, gastric, colorectal and prostatic. MVD has been suggested as a prognostic factor in some human studies, and has been suggested as a prognostic factor in canine osteosarcoma.[9192]Newer methods to assess tumor vascularity *in vivo* include various imaging modalities such as dynamic contrast-enhanced magnetic resonance imaging (DC-MRI), dynamic contrast-enhanced computed tomography (DC-CT), positron emission tomography (PET), single-photon emission

computed tomography (SPECT), optical imaging, and microbubble contrast-enhanced ultrasound.[93, 94, 95, 96] Serum biomarkers are another surrogate method of assessing angiogenesis *in vivo*. The advantage of using biomarkers are that they are non-invasive, simple and represent current status within the patient, quantitative, and relatively inexpensive; however serum levels may not accurately reflect the tumor microenvironment. The use of these modalities may also be valuable for monitoring response to therapy as the clinical use of anti-neoplastic drugs with anti-angiogenic properties increases.

2.5.2 VEGF-Structure and Function

Vascular endothelial growth factor (VEGF/ VEGF-A) is 45-47kD glycoprotein that is a specific mitogen for endothelial cells. It belongs to the VEGF/PDGF (platelet derived growth factor) cysteine-knot superfamily and is a tyrosine kinase receptor ligand. This superfamily is important in hormone and extracellular signaling molecule formation in eukaryotes. [97, 98] VEGF is produced from a DNA coding region (human chromosome 6p21.3) with eight exons. VEGF is highly conserved across species. At least six human isoforms (121, 145, 165, 183, 189, 206 amino acids) exist as a result of alternative splicing of the mRNA.[99, 100] (Figure 4) Alternative splicing is vital in regulating the bioavailability of VEGF. Most VEGF producing neoplastic cells preferentially express VEGF 121, VEGF 165 and VEGF 189. These three major isoforms of VEGF have different properties concerning receptor binding and extracellular localization. Isoform 121 is the shortest and most freely diffusible. Isoform 165 has the highest mitogenic efficacy, while 189 is primarily extracellular membrane-associated.[101, 102] The more soluble isoforms acts at distal sites to promote vascular recruitment, and the extracellular membrane-associated isoforms promote local expansion of capillary beds. VEGF is key in promoting normal physiologic as well as pathologic angiogenesis. VEGF has many effects including induction of proteases that cause extracellular matrix remodeling, increase in vascular permeability, and endothelial cell migration and growth. VEGF activates the VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1 or KDR) receptors on human endothelial cells and the VEGFR-2 receptor in dogs.[8, 103] VEGF works via inhibition of apoptosis, cell proliferation, sprouting, migration tube formation and increased vascular permeability. All of this occurs through the interaction of VEGF with its tyrosine kinase receptors. The mitogenic, angiogenic and permeability-enhancing effects of VEGF are primarily due to VEGFR-2. [22]

2.5.3 VEGF: Regulation

It is believed that VEGF works in a paracrine fashion via the heparinase driven release of the various isoforms that are found on the cell surface or extracellular matrix.[100]As previously noted, the major transcriptional regulator of VEGF is HIF-1. In normoxic conditions, HIF-1, a transcription factor comprised of an α and β subunit is hydroxylated, bound by von-Hippel Lindau (VHL)/ubiquitin complex, and targeted for ubiquitin degradation. In hypoxic conditions (such as the tumor microenvironment) HIF-1 α transcription is activated and is protein stabilized which results in active HIF-1 complex formation and subsequently increased VEGF transcription.[85, 104] (Figure 5)

Tumors often modulate VEGF expression through transcriptional regulation. VEGF expression has been correlated to high mitotic activity and cell proliferation.[22] VEGF receptors are also overexpressed in many tumor types in both people and dogs (OSA, gastric, esophageal, breast, renal, lung and colorectal carcinomas).[8, 103] Hypoxic induction of HIF-1α, subsequently elevated VEGF expression, and increased MVD have been documented in human OS, indicative of a more aggressive phenotype and higher rate of metastasis.[91, 105] As previously discussed oxygen tension is highly important in the regulation and expression of VEGF mRNA via HIF-1 α . Potential tumor-associated regulators of VEGF expression include various growth factors (EGF, TGF-α, TGF-β, FGF and PDGF), the inflammatory cytokines IL-1 and IL-6, and amplification or oncogenic mutations of Ras.[106] VEGF protein levels are highly influenced by post-transcriptional changes. The half-life of VEGF mRNA is under one hour, making regulation of its stability a priority. The principal method of post-translational regulation is N-linked glycosylation, which promotes protein secretion. VEGF undergoes N-linked glycosylation (fucosylation) at a specific asparagine residue. Non-glycosylated VEGF isoforms suffer from inefficient secretion and exhibit lower heparin binding affinity.[100, 101] The mechanism by which microtubule inhibitory drugs exert their effects on VEGF has not been well described. Microtubules regulate HIF-1 α via control of mRNA transport and ensuing HIF translation. Taxanes and other MT disrupting agents inhibit nuclear accumulation of HIF-1 α. HIF-1 α can be included with other transcription factors that utilize microtubules for transport during interphase. As HIF-1 α is regulated by MTs at both protein synthesis and trafficking levels, the efficacy of the MT inhibitors may be more significant than previously understood. [107] Inhibition of protein synthesis and/or trafficking of both HIF-1 α and/or VEGF may be potential

mechanisms by which MTIs, and possibly BZs, modulate VEGF levels. Further investigation is needed in this area.

2.5.4 VEGF: Receptors

Tumors may also promote angiogenesis by up regulating VEGF receptors, specifically VEGFR-1 (flt-1), VEGFR-2 (flk-1/KDR), and less importantly heparin sulfate proteoglycans, and neuropilins. [100] The VEGF receptors are structurally similar, having an extracellular ligand-binding region comprised of seven immunoglobulin-like loop domains attached with a short trans-membrane helix to a cytoplasmic catalytic domain. VEGFR-1 is expressed on both endothelial cells and monocytes while VEGFR-2 is limited to the endothelium. [108] VEGF is secreted by nearly all solid tumors in response to hypoxia. Upregulation of VEGF has been clearly demonstrated in human cancers and more recently in various canine tumors.[103, 109] In canine mammary gland tumors increased VEGF and VEGFR-2 were strongly correlated with a high histologic grade and tumor proliferation. [110] VEGF, VEGFR 1 and 2 staining is significantly stronger in hemangiosarcomas compared to hemangiomas, indicating that even in vascular tissues, the neoplastic process causes upregulation of VEGF and its receptors.[111]VEGFR-2 links with candidate genes associated with poor prognosis in canine OS on pathway analysis.[112]

2.5.5 VEGF: Inhibitors

Many anti-angiogenic and anti-vasculogenic drugs exist. Some inhibit endothelial cells directly while others inhibit the signaling cascade or impair the ability of endothelial cells to break down the ECM. Specific targeting of VEGF involves prevention of ligand-receptor interaction through ligand sequestration with a VEGF antibody, competitive inhibition of kinase activity or blocking the binding of VEGF-A with a monoclonal antibody to the receptor.[113] (Figure 6) One of the few FDA approved and best known VEGF inhibitors used in human medicine is bevacizumab (Avastin). Bevacizumab is a humanized monoclonal antibody that targets VEGF-A in the treatment of various cancers, including colorectal, lung, breast, kidney, and glioblastoma.[108, 114] Antibody therapy in veterinary medicine is lacking due to cost of development and functionality, however tyrosine kinase inhibitors (TKIs) have gained significant popularity over the last decade. TKIs work by blocking (reversibly or irreversibly) the ATP binding site of a kinase. If ATP cannot bind, the kinase cannot phosphorylate itself or

initiate downstream signaling.[98]VEGF, PDGRF and FGFR are members of the receptor tyrosine kinase family and their blockade prevents both auto-phosphorylation and signal transduction thereby stopping the angiogenic signal. (Figure 6) The advantage of a TKI is twofold in that it is effective against cancers that have upregulation of VEGF and/or VEGF receptors. The TKIs as small molecule inhibitors have been very successful as anti-cancer therapies both alone and in combination with tradition cytotoxic therapies. Some BZs also exhibit tyrosine kinase inhibition and this may be another mechanism of therapeutic activity in addition to MTI. ABZ appears to inhibit VEGF in malignant ascites formation[115 116] and more recently, a series of novel BZ derivatives have been shown to inhibit various growth factor receptors (EGFR, VEGFR-2 and PDGFR).[38] The molecular mechanism of these actions has not been well described. Further investigation into optimal anti-cancer BZ drug/dose as well as elucidation of the mechanism by which they inhibit tumor growth may open the door to a novel adjunctive therapy.

CHAPTER 3
IN VITRO EFFECTS OF BENZIMIDAZOLES ON THE CANINE OSTEOSARCOMA CELL LINE D17

3.1 INTRODUCTION

Canine osteosarcoma (OS) is a naturally occurring tumor that is highly malignant and causes severe pain in affected patients. The disease is considered nearly universally fatal in dogs. From a comparative oncologic standpoint the dog is an excellent model for human OS as it follows a similar clinical course, and effective treatment is hampered by challenges similar to those encountered in human medicine. Additional therapeutic options could not only improve the quality of life and survival in canine patients but may also contribute to the treatment of bone tumors affecting people. Novel therapeutic options for the treatment of canine OS have been limited over last two decades and in the quest for new agents we look to agents that exhibit both direct cytotoxicity as well as indirect effect on molecular targets such as vascular endothelial growth factor (VEGF). One avenue in investigating novel therapies is re-purposing of existing drugs with known toxicity profiles for their putative anti-cancer activity as a prudent use of limited research resources.

The benzimidazoles (BZs) are known microtubule (MT) inhibitors that have resurfaced as more than just anti-parasitics in both human and veterinary medicine. *In vivo* rodent work had initially shown promise for the BZs as anti-neoplastic agents and some engineered BZs are now in clinical trials for people.[34] It is believed that the BZs have both direct and indirect anti-neoplastic effects; the direct effects through MT inhibition[117] leading to growth arrest, and indirect effects via angiogenesis modulation.[18-21] Modulation of angiogenesis appears to primarily function through reduction in VEGF, a selective mitogen for endothelial cells that increases microvascular permeability and leakage. Microtubule inhibitors are a current mainstay class of anti-neoplastic drugs in both human and veterinary medicine. Pediatric OS is treated with paclitaxel and docetaxel [23, 118-120] and the vinca alkaloids and taxanes are used for the treatment of various tumors in veterinary medicine. Alteration of MT polymerization causes cell arrest in telophase, triggering apoptosis. Canine OS is an attractive *in vitro* tumor model for testing the effects of novel MTIs such as BZs based on reported sensitivity to MT inhibitors [1-3] and the *in vivo* role of VEGF in angiogenesis and tumor progression. [9, 10]

The hypothesis of this study was that BZs exert direct and indirect anti-cancer activity *in vitro* in canine osteosarcoma. Our objectives were to:

1. Investigate the direct *in vitro* anti-neoplastic effects of BZs on cell proliferation and apoptosis in a canine OSA cell line (D17).
2. Demonstrate that a primary mechanism of action of BZs is direct MT inhibition leading to cell cycle arrest in G2/M in D17 cells.
3. Investigate the effects of BZs on VEGF secretion from D17 cells (addressed in the next chapter).

3.2 MATERIALS AND METHODS

3.2.1 Cell lines

A canine osteosarcoma cell line (D17) was purchased commercially from American Tissue Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cell cultures were maintained at 37 °C in 5% CO2 and passaged twice weekly.

3.2.2 Reagents

Albendazole (ABZ), fenbendazole (FBZ) and mebendazole (MBZ) was obtained from Sigma Aldrich. Stock solutions were prepared in DMSO prior to each use. At the highest drug concentration used for experiments, vehicle was 1% DMSO (used for vehicle control).

3.2.3 *In vitro* cell proliferation

D17 cells were plated at a density of 7.5 x 10^4 cells per 200 µl of complete media in a 96-well microtiter plate incubated at 37°C and 5% CO2. After allowing cells to adhere for 24 hours, media was decanted and replaced with fresh media containing various concentrations of ABZ, FBZ and MBZ (0 -200µM). Following 24 and 48 hours of BZ treatment, adherent cells in microwells were washed twice with PBS. The plate was analyzed for cell proliferation using a tetrazolium salt-containing colorimetric cell proliferation assay (CellTiter 96® AQueous One, Promega Corporation, Madison, WI) after 100 µL of fresh medium was added to each well. Briefly, the assay uses a MTS tetrazolium compound that is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. Formazan as

measured by the absorbance at 490nm is directly proportional to the number of living cells. Results are expressed as percentage of viable cells compared to untreated cells. Dose response curves were used to calculate IC50 for all drugs.

3.2.4 *In vitro* flow cytometry for apoptosis and cell cycle distribution

To evaluate the effects of BZs on cell viability, apoptosis was measured via flow cytometry. D17 cells were initially plated at a density of 1 x 10^5 cells per T25 tissue culture flask in complete media and incubated at 37°C and 5% CO2. Cells were then grown in 6 well plates. After allowing cells to adhere for 24 hours, media was decanted and replaced with fresh media containing various concentrations of ABZ, FBZ and MBZ (0-25µM). Cells were allowed to grow for an additional 24 hours in the presence or absence of drug. D17 cultures were trypsinized, harvested and washed once in phosphate-buffered saline (PBS) and resuspended in 1X binding buffer in PBS. Apoptosis was assessed by flow cytometry using the Annexin V-FITC apoptosis detection kit as described by the manufacturer (BD Biosciences Pharmingen, San Diego, CA). Cells were incubated with Annexin V-FITC in the dark at room temperature for 30 minutes. Cells were then washed once with PBS and re-suspended in 1X binding buffer in PBS supplemented with propidium iodide (PI). Flow cytometry data was acquired with the BD Accuri ® C6 flow cytometry machine and cells were gated based on their forward and side scatter properties. Cells were determined to be viable, early apoptotic, late apoptotic, and necrotic based on absolute Annexin-V-FITC and PI staining. Annexin V-FITC binds to phosphatidylserine located on the outer leaflet of the cell membrane. In a normal cell the phosphatidylserine is located on the inner leaflet of the membrane and in the event of early apoptosis, the phosphatidylserine moves to the outer leaflet. Propidium iodide (PI) is a vital dye that is excluded by intact cell membranes. Propidium iodide binds to DNA by intercalation between bases and may be used to assess cell membrane integrity with Annexin V. It can also be used to label late apoptotic cells as they have already lost their membrane integrity as well as to evaluate DNA content for cell viability. All data were analyzed using FACS (DeNovo Software). A minimum of 100,000 cells were counted for each sample. Triplicates of each experimental condition were performed.

To evaluate the effects of BZs on cell cycle distribution, flow cytometry with PI was performed. After drug treatment, cells were harvested, washed twice with PBS, and re-suspended

to total volume of 1 mL of 70% ice-cold ethanol overnight at -20°C. The cells were washed twice in PBS, centrifuged, and the pellets were re-suspended in a total volume of 200 µL PBS containing RNase A (5µg) and PI (10 µg). Parameters including forward and side scatter and DNA content were obtained using a BD Accuri ® C6 flow cytometer. A 488 nm (blue laser) was used with a 585/40 filter for PI excitation/emission. FACS Express 4.0 (DeNovo Software) was used for gating and analysis of cell cycle using single cycle assessment of the diploid cells. A minimum of 12,000 cells were counted per sample. Triplicates of each experimental condition were performed.

3.2.5 *In vitro* tubulin polymerization

To evaluate the effects of BZs on tubulin polymerization necessary for MT assembly, a porcine brain tubulin polymerization assay was performed in 100-µl volumes at 37°C using a commercial assay (Cytoskeleton, Denver) according to the manufacturer's instructions. In brief, 10 µL of sample (drug or control) in G-PEM buffer [80 mmol/L PIPES, 2 mmol/L MgCl2, 0.5 mmol/L EGTA, 1 mmol/L GTP (pH 6.9), 5% (v/v) glycerol] were added to a 96-well plate and incubated at 37°C for 10 minutes. Then, 100 µL of 3.0 mg/mL reconstituted tubulin in G-PEM buffer at 4°C were pipetted into each well. The plate was incubated in a plate reader at 37°C and the absorbance was recorded immediately. The effects on polymerization of the test compounds were quantified by measuring the absorbance every 30 seconds at 340 nm (A340) for 60 minutes using a FLUOStar® Optima reader (BMG Labtech, Durham NC) was used to acquire data using the kinetic mode (two readings per minute). Duplicates of each experimental condition were performed and averaged.

3.3 STATISTICAL ANALYSIS

Normal distributed data sets were expressed as mean and standard deviation, and non-normal distributed data sets were expressed as median and range. All statistical analysis was performed using commercial computer software (GraphPad, Instat3). Significance was defined as $p < 0.05$.

For each drug, cell proliferation as assessed by change in cell count in treated and vehicle control (1% DMSO) cells in comparison to untreated cells (media control) was analyzed with a one-way ANOVA with Dunnett’s post-hoc test. Apoptosis as assessed by Annexin-FITC mean

fluorescent intensity in comparison to untreated (media control) cells was analyzed with a one-way ANOVA with Dunnett's post-hoc test. Cell cycle arrest as assessed by percent of cells in G2/M phase in comparison to untreated (media control) cells was analyzed with a one-way ANOVA with Dunnett's post-hoc test.

3.4 RESULTS

3.4.1 *In vitro* cell proliferation

All BZs induce a dose dependent and time dependent effect of cell proliferation compared to untreated media control. Vehicle control (1% DMSO) was not statistically different from untreated media control. For ABZ at 24hr incubation, $p<0.01$ for drug dose $\geq 5\mu M$ with a 25% inhibition of cell proliferation. There is a possible trend towards significance at 1μM ($p=0.06$). For MBZ at 24hr incubation, $p<0.01$ for drug dose $\geq 5\mu M$, with a 24% inhibition of proliferation. For FBZ at 24hr incubation, $p<0.01$ for drug dose $\geq 5\mu M$, with a 20% inhibition of proliferation. (Figures 7-10) The effect on cell proliferation is even greater after a 48 hour drug incubation period (data not shown). IC50 for BZs was calculated at 23.4 μM for ABZ, 17.5μM for MBZ, and 79.1 μM for FBZ.

3.4.2 *In vitro* flow cytometry for apoptosis and cell cycle arrest

Flow cytometric analysis of cells labeled with Annexin V-FITC demonstrated induction of apoptosis for all drugs. Vehicle control (1% DMSO) was not statistically different from untreated media control. Figure 11 depicts representative histograms for MBZ; all results were similar. There was efficacy at all doses ranging from 1.6 μM to 25 μM for all three BZs. Effects are dose-dependent with the high dose of 25 μM demonstrating a mean of nearly 33% of cells apoptotic when treated with FBZ, 22% with MBZ and 9% with ABZ. ABZ was least effective in inducing apoptosis, with apoptotic cells doubling compared to control at doses of ≥ 12.5 μM while apoptotic cells more than doubled compared to control at doses of ≥ 1.6 μM. (Figure 12, Table 3)

Flow cytometric analysis of cell cycle distribution demonstrated cell cycle arrest for all drugs. The histograms visually demonstrate the re-distribution between G1, S, and G2/M phases. (Figure 13) The gates depicted in Figure 13 were selected manually and are for visual demonstration only. Actual calculations were performed using the FACS Express 4.0 software

which more accurately calculates the true S phase component using mathematical modeling. (Figure 14) For all samples, no abnormal DNA content was noted and the S phase confidence was fair to good. For MBZ and FBZ, cells were predominantly (>80%) arrested in G2/M at doses of ≥1 μM ($p<0.01$). ABZ showed a slightly less robust response, with 51% of cells arrested in G2/M at ≥1 μM and >80% of cells arrested in G2/M at higher doses (($p<0.01$). (Figure 15, Table 4)

3.4.3 *In vitro* microtubule polymerization

Evaluation of the effect of ABZ, FBZ and MBZ on tubulin polymerization showed a dose independent inhibition of polymerization. Controls included no drug treatment (baseline polymerization), paclitaxel (1μM) a polymerization stabilizer (taxane), and vincristine (2μM) as a polymerization destabilizer (vinca alkaloid). Visually, there is an apparent difference in rate of polymerization between controls. To compare the curves of treatment groups to control, we used the slopes method in which the rate of polymerization is obtained graphically from the linear portion of the polymerization profile, which represents the elongation phase of microtubule assembly. (Figure 16) Drug treatments visually produce polymerization curves fitted between no drug treatment and the polymerization destabilizer vincristine. The depolymerization of the various treatments was calculated by comparison to control and vincristine. The difference between no drug treatment and vincristine was set as maximal depolymerization, or 100% depolymerization effect. Effects of drug treatment on depolymerization were compared to this. There was no apparent dose-dependent effect for any drug, in fact for all drugs, minimal response was seen at the highest dose of 50μM. The most robust response was seen with ABZ: doses of 10-25 μM demonstrated increased depolymerization compared to vincristine (128-160% of maximal response) while at a higher dose of 50μM, there was slightly less effect at 88%. (Figure 17) The least response was seen with FBZ: doses of 10-25 μM demonstrated modest depolymerization compared to vincristine (42-59% of maximal response) while a higher dose of 50μM demonstrate no effect and was similar to no drug (2.3% maximal response). (Figure 18) Mebendazole had similar and less robust response compared to ABZ, with doses of 10-25 μM demonstrated slightly increased depolymerization compared to vincristine (111-123% of maximal response) while at a higher dose of 50μM, there was slightly less effect at 64%. (Figure

19) Our data confirm that all three BZs do function as MT inhibitors via destabilization similar to vincristine. (Figures 16-19)

3.5 DISCUSSION

Benzimidazoles have been shown to have direct anti-neoplastic effects on cancer cells by inhibiting cell proliferation and inducing apoptosis. These effects have been well documented in both animal and human cancers. [14, 53, 55, 56, 121] Our *in vitro* results in the D17 OSA cell line demonstrate that all BZs tested do inhibit cell proliferation and that the cells undergo apoptosis following cell cycle arrest in G2/M. Proliferation was inhibited at doses of 5μM and above for all BZs, with the least response to FBZ treatment. Apoptosis was identified at similar concentrations, with BZ treatment ≥1.6 μM resulting in increased apoptosis. The apoptotic effect was most pronounced with MBZ and FBZ as compared to ABZ. Based on PK data for ABZ and FBZ all treatment doses from 1uM to 25 μM are biologically achievable (with oral doses of 20-125mg/kg/day) and are documented as well tolerated. Typical anti-parasitic doses are 25-50 mg/kg every12 hours for ABZ and every 24 hours for FBZ. Only ABZ at the higher doses for 25 μM and above have been documented to cause mild gastrointestinal upset. [36, 41, 46] While PK data for MBZ is not reported in dogs, we can only speculate as to whether these doses would be physiologically achievable in the dog. Based on similar chemical characteristics, bioavailability and molecular weight, we believe this a reasonable assumption. Flow cytometry confirmed that the inhibition of proliferation in these cells was not due to cellular necrosis and that the mechanism is G2/M arrest by MT inhibition. The least response was seen with ABZ at 1μM, however all other drug concentrations were similar with >80% cell cycle arrest. A dose dependent response may be present but would necessitate investigation of very low doses (≤1 μM BZ). The finding that cells arrested in G2/M supports the mechanism of microtubule inhibition. When microtubule polymerization is altered, the normal tension and relaxation of the kinetochore is absent. This is a critical signal to the cell and cellular arrest ensues. Polymerization assays confirmed the effect of all three BZs on polymerization inhibition. This effect was rapid, independent of dose and nearly identical between all drugs. Interestingly, at low concentrations both vinca alkaloids and taxanes are reported to act in an opposite manner at the microtubule; [66] perhaps BZs exhibit similar changes with concentration. Further investigation at additional concentrations would be needed to evaluate this.

Limitations of the study include the *in vitro* nature of these results, which may not be representative of naturally occurring disease. These are preliminary findings on the effects on cellular proliferation, microtubule inhibition and resulting cellular arrest and apoptosis. Effects appear to be dose-dependent except for tubulin polymerization. While all three BZs exhibited some effects, ultimately FBZ appears least effect overall. All three drugs exhibit similar G2/M cell cycle arrest. FBZ had the least effect on cellular proliferation and tubulin depolymerization. MBZ and ABZ have comparatively similar profiles, however ABZ appeared least effective at inducing apoptosis and demonstrated slightly reduced effect on cell cycle distribution at a low dose.

CHAPTER 4
IN VITRO EFFECTS OF BENZIMIDAZOLES ON VEGF SECRETION IN D17 CELLS

4.1 INTRODUCTION

Osteosarcoma (OS) is a highly malignant and severely painful disease that affects both people and dogs. Aggressive treatment with amputation and chemotherapy has improved comfort and survival. Ultimately, 90% of dogs will die from metastatic disease with an average of one year survival despite aggressive therapy, underscoring the negative effects of occult micro-metastatic disease at the time of diagnosis. While maximum tolerated dose chemotherapy with drugs such as doxorubicin and carboplatin delay metastasis, little has been achieved over the last two decades to significantly extend overall survival times. The long-term prognosis for dogs with OS is poor and thus novel adjuvant therapies which slow tumor growth (anti-angiogenic therapies) may be beneficial in combination with direct cytotoxic drugs.

Angiogenesis is necessary for continued primary tumor growth as well as establishing distant metastases. [88, 100, 122]Regulation of angiogenesis is primarily by vascular endothelial growth factor (VEGF). Given that sustained angiogenesis is necessary for tumor growth, therapeutic strategies which reduce or block the effects of tumor associated VEGF are currently being investigated for the treatment of various cancers and have improved survival in many cancers and may help to delay progression of micro-metastasis, which could significantly improve survival times of OSA. Serum VEGF levels are elevated in dogs with OS and pretreatment levels have been significantly correlated with disease free interval. In humans, overexpression of VEGF is linked to progression of disease and poor prognosis in carcinomas as well as OS. [7, 10] Based on osteosarcoma's sensitivity to microtubule inhibitors as well as the association with VEGF overexpression, investigation of putative BZs indirect effects via VEGF modulation are warranted.

The benzimidazoles are a class of anti-parasitics and anti-fungals that exert in vivo and in vitro anti-neoplastic effects. In one study, the discovery of the anti-neoplastic effects of FBZ was incidental and serendipitous, when rodents treated with routine FBZ dewormer experienced xenograft tumor failure. [13]Many synthetic BZ have been manufactured and these have been utilized more in human medicine for both antifungal and more recently anti-neoplastic effects [14,

[123] Additionally, several BZ derived compounds including ABZ have been shown to decrease VEGF secretion and microvessel formation. [124, 125] The advantages of the BZs for veterinary medicine are their long established use, wide margin of safety and low cost.

The purpose of this study was to investigate the *in vitro* effects of ABZ, FBZ and MBZ on VEGF secretion in an immortalized canine osteosarcoma cell line, D17. In light of microtubule inhibition, an observed decrease in VEGF may be due to the MTs role in protein synthesis and trafficking, perhaps at the level of HIF1-α or VEGF transcription and translation. Further investigation would be needed to investigate anti-angiogenic molecular mechanisms further.

4.2 MATERIAL AND METHODS

4.2.1 Cell lines

A canine osteosarcoma cell line (D17) was purchased commercially from American Tissue Culture Collection (Manassas, VA). Cells were cultured in in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell cultures were maintained at 37 °C in 5% CO2 and passaged twice weekly.

4.2.2 Reagents and antibodies

Albendazole (ABZ), fenbendazole (FBZ) and mebendazole (MBZ) was obtained from Sigma Aldrich. Stock solutions were prepared in DMSO prior to each use. A commercial canine ELISA (Canine VEGF DuoSet, R&D Systems) was used to quantify soluble VEGF. The kit was used as per manufacturer's instructions.

4.2.3 *In Vitro* VEGF Secretion

D17 cells were plated at a density of 7.5 x 10^3 cells per 200 µl of complete media in a 96-well microtiter plate incubated at 37 °C and 5% CO2. After allowing cells to adhere overnight, media was decanted and replaced with fresh media containing various concentrations of ABZ, FBZ and MBZ (0-200µM), and cells were allowed to grow for an additional 24 hours. Cell culture supernatants (in triplicate) were harvested and soluble VEGF determined with a commercially-available immunoassay (Canine VEGF DuoSet, R&D Systems). Differences in soluble VEGF secreted by D17 subsequent to treatment with BZs were normalized based upon

differences in cell proliferation through the use of a non-radioactive colorimetric proliferation assay (CellTiter96) in which optical density linearly correlates with viable cell numbers. (Described in Chapter 3) Specifically, normalized VEGF concentrations were based the average of triplicate samples for each experimental group expressed as the following ratio:
Normalized VEGF = [Calculated VEGF (pg/ml)]/optical density

4.3 STATISTICAL ANALYSIS

To assess the dose-dependent, biologic activity of ABZ, FBZ and MBZ in the D17 OS cell line, reductions in soluble VEGF secretion (normalized based on paired proliferation assay OD) were evaluated with a one way ANOVA with Dunnett's post-hoc test. Normally distributed data sets were expressed as mean and standard deviation. All statistical analysis was performed using commercial computer software (GraphPad, Instat3). Significance was defined as $p < 0.05$.

4.4 RESULTS

4.4.1 *In Vitro* VEGF Secretion

All BZs induce a dose dependent effect of VEGF secretion compared to untreated media control. Vehicle control (1% DMSO) was not statistically different from untreated media control. MBZ appeared to have the most robust response, with significant inhibition of VEGF secretion in D17 cells at doses ≥1μM ($p<0.01$) and ~50% reduction at doses ≥1μM. ABZ significantly inhibited VEGF secretion in D17 cells at doses ≥1μM ($p<0.01$), with >50% reduction at doses ≥10μM. FBZ was least effective, with significant inhibition of VEGF secretion in D17 cells at doses ≥5μM ($p<0.01$) and slightly less than 50% reduction at doses ≥50μM. (Figures 20-23)

4.5 DISCUSSION

Our results show that all three BZs decreased VEGF secretion in the D17 OS cell line. Fenbendazole had limited effectiveness in reducing VEGF secretion. While our results indicate a decrease in VEGF levels the underlying mechanism is unknown. Microtubules may play a role in protein accumulation and transcription HIF 1-α, the upstream regulator of VEGF; however it would be erroneous to assume this to be the most important mechanism. Further investigation is ongoing to evaluate VEGF and HIF 1-α protein expression via Western blot to elucidate where

in the pathway VEGF protein is modulated. In light of the significance of VEGF levels in predicting and tumor recurrence in various tumors including canine OS, the BZs effect on VEGF secretion may be auspicious to their other anti-neoplastic effects [7, 10] A drug that has both anti-proliferative, apoptotic and anti-angiogenic effects could be a useful addition in our treatment protocols for OS and possibly other tumors. Further investigation into the exact mechanism of VEGF inhibition is needed. Although the predictive value of this *in vitro* data is limited, evaluation of serum VEGF as a surrogate of BZ drug effect would be reasonable to determine in pilot studies *in vivo*.

Limitation of our study includes the use of a single OS cell line and a single read-out of angiogenic effect. This cell line may not be representative of all OSs or correlate with biologic effects in naturally occurring disease. Soluble VEGF may not be the major anti-angiogenic mechanism, and may not translate into a biomarker *in vivo* as global serum levels may not reflect the tumor microenvironment.

CHAPTER 5
DISCUSSION AND CONCLUSIONS

In this study we present data to support that ABZ, FBZ and MBZ, clinically useful BZs in canine medicine, exert direct and indirect effects against a canine osteosarcoma line *in vitro*. Direct effects of cell proliferation were apparent even at low doses (5μM) for all BZs, with the effect being least robust in FBZ. One molecular mechanism of action is tubulin depolymerization. All three BZs had a rapid dose independent effect, with ABZ having the strongest effect Results are very similar to the effects of vinca alkaloids. Cells arrest in G2/M even at very low doses (1 μM) of BZs, likely as a result of tubulin depolymerization resulting in MT inhibition. Strong responses were observed even with ABZ at 1μM, and all other drug concentrations demonstrated >80% cell cycle arrest. It is likely that cell cycle arrest is one mechanism by which BZs induce apoptosis in the D17 cell line. When MT polymerization is disrupted, the mitotic spindle does not sense tension, which is necessary for proper chromosome alignment and separation. This results in catastrophic failure of cellular division and cells will undergo apoptosis. Apoptosis was identified at similar low concentrations, with BZ treatment ≥1.6 μM resulting in increased apoptosis. The apoptotic effect was least pronounced with ABZ. Based on PK data for ABZ and FBZ treatment doses up to 25 μM are biologically achievable (with oral doses of 20-125mg/kg/day, anti-parasitic doses are 25-50mg/kg/day); these doses have minimal adverse effects. While PK data for MBZ is not reported in dogs, based on information in rodent species and the similarities in chemical characteristics to other BZs, plasma levels comparable to these *in vitro* concentrations may be achievable. While effects varied between drug and mechanism, overall ABZ and MBZ appear to be the most effective, and FBZ the least effective at equivalent micromolar concentrations. However, pharmacologic characteristics including absorption and bioavailability, metabolism, and excretion may play a role in dictating biologic activity *in vivo*.

Some BZs are also reported to have indirect anti-cancer effects via modulation of angiogenesis, particularly VEGF. In our study, all BZs reduced VEGF secretion *in vitro*, although FBZ was again least effective. As a surrogate marker for angiogenesis and a prognostic factor in canine OS, VEGF may also translate as a serum biomarker in pilot studies *in vivo*. The molecular mechanism by which microtubule inhibitory drugs exert their effects on VEGF has not

been well described. Microtubules regulate HIF-1 α via control of mRNA transport and ensuing HIF translation. Taxanes and other MT disrupting agents inhibit nuclear accumulation of HIF-1 α, and thus HIF-1 α can be included with other transcription factors that utilize microtubules for transport during interphase. HIF-1 α and/or VEGF may be regulated by MTs at both protein synthesis and trafficking levels.

Known regulatory functions of HIF-1 α include target genes, pro-angiogenic cytokines, transcription factors and heat shock factor. Various integrins and matrix metalloproteinases are also controlled by HIF-1α Heat shock protein 90 (HSP 90) is molecular chaperone that regulates the conformational stability and structure of an array of proteins. Known HSP90 dependents include oncoproteins, including EGFR family members, mutant p53, Kit, Akt, Bcr-Abl and Met. Inhibition of HSP90 leads to degradation of the dependent proteins via the ubiquitin proteasome pathway ultimately resulting in apoptosis. Accumulation of over-expressed or mutated dependent proteins is noted in neoplasms. HSP90 inhibitors, such as STA-1474 have recently shown measureable objective responses in various tumor types including dogs with osteosarcoma, mast cell tumors, melanoma and thyroid carcinoma**.** [126] Additional molecular effects of MTIs and the BZs may include alterations of HSP90 via HIF-1 α. The combination of BZs and HSP inhibitors should be investigated based on the common protein, HIF-1 α.

Aberrant expression of β tubulin is reported in multiple cancers including osteosarcoma and represents a more aggressive phenotype. It has also been implicated in drug resistance to taxols in OS cell lines.[74-76] Based on the mechanisms of action of the MTIs, their disruption of polymer formation and dynamicity may be an additional avenue to address aberrations in β tubulin expression. Investigations of BZs on specific tubulin isotypes should be considered for future evaluation.

The MTS proliferation assay showed that BZs do inhibit neoplastic OS cell proliferation. This was concordant with previous studies showing this effect in other tumor types following treatment with BZs both *in vitro* and *in vivo*. The doses at which this was achievable were those that are clinically achievable and well tolerated in dogs. The effect was indeed both dose and time dependent as was noted in previous work. Flow cytometry for apoptosis confirmed that the decrease in proliferation was indeed due to apoptosis and not necrosis. Additionally, flow cytometry with DAPI showed that the vast majority of cells were arrested in the G2/M phase of

the cell cycle. This supports the mechanism of microtubule inhibition as cells with defective MTs or who cannot form a functional mitotic spindle will ultimately arrest in G2/M.

The tubulin polymerization assay confirmed that the mechanism of action of the BZs is indeed due to tubulin inhibition. Our results showed a rapid destabilization of tubulin, similar to the vincristine, a known destabilize, however we observed a rapid plateau at all doses. Adjustments for time to collecting data points did not improve our results which are a limitation of both equipment capability and assay requirements. We believe that lower doses (< 1μM) would be needed to more specifically identify the rate of inhibition and to further classify the exact mechanism of the tubulin inhibition. While the overall trend is visually similar to vincristine, finer data points early in the polymerization curve may hold more information.

The treatment of OS cells with the BZs resulted in decreased serum VEGF. Decreases in VEGF have been reported following treatment with other MT inhibiting drugs. The mechanism of decrease is unknown and may be to be due to deregulation of HIF 1 α. HIF-1 α is the direct upstream regulator of VEGF. The MTs are crucial for many cellular regulatory functions including formation and movement of proteins, activation and movement of transcription factors and many, many others. The MT inhibition caused by the BZs has the potential to affect multiple other factors within cellular regulation. It is unknown if there is a single most important factor, such as HIF-1 α or if the BZs effects on multiple levels of cellular regulation lead to an overall decrease in neoplastic cell survival.

Overall, we have good evidence to suggest that the MTs do inhibit neoplastic cell growth via interference with MT formation, which leads to G2/M cell cycle arrest followed by apoptosis. More would is need to delineate the exact mechanism of the polymerization inhibition and the upstream effects of the BZs and how they impact VEGF secretion.

As previously stated, our study has several limitations including the use of a single OS cell line to represent the heterogeneous disease of canine OS and the overall *in vitro* nature of the study which limits the immediate translational significance. In addition, this study relies on a single read-out of angiogenic effect (VEGF) without knowledge of the underlying molecular mechanism by which it is modulated by BZs. Soluble VEGF may not be the major anti-angiogenic mechanism, and may not translate into a biomarker *in vivo* as global serum levels may not reflect the tumor microenvironment. Three candidate BZs were selected based on

clinical use in dogs, however there are hundreds of BZ-like chemical compounds and thus ABZ and MBZ may not be most effective.

Within these limitations, our data shows that BZs (ABZ, FBZ, MBZ) exert direct and indirect anti-cancer effects in D17 OS cells. Specifically, tubulin depolymerization appears to lead to altered microtubule function and cell cycle arrest. The cell cycle arrest demonstrated may result in inhibition of proliferation and induction of apoptosis seen *in vitro*. Indirectly, BZs modulate soluble VEGF protein levels, possibly because the MT is an important functional element in subcellular location of HIF 1-α, the upstream regulator of VEGF.

Taken in context of the demonstrated *in vitro* activities and combined with the clinical information already available on these BZs in regards to dosage, safety, toxicity, and pharmacokinetics as well as the relative in expense of BZ drugs compared to traditional chemotherapy, BZs, particularly ABZ and MBZ, are attractive novel therapies. They may prove to be beneficial as adjunctive therapy and an alternative mechanism to combat drug resistance. Given the heterogeneous nature of cancer and the multiple mechanisms of resistance, BZs are likely to be most useful when used in conjunction with traditional (maximum tolerated dose) chemotherapy in osteosarcoma. In this setting, the limited toxicity is of particular benefit, as combination chemotherapy is often limited to additive toxicity of the drugs. This study provides preliminary evidence supporting the transition of ABZ and MBZ from the bench top to live patients. Further study is needed to evaluate PK and safety in cancer-bearing dogs, particularly if used in a combination chemotherapy setting given the potential for adverse hematologic with ABZ. Cell function effects were seen even at low levels *in vitro*, and thus treatment to MTD may not be necessary to exert biologic effect *in vivo*. Evaluation of serial levels of serum VEGF as a surrogate and transitional biomarker of biologic drug activity may aid in the estimation of drug effect in cancer-bearing dogs.

CHAPTER 6

FIGURES AND TABLES

Figure 1. The basic benzimidazole structure of a benzene and imidazole.

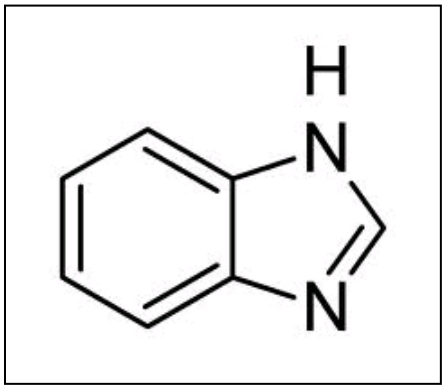

Figure 2. Microtubule structure of αβ-tubulin heterodimers. There are 13 protofilaments that form a cylinder that is 24 nm in diameter. The exposed β-subunit is the plus end and the α-subunit is the minus end. Adapted from Current Cancer Drug Targets, Jordan, 2007.

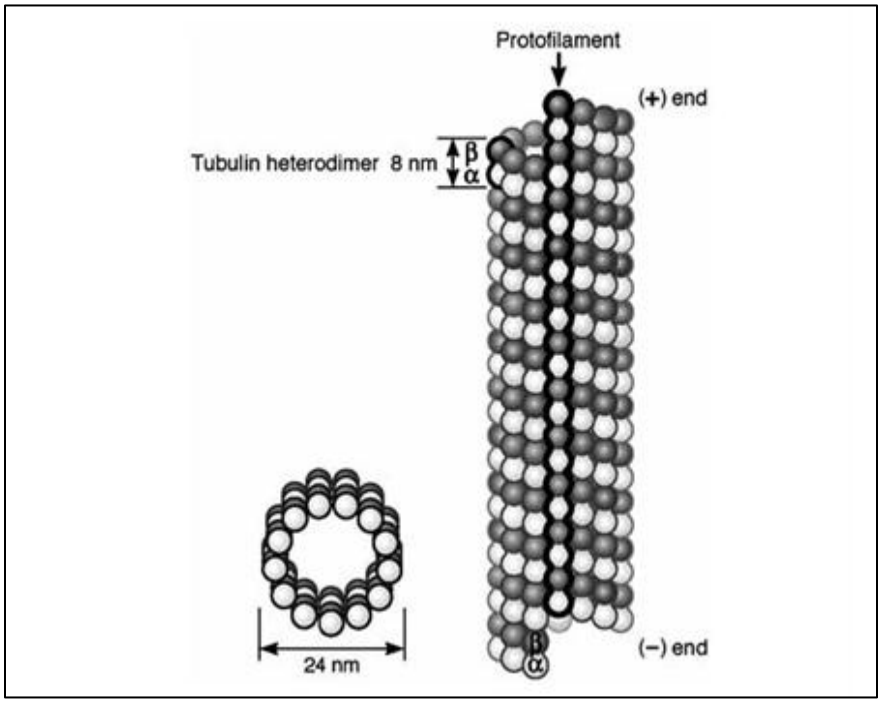

Figure 3. Schematic representation of the curved configuration of two tubulin dimers, α1β1 and α2β2. Colchicine has been shown to bind between subunits of the same dimer whereas vinblastine binds between dimers. Adapted from Mol. Biotechnol. Wade, 2009.

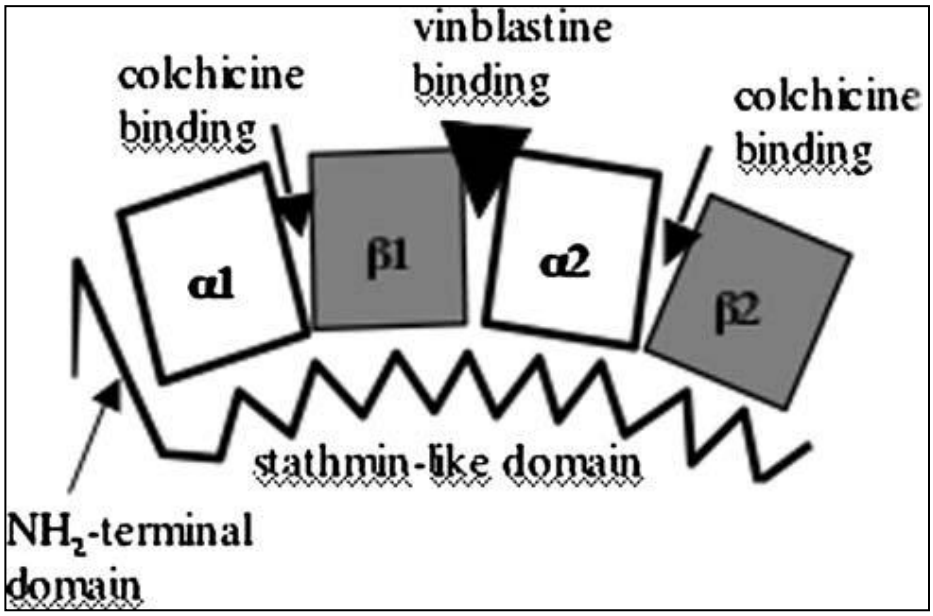

Figure 4. Isoforms produced from alternative exon splicing of VEGF. Isoform 165 is the most mitogenic. Adapted from J Investig Dermatol Symp Proc. 2006, Courtesy of J. Wypij.

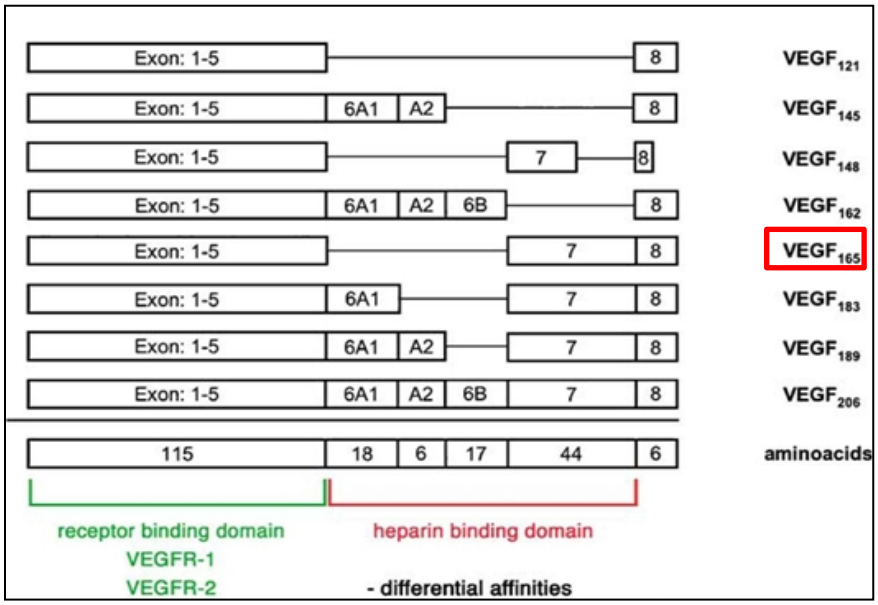

Figure 5. Transcriptional regulation of VEGF by HIF-1 complex. Atlas of Genetics and Cytogenetics in Oncology and Haematology (http://AtlasGeneticsOncology.org, 2008). Courtesy of J. Wypij.

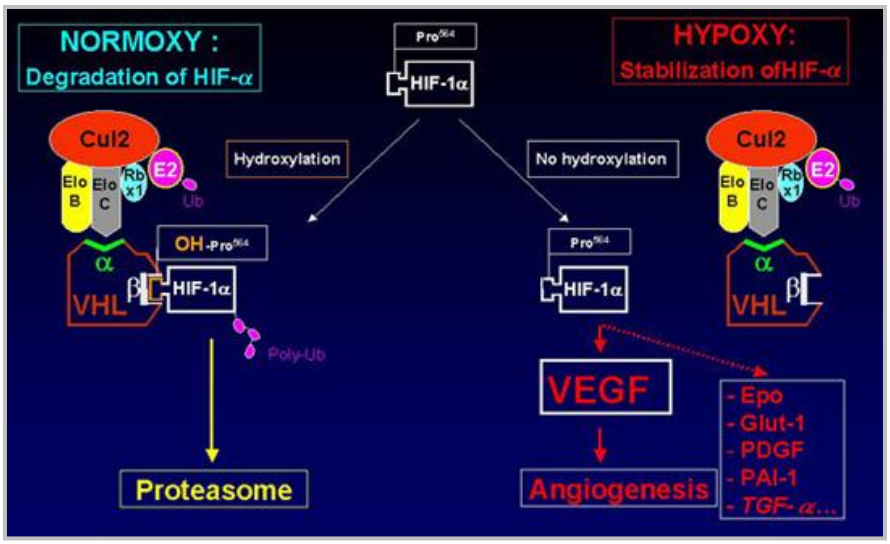

Figure 6. VEGF Signal Inhibition, specifically TKIs and monoclonal antibodies. From http://img.medscape.com/fullsize/migrated/editorial/cmelive/2007/6165/images/slide31.gif

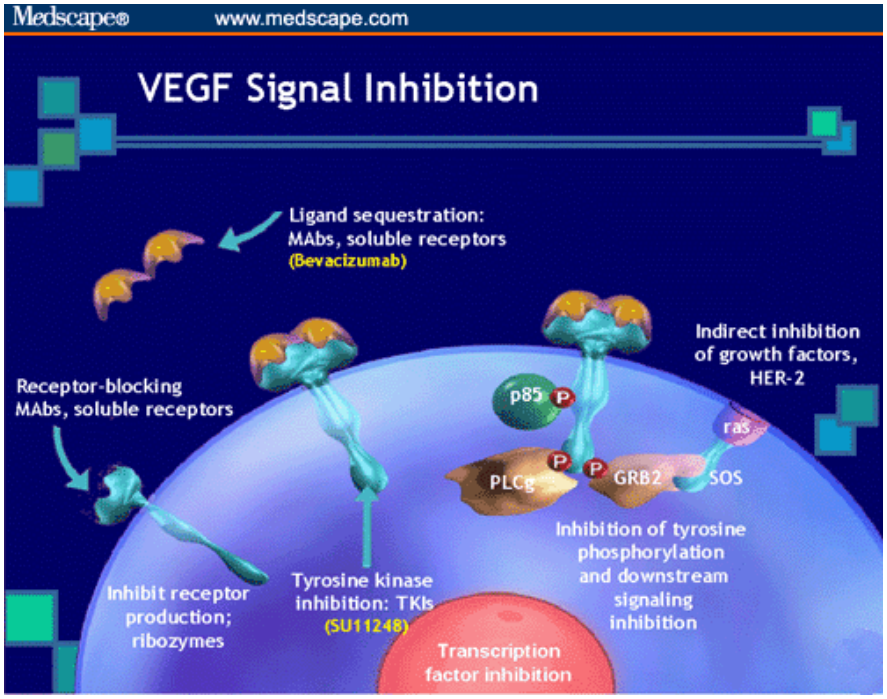

Figure 7. Dose dependent inhibition of cell proliferation of D17 cell line when treated with albendazole at doses of 0-200µM for 24 hours.

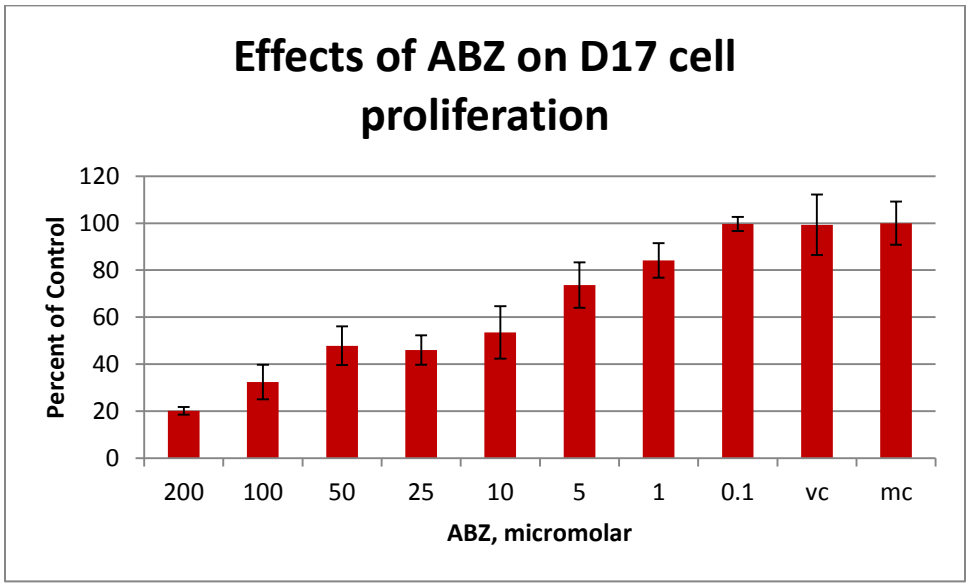

Figure 8. Dose dependent inhibition of cell proliferation of D17 cell line when treated with fenbendazole at doses of 0-200µM for 24 hours.

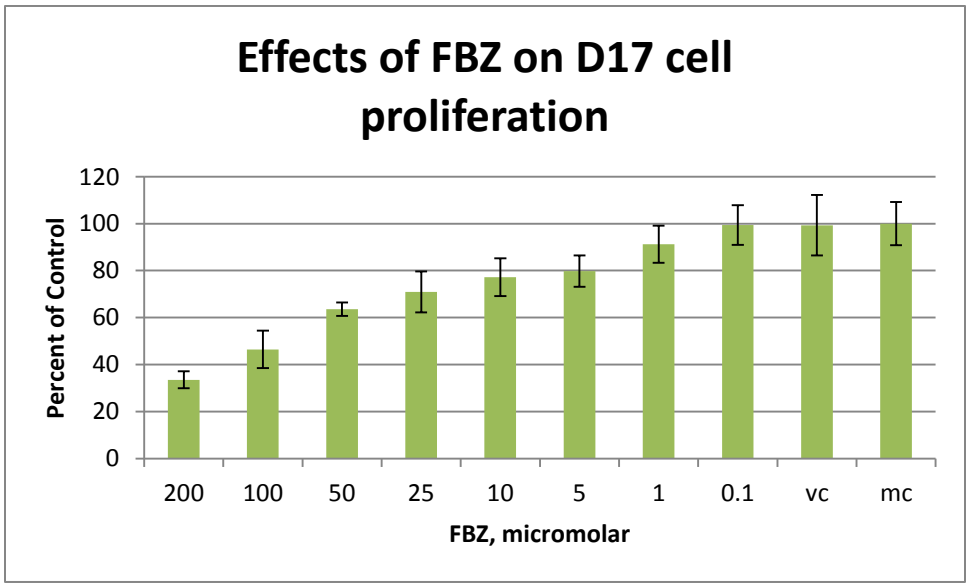

Figure 9. Dose dependent inhibition of cell proliferation of D17 cell line when treated with mebendazole at doses of 0-200µM for 24 hours.

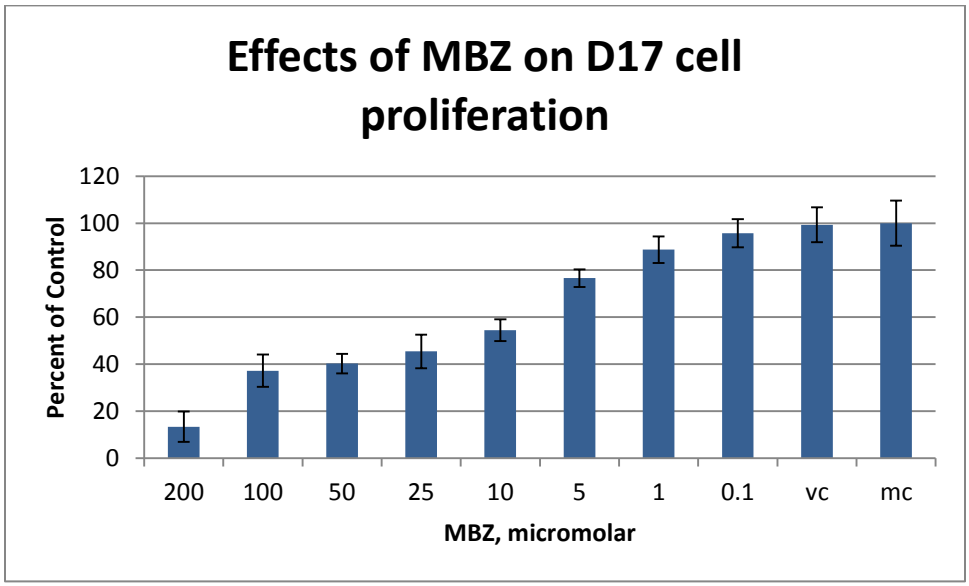

Figure 10. Comparison of albendazole, fenbendazole and mebendazole on inhibition of cell proliferation of D17 cell line.

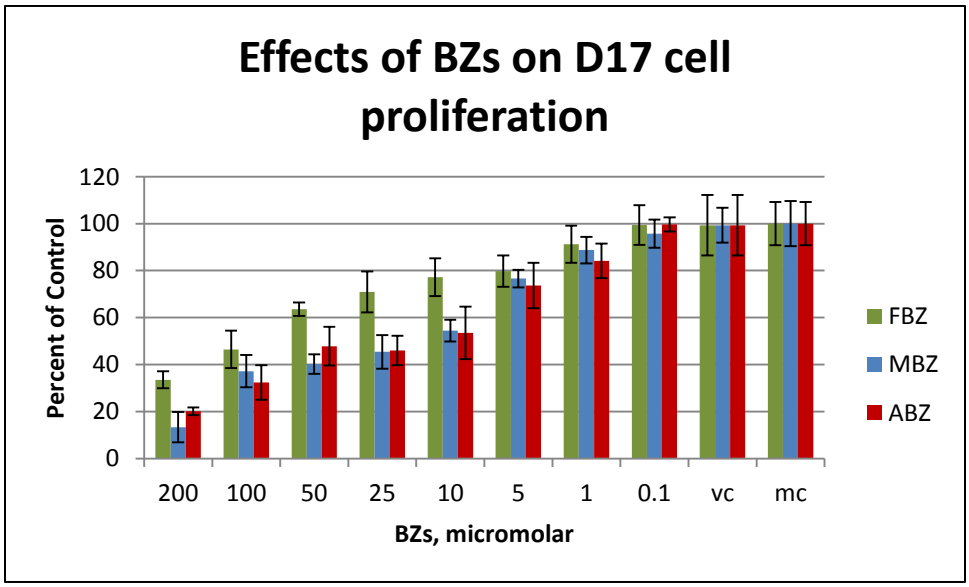

Figure 11. Flow cytometry utilizing Annexin V-FITC. Increased numbers of apoptotic cell are noted at increasing concentrations (0-25µM) of mebendazole.

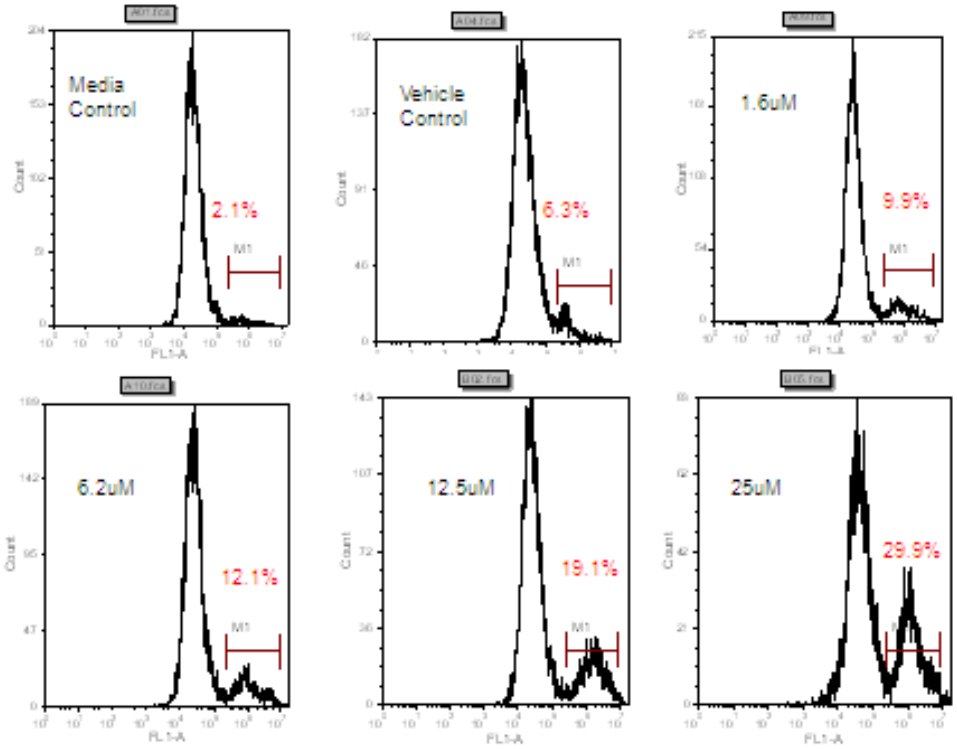

Figure 12. Comparison of effect of albendazole, fenbendazole and mebendazole on induction of apoptosis in cells treated at varying concentrations for 24 hours.

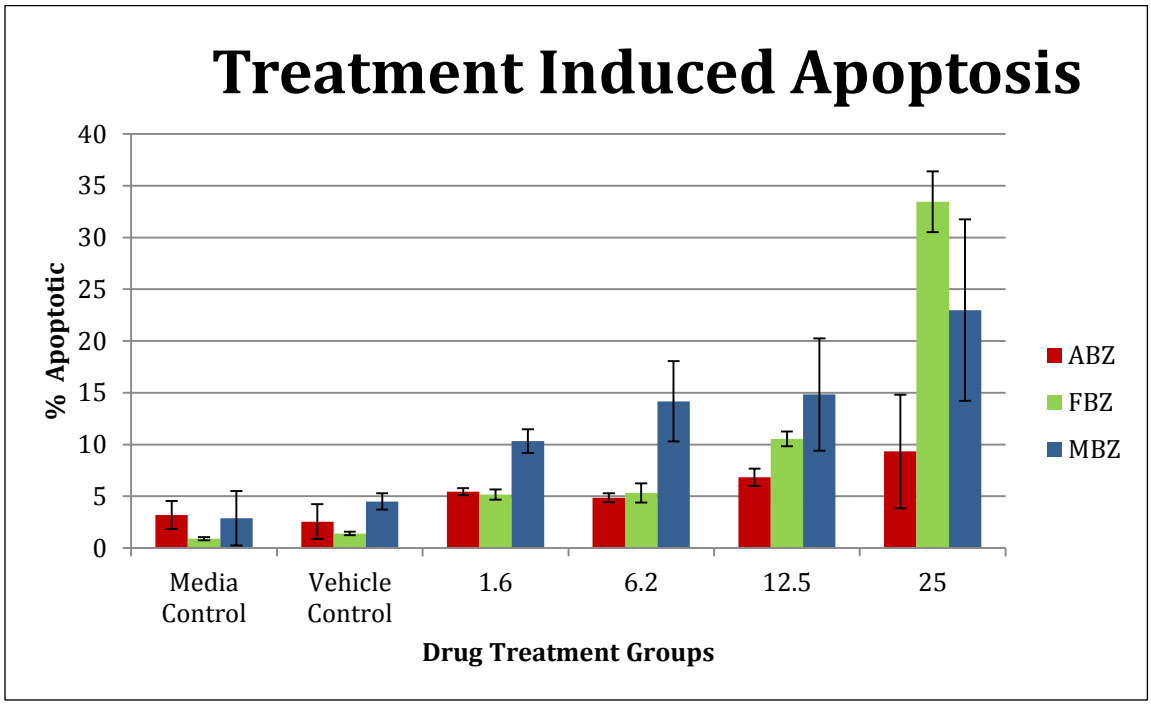

Figure 13. Cell cycle arrest gating was based on data as shown here for ABZ at 5 μM.

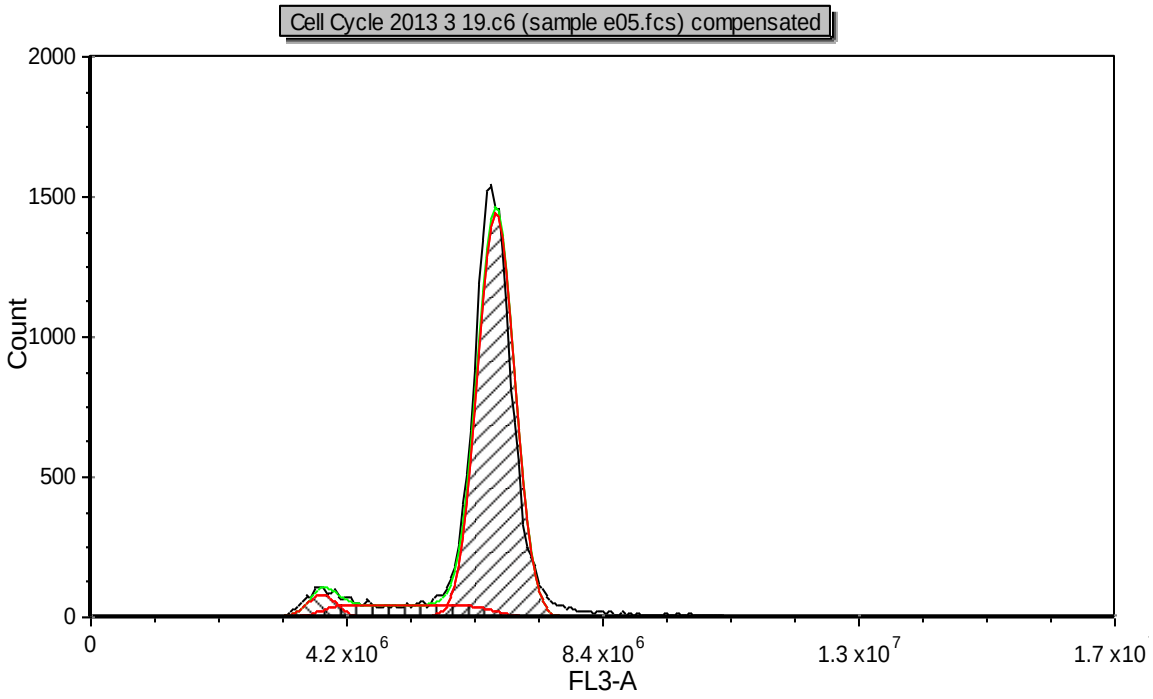

Figure 14. Flow cytometric visualization of effect of ABZ on cell cycle arrest. Cells treated with ABZ arrested in G2/M at doses all dose, with most pronounced effect at 5μM-25μM.

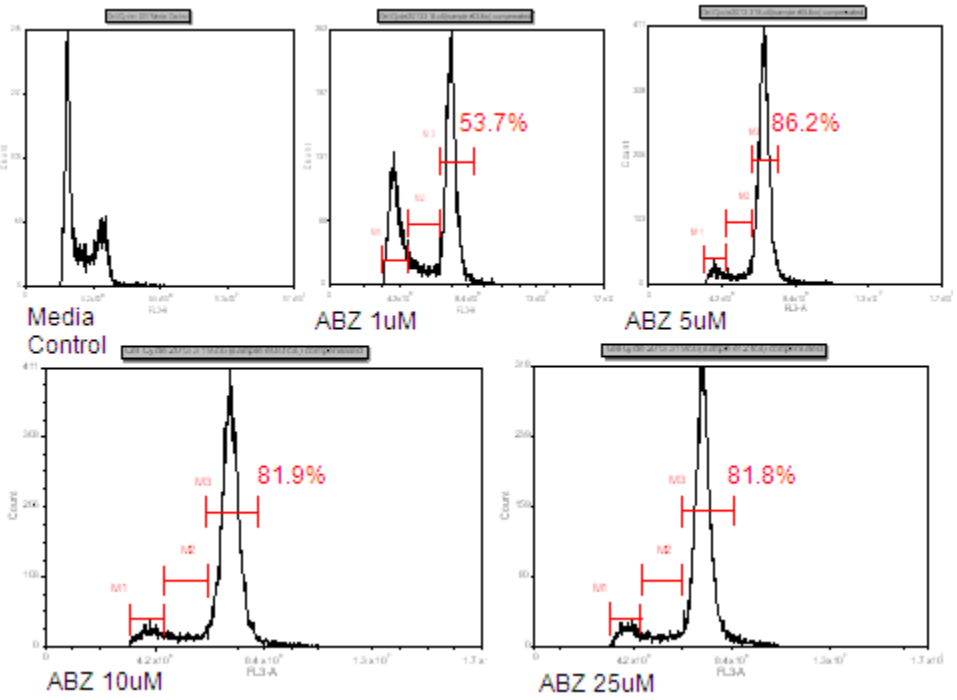

Figure 15. Flow cytometric analysis of cell cycle arrest. Cells treated with ABZ, FBZ and MBZ arrested in G2/M of cell cycle at doses 1μM and greater for all three drugs.

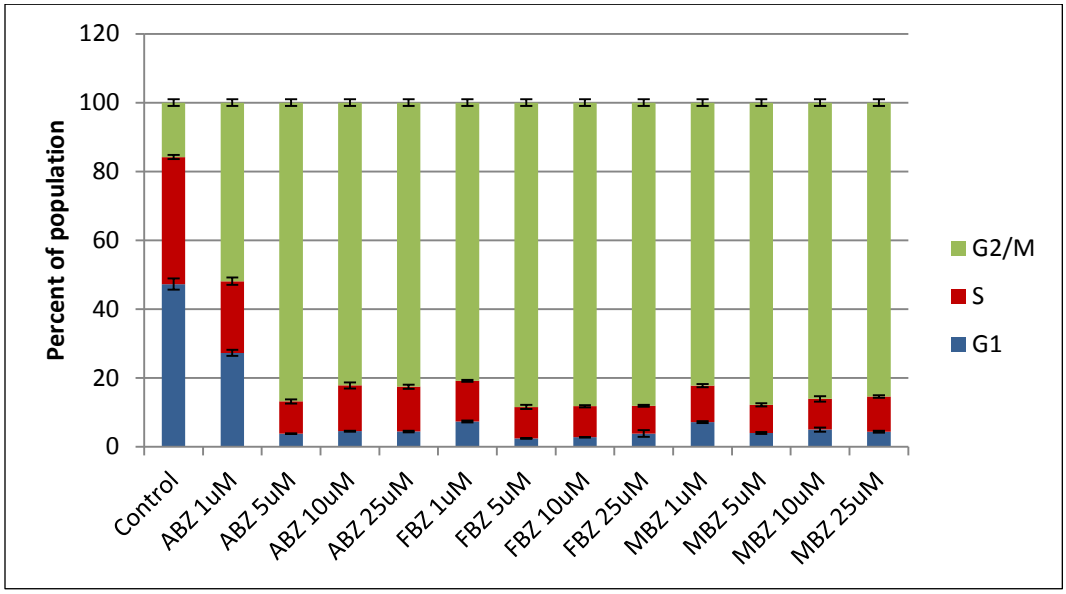

Figure 16. Example of Tubulin Assay. Phases of tubulin polymerization are indicated for the control curve; I: nucleation, II: growth III: steady state equilibrium.
Courtesy of Cytoskeleton, Denver CO.

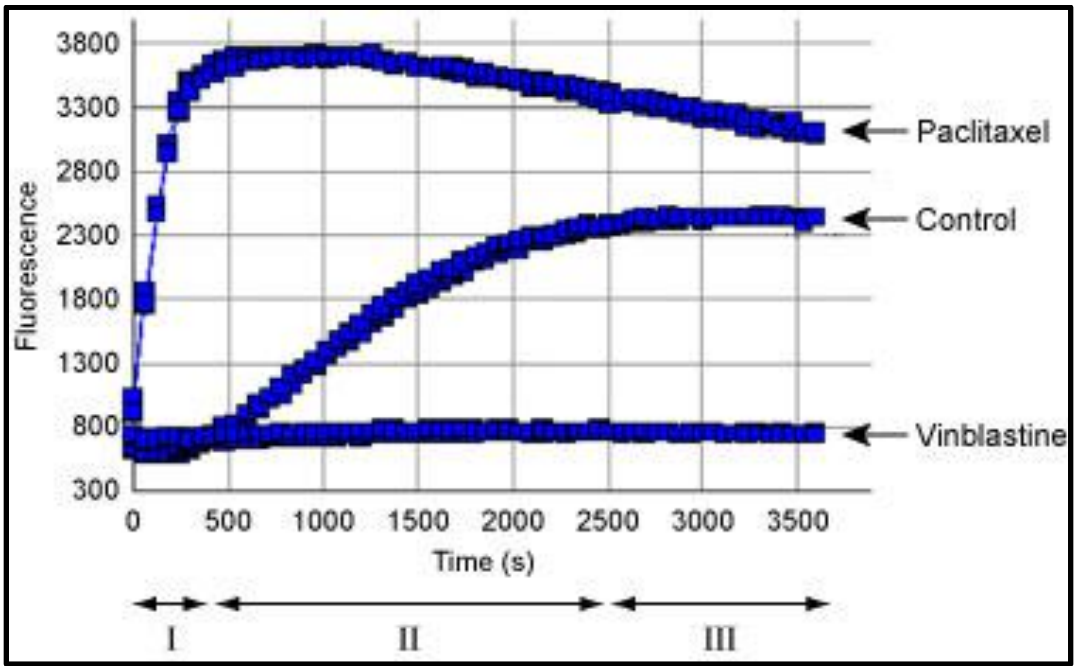

Figure 17. Albendazole inhibits tubulin polymerization at doses from 10µM-50µM. Results are similar to vincristine, a tubulin destabilizer.

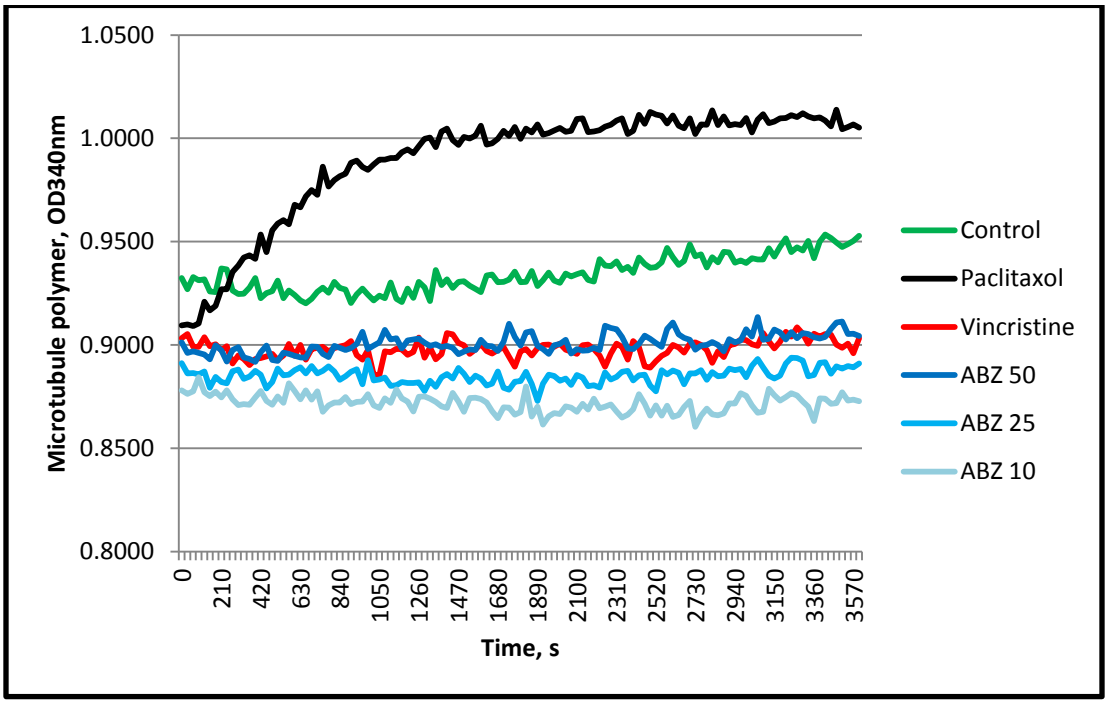

Figure 18. Fenbendazole inhibits tubulin polymerization at doses from 10µM-50µM. Results are similar to vincristine, a tubulin destabilizer at lower doses, while the highest dose has minimal effect.

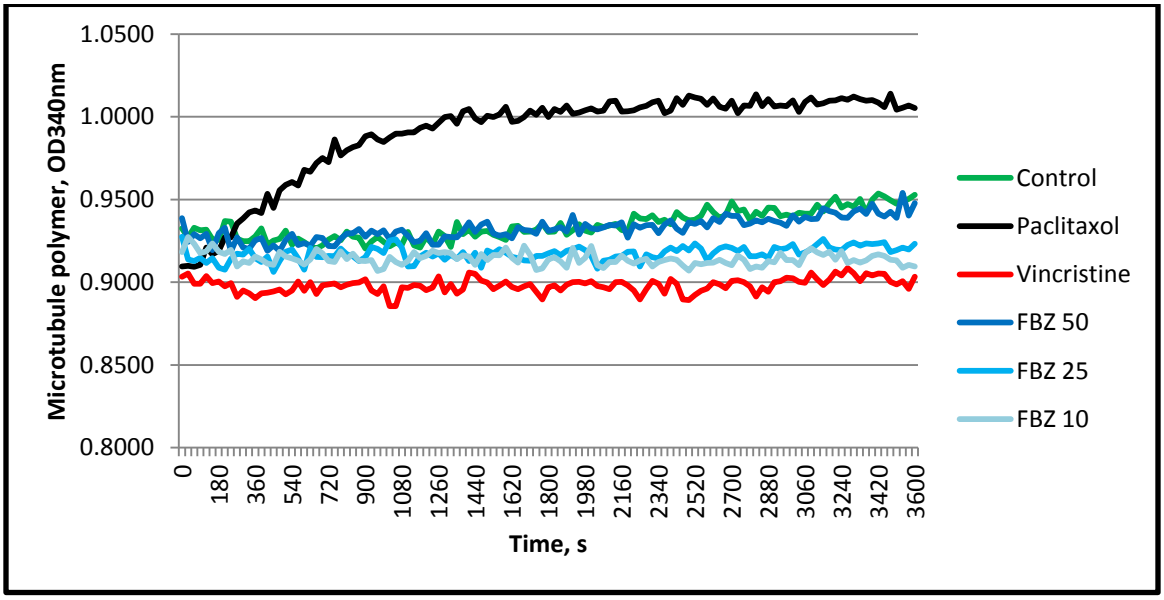

Figure 19. Mebendazole inhibits tubulin polymerization at doses from 10µM-50µM. Results are similar to vincristine, a tubulin destabilizer at all doses.

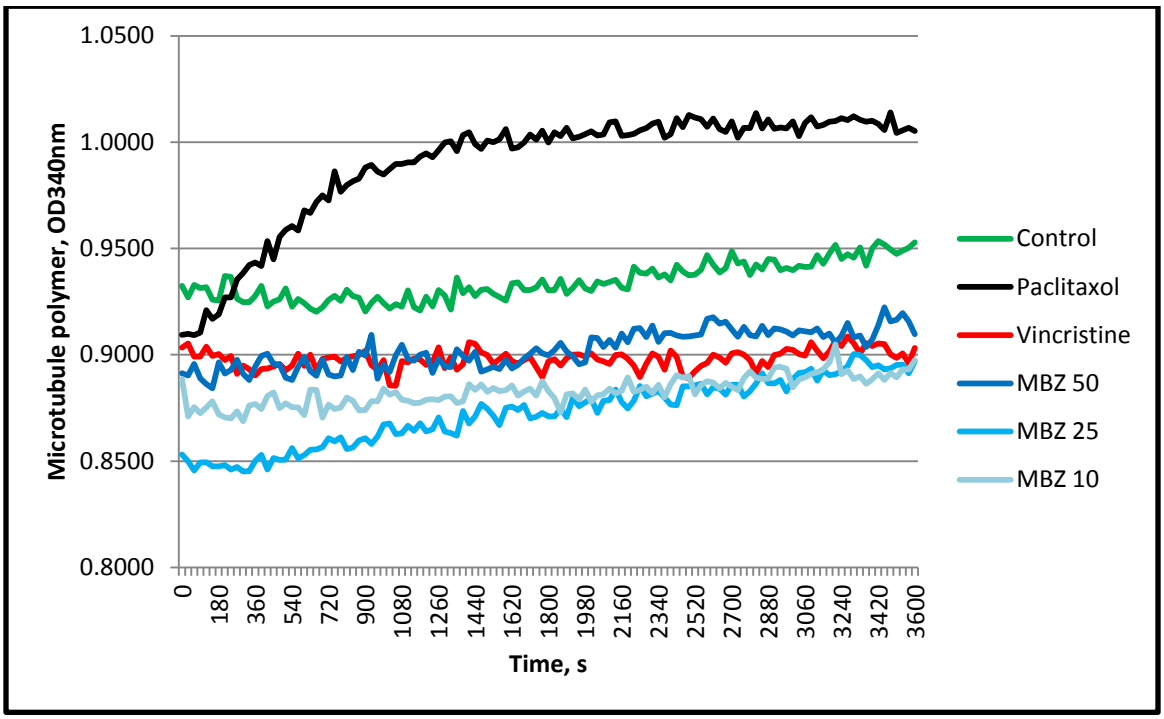

Figure 20. Albendazole inhibits VEGF secretion at doses ≥1µM ($p<0.01$).

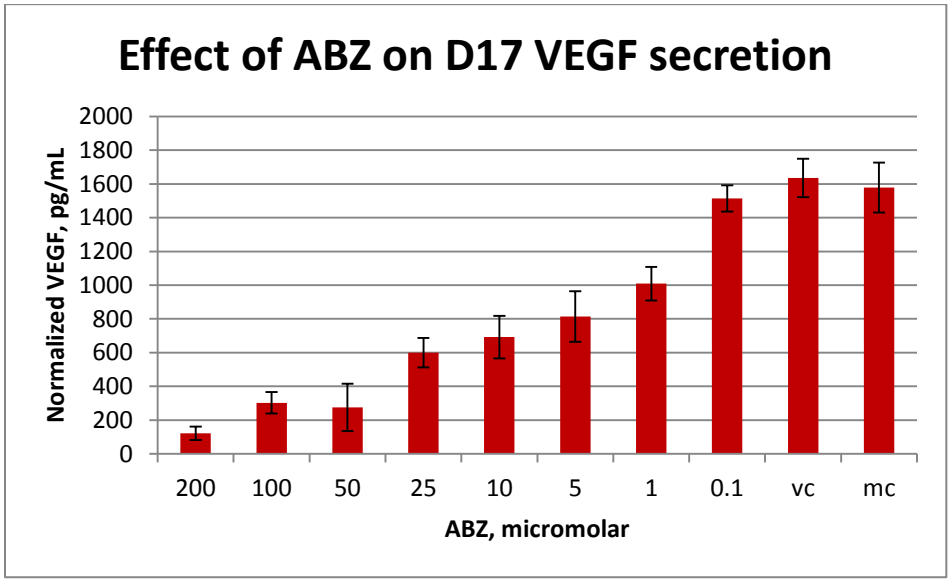

Figure 21. Fenbendazole inhibits VEGF secretion at doses ≥5μM ($p<0.01$).

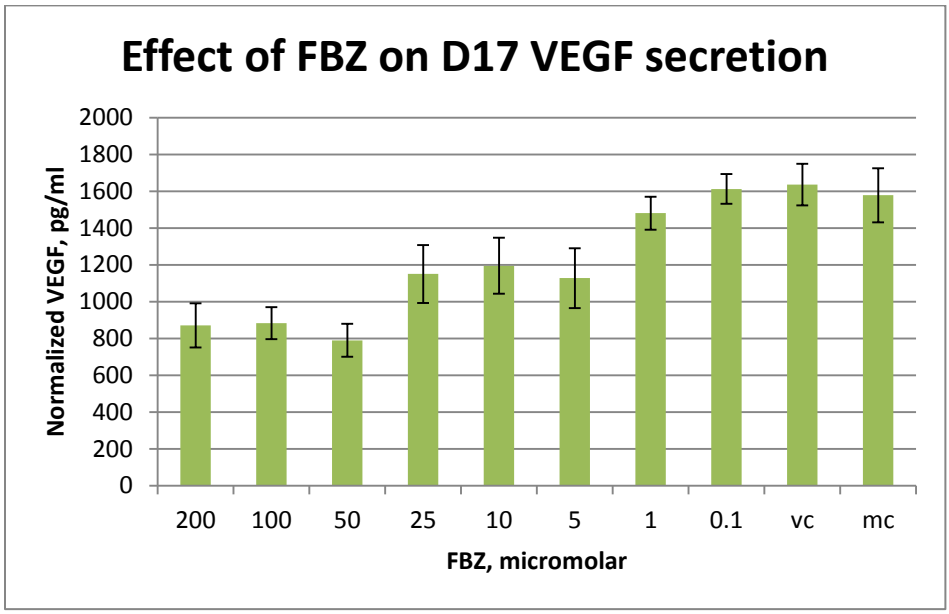

Figure 22. Mebendazole inhibits VEGF secretion at doses ≥ 1 μM ($p<0.01$).

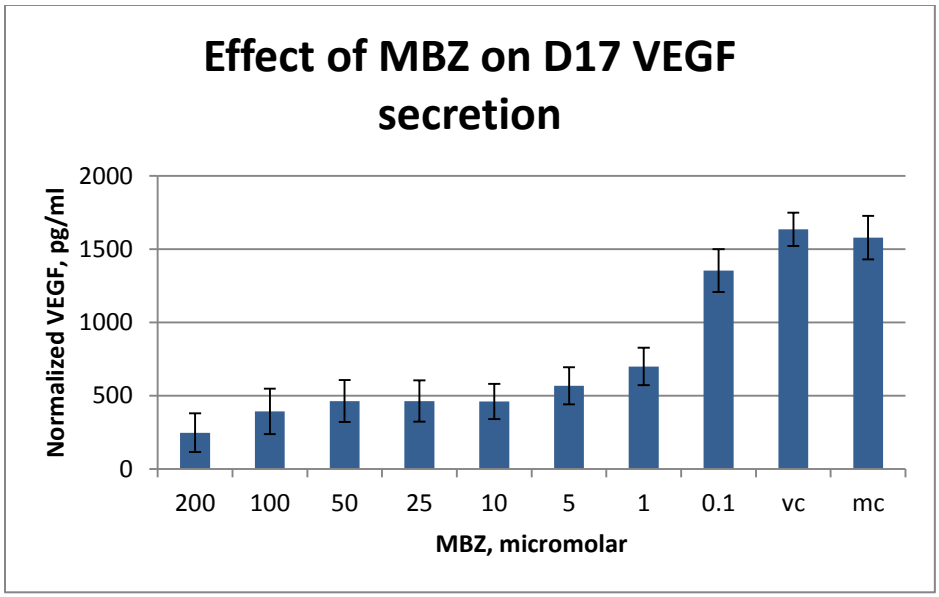

Figure 23. Comparison of albendazole, fenbendazole and mebendazole inhibition of VEGF secretion at doses from 0-200μM.

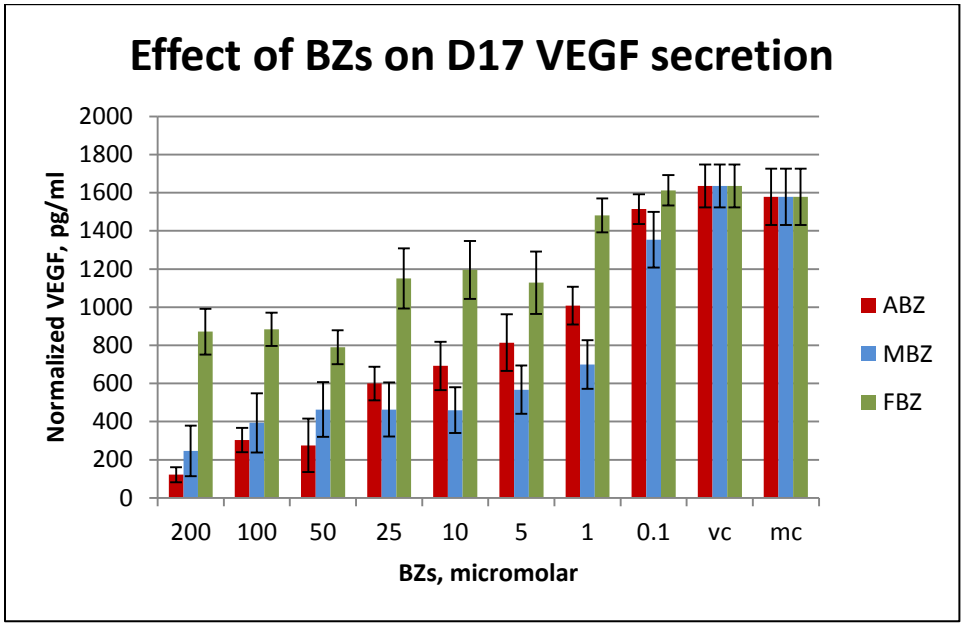

Table 1. Benzimidazole metabolites and their established plasma concentrations.
References*11, **45, ***41,****126, *****127

	Active metabolite	Cmax	Tmax
ABZ	Albendazole Sulfoxide	Canine:1.81ug/ml at 50mg/kg****	4.4 hrs
	Albendazole Oxide= ricobendazole	Human: 400mg/human=0.16mg/L*****	4.2 hrs
FBZ	Fenbendazole Sulphone and Sulphoxide	Canine: 0-0.53ug/ml at 20mg/kg*	Unknown
	Oxfendazole (sulfoxide and sulphone forms)	0.42ug/ml at 20mg/kg**	12hrs
MBZ	None known in animals	Canine: Unknown	Unknown
	Active metabolite in humans the 2-amine form	Humans: 1.5 g achieved levels >5 μg/L (no food) 1.5g achieved 27-42ug/L (high fat meal)***	Unknown
		100mg BID achieved 0.03mcg/ml MBZ and 0.09mcg/ml of 2-Amine MBZ	Unknown
		Rats: 40mg/kg achieved 349 ng/ml Wistar rat***	Unknown
OFZ	Exists as a sulfoxide	Canine: 2.5- 80mg/kg achieved 0.07ug-0.14 ug/ml*	12 hr

Table 2. A summary of similarities and differences between the mechanisms of antimitotic drugs. Adapted from Jordan, Current Cancer Drug Targets, 2007.

Drug	Mitotic Arrest at low and high drug concentrations	MT Dynamics	High Concentration effects on MT polymer mass
Taxanes, epothilones	Yes	Suppressed	Enhanced
Vincas, colchicine, dolastains	Yes	Suppressed	Depolymerized

Table 3. Effect on cell survival as assessed by flow cytometry. Results are reported as an average of the experiments which were performed in triplicate.

Drug	Dose μM	% Survival
ABZ	Media Control (1% DMSO)	96.54
	1	90.21
	5	86.39
	10	87.08
	25	85.19
FBZ	Medial Control (1% DMSO)	95.33
	1	88.70
	5	89.24
	10	87.70
	25	59.55
MBZ	Medial Control (1% DMSO)	95.21
	1	86.66
	5	89.03
	10	87.92
	25	85.62

Table 4. Effect on cell cycle arrest when treated with BZ assessed by flow cytometry. Results are reported as an average of the experiments which were performed in triplicate. 5μM -25μM. All results were significant at $p<0.01$.

Drug	Dose μM	% Arrested in G2/M of cell cycle
ABZ	Media Control (1%DMSO)	15.79
	1	51.88
	5	86.81
	10	82.17
	25	82.53
FBZ	1	80.8
	5	88.4
	10	88.2
	25	88.0
MBZ	1	82.23
	5	87.82
	10	86.07
	25	85.35

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