Activation of the AKT and mammalian target of rapamycin pathways and the inhibitory effects of rapamycin on those pathways in canine malignant melanoma cell lines

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Objective—To investigate the activation of the AKT and mammalian target of rapamycin (mTOR) pathways and assess the inhibitory effects of rapamycin on those pathways in canine malignant melanoma cells.

Sample Population—3 established primary canine melanoma cell lines generated from naturally occurring tumors.

Procedures—Expressions of total and phosphorylated AKT, mTOR, and p70 ribosomal S6 kinase 1 (p70S6K) in canine melanoma cells that were or were not exposed to 10nM rapamycin were assessed via western blot analysis. Clonogenic assays were performed to determine the surviving fraction of melanoma cells after exposure to 0.1, 1, 10, or 100nM rapamycin.

Results—Expressions of total and phosphorylated AKT, mTOR, and p70S6K proteins were detected (ie, the AKT and mTOR pathways were activated) in all 3 cell lines. Rapamycin treatment resulted in decreases in phosphorylated mTOR expression and phosphorylated p70S6K expression but no change in phosphorylated AKT expression. Expression of total AKT, mTOR, and p70S6K persisted after rapamycin treatment. There was a significant dose-dependent decrease in surviving tumor cell fraction for each cell line following treatment with rapamycin.

Conclusions and Clinical Relevance—These data indicated that AKT and mTOR, as well as their downstream product p70S6K, are present and active in canine melanoma cells. Activation of the mTOR pathway can be inhibited by rapamycin; treatment of melanoma cells with rapamycin decreased the surviving tumor cell fraction. Use of mTOR inhibitors as antineoplastic treatments in dogs with melanoma warrants investigation. Furthermore, these data support the use of canine melanoma cells as a molecular model for melanoma in humans. (*Am J Vet Res* 2009;70:263–269)

Malignant melanoma is a common tumor in dogs; it is the most common malignancy of the oral cavity and a common tumor of the digits.^{1–3} Although local control of this disease by use of surgery and radiation therapy is possible, most dogs die as a result of metastasis within 1 year of diagnosis. There are currently few effective systemic treatments for malignant melanoma in dogs, although immunotherapy and the use of carboplatin chemotherapy appear to have some usefulness.^{4–6} Because of the resistance of this type of tumor to most treatments to date, specific molecular inhibitors are needed to effect better outcomes. One of the more promising areas of research is aimed at protein kinase inhibition because of the important regulatory role that those enzymes have in the metabolism, division,

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ABBREVIATIONS

DMEM	Dulbecco modified Eagle medium
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco phosphate-buffered saline
mTOR	Mammalian target of rapamycin
p70S6K	p70 ribosomal S6 kinase 1
PTEN	Phosphatase and tensin homologue
TBS	Tris-buffered saline

and proliferation of cancer cells. The AKT pathway is 1 pathway that can be targeted through kinase inhibition. The AKT pathway is activated in most melanomas of humans, and that activation is associated with a worse prognosis.⁷ One of the regulatory protein kinases located downstream in the AKT pathway, mTOR, has been suggested to be of key importance in regulating translation of proteins involved in cell cycle progression and cellular proliferation.⁸

The mTOR protein is a polypeptide that is present in the cytoplasm and is highly conserved among species.^{9–11} It is detected in almost all mammalian cells; normally, it is only active during cell division and

growth and is stimulated by growth factors and mitogens.^{12–14} When phosphorylated, mTOR interacts with 2 distinct proteins to promote protein synthesis and cell growth. The first of these proteins is p70S6K, which is phosphorylated by mTOR and consequently activated. Phosphorylation of p70S6K causes increased translation of specific 5' terminal oligopyrimidine mRNAs that code for ribosomal proteins, elongation factors, and growth factors and their receptors; thus, protein synthesis and growth of the cell are increased. High amounts of p70S6K have been detected in melanomas from humans.¹⁵ One concern regarding use of mTOR inhibitors for the treatment of tumors is that mTOR inhibition may lead to phosphorylation of the AKT protein.¹⁶

Rapamycin is a commercially available inhibitor of mTOR. This bacterial macrolide causes decreased phosphorylation of the mTOR targets, which potentially blocks the growth, proliferative, and survival actions of mTOR in malignancies in dogs.^{14,17} Previously, we determined that rapamycin is effective in inhibiting canine osteosarcoma cells in an in vitro clonogenic assay.¹⁸

Numerous mutations have been identified in melanomas of both humans and dogs^{1,14,19} that could lead to constitutive activation of the AKT pathway. The purpose of the study reported here was to investigate the activation of the AKT and mTOR pathways and assess the inhibitory effects of rapamycin on those pathways in canine malignant melanoma cells. Our hypothesis was that the AKT pathway is activated in canine melanoma cells and that, in turn, mTOR is also present and constitutively activated. We further hypothesized that rapamycin blocks the mTOR pathway despite continued activation of the AKT pathway, which results in decreased survival of canine melanoma cells.

Materials and Methods

Cell lines—The melanoma cell lines (designated as cell lines 12, 23, and 50) were isolated from primary tumors of dogs with melanoma of the oral cavity that were evaluated at the Veterinary Medical Teaching Hospital of the University of California, Davis. The development, characterization, and authentication method for osteosarcoma cell lines have been previously described,²⁰ and these melanoma cell lines were derived by use of those same protocols. After 20 passages, cells were pelleted via centrifugation, fixed in formalin, and then embedded in paraffin. To confirm that the cells originated from melanomas, the cells were stained immunohistochemically to detect S-100 protein, microphthalmia transcription factor, and melan A by use of methods described elsewhere.^{21,22} Cells were maintained in DMEM with a high glucose concentration,^a 1% nonessential amino acids,^b and 10% heat-inactivated fetal bovine serum.^c Cells were grown in filter-top flasks^d at 37°C in 5% CO₂ and passaged at least once per week or when they reached > 90% confluence.

Inhibitor—The mTOR inhibitor rapamycin^e was purchased as a lyophilized powder (10 nmol) and stored at –20°C until resuspended with 100 µL of DMSO.^f Following resuspension, rapamycin was used as treatment or stored at –20°C until use.

Cell preparation for protein analysis—For protein analysis, cell lines 12, 23, and 50 each were rinsed twice with D-PBS solution. Cells were starved of serum by placing them in serum-free medium (DMEM with 1% nonessential amino acids) at 37°C in 5% CO₂ for 4 hours. Lyophilized rapamycin powder was solubilized in DMSO to create a stock solution (100 µL), according to the manufacturer's recommendations. Dimethyl sulfoxide was added to the medium used for the cells that received no rapamycin and the cells in the positive and negative control groups to achieve a concentration of DMSO equivalent to that in the medium used for the rapamycin-treated cells. After the 4-hour period in serum-free medium, the cells were incubated with 0 or 10nM rapamycin for 1 or 24 hours prior to protein extraction. Cells were then washed twice in D-PBS solution and lysed in a lysis buffer (10mM Tris-HCl; 137mM NaCl; 10% glycerol; 1% nonionic, nondenaturing detergent^g; and 10mM EDTA) with 10mM sodium fluoride, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, 1 µg of aprotinin/mL, 1 µg of leupeptin/mL, and 1 µg of pepstatin A/mL added. The flasks were then agitated for 2 minutes and the solution transferred to Eppendorf tubes and heated at 95°C for 6 minutes. Total protein concentration was assayed via spectrophotometry.^h

Detection of proteins—For each of the 3 cell lines, there were 3 treatment groups: cells exposed to 10nM rapamycin for 1 or 24 hours and cells that were not exposed to rapamycin. Tris-acetate gelsⁱ and tris-acetate running buffer^j were used for western blot analysis for mTOR and phosphorylated mTOR. Molecular weight indicators were used for all western blots to ensure that the resulting bands were at the expected molecular weight. Bis-tris gels^k and morpholinoethane sulfonic acid (MES)-SDS running buffer^l with antioxidant^m were used for western blot analysis of p70S6K and phosphorylated p70S6K. Aliquots of protein (50 µg) were mixed with sample bufferⁿ and deionized water and then heated at 95°C for 5 minutes; 5 µL of sample mixture was loaded into each well. For p70S6K and phosphorylated p70S6K analysis, 2 µL of reducing agent^o was added to the sample mixture before placing in the well. After running the gels at 100 V, proteins were transferred by use of a dry transfer system,^p according to the manufacturer's recommendations. Membranes were then blocked by incubation with 1× TBS solution (20× TBS^q solution diluted) and 0.1% Tween-2016^r with 5% nonfat milk.

The membranes were then blocked by incubation with 5% bovine serum albumin in TBS solution (0.5 mol/L NaCl with 20 mmol/L Tris; pH, 7.5) for 1 hour at room temperature (approx 20°C). Antibodies against either AKT (1:1,000),^s phosphorylated AKT (1:1,000),^t mTOR (1:1,000),^u phosphorylated mTOR (1:1,000),^v p70S6K (1:1,000),^w and phosphorylated p70S6K (1:2,000)^x were then added, and membranes were incubated overnight (approx 16 hours) in TBS solution with Tween-20 and 5% bovine serum albumin with gentle agitation at 4°C. The membranes were washed and incubated with horseradish peroxidase conjugated secondary antibody^y (1:5,000 concentration). The membranes were again washed and incubated with a substrate for

detection of horseradish peroxidase^z for 5 minutes. Radiographic film^{aa} was exposed to the membranes and then developed. Each gel was subsequently stripped by use of a western blot stripping buffer^{bb} in accordance with manufacturer instructions, and a polyclonal antibody against β -actin^{cc} was used to verify uniform total protein loading of each well on each gel.

For all western blot experiments, NIH 3T3 cells that are known to express mTOR were used as positive control samples.^{23–25} Wells were loaded with all reagents except for the protein aliquots for use as negative control samples.

Clonogenesis assay—Cell lines were grown in flasks^d until 70% to 80% confluent on the day of treatment. Medium was removed from each flask, and cells were washed once with D-PBS solution.^{dd} Cells were individualized through the addition of 3 mL of trypsin^{ee} and kept at room temperature (approx 20°C) for 5 minutes. Seven milliliters of fetal bovine serum was added to each flask to neutralize the trypsin, and cell concentration (number of cells/mL) was assessed by use of a hemocytometer and the trypan blue exclusion test. Cells in the untreated group were plated and returned to the previous growth conditions with the addition of a volume of DMSO equivalent to that of the rapamycin added to treated cells. This addition of DMSO to the untreated cells was to control for any effect of DMSO on cell growth. Cells in the other treatment groups were plated in Petri dishes in medium with differing concentrations of rapamycin (0.1, 1, 10, and 100 nmol in DMEM-10). For all plates, a sufficient number of cells were plated to give an expected count of 50 to 100 colonies. All plates were incubated at 37°C in 5% CO₂ for 8 days. Plates were removed from the incubator, washed twice with room-temperature D-PBS solution, and stained with crystal violet solution.^{ff} Only colonies composed of more than 50 cells were counted. The plating efficiency was defined as the number of colonies divided by the number of cells plated in the untreated group. The surviving tumor cell fraction was calculated by dividing the number of colonies that were counted by the number of cells that were plated. Experiments were performed in triplicate.

Statistical analysis—Results of the clonogenic assay were expressed as a surviving fraction equal to the number of colonies that were counted divided by the number of cells that were plated divided by the plating efficiency. Surviving fraction for each cell line was analyzed separately. Because the data were not normally distributed, comparisons of the surviving fractions within each cell line at the various rapamycin concentrations were made by use of a Kruskal-Wallis test. Values of $P < 0.05$ were considered significant. Statistical analyses were performed by use of a commercially available software program.^{gg}

Results

Activation of the AKT pathway in canine melanoma cells—Western blot analysis of all 3 canine melanoma cell lines revealed the presence and phosphorylation of AKT. Treatment with 10nM rapamycin for 1 or

24 hours had no effect on either the total amount of AKT protein or on the phosphorylated portion of the AKT protein in any cell line (Figure 1). Results of the western blot analysis for β -actin confirmed uniform protein loading.

Expressions of mTOR and p70S6K proteins in canine melanoma cells—Western blot analysis revealed that mTOR and P70S6K were present in all 3 canine melanoma cell lines (Figure 2). The amount of total mTOR and total p70S6K proteins remained relatively constant in each of the 3 cell lines regardless of whether the cells were or were not exposed to rapamycin. Phosphorylated mTOR and phosphorylated p70S6K were also confirmed to be present in all 3 cell lines, which indicated that the active form of each protein was present. The expressions of these proteins were confirmed by the use of molecular-weight indicators and positive control samples. Results of the western blot analysis for β -actin confirmed uniform protein loading.

Western blot analyses revealed that after treatment with 10nM rapamycin for 1 or 24 hours, there were continued expressions of mTOR and p70S6K. In the rapamycin-treated cells, the intensity of phosphorylated mTOR staining decreased for each cell line, compared with the intensity of staining in the untreated cells from the same cell line. The extent of decreased expression of phosphorylated mTOR was dependent on the time of exposure to rapamycin and decreased from 1 through 24 hours of exposure. The phosphorylated portion of p70S6K was partially inhibited at 1 hour of exposure to 10nM rapamycin and revealed a further decrease in expression at 24 hours of exposure (Figure 2). Results of the western blot analyses for β -actin confirmed uniform total protein loading.

Figure 1—Results of western blot analyses of total and phosphorylated AKT in 3 canine melanoma cell lines (cell line 12 in lanes numbered 1; cell line 23 in lanes numbered 2; and cell line 50 in lanes numbered 3) that were treated with 10nM rapamycin (+) or remained untreated (–) for 1 or 24 hours. The AKT protein was present and phosphorylated in all cell lines. Amounts of protein were not affected by treatment with rapamycin. Lane PC was a positive control sample composed of NIH 3T3 cells, and lane NC was a negative control sample with no protein loaded. The expression of β -actin was evaluated as a loading control. All western blot analyses were run in triplicate to ensure that results were consistent. Phospho-AKT = Phosphorylated AKT.

Figure 2—Results of western blot analyses of total and phosphorylated mTOR and p70S6K in 3 canine melanoma cell lines (cell line 12 in lanes numbered 1; cell line 23 in lanes numbered 2; and cell line 50 in lanes numbered 3) that were treated with 10nM rapamycin (+) or remained untreated (-) for 1 or 24 hours. Notice that mTOR and p70S6K are present and phosphorylated in canine melanoma cells. Results of analyses of total mTOR and p70S6K at the 24-hour time point were similar to results at the 1-hour time point (data not shown). Treatment with rapamycin can decrease this phosphorylation with 1 hour of exposure to the drug and to a greater degree with 24 hours of exposure to the drug. Lane PC was a positive control sample composed of NIH 3T3 cells, and lane NC was a negative control sample with no protein loaded. The expression of β -actin was evaluated as a loading control. All western blot analyses were run in triplicate to ensure that results were consistent. Phospho-mTOR = Phosphorylated mTOR. Phospho-p70S6K = Phosphorylated p70S6K.

Clonogenic inhibition with rapamycin—For each of the 3 canine melanoma cell lines, the results of the clonogenic assay performed on untreated cells and cells exposed to rapamycin (0.1, 1, 10, or 100.0nM) were compared (Figure 3). For cell lines 12, 23, and 50, there were significant ($P = 0.015$, $P = 0.015$, and $P = 0.014$, respectively) dose-dependent decreases in the surviving fraction of cells. At the 0.1nM concentration of rapamycin, mean surviving fraction for 2 of the 3 cell lines (cell lines 12 and 23) ranged from 0.28 to 0.38. The surviving fraction in cell line 50 did not decrease until cells were exposed to a 1nM concentration of rapamycin. Following treatment with a 1nM concentration of rapamycin, mean surviving fraction was 0.1 and 0.25 in cell lines 12 and 23, respectively, whereas cell line 50 had a surviving fraction of 0.9. Following treatment with a 100nM concentration of rapamycin, sur-

Figure 3—Results (mean $\pm$ 95% confidence limits) of a clonogenic assay performed on 3 canine melanoma cell lines (cell lines 12 [circles; solid line], 23 [squares; short-dashed line], and 50 [triangles; long-dashed line]) that were each exposed to rapamycin at concentrations of 0, 0.1, 1, 10, and 100nM for 8 days. The experiments were repeated in triplicate, and the mean of the surviving tumor cell fraction was plotted against the concentration of rapamycin on a logarithmic scale for each cell line. For each of the 3 cell lines, the dose-dependent decrease in surviving fraction was significant ($P = 0.015$, $P = 0.015$, $P = 0.014$ for cell lines 12, 23, and 50, respectively).

Figure 4—Photomicrographs of plates of canine melanoma cell lines 12 (A and B), 23 (C and D), and 50 (E and F) that were not exposed to rapamycin (A, C, and E) or that were exposed to 10nM rapamycin (B, D, and F). Because rapamycin was dissolved in DMSO, the untreated cells were exposed to an equivalent concentration of DMSO. The cells were allowed to grow for 8 days and then fixed with ethanol and stained with crystal violet solution. Notice that the cells that were treated with 10nM rapamycin formed smaller colonies that were composed of less densely arranged cells, compared with untreated cells. This effect was evident following treatment with rapamycin at concentrations of 1nM and greater; the effect was more pronounced as the drug concentration increased. Bar = 500 μ m.

viving fractions were 0.1 and 0.15 in cell lines 12 and 23, respectively, and 0.6 in cell line 50.

During microscopic evaluation of the culture plates used in the clonogenic assay, it appeared subjectively that the colonies formed on the plates treated with the higher concentrations of rapamycin (1, 10, and 100nM) were fewer in number and consisted of smaller, less dense colonies of cells, compared with characteristics of the colonies on the plates that were untreated or treated with the low concentration of rapamycin (0.1nM; Figure 4).

Discussion

The present study was designed to evaluate the phosphorylation of the AKT pathway, including the presence and activity of mTOR, in canine melanoma cells and assess the susceptibility of the AKT pathway to inhibition with rapamycin. The study results indicated that the AKT pathway is phosphorylated and that mTOR is present and active in canine melanoma cells. In addition, phosphorylation and subsequent activation of mTOR and its downstream target p70S6K can be inhibited by the addition of rapamycin. Rapamycin-induced inhibition of this pathway suppressed clonogenicity among canine melanoma cells; by use of a clonogenic assay, a rapamycin-associated decrease in surviving tumor cell fraction as well as a subjective decrease in cell colony size and density were identified. The alterations in colony size and density were subjective findings but are indications that in addition to decreasing colony formation, rapamycin has other effects on the growth and maturation of canine melanoma cells. It is thought that rapamycin causes a delay in the G1 phase or arrest in the cell cycle,¹³ which could account for decreased proliferation of the cells in the colonies that developed in the clonogenic assay in our study.

In the present study, the amount of phosphorylated AKT protein in canine melanoma cells did not increase as a result of rapamycin treatment. This finding is important because there is some evidence that inhibition of the mTOR protein can lead to activation of the AKT pathway.¹⁶ It is possible, however, that an interval longer than the 24-hour treatment period used in our study would be required for this effect to become apparent.

Also of interest was the effect of rapamycin exposure on the amount of phosphorylated mTOR protein in cell line 50. Although exposure of cell line 50 to rapamycin resulted in an apparent decrease in the amount of phosphorylated mTOR protein, compared with the amount in the untreated cells of this line, the amount of phosphorylated mTOR protein was relatively increased, compared with the amounts in cell lines 12 and 23. The comparatively decreased inhibition of phosphorylated mTOR in cell line 50 could explain the increased survival of that cell line in the clonogenic assay, compared with survival of the other 2 cell lines. This provides further evidence that the mTOR pathway is involved in canine melanoma cell survival and growth.

Activation of the AKT signaling pathway is important in the development of melanoma in humans. This pathway can be activated by mutations in the PTEN tumor suppressor gene. Loss of PTEN protein expression has been detected in melanomas in dogs.¹ Mutations in the PTEN gene and epigenetic changes can both result in loss of functional PTEN protein and activation of

the AKT pathway in melanomas in humans.²⁶ Whether there is functional PTEN protein present in the cell lines used in the experiments of the present study is unknown, but loss of PTEN protein could account for activation of the AKT pathway.

Mammalian target of rapamycin is a central signaling molecule in the AKT pathway that integrates extracellular signals by growth factors, hormones, and nutrient status to regulate the cellular protein expression and cell growth. The main activities of mTOR include translational control of protein synthesis, apoptosis modulation, cell cycle regulation, and metabolic regulation via the role of AKT pathway in insulin signaling and glucose homeostasis.^{13,19} Blockade of the AKT-mTOR pathway by use of rapamycin and its derivatives results in inhibition of critical cellular proteins and cell cycle arrest.⁸ In several types of neoplasms in humans, mTOR signaling is increased because of increased amounts of AKT, because of loss of PTEN protein, or via other regulatory pathways. It is also known that tumors with high levels of phosphorylated AKT and phosphorylated p70S6K expression are more susceptible to mTOR inhibition.²⁷ Inhibition of the mTOR pathway inhibits growth of canine osteosarcoma cells¹⁸ and cells of several types of tumors in humans. Inhibition of the mTOR pathway in melanoma and other cancers may be of clinical use in inhibiting tumor cell growth and metastasis *in vivo*. It is also possible that use of inhibitors of the mTOR pathway in combination with more traditional chemotherapy agents may be more effective as cancer treatments than mTOR inhibition or chemotherapy alone.²⁸ The experimental design used in the present study could be used to further investigate this speculation.

Rapamycin has been used as an immunosuppressive agent in cats and dogs; however, reported data concerning the use of mTOR inhibitors as potential antineoplastic drugs in veterinary medicine are minimal.²⁹⁻³² One concern regarding clinical use of rapamycin is the incidence of severe adverse effects associated with immunosuppressive doses administered to dogs. These adverse effects included vasculitis, peritonitis, diarrhea, intussusception, oral ulceration, and emaciation.^{29,33} Another potential adverse effect of concern associated with chemotherapeutic inhibition of mTOR is the possible impact on insulin and glucose homeostasis.³⁴ However, these adverse effects likely develop only at the higher concentrations of rapamycin needed for immunosuppression in dogs. In humans, the doses of rapamycin needed to inhibit mTOR in tumors are lower or can be given intermittently, compared with rapamycin administration for immunosuppression in transplant recipients.³⁵ A dosage of 0.1 mg of rapamycin/kg given every other day resulted in no clinically important adverse effects in healthy experimental dogs that received renal allografts.³¹ Given that mTOR pathway activity is increased in tumors and that their susceptibility to rapamycin is subsequently increased, compared with nonneoplastic tissues, it is possible that low concentrations of rapamycin can be used as antineoplastic agents without toxic effects on the rest of the body.¹⁹

To our knowledge, reports on the pharmacokinetics of rapamycin in dogs are limited. One study³² re-

vealed that administration of rapamycin (0.05 mg/kg/d, IM) was clinically tolerated and resulted in whole blood concentrations of 9 to 14 µg/L (9.6 to 15.3nM). This range of concentrations includes the concentrations at which decreased phosphorylation of mTOR and p70S6K as well as decreased survival of melanoma cells in the clonogenic assay were detected in the present study. However, further studies to determine bioavailability, pharmacokinetics, and dose tolerance in dogs would be needed to verify that rapamycin can be administered safely and achieve blood concentrations that are potentially therapeutic with minimal adverse effects.

In human medicine, it is known that inhibitors of mTOR have potent antitumor activity in several tumor types, including lung cancer, melanoma, lymphoma, gastrointestinal cancer, renal cell carcinoma, and hepatocellular cancer.³⁶ The potential benefits of rapamycin and its derivatives as chemotherapeutics are known; administration as monotherapy or as adjunct measures in conventional treatments, such as cisplatin, appears to have promising clinical application.¹⁹

Limitations of the present study include the small number of cell lines that were evaluated and the lack of a more quantitative measure of protein expression. Furthermore, the relationship of in vivo efficacy of rapamycin to these in vitro findings is unknown at this time. The study oversimplified the complex interactions of several signaling pathways both upstream and downstream of mTOR that may play a role in controlling the susceptibility or resistance of a tumor to mTOR inhibition in vivo. We attempted to evaluate the most prominent proteins in this signaling cascade including AKT, mTOR, and p70S6K; however, these are only a few of the effectors in the AKT-mTOR pathway. Further investigation of the effects of rapamycin on these pathways and in vivo studies of rapamycin in dogs with melanoma are warranted to further assess this potentially effective targeted chemotherapeutic.

Results of the present study have further validated the use of canine melanoma cell lines as a model for the disease in humans. In the canine melanoma cell lines, phosphorylation of AKT and mTOR was similar to that detected in human melanoma cells and the mTOR pathway was also inhibited with rapamycin. Inhibition of mTOR is becoming a promising targeted treatment of cancer in both human and canine medicine. Further characterization of this pathway in tumors in dogs and clinical trials of the antineoplastic effects of rapamycin may prove to have direct clinical benefits for that species and ultimately for humans.

- a. Dulbecco Modified Eagle Medium, Invitrogen, Carlsbad, Calif.
- b. Non-Essential Amino Acids, Invitrogen, Carlsbad, Calif.
- c. Fetal Bovine Serum, Omega Scientific, Tarzana, Calif.
- d. T75 flasks, Nunc, Rochester, NY.
- e. Rapamycin, Cell Signaling, Danvers, Mass.
- f. Dimethyl Sulfoxide, Sigma, St Louis, Mo.
- g. IGEPAL CA 630, Sigma-Aldrich, St Louis, Mo.
- h. Biophotometer, Eppendorf, Westbury, NY.
- i. NuPAGE Novex Tris-Acetate gels (3% to 8%, 15 well), Invitrogen, Carlsbad, Calif.
- j. NuPAGE Tris-Acetate running buffer, Invitrogen, Carlsbad, Calif.
- k. NuPAGE Novex Bis-Tris Gels (4% to 12%, 12 well) Invitrogen, Carlsbad, Calif.
- l. NuPAGE MES SDS running buffer, Invitrogen, Carlsbad, Calif.

- m. NuPAGE antioxidant, Invitrogen, Carlsbad, Calif.
- n. NuPAGE LDS sample buffer, Invitrogen, Carlsbad, Calif.
- o. NuPAGE Reducing Agent (10X), Invitrogen, Carlsbad, Calif.
- p. iBlot dry transfer system, Invitrogen, Carlsbad, Calif.
- q. Tris-Buffered Saline, USB Corp, Cleveland, Ohio.
- r. Tween-2016, Bio-RAD, Hercules, Calif.
- s. Anti-AKT (Pan) monoclonal rabbit antibody, Cell Signaling, Danvers, Mass.
- t. Anti-phospho-AKT (ser 473) monoclonal rabbit antibody, Cell Signaling, Danvers, Mass.
- u. Anti-mTOR monoclonal rabbit antibody, Cell Signaling, Danvers, Mass.
- v. Anti-phospho mTOR polyclonal rabbit antibody, Cell Signaling, Danvers, Mass.
- w. Anti-p70S6 Kinase polyclonal rabbit antibody, Cell Signaling, Danvers, Mass.
- x. Anti-phospho p70S6 Kinase monoclonal mouse antibody, Cell Signaling, Danvers, Mass.
- y. Goat anti-rabbit HRP conjugate antibody or goat anti-mouse HRP conjugate antibody, Pierce Biotechnology, Rockford, Ill.
- z. Super Signal West Dura Extended Duration Substrate, Pierce Biotechnology, Rockford, Ill.
- aa. Amersham Hyperfilm MP, GE Healthcare Limited, Buckinghamshire, England.
- bb. Restore Western Blot Stripping Buffer, Pierce Biotechnology, Rockford, Ill.
- cc. β-Actin, Santa Cruz Biotechnology, Santa Cruz, Calif.
- dd. Dulbecco-Phosphate Buffered Saline, Invitrogen, Carlsbad, Calif.
- ee. Trypsin, Invitrogen, Carlsbad, Calif.
- ff. Crystal violet, Fischer Scientific, Pittsburgh, Pa.
- gg. Stata 9.0, StataCorp, LP, College Station, Tex.

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