In vitro effects of doxorubicin and tetrathiomolybdate on canine hemangiosarcoma cells

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OBJECTIVE
To assess the in vitro effects of doxorubicin and tetrathiomolybdate (TM) on cells from a canine hemangiosarcoma cell line.

SAMPLE
Cultured cells from the canine hemangiosarcoma–derived cell line DEN-HSA.

PROCEDURES
Cells were treated with TM (0 to 1.5µM), doxorubicin (0 to 5µM), or both with or without 24 hours of pretreatment with ascorbic acid (750µM). Degree of cellular cytotoxicity was measured with a colorimetric assay. Long-term growth inhibition was assessed with a 10-day colony-formation assay. Induction of apoptosis was quantitated by fluorometric assessment of caspase-3 and -7 activation. Formation of reactive oxygen species (ROS) was also detected fluorometrically.

RESULTS
Exposure of cells to the combination of TM and doxorubicin resulted in a greater decrease in proliferation and clonogenic survival rates than exposure to each drug alone. This treatment combination increased ROS formation and apoptosis to a greater extent than did doxorubicin or TM alone. Ascorbic acid inhibited both TM-induced ROS formation and apoptosis.

CONCLUSIONS AND CLINICAL RELEVANCE
Results suggested that the enhancement in cytotoxic effects observed with DEN-HSA cell exposure to the combination of doxorubicin and TM was achieved through an increase in ROS production. These findings provide a rationale for a clinical trial of this treatment combination in dogs with hemangiosarcoma. (*Am J Vet Res* 2018;79:219–225)

Hemangiosarcoma is a malignant tumor of endothelial cells that commonly arises from the spleen, heart, liver, and subcutis in dogs, accounting for 7% of all canine malignant tumors.[1–4] Such tumors are extremely aggressive owing to their high metastatic rate and local tissue invasion, both of which occur early in development of the disease.[5]

Current standard-of-care treatments include excision in conjunction with chemotherapy.[1–3] Mean survival time for dogs with splenic hemangiosarcoma is 2 to 3 months with surgical treatment alone and 5 to 7 months with surgery and chemotherapy, and this dismal prognosis has not improved in nearly 30 years.[5–7] Therefore, discovery of new or additional treatments that might extend survival time in affected dogs is important.

Numerous chemotherapy protocols exist for hemangiosarcoma in dogs, and all of them involve doxorubicin as the cornerstone.[3,7,8] Doxorubicin has 2 major cytotoxic modes of action: inhibition of topoisomerase II, which is an enzyme necessary for DNA synthesis, and intracellular formation of ROS. Because high concentrations of intracellular ROS can induce cell death, cancer cells adapt to increased intracellular concentrations of ROS through the upregulation of intracellular antioxidant proteins such as superoxide dismutase 1 to degrade intracellular ROS that allow protumorigenic signaling without inducing cell death.[9–14] This reliance on antioxidants potentially makes cancer cells selectively vulnerable to antioxidant inhibition, as nontransformed cells generate lower basal amounts of intracellular ROS and are therefore less dependent on ROS detoxification. As a result of this difference, exposure of cells to a molecule that inhibits ROS scavenging might differentially target cancer cells.

Tetrathiomylobdate is an FDA-approved, commercially available, nontoxic copper-binding agent used in the treatment of Wilson disease (a copper accumulation disorder). This agent has additional anticancer and antiangiogenic effects. Specifically, the binding of TM to copper, which is a cofactor for the cellular antioxidant CuZn superoxide dismutase,

ABBREVIATIONS

MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
RFI	Relative fluorescent intensity
ROS	Reactive oxygen species
TM	Tetrathiomolybdate

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inhibits this important enzyme's protective function against oxidative insult. As previously alluded to, use of an antioxidant-inhibiting drug, such as TM, could sensitize hemangiosarcoma cells to the effects of and delay the resistance to an ROS-generating drug, such as doxorubicin.

The combination of doxorubicin and TM is reportedly more effective at inducing apoptosis and decreasing cellular proliferation in endometrial, ovarian, and breast cancer cells than doxorubicin alone when assessed in vitro and in mice.[15–17] Additional important and potentially clinically useful attributes of TM include its ability to ameliorate the cardiotoxic effects of doxorubicin and inhibit angiogenesis.[18] Tumor metastasis requires the formation of new blood vessels. Copper is a required cofactor for proangiogenic mediators such as vascular endothelial growth factor, fibroblast growth factor 2, interleukin-1α, interleukin-6, and interleukin-8.[19] Inhibition of angiogenesis by TM has been demonstrated in vitro and in vivo.[19,20] We hypothesized that the combination of doxorubicin and TM would induce more apoptosis and decrease proliferation of cells from the canine hemangiosarcoma cell line DEN-HSA[21,22] than would doxorubicin or TM alone through induction of ROS.

Materials and Methods

Hemangiosarcoma cell culture

Cells from the canine hemangiosarcoma–derived cell line DEN-HSA were obtained.[a] The passage number of these cells was 7 at the time of removal from liquid nitrogen storage. Cells were thawed for 5 minutes in a 37°C water bath and then grown in Dulbecco modified Eagle medium[b] in 25-mL plastic flasks.[c] All medium was supplemented with 10% fetal bovine serum[d] and 1% penicillin-streptomycin.[b] Cells were maintained in a humidified incubator at 37°C in 5% CO_2.

Doxorubicin and TM working solutions

Doxorubicin[d] and TM[e] were dissolved in cell culture medium to create a working stock solution (0.5M) and maintained frozen (–20°C) in 1-mL aliquots. The aliquots were thawed once as needed and then further diluted with cell culture medium to the desired working concentrations. Working concentrations were made fresh prior to use in experiments.

Working drug ranges

Tetrathiomolybdate has been evaluated in vitro by use of epithelial cell lines, and this agent alone did not appear to induce cell death in those studies.[15–20] However, in preliminary experiments involving exposure of the authors' mesenchymal cell line to TM concentrations similar to those used in the other studies, TM induced cell death in both MTS and clonogenic assays (data not shown). Therefore, preliminary experiments were conducted with TM alone in a broad range of concentrations (serial dilutions over 3 logarithmic orders) to identify a working range of TM for the DEN-HSA cell line specifically (data not shown); the concentrations of TM that provided a measurable output were then used in the experiments reported here. Concentrations used in the MTS and clonogenic assays are by necessity different owing to the duration of cell exposure to TM (ie, 3 days vs 10 days, respectively).

MTS cellular cytotoxicity assay

To determine the effects of doxorubicin, TM, and doxorubicin plus TM on short-term cytotoxicity of canine hemangiosarcoma cells in vitro, DEN-HSA cells were seeded into 96-well microtiter plates[d] (800 cells/200 µL/well) in drug-free cell culture medium for 24 hours and allowed to attach. After attachment, drug-free cell culture medium was removed by careful aspiration and replaced with 200 µL of cell culture medium containing the desired final concentrations of doxorubicin (0, 0.07, 0.15, 0.3, and 0.6µM) alone or in combination with TM (0, 1, and 1.5µM) for 24 hours. Cells were again washed free of drug with careful aspiration of cell culture medium, which was then replaced with 200 µL of doxorubicin-free cell culture medium containing the desired final concentration of TM (0, 1, or 1.5µM) for an additional 72 hours.

A commercial assay[f] was used to assess cellular cytotoxicity in accordance with the manufacturer's specifications. Absorbance of formazan, the reduced MTS product, was recorded by use of a 96-well plate reader[g] and a wavelength of 490 nm. Values for the untreated control wells (doxorubicin, 0µM; TM, 0µM) were normalized to 100%. Relative cellular cytotoxicity was determined by comparing mean values of untreated control cells with those of cells exposed to 0µM doxorubicin plus 1.0µM TM and 0µM doxorubicin plus 1.5µM TM. Cells in doxorubicin-free, TM-containing wells were compared with those in wells that contained increasing concentrations of doxorubicin but similar concentrations of TM. Experiments in quadruplicate were repeated twice.

Clonogenic assay

To determine the effects of doxorubicin, TM, and doxorubicin plus TM on the long-term cytotoxicity of canine hemangiosarcoma cells in vitro, DEN-HSA cells (1,000 cells/2 mL/well) were seeded into 6-well plates[d] for 24 hours. The drug-free cell culture medium was removed by careful aspiration and replaced with 2 mL of cell culture medium containing the desired final concentrations of doxorubicin (0, 0.03, 0.07, 0.15, and 0.3µM) alone or with TM (0, 0.75, and 1µM) for 24 hours. Subsequently, the drug-free cell culture medium was removed by careful aspiration and replaced with 2 mL of cell culture medium containing the desired final concentration of TM (0, 0.75, and 1µM) for an additional 10 days. To minimize jostling of plates containing the delicate cell colonies, plates were evaluated every other day to ensure that the medium was not depleted (ie, turned yellow).

To stain the cell colonies for scanning, plates were placed and maintained on wet ice, and wells

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were washed twice with 2 mL of ice-cold PBS solution. Colonies were fixed with 2 mL of ice-cold 100% methanol for 10 minutes and then stained with 0.5% crystal violet (wt/vol) in 75% PBS solution and 25% methanol. Plates were scanned with a commercial scanner.[h] Experiments were performed at least twice.

Caspase-3 and -7 apoptosis assay

To determine activation of the apoptotic cascade in the DEN-HSA cells in response to doxorubicin, TM, and doxorubicin plus TM, DEN-HSA cells (100,000 cells/100 µL/well) were seeded into 96-well black, clear-bottomed microtiter plates[d] and allowed to adhere for 24 hours. The drug-free cell culture medium was removed from all wells by careful aspiration and replaced with an equal volume of drug-free cell culture medium (vehicle) or with cell culture medium containing the desired final concentration of TM (1µM), doxorubicin (5µM), or doxorubicin (5µM) plus TM (1µM) for 24 hours. Apoptosis was then assessed by use of a caspase-3 and -7 commercial kit.[i] Negative and positive control solutions, provided with the kit, were included with each experiment and treated as directed by the kit instructions. These control wells were used to confirm that the experiment worked and were not included in any further analysis. The RFI of these wells was set to 1 ± 0.6, which reflected background apoptosis

To determine whether an ROS scavenger such as ascorbic acid could affect the activation of the apoptotic cascade, identical experiments were conducted as described for the caspase assay with the following change. Cells were incubated with culture medium containing a final ascorbic acid[d] concentration of 750µM for 24 hours prior to testing in each of the same conditions as those tested in the absence of ascorbic acid after they had been allowed to adhere to the plastic overnight.

In both sets of experiments (without or with ascorbic acid), at the end of the incubation period, wells were developed in accordance with the kit manufacturer's specifications, and fluorescence was detected at an excitation wavelength of 380 nm and emission wavelength of 500 nm by use of a 96-well plate reader.[j] Mean RFI of vehicle-treated control cells was normalized to 1 (arbitrary units). Mean ± SD RFI of treated wells was recorded as fold change relative to vehicle control. Two experiments in triplicate were completed.

ROS assay

To determine whether intracellular ROS concentrations changed in response to treatment with doxorubicin, TM, or doxorubicin plus TM, DEN-HSA cells (2,500 cells/100 µL/well) were seeded into 96-well black, clear-bottomed microtiter plates[d] and allowed to adhere for 24 hours. A commercial assay[k] for of ROS detection was used, and in accordance with the manufacturer's recommendations, cells were then left untreated (vehicle-treated control cells) or exposed to the desired test conditions of doxorubicin alone (5µM), TM alone (1µM), or doxorubicin plus TM for 5 hours. Each experiment also included positive and negative control wells, which were treated with reagents provided by the manufacturer but not included in analysis.

After development of the plate, fluorescence was measured by use of a 96-well plate reader.[j] Mean RFI (total ROS production) of the vehicle-treated control cells was normalized to 1 (arbitrary units). Mean RFI of treated wells was recorded as fold change relative to vehicle control. Two independent experiments in sextuplicate were completed. To determine whether an ROS scavenger such as ascorbic acid could decrease the production of ROS in our experimental system, identical experiments to the other ROS assays were conducted in which the cells were first incubated for 24 hours with culture medium containing a final ascorbic acid concentration of 750µM prior to testing. Two independent experiments conducted in sextuplicate were again completed.

Statistical analysis

All statistical analyses were performed with statistical software.[l] Mean values of each condition tested were analyzed via 1-way ANOVA, followed by Tukey post hoc tests when a significant ($P < 0.05$) difference was detected. Specifically, for the MTS assay, mean absorbance of the control treatment (0µM doxorubicin and TM) was normalized to 100%, and then the mean absorbance (percentage of the control value) of each treatment group was compared with the control value or mean absorbances were compared with one another. Because TM treatment had an inhibitory effect on the cells, the 0µM doxorubicin, TM-treated wells served as control specimens for the TM treatments. For the ROS assay, mean RFI was compared between cells treated with drug-free medium (vehicle control) and those treated with doxorubicin, TM, and doxorubicin plus TM in the presence or absence of ascorbic acid. Results are reported as mean ± SD.

Results

Cellular cytotoxicity

Doxorubicin treatment of DEN-HSA cells resulted in a dose-dependent increase in cellular cytotoxicity **(Figure 1)**. This dose-dependent effect of doxorubicin was enhanced with exposure of cells to TM. Cells exposed to 1µM TM alone had a mean ± SD minor increase in cell killing of 4.5 ± 18.3%, which was not different than that observed for the untreated control (vehicle) cells ($P = 0.60$). In contrast, cells exposed to 1.5µM TM alone had an increase in cellular cytotoxicity of 30.9 ± 5.0%, and this difference was significant ($P < 0.001$). Cells exposed to 0.07µM doxorubicin alone had a mean increase in cellular cytotoxicity of 6.4 ± 11%, whereas cells exposed to 0.07µM doxorubicin plus 1 or 1.5µM TM had a mean increase of 29.2 ± 5.2% or 48.3 ± 5.3%, respectively ($P < 0.001$

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for both). Cells exposed to 0.15µM doxorubicin alone had a mean increase in cytotoxicity of 14.4 ± 14.0%, whereas cells exposed to 0.15µM doxorubicin plus 1 or 1.5µM TM had a mean increase in cytotoxicity of 40.7 ± 10.4% or 57.0 ± 4.9%, respectively ($P < 0.001$ for both). Cells exposed to 0.30µM doxorubicin alone had a mean increase in cytotoxicity of 40.4 ± 20.9%, whereas cells exposed to 0.30M doxorubicin plus 1 or 1.5µM TM had a mean increase of 57.1 ± 11.3% or 64.1 ± 6.3%, respectively ($P = 0.005$ and $P < 0.001$, respectively). Cells exposed to 0.60µM doxorubicin alone had a mean increase in cytotoxicity of 66.8 ± 13.7%, whereas cells exposed to 0.60µM doxorubicin plus 1 or 1.5µM TM had a mean increase in cytotoxicity of 71.8 ± 8.3% or 74.6 ± 6.5%, respectively ($P = 0.60$ and $P = 0.30$, respectively).

Colony formation

Colony formation as determined by means of a standard 10-day clonogenic assay was inhibited in a concentration-dependent manner in both the absence and presence of doxorubicin **(Figure 2)**. Doxorubicin alone had an estimated 50% inhibitory concentration between 0.07 and 0.15µM. With 0.3µM doxorubicin, colony formation was inhibited the most. The 0.75 and 1µM concentrations of TM appeared to have similar inhibition of colony formation when the doxorubicin concentration was 0, 0.03, or 0.07µM. At a doxorubicin concentration of 0.15µM, the 1µM concentration of TM inhibited colony formation to a greater extent than did the 0.75µM concentration of TM. This difference in inhibition of colony formation was most pronounced at a doxorubicin concentration of 0.3µM; at this concentration, some colonies were visible in the 0.75µM-TM wells and 0 to 2 colonies were visible in the 1µM-TM wells.

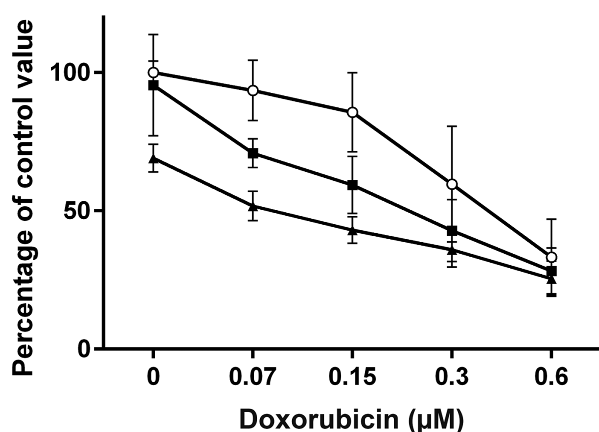

Figure 1—Mean proliferation values (percentage relative to control treatment) as determined by MTS inhibition assay for DEN-HSA cells treated with doxorubicin (0 to 0.6µM) plus TM at 0µM (circles), 1µM (squares), or 1.5µM (triangles). Values obtained for the control treatment (0µM doxorubicin and 0µM TM) were used to normalize the data for doxorubicin; because TM alone had a growth inhibitory effect, values for TM alone at 1µM or 1.5µM were also normalized to values obtained for the control treatment. Error bars represent SD; means represent the results of 2 experiments, each conducted in quadruplicate.

Caspase-3 and -7 activity

In the assay involving cleavage of caspase-3 and -7 as a measure of induction of apoptosis, the negative and positive control cells had a mean ± SD fold increase in apoptosis (RFI) of 1.0 ± 1.4 and 7 ± 1.5, respectively, confirming validity of the experiments. Treatment of DEN-HSA cells with 1µM TM resulted in a mean fold increase in apoptosis of 2 ± 1.7, which was not significantly different from the value for untreated cells **(Figure 3)**. Treatment with 5µM doxorubicin resulted in a mean fold increase in apoptosis of 5.2 ± 1.8, which was greater than that observed for vehicle-treated control cells ($P = 0.004$) and similar to that for TM-treated cells ($P = 0.16$). Treatment with doxorubicin plus TM resulted in a mean fold increase in apoptosis of 7.9 ± 1.5, which was greater than that observed for vehicle-treated control cells or TM-treated cells ($P < 0.001$ for both). Finally, treatment with doxorubicin plus TM resulted in a greater fold increase in apoptosis than did treatment with doxorubicin alone, and this difference was significant ($P = 0.02$).

When ascorbic acid was added to the cell cultures prior to treatment, fold changes in apoptosis for vehicle-treated control cells (1.8 ± 0.6), negative control cells (1.1 ± 1.1), positive control cells (7 ± 1.2), and doxorubicin-treated cells (5.0 ± 15) were similar ($P > 0.05$) to those observed in ascorbic acid–free conditions. In contrast, when ascorbic acid was present prior to cell treatment with TM (0.9 ± 1.2) or doxorubicin plus TM (5.5 ± 1.5), the fold change in apoptosis was less, and the difference between cells exposed versus not exposed to ascorbic acid was significant ($P < 0.02$ and $P < 0.01$, respectively).

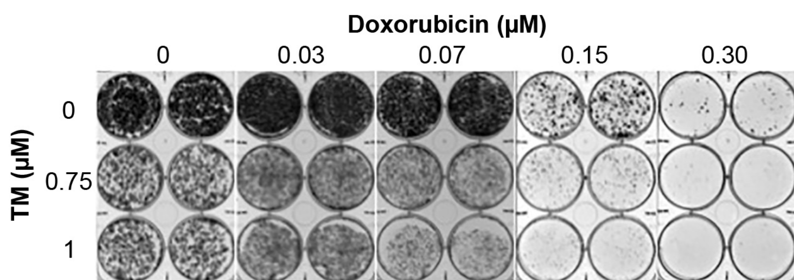

Figure 2—Representative photograph of results of a standard 10-day clonogenic assay showing inhibition of colony formation in DEN-HSA cells after treatment with doxorubicin alone, TM alone, or both at various concentrations. Cells were stained with 0.5% crystal violet (w/v) in 75% PBS solution and 25% methanol. The darker the wells appear, the greater the number of colonies present in the wells.

ROS production

Treatment of DEN-HSA cells with conditioned medium exchanged for fresh, drug-free medium (ie, vehicle-treated control cells) had a low basal level of ROS production, as inferred through the mean ± SD RFI value (1.1 ± 0.6; **Figure 4**). Cells treated with

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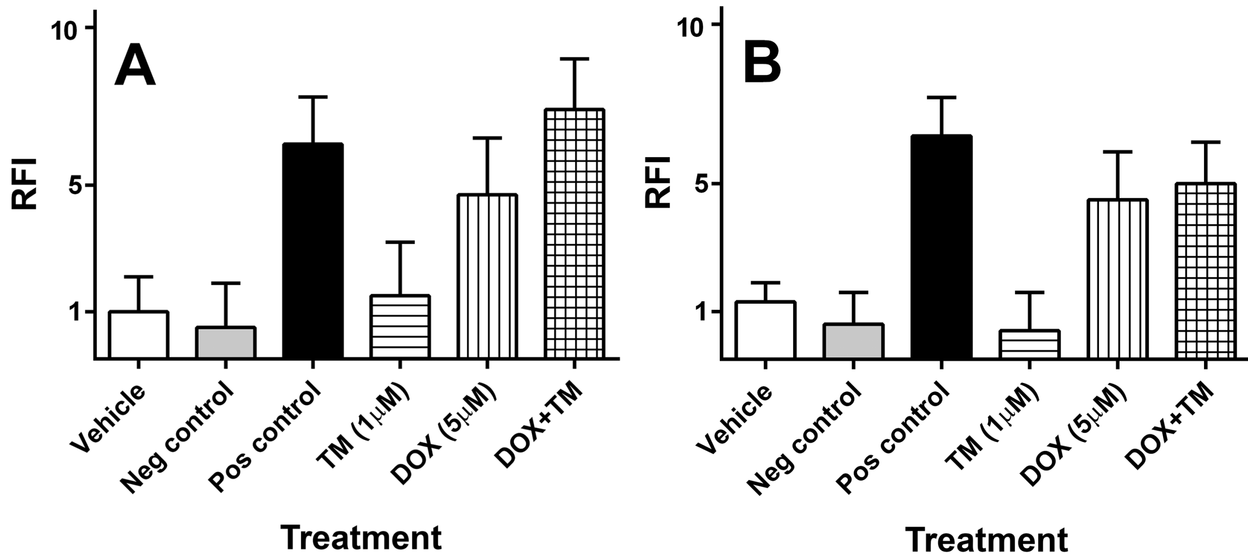

Figure 3—Mean RFI (arbitrary units) as determined via caspase-3 and -7 assay of apoptosis in DEN-HSA cells after exposure to 5μM doxorubicin, 1μM TM, or both without (A) or with (B) 24 hours of pretreatment with ascorbic acid (750μM). In both panels, values for vehicle-treated control cells were set to 1 and all other values evaluated as a fold change in RFI. Negative (Neg) and positive (Pos) control solutions were included in each experiment to confirm validity of the experiment but were not used for analysis. Error bars represent SD; mean values were derived from 2 experiments conducted in triplicate.

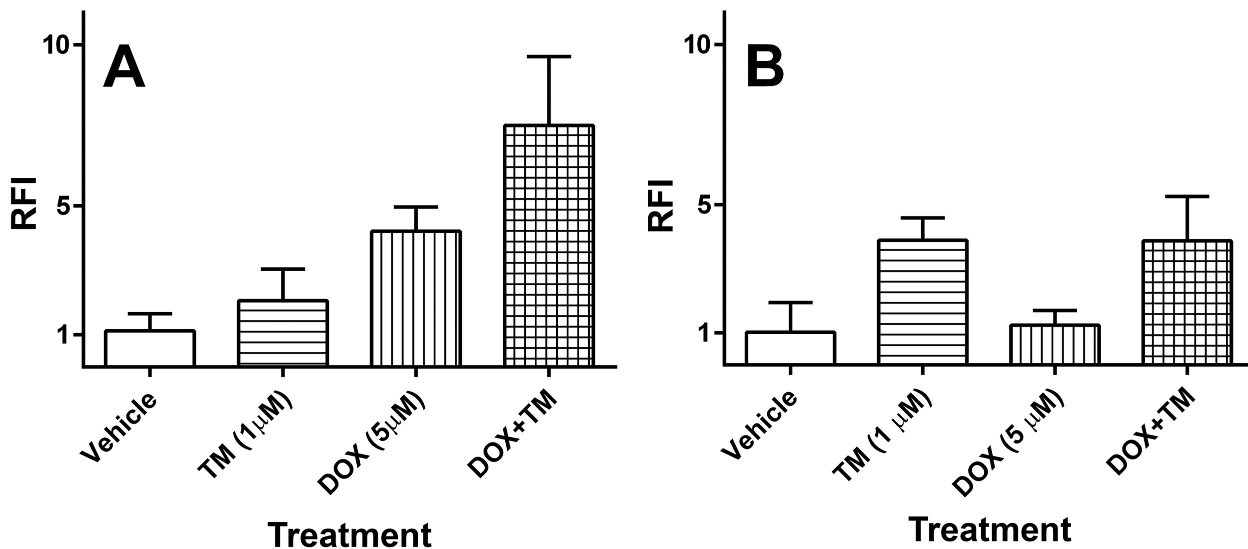

Figure 4—Mean RFI (arbitrary units) as determined via assay of ROS production by DEN-HSA cells after exposure to 5μM doxorubicin, 1μM TM, or both without (A) or with (B) 24 hours of pretreatment with ascorbic acid (750μM). ***See*** Figure 3 for remainder of key.

1μM TM alone had a mean fold increase in ROS production of 2.1 ± 0.8, which was similar to the value for vehicle-treated control cells (P = 0.99). Treatment of cells with 5μM doxorubicin only resulted in a mean fold increase in ROS production of 4.2 ± 0.8, and this value was greater than that for treatment with vehicle or TM alone (P < 0.001 for both). The combination of doxorubicin plus TM resulted in a mean fold increase in ROS production of 7.5 ± 1.7, and this value was greater than that observed for vehicle, doxorubicin alone, or TM alone (P < 0.001 for all).

When ascorbic acid was added to the cell cultures prior to treatment, the mean fold increase in ROS production for untreated control cells (1.0 ± 0.6) was similar to the value obtained for ascorbic acid–free conditions (P = 0.54). In contrast, the values obtained for doxorubicin (1.2 ± 1.0) were lower than those observed in the ascorbic acid–free conditions (P < 0.001). This exposure resulted in an increase in ROS production by TM-treated cells to 3.9 ± 1.2 and by doxorubicin plus TM–treated cells to 3.9 ± 1.6, and these differences were significant (P = 0.02 for both). The ROS production for cells treated with doxorubi-

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cin plus TM but no ascorbic acid was greater than that for cells treated with doxorubicin plus TM and ascorbic acid (*P* = 0.002).

Discussion

Current treatment approaches for hemangiosarcoma in dogs, which consist of a combination of surgery followed by a doxorubicin-based chemotherapy protocol, improve survival times over surgery alone; however, these stage-dependent survival times have not improved in the last 30 years.[5–7] One reported mechanism of the cytotoxic effects of doxorubicin is the formation of ROS. Cells destroy ROS through the copper-containing antioxidant enzyme superoxide dismutase 1.[9–14] Tetrathiomylobdate, a copper chelator, decreases intracellular amounts of this enzyme and consequently increases intracellular ROS concentrations. We postulated that cytotoxic effects would be enhanced in DEN-HSA cells with exposure to the combination of doxorubicin and TM (antioxidant suppressor) via a multimodal increase in ROS. To test this hypothesis, the effects of TM plus doxorubicin were explored in cultured canine DEN-HSA cells in vitro.

Inhibition of DEN-HSA cell proliferation after exposure to doxorubicin alone occurred in a dose-dependent manner in the present study. This inhibition was greater when DEN-HSA cells were exposed to doxorubicin plus TM and was significant when the doxorubicin concentration was 0.07, 0.15, and 0.30μM. Exposure of DEN-HSA cells to 0.60μM doxorubicin plus TM resulted in a nonsignficant increase in inhibition of proliferation, compared with exposure to doxorubicin alone. Significant inhibition of cellular proliferation was not observed with exposure to 0.60μM doxorubicin because this high concentration resulted in a marked (66.8%) cell kill when used alone. Similarly, the clonogenic assay qualitatively revealed the dose-dependent increase in cellular cytotoxicity, as depicted by decreased colony formation, in cells exposed to doxorubicin plus TM versus doxorubicin alone. However, at higher concentrations of both drugs, there was apparent complete or nearly complete inhibition of colony formation, which also impeded further analysis. It would be tempting to speculate that perhaps at these higher doses, doxorubicin and TM act additively or synergistically. Additional experiments such as those reported by Chou[23,24] involving evaluation of the additive, synergistic, or antagonistic effects of drug combinations might help to investigate this possibility.

Apoptosis of DEN-HSA cells in the study reported here was indirectly analyzed by means of a caspase-3 and -7 assay. Cells treated with doxorubicin plus TM had increased activation of these enzymes, as reflected by RFI values, compared with cells treated with doxorubicin alone. This finding was consistent with the results from the clonogenic assay and MTS analysis.

Lastly, total ROS production was markedly increased in cells exposed to doxorubicin plus TM, compared with production in cells exposed to vehicle, TM alone, or doxorubicin alone. This significant finding indicated that treatment with the combination of doxorubicin and TM induced a greater amount of ROS production in DEN-HSA cells than did treatment with doxorubicin alone.

Finally, to determine whether the observed cytotoxic effects in the DEN-HSA cells were achieved through an ROS-mediated mechanism, the ROS-scavenger ascorbic acid was added prior to cell exposure to TM alone, doxorubicin alone, or doxorubicin plus TM. The data suggested a decrease in ROS production to levels achieved in the absence of TM. Additionally, activation of proapoptotic caspase-3 and -7 was also decreased in the presence of ascorbic acid, suggesting that the cytotoxic effects observed with the MTS and clonogenic assays were through ROS-mediated cell death.

Overall, the in vitro findings reported here suggested that TM sensitizes canine DEN-HSA cells to the cytotoxic effects of doxorubicin and that this enhancement is mediated through increases in ROS formation. Additional studies are warranted to determine the effects of TM and doxorubicin on various canine and feline neoplasias in vitro. Given the lack of improvement in survival times of dogs with hemangiosarcoma in the last quarter century, we also suggest a postoperative clinical trial involving dogs with visceral hemangiosarcoma to compare the effects of doxorubicin with those of doxorubicin plus TM.

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Footnotes

a. Provided by Dr. Douglas H. Thamm, Colorado State University, Fort Collins, Colo.
b. Life Technologies, Grand Island, NY.
c. Corning cell culture flasks (25 mL), Sigma-Aldrich, St Louis, Mo.
d. Sigma-Aldrich, St Louis, Mo.
e. Provided by Dr. George Brewer, University of Michigan, Ann Arbor, Mich.
f. CellTiter 96 AQ_{ueous} One Solution cell proliferation assay, Promega, Madison, Wis.
g. SpectraMax 190 microplate reader, Molecular Devices, Sunnyvale, Calif.
h. FluorChem E System, ProteinSimple, San Jose, Calif.
i. SensoLyte homogeneous AFC caspase 3/7 assay kit, AnaSpec, Fremont, Calif.
j. Gemini EM microplate reader, Molecular Devices, Sunnyvale, Calif.
k. Total ROS/superoxide detection kit, Enzo Life Sciences, Farmingdale, NY.
l. GraphPad Prism, version 6.05, GraphPad Software, La Jolla, Calif.

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