Use of certain carotenoids as cancer preventative agents has been evaluated extensively for a handful of natural carotenoids, with β-carotene being the most widely evaluated. 1,2 Although associations have been made between vegetable consumption (carotenoid-rich vegetables) and a reduction in neoplasia in humans and dogs, there is little evidence that single dietary carotenoids are beneficial.3 Furthermore, carotenoid-rich nutraceuticals are available as over-the-counter products in both the veterinary and human market, and the incidence of nutraceutical use in pets with cancer is approximately 40%; therefore, understanding the functional importance of such carotenoids is extremely important, particularly when other chemotherapeutic agents are used.

The carotenoid lycopene has gained tremendous popularity because of its association with a diminished incidence of prostate cancer in humans.5–7 Lycopene has modest antioxidant potential, but it has more bio-potent activities as a regulator of cell proliferation.7,8 Lycopene molecules are highly hydrophobic, yet they are absorbed intact and can reach the bloodstream with minimal alteration of the compound, in contrast to β-carotene, which is rapidly converted to retinal (also called retinaldehyde or vitamin A aldehyde) by the intestinal epithelium in dogs.9 Two studies10,11 in dogs revealed that single oral administration of lycopene increases serum lycopene concentrations to a maximum of between 50 to 500nM in the bloodstream with a half-life of approximately 30 hours. Further examination of daily dosing at 30 mg/kg for 28 days resulted in serum concentrations of 1µM, and lycopene concentrations in most tissues ranged from 100nM to 5µM/kg of tissue.

The native poliene structure from which all other carotenoids are synthesized in plant material. Lycopene has modest antioxidant potential, but it has more bio-potent activities as a regulator of cell proliferation.7,8
sue, depending on the tissue examined. Although the amount of lycopene uptake by naturally occurring tumor tissue is not known, it is conceivable, on the basis of serum and tissue concentrations, that low micromolar to high nanomolar concentrations of lycopene may be achievable.

One of the most successful in vitro techniques for examining the effects of lycopene has been the use of prostatic and colon carcinoma cell cultures. Lycopene inhibits cellular growth and in some instances can induce apoptosis. Additionally, supplemental lycopene can decrease tumor burden and cellular proliferation in mice with induced prostate cancer and ferrets with induced lung carcinogenesis, which makes lycopene an interesting bioactive molecule that could play a role in preventing or treating neoplastic diseases. The exact mechanism by which lycopene exerts its effects has been debated and may differ, depending on the cell type examined. Traditionally, lycopene is thought to be an antioxidant, but it can also induce upregulation of antioxidant response elements through nuclear signaling. Lycopene has also been associated with alteration of the cell cycle through diminished cyclin D signaling. Lycopene has also been associated with alteration of the cell cycle through diminished cyclin D activity, which causes an increase in G0, and, hence, inhibits cell replication capabilities. In other cellular signaling, lycopene enhanced mitochondrial-based apoptosis through mitochondrial permeability changes and alterations in Bcl-2 and Bax family protein homeostasis at the level of the mitochondrial membrane, as well as AKT-mediated cell survival signaling in colon carcinoma cells.

The potential use of lycopene as an antineoplastic or preventative agent is intriguing; however, prostatic carcinoma is rarely observed in dogs, whereas other neoplastic diseases of dogs are more common and translatable to cell culture systems. One devastating and much more common neoplastic disease in dogs is osteosarcoma, which is associated with a poor post-amputation survival rate; in addition, chemotherapeutic intervention provides limited increases in survival times. To better understand the effects of lycopene on canine osteosarcoma cell growth, the study reported here was conducted with 3 osteosarcoma cell lines, which were treated with lycopene to determine changes in cellular proliferation. In addition, we examined potential mechanisms of cell cycle dynamics and the cell death response. Influences of lycopene on doxorubicin-induced cell death were evaluated to determine whether lycopene would have protective functions during a standard chemotherapeutic cell death response.

Materials and Methods

Sample population—Osteosarcoma cells were obtained from 3 sources, including OS 2.4 cells, HMPOS cells, and the D17 cell line. For cell cultures, RPMI media with 10% FBS plus 1% antimicrobial-antimycotic solution was used for cell proliferation assays. Growth curve assays were performed in RPMI media supplemented with 2% FBS. Lycopene was diluted in THF and stored under nitrogen gas at −80°C with a fresh reconstitution of the 5mM solution biweekly, which was completely soluble when added to aqueous media. Final concentrations of THF were always ≤ 0.5% (vol/vol) because it has been suggested that higher concentrations adversely affect cell growth. Cell lines were maintained in RPMI medium with 10% FBS plus 1% antimicrobial-antimycotic solution at 37°C and 5% CO2 for all experiments and for passage of cells.

Growth curves—Lycopene was solubilized in media at concentrations of 10, 5, 2.5, 1.25, 0.63, and 0.31µM; there were also media containing vehicle (ie, THF) and media alone. Cells were plated at 1,000 cells/well in 96-well tissue culture–treated plates in RPMI with 2% FBS and 1% antimicrobial-antimycotic. The MTT assays were performed every 2 days for a total of 8 days by adding 20 µL of a solution of MTT dye (5 mg/mL) to the incubating cells at each time point and allowing incubation for 1 additional hour. Media were then decanted, and the cells were washed with sterile PBS solution, immediately solubilized in 200 µL of isopropanol, and evaluated by use of a plate reader at a wavelength of 540 nm. Each concentration was assayed in triplicate during 3 separate experiments, and a mean value was calculated for each time point.

Doxorubicin cytotoxicosis—Cells were treated with vehicle (ie, THF) or various concentrations of lycopene on the basis of previous growth curve evaluations for cells cultured in RPMI with 2% FBS plus 1% antimicrobial-antimycotic. The HMPOS cells were treated with similar concentrations of lycopene as in the aforementioned cell growth assays. Cells were then incubated with various concentrations of doxorubicin (67nM for HMPOS cells, 167nM for OS 2.4 cells, and 1.25µM for D17 cells) for 4 hours. Media were then changed to the initial concentrations of lycopene for the remainder of the 48-hour incubation. Cell proliferation was compared with results for lycopene-alone control values throughout the 48-hour treatment period. Cytotoxic exposures of doxorubicin, compared with control values at approximately 40% to 70% of proliferation inhibition, were examined as representative of mild to modest doxorubicin cellular insult, where any effects of lycopene treatment might have enhanced or diminished proliferation rates. All experiments were performed 3 times in triplicate, and a mean value was calculated.

Flow cytometry—All 3 osteosarcoma cell lines were treated with THF or 5µM lycopene for 48 hours. Cells were then trypsinized, washed twice with PBS solution, and fixed with cold ethanol. Fixed cells were stained with propidium iodide, treated with RNase, and subjected to flow cytometry as described elsewhere. Each treatment was performed in triplicate for each cell line. Cell populations were identified by their distinctive position on forward- and side-scatter plots. Each cell line was gated to count 10,000 events via the flow cytometer. Data were analyzed for total counts within the subG0, G1, S, and G2-M phases, and a mean value was calculated for each treatment to provide a final percentage of cells within each phase of the cell cycle, except for subG0, which was assessed in comparison with all cells in the cell cycle.

Cell lysis and western blot analysis—Cells were grown and treated for variable amounts of time with...
vehicle (ie, THF) and 10µM lycopene. At 48 hours of incubation, cells were lysed in accordance with a protocol described elsewhere. Cell lysates were collected, and protein determination was performed on each sample by use of the Bradford technique. Lysates were equilibrated to a common volume (µg/mL basis) in lysis buffer and loading buffer. Western blot analysis was performed with 8%, 12%, or 15% SDS-PAGE by loading 30 µg of protein/well, with transfer to polyvinylidene fluoride membranes and immunoblotting. Because no cell cycle changes could be identified, lysates were immunoblotted for caspase 3, Bid, Bax, Bcl-2, β-actin, ser473 phospho-AKT, AKT, and caspase 8. After membranes were blocked with 10% nonfat dried milk, cells were incubated with a primary antibody overnight at 4°C. All primary antibodies were diluted 1:1,000 in TBST, except for anti–β-actin antibody, which was used at a dilution of 1:10,000. Membranes were washed twice with TBST and then incubated at 37°C for 1 hour by use of appropriate 1:5,000 dilutions of anti-rabbit or anti-mouse antibodies. Blots were again washed 3 times with TBST and examined by use of an imaging station, with densitometry performed when necessary.

Soft agar analysis—Soft agar analysis was performed in accordance with the procedure described in another study. Briefly, cells were plated in soft agar at a concentration of 5,000 cells/well and treated with 5µM lycopene, vehicle control (ie, THF), or control media alone. Agar was refreshed every 3 days for 16 days. On day 16, the number of colonies was counted in triplicate for each treatment, and a mean value was calculated.

Mitochondrial permeability assays—A staining kit was used to evaluate intact versus permeable mitochondria. The kit was used in accordance with the manufacturer’s suggestions on HMPOS cells obtained at 3 time points (24, 36, and 48 hours). Briefly, 10,000 cells were plated on chamber slides and allowed to incubate in growth media overnight. The next morning, cells were treated with THF or 10µM lycopene. After incubation for 24, 36, and 48 hours, 5 µL of stain was added to the cell culture media, and cultures were incubated at 37°C for 20 minutes. The chamber slides were then removed from the incubator and washed 2 times (60 s/wash) by use of the wash buffer provided with the kit. A coverslip was immediately applied to each chamber slide, and slides were examined via a fluorescent microscope. A total of 400 cells was counted on each chamber slide; cells were quantified on the basis of staining characteristics (ie, red or green), and the percentage of green cells was determined at each time point for vehicle-treated control cells and lycopene-treated cells. Each experiment was repeated 3 times, and a mean value was calculated.

DNA staining—To identify end-stage apoptosis through altered nuclear morphology, HMPOS cells treated with lycopene or THF were obtained at 36 and 48 hours of incubation and stained with a DNA stain. Briefly, 10,000 cells were plated on chamber slides and allowed to incubate in growth media overnight. The next morning, cells were treated with THF or 10µM lycopene. After incubation for 36 or 48 hours, cells were removed from the incubator and immediately fixed in neutral-buffered 3.7% formalin for 20 minutes. Cells were permeabilized for 5 minutes by incubation with 0.02% triton in PBS solution. Cells were then washed twice with PBS solution and incubated in 0.1% Hoechst.
33342 staincc for 15 minutes on a platform rocker. The cells were then washed twice (10 min/wash), and a coverslip was applied by use of fluorescent mounting media. A total of 400 cells was counted on each chamber slide; cells were quantified as apparently normal or apoptotic (ie, pyknotic condensed nuclei, nuclear fragments, and blebs). Mean percentages of apoptotic nuclei were calculated on the basis of total cell counts. Each experiment was repeated 3 times, and a mean value was calculated.

**Statistical analysis**—All soft agar results and growth curves at day 8 were evaluated to determine significant reductions in growth kinetics. Evaluations were performed by use of an ANOVA with Tukey post hoc comparisons, with α set at P = 0.05. The tBid-to-total Bid ratios were assessed on the basis of densitometry (in triplicate) and evaluated by use of an ANOVA with Tukey post hoc comparisons, with α set at P = 0.05. Results of cytotoxicity testing that compared lycopene alone with lycopene and doxorubicin were analyzed at each lycopene concentration by use of a nonpaired Student t test, with α set at P = 0.05. All data for DNA staining, flow cytometry, and mitochondrial permeability apoptosis were compared with data for THF (vehicle) control groups by use of a nonpaired Student t test to determine significant differences at each time point.

**Results**

**Growth curves**—After 8 days of incubation, all 3 cell lines had a significant reduction in cell proliferation when treated with lycopene, compared with results for the media-alone and THF-treated cells (Figure 1). Manual cell counts after 6 days of incubation with various concentrations of lycopene revealed results similar to those for MTT assays (data not shown). The D17 cells had significant reductions in viable cell numbers for lycopene concentrations between 2.5 and 10 µM, whereas OS 2.4 cells had significant decreases in viable cell numbers for the full range of lycopene concentrations (ie, 0.31 to 10 µM). The HMPOS cells were the most sensitive because they had significant decreases in viable cell numbers for the full range of lycopene concentrations and also had a loss of cellular mass at both 5 and 10 µM, which suggested a lycopene-induced cell death response.

**Doxorubicin cytotoxicosis**—Doxorubicin inhibited cell proliferation in all 3 cell lines within 48 hours after the onset of incubation with doxorubicin. Optimal inhibitory concentrations were 67nM for HMPOS cells, 167nM for OS 2.4 cells, and 1.25µM for D17 cells. All 3 cell lines, when treated with concentrations of lycopene between 0.33 and 10µM, had diminished cell viability; however, when the effects of lycopene were examined by use of half-maximal inhibitory concentrations of doxorubicin, there were no additive or antagonist effects during doxorubicin treatment, regardless of lycopene concentration (Figure 2).

**Flow cytometry**—Analysis of all 3 cell lines revealed no shifts in cell cycle dynamics during G<sub>1</sub>, S, or G<sub>2</sub>-M attributable to treatment with the vehicle (ie, THF) or 5µM Lycopene (Table 1). All cell lines ranged between 67% and 74% for G<sub>1</sub>, 12% and 19% for S, and 10% and 15% for G<sub>2</sub>-M. Treatment of HMPOS cells with 5µM lycopene induced a significant increase in subG<sub>1</sub> nuclear debris (apoptosis), compared with results for the THF-treated cells.

![Figure 2](image-url)
Western blot analysis—Western blot analysis for markers of apoptosis revealed activation of caspase 3 (ie, the terminal caspase) by 48 hours of incubation (Figure 3). Examination of caspase 8 activation (10-kDa fragment) did not reveal prominent activation of caspase 8, with a modest increase in signal concurrent with caspase 3 activation, which suggested little to no activation of the extrinsic cascade for apoptosis.

Soft agar analysis—All 3 cell lines grew successfully in soft agar and had a significant decrease in colony formation when treated with 5µM lycopene, compared

Table 1—Mean ± SD percentage of cells in various phases of the cell cycle for each of 3 osteosarcoma cell lines incubated with THF (ie, vehicle) or 5µM lycopene for 48 hours.

<table>
<thead>
<tr>
<th>Osteosarcoma cell line</th>
<th>Treatment</th>
<th>SubG&lt;sub&gt;1&lt;/sub&gt;</th>
<th>G&lt;sub&gt;1&lt;/sub&gt;</th>
<th>S</th>
<th>G&lt;sub&gt;2-M&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMPOS</td>
<td>THF</td>
<td>3.4 ± 0.4</td>
<td>72.6 ± 1.5</td>
<td>15.2 ± 0.9</td>
<td>12.2 ± 1.0</td>
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<tr>
<td></td>
<td>Lycopene</td>
<td>5.9 ± 0.5*</td>
<td>72.4 ± 1.9</td>
<td>17.4 ± 1.6</td>
<td>10.2 ± 1.2</td>
</tr>
<tr>
<td>D17</td>
<td>THF</td>
<td>6.7 ± 0.3</td>
<td>67.1 ± 3.8</td>
<td>19.2 ± 1.7</td>
<td>13.7 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Lycopene</td>
<td>6.3 ± 0.6</td>
<td>69.8 ± 1.7</td>
<td>17.8 ± 0.9</td>
<td>12.4 ± 1.4</td>
</tr>
<tr>
<td>OS 2.4</td>
<td>THF</td>
<td>1.7 ± 0.3</td>
<td>73.8 ± 1.5</td>
<td>11.6 ± 3.1</td>
<td>14.6 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Lycopene</td>
<td>2.1 ± 0.6</td>
<td>71.7 ± 1.6</td>
<td>14.8 ± 2.0</td>
<td>13.5 ± 1.9</td>
</tr>
</tbody>
</table>

*Within a cell line, value for this phase differs significantly (P < 0.05) from the value for this phase for the THF treatment.

Figure 3—Apoptosis and cell signal perturbations in HMPOS cells incubated with media alone, THF, or 10µM lycopene for 48 hours. A—Western immunoblots reveal activation of caspase 3 by 48 hours of incubation with 10µM lycopene treatment with diminished ser473 AKT phosphorylation by 36 hours of incubation with 10µM lycopene. The results for β-actin indicate equal loading of wells. B—Photomicrograph revealing pyknotic nuclei with nuclear condensation, blebs, and nuclear fragments (arrows). Hoechst 33342 stain; bar = 10 µm. C—Mean ± SD percentage of apoptotic HMPOS cells after incubation with THF (gray bars) or 10µM lycopene (black bars) for 36 or 48 hours. *Within a time point, value differs significantly (P < 0.05) from the value for the THF treatment. Lyco = Lycopene. Trt = Treatment.
with results when treated with THF (Figure 4). Interestingly, OS 2.4 and HMPOS cells were significantly more sensitive than were D17 cells because lycopene completely abolished any colony formation for those 2 cell lines.

Mitochondrial permeability assays—Western blot analysis for markers of mitochondrial permeability determined by use of Bcl-2, Bax, and Bid immunoblotting revealed that Bax (a proapoptotic protein involved in mitochondrial permeability) expression did not change among treatments, whereas Bcl-2 expression decreased slightly during lycopene treatment (Figure 5). Examination of the ratio of Bcl-2 to Bax by use of densitometry revealed that there were no significant changes in the ratio of Bcl-2 to Bax during lycopene treatment. However, there was a modest increase in tBid expression, with an approximate 1-fold increase in the ratio of tBid to total Bid from the ratio for the control lysates at 36 and 48 hours of incubation. In conjunction with the increase in tBid expression, mitochondrial staining of lysates obtained at 24, 36, and 48 hours of incubation
revealed a prominent increase in permeable mitochondria prior to activation of caspase 3 at 48 hours (Figure 6).

Hoechst 33342 DNA staining—Examination of end-stage apoptosis via staining of condensing nuclei revealed an extremely mild increase at 36 hours of incubation and a prominent increase in nuclear fragments and blebs at 48 hours of incubation. Both of these time points coincided with the timing for activation of caspase 3 (Figure 3).

Discussion

Lycopene has antiproliferative properties in numerous cell cultures, whereas other cell cultures have revealed little or no response to lycopene treatment. There has been considerable attention given to lycopene as a preventative or therapeutic agent for prostate cancer. In contrast to many other carotenoids, lycopene has been found to induce apoptosis in prostate and colon carcinoma cells in vitro. However, to our knowledge, lycopene has not been investigated as an antiproliferative agent in sarcoma cell lines. Analysis of data from the study reported here suggested that lycopene can perturb the proliferation of sarcoma cell lines, specifically osteosarcoma cell lines, by 70% to 100%, depending on the cell line examined at the highest concentration of lycopene used (10 μM). However, regardless of the similar biological nature of osteosarcoma cells clinically, their reactivity to antineoplastic agents in vitro and in soft agar assays can differ dramatically from their in vivo reactivity, and further investigation into the antiproliferative effects of lycopene in vivo would be needed to prove the potential efficacy of such treatment.

Of notable interest is the fact that there was significant retardation of cellular proliferation for the high nanomolar concentrations of lycopene in both the OS 2.4 and HMPOS cell lines and complete ablation of growth in soft agar assays at modest supraphysiologic concentrations of lycopene. This may be relevant because serum concentrations have approached 1 μM in canine plasma after oral administration of 30 to 50 mg of lycopene/d. Lycopene concentrations in tissue
range from 100nM to 3µM, which makes tissue distribution highly variable but promising depending on tumor location. However, without knowing tissue concentrations of lycopene in tumors and having knowledge about cellular distribution, any suggestions of physiologic relevance are merely speculative.

Apoptosis in HMPOS cells was confirmed through western blot analysis for cleaved caspase 3, which is the terminal caspase involved in the apoptotic response, as well as nuclear staining with Hoechst 33342, which allowed observation of condensed, fragmented, pyknotic nuclei. Currently, only prostate and colon cell lines have been found to undergo apoptosis after lycopene treatment. The exact mechanisms for the apoptotic response in cells after lycopene treatment appear variable, including changes in mitochondrial permeability and suppression of AKT phosphorylation. Flow cytometry results from all 3 cell lines revealed no significant alterations in cell cycle dynamics, with all 3 cell lines having relatively consistent percentages for all phases of the cell cycle. This does not completely rule out any changes in activation of cyclin D and E because cell synchronization might have revealed some changes for the respective phases within the cell cycle. However, we did not observe complete loss of any specific phase of the cell cycle, which is similar to the results of another study conducted by our laboratory group by use of similar methods. Additionally, we detected a significant mild increase in the subG1 phase in HMPOS cells in the study reported here, which provided additional evidence of an apoptotic response to lycopene in this cell line.

Interestingly, similar to the results for human colon carcinoma cells, there was a pronounced decrease in phospho-AKT concentrations, particularly at 36 and 48 hours of lycopene treatment, which was not associated with overall AKT protein concentrations in the cells. The AKT signaling is an important part of cell survival, and it has been reported that inhibition of AKT phosphorylation can promote apoptosis through many mechanisms, such as diminishing nuclear signaling by nuclear factor-kB and attenuating other prosurvival proteins.

One prosurvival protein involved in mitochondrial-induced apoptosis is Bcl-2 and other members of that protein family. Although we did not detect a significant decrease in Bcl-2 expression when attempting to quantify the ratio for expression of Bcl-2 to Bax, analysis of the western blots revealed a pattern of slightly less expression of Bcl-2. This may result in a slight imbalance of Bcl-2 to Bax, which is a potential signal for changing mitochondrial permeability. We detected no definitive changes in Bax expression, but Bax expression may be associated with the amount of Bax that transmigrates to the mitochondria during cell stress and apoptosis, which was not investigated in the present study. Further promising evidence for changes in mitochondrial permeability was the activation of Bid, which, when cleaved via caspase 8, 10, 10, allows exposure of the BH3 domain; in turn, this results in binding and activation of Bax and promotes apoptosis. When comparing Bid expression to total Bid expression, it was evident that there was a significant increase in Bid expression from baseline values at the 36- and 48-hour time points, which suggested that mitochondrial permeability may have been inducing apoptosis. Additionally, mitochondrial staining was performed. The cationic fluorophore in the mitochondrial stain we used will oligomerize within mitochondria to yield red fluorescence; however, when permeability changes within mitochondria, the fluorophore is released as a monomer into the cytoplasm, which causes green fluorescence. At the 36- and 48-hour time points in lycopene-treated cells, there was a transition to a greater number of green-fluorescing cells, which suggested a similar timeframe for mitochondrial permeability and cleavage of Bid to tBid.

Lycopene at presumed physiologic concentrations caused a pronounced and potent attenuation of cell proliferation in all 3 cell lines; therefore, the question arises as to how nontraditional nutraceuticals might affect traditional chemotherapeutic interventions. Lycopene is considered a moderate antioxidant and also has the ability to upregulate nuclear antioxidant response elements, which may be part of its protective effect in prevention of cellular transformation. As such, many oncologists do not recommend the use of nutraceuticals because of a lack of knowledge regarding whether a product will hinder or augment a chemotherapy intervention. Common chemotherapeutic agents used for treatment of osteosarcomas are cisplatin, carboplatin, and doxorubicin. Considering doxorubicin has the ability to promote production of free radicals in cells, we decided to determine whether lycopene acted as an agonist or an antagonist to doxorubicin treatment in vitro. Surprisingly, cell proliferation assays revealed no observable augmentation or decrease in the chemotherapeutic response to doxorubicin.

Overall, lycopene treatment at concentrations between 0.33 and 10µM provided a modest to strong antiproliferative signal for osteosarcoma cells; however, the extent of that response was dependent on the osteosarcoma cell line. In fact, the use of lycopene may actually be able to induce apoptosis in canine osteosarcoma cells, depending on the cell line examined. Furthermore, soft agar assays revealed complete ablation of colony formation in 2 of the 3 cell lines at high concentrations of lycopene, which suggested a potential role for prevention of anchorage-independent growth or slowing of neoplastic growth. Lycopene did not appear to affect concurrent treatment of cells with the chemotherapeutic agent doxorubicin, which makes lycopene a potential treatment for concurrent administration during chemotherapeutic intervention. The mechanisms of lycopene-induced apoptosis are partly attributable to changes in mitochondrial permeability and diminished AKT activity; however, the mechanisms involved in causing these changes have yet to be elucidated. The fact that physiologic doses of lycopene may be achievable in dogs, in conjunction with the efficacy of the in vitro study reported here, warrants future investigation into the use of lycopene as a chemopreventative, and possibly even as a chemotherapeutic, agent in the treatment of osteosarcoma in dogs.
References


