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Journal

Veterinary and Comparative Oncology, 15(3)

ISSN

1476-5810

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Publication Date

2017-09-01

DOI

10.1111/vco.12250

Peer reviewed

Published in final edited form as:

Vet Comp Oncol. 2017 September ; 15(3): 1087–1100. doi:10.1111/vco.12250.

Enrofloxacin enhances the effects of chemotherapy in canine osteosarcoma cells with mutant and wild-type p53

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Abstract

Adjuvant chemotherapy improves survival time in dogs receiving adequate local control for appendicular osteosarcoma, but most dogs ultimately succumb to metastatic disease. The fluoroquinolone antibiotic enrofloxacin has been shown to inhibit survival and proliferation of canine osteosarcoma cells *in vitro*. Others have reported that fluoroquinolones may modulate cellular responses to DNA damaging agents and that these effects may be differentially mediated by p53 activity. We therefore determined p53 status and activity in three canine osteosarcoma cell lines and examined the effects of enrofloxacin when used alone or in combination with doxorubicin or carboplatin chemotherapy. Moresco and Abrams canine osteosarcoma cell lines contained mutations in p53, while no mutations were identified in the D17 cells or in a normal canine osteoblast cell line. The addition of enrofloxacin to either doxorubicin or carboplatin resulted in further reductions in osteosarcoma cell viability; this effect was apparent regardless of p53 mutational status or downstream activity.

Keywords

chemotherapy; comparative oncology; oncology; small animal; tumour biology

Introduction

The best clinical outcomes for dogs with osteosarcoma (OSA) are achieved through surgical excision followed by chemotherapy with carboplatin (Carbo) and/or doxorubicin (Dox).[1,2] Although adequate local control for appendicular OSA can usually be attained with amputation or a surgical limb-salvage procedure, the overwhelming majority of dogs still

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succumb to metastatic disease. As such, there is a definite need for novel therapies that can improve the prognosis for these canine patients.

Fluoroquinolones are a class of antibiotics that have demonstrated independent anti-tumour activity *in vitro* against a variety of cancers including colon,[3,4] prostate,[5–7] lung,[8,9] bone,[10,11] leukaemia,[2,12] and bladder,[13] and *in vivo* against three colon cancer cell lines.[4] We have earlier reported that the fluoroquinolone antibiotics enrofloxacin (Enro) and ciprofloxacin (Cipro) have *in vitro* activity against canine OSA cells, causing decreased cell viability, induction of S-G2/M cell cycle arrest and apoptosis.[11] Cipro has been shown to have similar effects in human cancer cell lines.[5,13,14] Furthermore, several other mechanisms have been described for fluoroquinolone family members including regulation of microRNAs, inhibition of Metnase-dependent DNA repair, and disruption of mitochondrial calcium homeostasis (mitochondrial depolarization).[4,9,15]

Multiple studies have reported that the cytotoxic effects of fluoroquinolones may be cancer cell-specific, having little or no cytotoxic effect on a variety of normal cells such as fibroblasts, lymphoid cells and prostate epithelial cells.[4,5,13,14] Another recent study found that Cipro treatment prior to irradiation resulted in enhanced tumour cell death, while at the same time conferring a protective effect on normal peripheral blood mononuclear cells (PBMCs).[16] This differential effect was associated with opposing changes in p53 phosphorylation, suggesting a possible role for the p53 pathway in this protective response.

There is conflicting data, however, regarding the exact role of the p53 pathway in fluoroquinolone-induced apoptosis and cell death. Although one study found that Cipro-induced apoptosis was decreased in B-cell leukaemia cell lines lacking functional p53 protein,[15] a recent study reported no difference in fluoroquinolone-induced apoptosis between p53+/+ and p53-/- human colon cancer cell lines.[7] One possible explanation for this difference is that the influence of the p53 pathway in the response to fluoroquinolones may be tumour specific or cell line dependent. As such, it is unclear whether a functional p53 pathway mediates the response of canine OSA cells, specifically, to fluoroquinolones. Therefore, because approximately 40% of canine OSA patients harbour p53 mutations within their tumour,[17] we felt it critical to determine whether p53 mutations resulting in reduced p53 pathway signalling could mediate the effects of Enro on canine OSA cells when used in combination with Dox or Carbo. We hypothesized that Enro would enhance the effects of chemotherapy in p53 wild-type canine OSA and normal cells but that this effect would be muted in OSA cells harbouring mutated p53.

To test this hypothesis, the p53 mutational status of three canine osteosarcoma cell lines (Abrams, D17 and Moresco) was determined prior to interrogating p53 downstream signalling at the protein level. Alterations in the expression of p53, MDM2, p21, and γH2AX proteins after exposure to Enro or chemotherapy alone and in combination were used to evaluate p53 pathway function and were correlated with each cell line's p53 mutational status. Lastly, we examined the effects of Enro treatment in combination with Dox or Carbo on cell viability in canine OSA cells and normal canine foetal osteoblasts and found significant decreases in viability of all cells, including those cell lines harbouring mutated p53.

Material and methods

Cell lines

Three canine OSA cell lines (Abrams, D17, and Moresco) and cultured canine foetal osteoblasts were used in this study. The Abrams and Moresco cell lines were a gift from Dr. Douglas Thamm and Colorado State University.[11,18,19] The D17 cell line is derived from an osteosarcoma lung metastasis and was purchased from ATCC (Manassas, VA, Cat# CCL-183).[11] Canine OSA cell lines were grown in complete media (CM) which contained Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal Bovine Serum (FBS) and 1× penicillin/streptomycin (all from Thermo Fisher Scientific, Waltham, MA), and incubated at 37 °C in 5% humidified CO_2. Canine foetal osteoblasts (CaOB) were isolated and cultured using a modified protocol described by others.[20] Foetal canine calvaria were obtained from discarded tissue following a pregnant spay performed at the University of California Davis Veterinary Medicine Teaching Hospital. Under sterile conditions, calvaria were removed from four foetal pups, washed in cold PBS, and transferred to a tube containing 6 mL of 1 mg/mL Collagenase/Dispase solution (Sigma-Aldrich, St. Loius, MO) and incubated with shaking for 20 min. After 20 min, the collagenase solution was removed and 6 mL of fresh collagenase solution was added and again incubated for 20 min. This process was repeated seven times; the first and second fractions were discarded, while the subsequent five fractions were filtered through a 70-μM cell strainer, pelleted with centrifugation, plated into T75 flasks with CM, and incubated at 37 °C in 5% humidified CO_2. When flasks reached 90% confluence, cells were harvested with 0.05% Trypsin/EDTA and processed for cryogenic storage using CM with 10% DMSO. Primary CaOB cultures were considered passage 0 and CaOB cells used for all experiments were from passage 1.

RNA extraction and sequencing

RNA was extracted from the CaOB and canine OSA cell lines using the RNeasy Plus kit (Qiagen, Valencia CA). 2 μg of RNA was converted to cDNA using the Thermoscript RT PCR kit (Thermo Fisher) and PCR amplification was carried out in 20 μL reactions using the Taq PCR Core Kit (Qiagen). Reactions were performed using a MJ Mini Personal Thermocycler (BioRad, Hercules, CA) set to 4 min at 95 °C followed by 36 cycles of 1 min at 95 °C (denaturing), 1 min at 60 °C (annealing) and 1 min at 72 °C (elongation). A final elongation period of 10 min at 72 °C completed the amplification. In order to amplify the entire canine *p53* open reading frame (ORF), primers that mapped to the 5′ and 3′ untranslated regions flanking the canine *p53* mRNA ORF were used for initial PCR amplification: AAGTCCAGAGCCACCATCC (sense) and CAGGGAAGGAGGACGAGA (anti-sense). Quality of PCR amplicons consisting of a 1.3 kb band were analysed with agarose gel electrophoresis and quantity was estimated by comparison to a 1 kb + ladder (Thermo Fisher). Unincorporated primers and dNTPs were removed from PCR products using ExoSAP-IT (USB, Cleveland, OH) according to manufacturer's instructions. For sequencing reactions, four different nested primers were used to provide optimal coverage of the *p53* ORF: CTTCCCAGGACGGTGACAC (sense), CGCTGCTCTGACAGTAGTGA (sense), TGTTGGGGGAGGACAGGAA (anti-sense), and TTCAGCTCCAAGGCTTCATT (anti-sense). Sequencing reactions were performed by the UC DNA Sequencing Facility (UC Davis, College of Biological Sciences) using the BigDye Terminator v. 3.1 Cycle

Sequencing Kit and ABI Prism 3730 Genetic Analyzer and Software. Sequences were aligned, analysed, and translated using Sequencher v. 5.1 software (Gene Codes Corp, Ann Arbor, MI).

Drugs

Enro was purchased from Sigma Aldrich and dissolved in 0.1 N HCl for a stock concentration of 20 mg/mL. Dox (2 mg/mL, 3.448 mM) and Carbo (10 mg/mL, 26.94 mM) were purchased through the UC Davis Veterinary Medical Teaching Hospital Pharmacy. Working concentrations for all drugs were achieved with further dilution in complete media.

MTT cell proliferation assays

MTT assays were used to assess proliferation of canine cell lines following treatment with Enro, Dox, or Carbo alone, or in combination. Drug concentrations used were based on published studies,[21,22] and for single treatment groups concentrations used were: Dox (10, 30, 100 and 300nM) and Carbo (10, 30, 100 and 300 uM). For combination treatment groups, drug concentrations used were: Enro (10, 20, 40ug/mL), Dox (3, 10, 30nM) and Carbo (10, 30, 100uM). For all experiments, 500 cells were seeded into 96-well plates and incubated in complete media for 24 h. Drugs were added (alone or in combination) to appropriate wells and incubated for an additional 72 h. Vehicle controls included HCl (Enro), saline (Dox), and water (Carbo). Additional controls included untreated (UT) cells (no veh or drug) and wells containing only complete media to assess background absorbance. Briefly, MTT solution was added to each well at a final concentration of 0.5 mg/mL and incubated at 37 °C for 4 h. 200 μL of DMSO was added to dissolve formazin crystals and absorbance was measured at 570 and 630 nM (reference wavelength) with a Spectramax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA). Four wells per treatment group were used and all experiments were repeated at least twice. Statistical analysis, including IC50 calculations, was performed using GraphPad Prism software (version 6.0 g) and statistical significance was determined using ANOVA with Tukey's multiple comparisons test.

Protein extraction and western blot experiments

For evaluating p53 function, canine cell lines were treated with increasing concentrations of Dox (0, 100, 300, and 1000 nM) for 24 h and protein was extracted as described below. For evaluating changes in protein expression in canine OSA cells and control CaOBs following combination Enro and Dox or Carbo treatment, cells in single treatment groups were treated for 6 or 24 h with Enro (40 μg/mL), Dox (100nM), or Carbo (100 μM) and combination treatment groups received simultaneous treatment of either Enro + Dox or Enro + Carbo. Vehicle control was HCl (2 μL of 0.1 N HCl per mL CM, no measureable change in pH observed).

For protein extraction, experimental media was removed and plated cells were washed three times with ice-cold PBS and then lysed with RIPA buffer. Protein lysates were clarified with centrifugation and total protein was quantified using a BCA Protein Assay. 15–20 μg of protein was then separated using polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Membranes were blocked with 5% milk in PBST and

incubated with primary antibodies overnight (14–18 h) at 4 °C. The primary antibodies used were rabbit anti-p53 (1:1000, Santa Cruz #6243), rabbit anti-p21 (1:2000, Santa Cruz #397), mouse anti-MDM2 (1:1000, Santa Cruz #965), mouse anti-γH2A.X (1:5000, abcam #26350, phospho S139), rabbit anti-histone H2A.X (1:2000, abcam #10475), and mouse anti-actin (1:20,000, Santa Cruz #56459). Membranes were then washed and incubated with an HRP-conjugated anti-rabbit (Thermo Fisher Cat# 31464) or anti-mouse (Thermo Fisher Cat# 31450) secondary antibody for 2 h, washed again, then visualized with Super-Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher #34095). For H2Ax, blots were first probed with the γH2Ax antibody and imaged as described above, then stripped using Restore Western Blot Stripping Buffer (Thermo Fisher #21059) twice for 20 min. each, and then blocked and probed with the histone-H2Ax (H-H2Ax) antibody and visualized as described above. Images were captured and exposure was optimized using Protein Simple FluorChemE (Bio-Techne, San Jose, CA). Figures were arranged using Adobe Photoshop software (version 11.0.2).

Results

p53 sequencing in canine OSA cell lines

The p53 mRNA sequence of the three canine OSA and control CaOB cell lines were determined using Sanger sequencing, which were then translated to protein sequences for alignment (Fig. 1). Both the D17 and CaOB p53 sequences aligned precisely with the published canine p53 ORF sequence from NCBI accession number AB537893.1 and was determined to be wild type. In contrast, both Abrams and Moresco contained mutations. The Abrams p53 sequence showed two missense mutations in base pair 709 (C > C/T, exon 7) and 795 (G > G/T, exon 8) of the canine p53 ORF, resulting in amino acid changes from arginine to tryptophan (R237W) and cysteine to phenylalanine (C265F), respectively. Both a mutated and wild-type base pair showed up on sequencing at each mutation site for Abrams, suggesting that each mutation was present in only one allele (heterozygous). The Moresco p53 sequence showed a single missense mutation at base pair 707 (G > A, Exon 7) of the canine p53 ORF, resulting in an amino acid change from arginine to glutamine (R236Q). Only the mutated base pair showed up on sequencing for Moresco.

Functional status of the p53 pathway in canine OSA cell lines

In order to interrogate p53 activity, canine OSA and CaOB cells were treated with increasing concentrations of Dox for 24 h and levels of p53, p21, and MDM2 were determined using western blot (Fig. 2). To highlight the differences in p53 induction in the canine OSA cell lines, two p53 blots from independent western blot assays using shorter and longer exposure times are shown. Constitutive p53 expression was very high in both Abrams and Moresco (mutated p53), relative to D17 and CaOBs (wild-type p53), and p53 expression increased in all cell lines following Dox treatment. For p21, two bands of approximately 23 and 19 kD were observed, which is consistent with what has been reported previously for canine p21 expression on western blot.[23] Higher levels of both MDM2 and p21 were constitutively expressed in D17 cells, compared to Abrams and Moresco. Following Dox treatment, D17 and CaOB cells increased MDM2 expression, while no change was observed in Abrams or Moresco. Similarly, a marked increased in p21 expression was observed in D17 and CaOB

cells beginning at 100 nM Dox treatment, while notably weaker increases in p21 expression were observed in Abrams, beginning at 100 nM Dox, and Moresco beginning at 300 nM Dox. These results are consistent with the sequencing data and show decreased p53 pathway function in the mutated Abrams and Moresco cell lines, compared to wild-type D17 and CaOBs.

Dox and Carbo treatment decreases canine OSA cell proliferation *in vitro*

When canine OSA cell lines (Abrams, D17, and Moresco) were treated with either Dox or Carbo alone for 72 h, a concentration-dependent decrease in cell viability was observed in all three cell lines as measured by MTT assay (Fig. 3). Abrams showed significantly more resistance to Dox treatment (IC50 = 53.95 nM) compared to D17 (IC50 = 27.85 nM, $P < 0.0001$) or Moresco (IC50 = 18.37 nM, $P < 0.0001$) and although D17 had a higher IC50 for Dox treatment compared to Moresco, this difference was not statistically significant. Conversely, D17 showed significantly more resistance to Carbo treatment (IC50 = 80.47 μM) compared to Abrams (IC50 = 21.41 μM, $P < 0.0001$) or Moresco (IC50 = 22.82 μM, $P < 0.0001$), and no significant difference in response to Carbo treatment was observed between Abrams and Moresco.

Enro treatment alters gH2Ax, p53, and p21 protein expression in canine OSA cell lines

The effects of treatment with Enro (40 μg/mL) alone, or in combination with Dox (100nM) or Carbo (100 μM) on H2Ax and p53 pathway proteins was evaluated in canine OSA cell lines and control CaOB cells using western blot (Fig. 4).

Following 6-h treatment, γH2Ax protein levels increased in the canine OSA cell lines treated with Enro alone and in all cell lines exposed to Dox (Fig. 4). γH2Ax levels further increased or remained the same in all cell lines when Enro was added to the Dox treatment. When treated with Carbo alone for 6 h, a relatively weak induction of γH2Ax was observed in D17, Moresco, and CaOBs, but not Abrams. When treated with the combination of Enro and Carbo for 6 h, an increase in γH2Ax levels was observed only in the D17 cell line, relative to Carbo treatment alone.

At 24 h, similar increases in γH2Ax were observed in all cell lines following Dox treatment as well as Carbo treatment, most notably in CaOBs. The 24-h single Enro treatment increased γH2Ax levels in Abrams, but none of the other cell lines at this time point. Conversely, the addition of Enro reduced γH2Ax levels in Abrams cells treated with Dox for 24 h. In D17, Moresco, and CaOBs, however, the addition of Enro appeared to exacerbate the Dox-induced increase in γH2Ax levels. In the Enro and Carbo combination treatment groups, a dramatic decrease in γH2Ax was observed in Abrams, relative to Carbo alone, while relatively weak decreases were observed in D17 and Moresco, but not CaOBs.

Although the relatively low concentrations of Dox (100nM) and Carbo (100 μM) used in this experiment were not enough to induce detectable increases in p53 expression in D17 or CaOBs, changes in p21 and MDM2 expression following Dox or Carbo treatment were consistent with previous results using Dox (Fig. 4). In Abrams cells treated for 6 h, Enro single treatment appeared to increase p53 expression, but when combined with Carbo, Enro inhibited the Carbo-induced increase in p53. In Moresco cells treated for 24 h, Enro

appeared to decrease p53 expression when treated alone and when combined with Carbo. When combined with Dox at either time point, Enro appeared to increase p53 expression in both Abrams and Moresco. At 24 h, the addition of Enro resulted in decreased p21 expression in Dox or Carbo treated Abrams, D17 and CaOB cells, relative to matched single Dox or Carbo treatment, but had no effect on MDM2 expression in any cell line.

Combination Enro +Dox or Carbo treatment has increased anti-proliferative activity against canine OSA cells *in vitro*, compared to single agent treatment

For combination treatments, the CaOB and canine OSA cell lines were exposed to varying concentrations of Enro alone and in combination with either Dox or Carbo for 72 h and cell viability was measured using an MTT assay. Alone, Enro treatment (10, 20, 40 μg/mL) resulted in a significant concentration-dependent decrease in cell viability in all OSA cell lines, consistent with previous data,[11] and in the CaOB cells (Fig. 5).

When combined with Dox, Enro treatment resulted in additional, significant decreases in cell viability beginning at 3 nM Dox for D17 and CaOB, and 10 nM Dox for Abrams and Moresco (Fig. 5). In Abrams and Moresco, cell viability in the 3 nM Dox and Enro combination groups was not significantly different from concentration-matched single-treatment Enro groups. Furthermore, the addition of Enro to CaOB cells treated with 10 or 30 nM Dox did not significantly alter cell viability compared to Dox treatment alone.

When combined with Carbo, Enro treatment resulted in additional, significant decreases in cell viability beginning at 10 μM Carbo for Moresco and CaOBs, and 30 μM Carbo for Abrams and D17 (Fig. 5). In Abrams and D17, cell viability in the 10 μM Carbo and Enro combination groups was not significantly different from concentration-matched single-treatment Enro groups. Furthermore, the addition of Enro did not significantly alter cell viability in CaOB cells treated with 30 or 100 μM Carbo, or in Moresco cells treated with 100 μM Carbo.

When we compare the effects of drug treatment between cell lines, D17 showed significantly more resistance to Enro treatment (40 μg/mL), compared to that of Abrams ($P < 0.01$) and Moresco ($P < 0.0001$, Fig. 6). The effect of Enro on CaOBs, however, was also not significantly different from any of the OSA cell lines. At the highest concentrations used for the combination treatments, D17 showed significantly more resistance to Enro (40 μg/mL) + Dox (30 nM) compared to that of Abrams ($P < 0.05$), Moresco ($P < 0.001$) and CaOBs ($P < 0.05$), while no significant difference in response was observed between the other cell lines in the same treatment conditions (Fig. 6). In addition, D17 showed significantly more resistance to Enro (40 μg/mL) + Carbo (30 nM) compared to all the other cell lines ($P < 0.0001$), and Abrams showed significantly more resistance compared to Moresco ($P < 0.01$) and CaOBs ($P < 0.0001$) under the same conditions (Fig. 6). No difference in response was observed between Moresco and CaOBs with the same Enro + Carbo treatment concentrations.

Discussion

Fluoroquinolone anti-cancer properties are due, in part, to their ability to trigger SG_2/M cell cycle arrest and apoptosis.[11,13,14] The p53 pathway has been suggested to play a role in mediating response to fluoroquinolones due to an observed correlation between apoptosis and decreased p21 expression.[7,14,16] However, the dependence of fluoroquinolones on the presence of wild-type p53 to elicit cell death has not been definitively elucidated.[7,14,16] In addition, several studies have reported that the cytotoxic effects of fluoroquinolones may be cancer specific, having little or no cytotoxic effect on a variety of non-cancerous cells.[4,5,14] Furthermore, fluoroquinolones can protect PBMCs from cytotoxic effects of ionizing radiation.[16] Therefore, we set out to further evaluate the effect p53 function has on the ability of Enro to treat canine OSA cell lines alone, or when given in combination with standard chemotherapy Dox or Carbo. Although two cell lines, Abrams and Moresco, were identified as having p53 mutations and dramatically decreased downstream p53 signalling, this did not correlate with a resistance to Enro-mediated cytotoxicity when used alone or in combination with Dox or Carbo. Furthermore, Enro was unable to protect non-cancerous CaOB cells from Dox- or Carbo-induced cytotoxicity.

Sequencing data revealed that both the CaOB and D17 p53 sequences aligned precisely with the canine p53 ORF originally published by Chu *et al.*,[24] which also matches NCBI accession number AB537893.1. Similar to Chu *et al.*,[24] our observed wild-type p53 sequence differed at the carboxyl and amino terminal ends of the sequence published by Veldhoen and Milner,[25] which corresponds to NCBI accession number NM_001003210.1. We agree with Chu *et al.* in that these discrepancies likely result from Veldhoen and Milner's use of mismatched primers that overlapped the ends of the p53 ORF sequencing, resulting in mispriming events during PCR amplification. Therefore we recommend that future studies evaluating canine p53 sequences refer to AB537893.1 for wild-type template and primer design, rather than NM_001003210.1.

CaOBs and D17 showed a wild-type p53 response, characterized by increases in p21 and MDM2 expression on western blot in response to treatment with DNA damaging chemotherapy. Abrams and Moresco showed a notably weaker response, observed only as slight increases in p21 expression and no increase in MDM2. This correlates with the sequencing data that revealed a wild-type p53 sequence from D17 and CaOBs, and mutations in both Abrams and Moresco cell lines. The wild-type p53 pathway response in D17 is consistent with what has been reported previously in this cell line.[23] Because both of the p53 missense mutations identified in Abrams were heterozygous, the partial p53 response observed in Abrams may result from having one allele that is able to produce a wild-type p53 protein. As such, a potentially dominant negative mutated p53 protein produced by the other allele may reduce the function of the wild-type p53 protein,[26] resulting in the weak induction of p21 observed here. Notably, one of the p53 mutations observed here in Abrams, C265F, matches what has been reported previously in this cell line.[27] The Moresco p53 sequence histogram, however, showed only a mutated base pair. This suggests that it was present on both alleles, or that only a single mutated p53 allele is present in the Moresco p53 genome (loss of heterozygosity). Of note, the mutation observed in Moresco exactly matches one of the p53 mutations observed in the Gracie canine OSA

cell line reported by others.[27] The weak induction of p21 in Moresco cells treated with the highest concentrations of Dox used may suggest some level of p53 protein function. Alternatively, the p21 expression observed in the Moresco and Abrams may be p53-independent, resulting from activation of related proteins such as p65, which has been shown to induce p21 expression in a p53 null setting.[28] Additional studies would be needed to further evaluate the extent of p53 function in these p53 mutated cell lines and determine the influence of p65 and related proteins in that setting.

A previous study reported delayed onset of Cipro-induced apoptosis in lymphoblast cells that are p53 null (NH32), as compared to their p53 wild-type counterpart (TK6).[14] We also saw a trend for a weaker induction of γH2Ax following 6 h of Enro treatment in the Abrams and Moresco cell lines with p53 mutations compared with D17. This trend was not observed, however, in the CaOB cell line with wild-type p53, suggesting that the correlation between p53 status and Enro-mediated changes in γH2Ax may be specific to neoplastic cell types. In D17 cells, an additional increase in γH2Ax induction was observed when Enro was added to Carbo treatment for 6 h. Similarly, although this phenomenon was not observed in the OSA cell lines with p53 mutations, it was also absent from the CaOBs with wild-type p53, suggesting a p53-independent mechanism or an effect unique to D17, possibly related to its relative resistance to Carbo treatment. Furthermore, at 24 h, there was no consistent pattern for γH2Ax induction or inhibition in cell lines with and without p53 mutations, which is consistent with a more recent study that found no significant difference in Cipro-induced induction of apoptosis in p53 wild type and p53 null HCT-116 colon cancer cells after 24-h treatment.[7]

Our data add to the pool of literature suggesting fluoroquinolones may improve the cytotoxic effects of standard anti-cancer treatments such as chemotherapy and radiation therapy. Specifically, studies have shown that Cipro combined with cisplatin or 5-FU was more effective at decreasing proliferation of A549 lung cancer cells and SW48 colon cancer cells, respectively, compared either drug alone,[9] and Cipro-sensitized TK6 cells to the cytotoxic effects of ionizing radiation.[16] In addition, although limited to three canine OSA cell lines, our data also suggest that the anti-proliferative effects of Enro and combination chemotherapy are not dependent on a wild-type p53 pathway response. In fact, the p53 mutants (Abrams and Moresco) were significantly more susceptible to combination treatments compared to their p53 wild-type counterpart (D17). While this may suggest that p53 mutations in OSA can produce a more favourable response to chemotherapy, the small number of samples in this study precludes such conclusions.

The observation that Enro had significant anti-proliferative effects on CaOB cells was not unexpected given that Enro has been shown to decrease proliferation of canine chondrocytes *in vitro*[29] and Cipro damages cartilage in young, growing dogs.[30] However, this observed cytotoxic effect on normal osteoblasts is contrary to several studies reporting little or no cytotoxic effects of fluoroquinolone treatment of other non-cancerous cell types including PBMCs, fibroblasts, lymphoid cells and prostate epithelial cells.[4,5,14] Furthermore, Enro was unable to protect CaOBs from the cytotoxic effects of chemotherapy, as has been reported with Cipro protecting human PBMCs from the cytotoxic effects of ionizing radiation.[16] These discrepancies may be explained by differences in the specific fluoroquinolone used,

the species from which the cells were derived, or the cell types themselves. In support of the latter points, a recent study comparing the anti-proliferative effects of Cipro found that, at matched concentrations, Cipro was more effective at eradicating A549 (human) lung cancer cells compared to A375.S2 (human) or B16 (mouse) melanoma cells, and had no effect on C6 (rat) glioma cells.[8] It is possible that cells of osteoid lineage are uniquely susceptible to the anti-proliferative effects of fluoroquinolones compared to other non-cancerous cell types, and additional studies comparing the effects of fluoroquinolones on osteoid and other normal cell types might shed light on this phenomenon. Together these results suggest that Enro's anti-proliferative effects are not cancer-specific, and highlight the different effects fluoroquinolones may have on different tissue types.

In conclusion, we show that p53 mutation status does not inhibit Enro's anti-proliferative effect on canine OSA cells or its ability to enhance the cytotoxic effects of chemotherapy. In contrast to what has been reported for other non-cancerous cell types treated with other fluoroquinolones, canine foetal osteoblast cells are susceptible to the anti-proliferative effects of Enro and are not protected from the cytotoxic effects of Dox or Carbo chemotherapy. Although these results may be specific to canine cells or possibly Enro itself, the purpose of this study was to evaluate the potential effects of a routinely used antibiotic in dogs. While these data suggest that Enro may be beneficial to canine OSA patients currently undergoing chemotherapy, these benefits may be limited with respect to protecting normal tissues from cytotoxic damage and could potentially even exacerbate unwanted systemic toxicity. It should also be noted that, while Enro is known to concentrate in pulmonary tissues and within leukocytes and sites of inflammation,[31–34] concentrations of Enro that might effectively reach OSA cells *in vivo* are unknown. Lastly, it should be stressed that the judicious use of antimicrobials precludes simply prescribing an antibiotic to OSA patients undergoing chemotherapy as this could negatively impact the patient's microbiome and could contribute to the epidemic of antimicrobial resistance. As such, additional, *in vitro and in vivo* studies are warranted prior to evaluating a potential role for Enro in treating OSA in clinical patients.

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A

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D17             361 KGQSTSRHKKLMFKREGPDSD 381
Moresco         361 KGQSTSRHKKLMFKREGPDSD 381
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B

	Exon (nucleotide[a])		
Sample	Exon 7 (+707)	Exon 7 (+709)	Exon 8 (+795)
Wild Type[b]	G	C	G
Abrams		C/T	G/T
Moresco	A		
Amino Acid Change	R236Q	R237W	C265F

Figure 1.

p53 protein sequence alignment of canine OSA cell lines. (A) p53 protein sequence of canine OSA cell lines Abrams, D17, and Moresco aligned to published wild-type p53 protein sequences corresponding to NCBI accession numbers AB537893.1 and NP_001003210.1. (B) Table showing exon and nucleotide positions of *p53* missense mutations observed in Abrams and Moresco along with resulting nucleotide and amino acid changes.[a] nucleotides are numbered from the ATG translation initiation codon, A = +1.[b] wild-type sequence corresponds to NCBI accession number AB537893.1

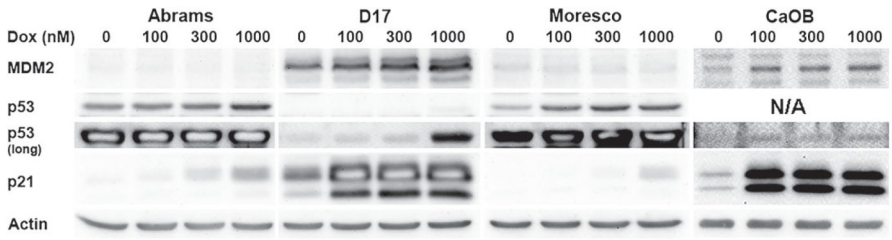

Figure 2.

p53 pathway activation in canine OSA cell lines. MDM2, p53, and p21 expression in canine OSA cell lines and CaOBs following 24 h of exposure to increasing concentrations of Dox. For canine OSA cell lines, two blots of p53 are shown from two independent assays in which longer incubation and exposure times were used in the second run. For CaOBs, weak p53 induction could only be observed in one blot with a very long exposure time.

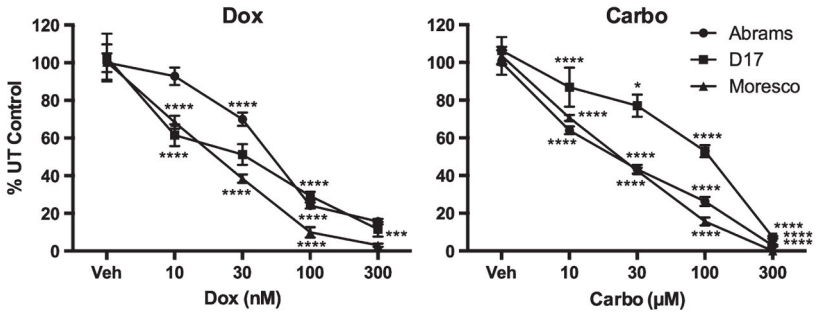

Figure 3.

Concentration dependent decrease in proliferation of OSA cell lines following Dox and Carbo treatment. Absorbance data from a representative MTT assay is shown as a percent of untreated (UT) control cells. Error bars reflect SD and statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test. *P*-values reflect significance as compared to preceding drug concentration for each cell line and drug. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

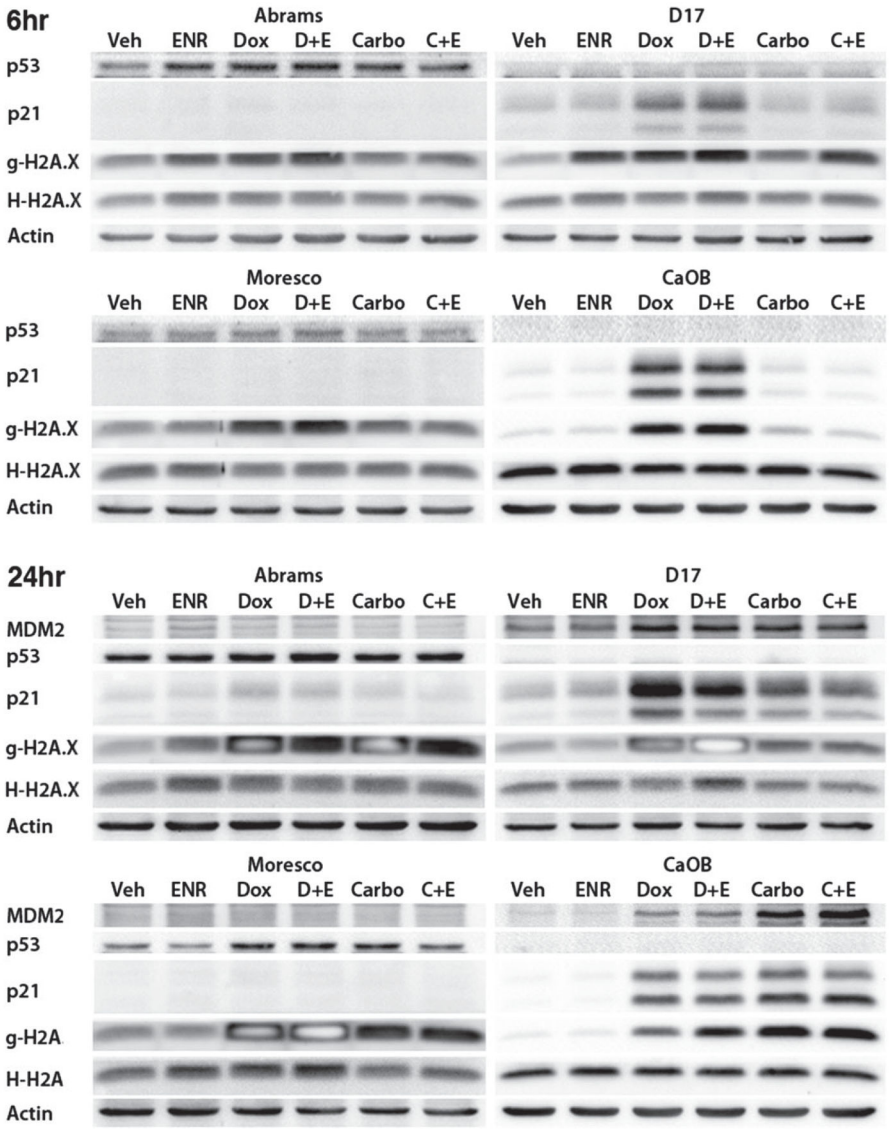

Figure 4.

p53 pathway and H2Ax protein expression in canine OSA cell lines following combination Enro and chemo treatment. Abrams, D17, Moresco, and CaOBs were treated with Enro (40 μg/mL), Dox (100nM), or Carbo (100 μM) alone and in combination. Protein was extracted at 6 and 24 h after initiation of treatment and analysed by western blot.

D + E = Dox and Enro combination treatment, C + E = Carbo and Enro combination treatment.

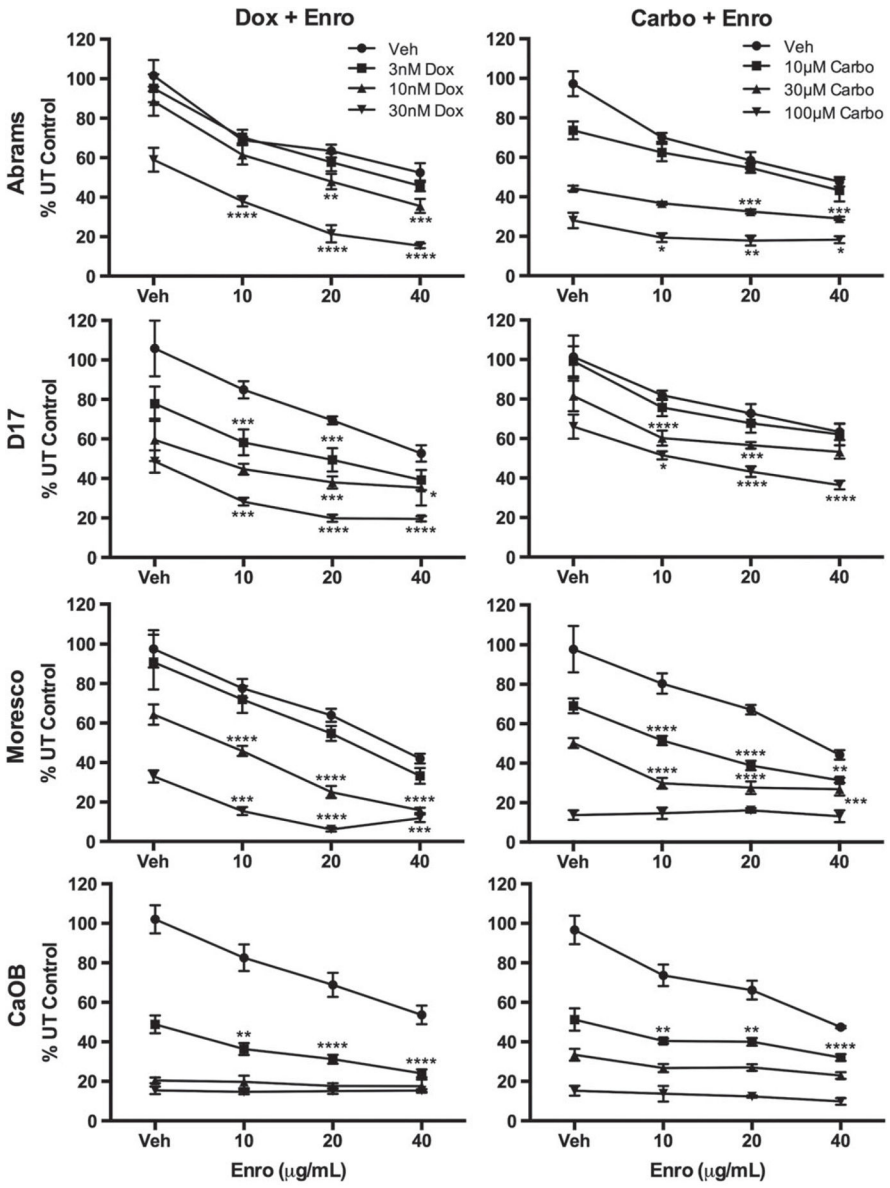

Figure 5.

Decreased cell proliferation in canine OSA and control normal CaOB cells following combination Enro and chemo treatment. Abrams, D17, Moresco and normal CaOBs were treated with increasing concentrations of Enro, Dox, or Carbo alone and in combination for 72 h. Cell proliferation was measured with an MTT assay and absorbance data is shown as a percentage of untreated (UT) control cells. Error bars reflect SD and statistical analysis was performed using two-way ANOVA with Tukey's multiple comparisons test. *P*-values are displayed for combination treatment groups and reflect the lower significance value when compared to both concentration matched single treatment groups (Enro and corresponding Dox or Carbo). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

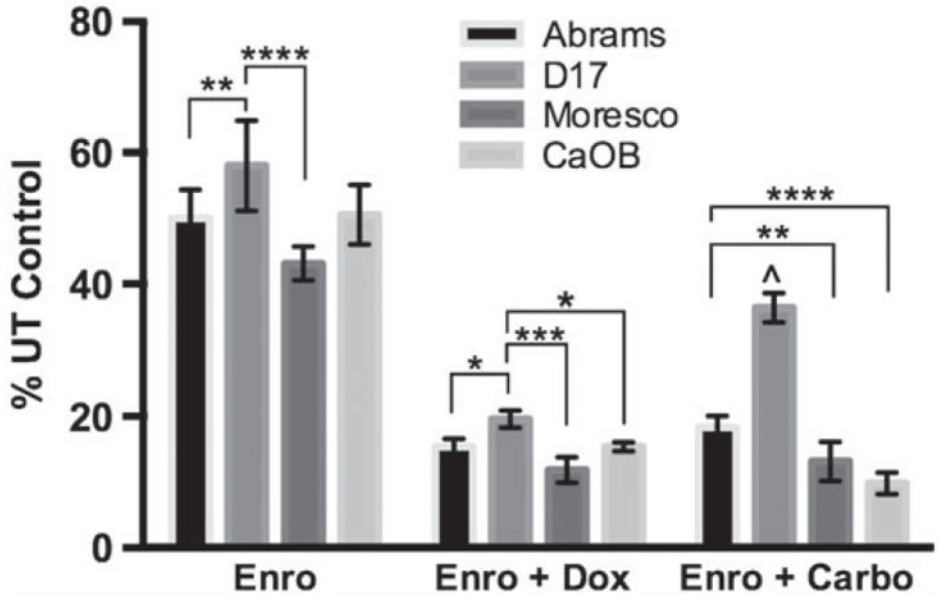

Figure 6.

Comparison of high concentration Enro and Dox or Carbo combination treatment effects on cell proliferation in canine OSA cell lines and CaOBs. MTT assay data from the highest concentrations of drugs used (same as represented in Figure 5) were compared between cell lines. Measurements, error bars, and statistical analysis were done similarly as in Figure 5. Concentrations of drugs were: Enro (40 μg/mL), Dox (30 nM) and Carbo (100 μM). *$P <$ 0.05, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$