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The radiosensitizing effect of the aurora kinase inhibitors, ENMD-2076, on canine mast cell tumours *in vitro*

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Abstract

ENMD-2076 is an aurora kinase inhibitor that also has multi-target tyrosine kinase inhibitor properties. In this study, the mRNA and the protein expression of aurora-A and aurora-B were evaluated in three canine mast cell tumour cell lines. Dose-dependent cytotoxicity was seen in the cells treated, and it affected the cell cycle with cells in the G2/M phase being selectively killed. The cells were also evaluated for radiosensitivity with/without ENMD-2076, and radiosensitization was seen after 3 Gy and 6 Gy exposures with ENMD-2076 for 48 h. Protein expression of caspase-3 was gradually increased, and the expression intensity was highest at 24 h post irradiation in cells without ENMD-2076 treatment, which indicates that radiation exposure with ENMD-2076-induced cell death faster than radiation treatment alone. Our study results suggest the potential usefulness of treating canine mast cell tumours with aurora kinase inhibitors alone or in conjunction with radiation therapy.

Keywords
aurora kinase inhibitors, canine mast cell tumours, ENMD-2076, radiosensitivity

Introduction

Canine mast cell tumours are the most common cutaneous malignant tumours in dogs.[1] While histological grades, I, II and III provide useful information regarding a patient's prognosis,[2,3] inter-observer variations have been reported,[4] as well as the observation that grade II tumours, which represent the majority of canine mast cell tumours, have varied outcomes. Other factors such as mitotic index, Ki67, nucleolar organizing regions (AgNOR), and/or c-KIT expression also have been reported to correlate with prognosis.[1,5–7] Treatment options depend on staging, but in general surgery and radiation therapy are very effective.[1,8–10] It has been reported that for even one loco-regional lymph node metastasis with an incompletely excised mast cell tumour in dogs, definitive radiation therapy for both primary surgically excised and metastatic lymph node sites still can provide long-term disease control and a disease-free survival time of 1240 days.[10] Chemotherapy is recommended if the patient has either systemic disease, a grade II mast cell tumour with negative histological prognostic factors, or a grade III mast cell tumour.[11–13] However, no clear evidence of prolonging survival time has been reported yet. The most commonly used chemotherapy drugs for mast cell tumours are CCNU and vinblastine.[1,13,14] Targeted therapy also has been attempted for canine mast cell tumours because this tumour expresses c-kit and contains a mutated constitutively activated form.[15,16] The c-kit receptor is a one of a family of tyrosine kinase receptors that regulate cell signal transduction, cell survival, cell proliferation, and differentiation.[17] Tyrosine kinase inhibitors have been reported to be radiosensitizers in humans. Some of the most well-known radiosensitizing agents are epidermal growth factor receptor (EGFR) inhibitors.[18] It has been reported that the presence of EGFR inhibitors enhances DNA damage, which could lead to lethal damages.[19] Since tyrosine kinase inhibitors are starting to be

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used intensively in veterinary medicine,[16,20,21] it is very important to investigate their potential use as radiosensitizing agents. Another justification of investigating radiosensitizing agents would be that, although radiation therapy alone for gross mast cell tumours in dogs has a favourable response duration (reported as 12 months),[21] using a radiosensitizing agent for non-surgical canine mast cell tumours might result in a longer tumour control and survival time over radiation therapy alone.

Aurora kinases are serine/threonine kinases that regulate the cell cycle and mitotic spindle assembly by centrosome duplication and separation.[22] There are three types of aurora kinases: aurora-A, aurora-B and aurora-C. Aurora-A and aurora-B have been investigated in human medicine. Aurora-A and aurora-B are expressed in most normal cells, but the localization and the timing of the activation of aurora-A and aurora-B are different during the cell cycle within normal cells. Aurora-A is ubiquitously expressed and regulates cell cycle events occurring from late S-phase through the M-phase.[23] Aurora-B localizes to the centromere during the early stages of mitosis, and then after anaphase begins, Aurora-B relocates gradually to the midzone and persists throughout cytokinesis.[22] Overexpression of the protein and mRNA of both aurora-A and aurora-B has been documented in multiple human tumours.[22] We recently confirmed both the protein and mRNA expression of aurora-A and aurora-B in normal canine endothelial cells and canine malignant lymphoid cell lines in our laboratory.[24] We have reported that canine aurora-A has a 90% homology to human aurora-A, and canine aurora-B has a 92% homology to human aurora-B by performing sequence alignment.[24] This result indicates that these genes are highly conserved between humans and canine species. ENMD-2076 is a novel, orally bioavailable, aurora kinase inhibitor that also targets various tyrosine kinases such as Flt-3 (FMS-like tyrosine kinase-3), Src (Sarcoma), VEGFR-2 (Vascular endothelial growth factor receptor-2) and FGR1 (Fibroblast growth factor receptor-1).[25]

In this study, we evaluate the protein and mRNA expression of aurora-A and aurora-B in the canine mast cell tumours cell lines (CoMS, CM-MC1 and VI-MC1). We also investigate the cell survival, the drug's effect on the cell cycle, and the enhancement of radiosensitivity with aurora kinase inhibitors. Our hypothesis is that ENMD-2076 enhances radiosensitivity and leads to cell death through apoptosis for canine mast cell tumour cells *in vitro*.

Materials and methods

Cell culture

Three malignant canine mast cell tumour cell lines, CoMS, CM-MC1 and VI-MC1, were used in this study, and all three cell lines have been previously characterized.[26–28] CoMS originated from a mucosal mast cell tumour in a dog.[26] CM-MC1 and VI-MC1 originated from visceral mast cell tumours in dogs.[27,28] All three canine mast cell tumours were kindly donated by Dr Nakagawa from Tokyo University, Tokyo (Japan) and Dr Kadosawa from Rakuno Gakuen University, Hokkaido (Japan). All three cell lines were maintained in RPMI-1640 [American Type Cell Culture Collection (ATCC), Manassas, VA, USA) supplemented with 10% foetal bovine serum (FBS, ATCC) and 2 mM L-glutamine (ATCC). All cells were maintained in 5% CO_2 at 37 °C in a humidified incubator. The same incubation conditions were used for the MTT and cell cycle assays.

Drug

ENMD-2076 (aurora kinase and multi-targeted tyrosine kinase inhibitor, Selleck chemicals, Houston, TX, USA) was purchased as a powder. ENMD-2076 was dissolved in dimethyl sulfoxide (DMSO) as a 10 mM stock solution and stored at −20 °C.

RNA preparation

RNA was extracted from malignant canine mast cell tumour cells (CoMS, CM-MC1, VI-MC1) using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The cells were counted manually with a haemocytometer, and 5×10^6 cells were used. Approximately 500 ng μL^{-1} of RNA was collected and the concentration of the RNA was measured by NanoDrop® ND-1000 (Wilmington, DE, USA). The total RNA was treated with TURBO DNA-free™ to remove

contaminating residual genomic DNA. For cDNA synthesis, the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA, USA) was used following the manufacturer's protocol. Briefly, 10× reverse transcription (RT) buffer, 25× dNTP mix, 10× RT random primers, MultiScribe reverse transcriptase, Nuclease-free H_2O, and 20 μL of total RNA were mixed gently in a total volume of 40 μL in a PCR tube, and then incubated in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems) under the following conditions: 25 °C for 10 min, 37 °C for 2 h followed by enzyme inactivation at 85 °C for 5 min.

Reverse transcription-polymerase chain reaction (RT-PCR)

Canine-specific aurora-A and aurora-B cDNA sequences were determined by our group previously.[24] The cDNA gene sequence of canine-specific aurora-A (GeneBank Accession: KC127668), canine-specific aurora-B (GeneBank Accession: KC137547), and canine-specific GAPDH (GeneBank Accession: NM_001003142.1) were used to generate primers using the Primer 3 program (http://frodo.wi.mit.edu/primer3/). The primers of aurora-A, aurora-B and GAPDH are as follows; aurora-A (forward/reverse sequences/amplicon size; CAGCCATAAACCGGC TCAGA/TCTCCTGGAGGATGGAGCAT/661 bp), aurora-B (CCTCATGGAGCCGCTCCAAT/CCTC CATGATCGTGGCTGTT/ 428 bp) and GAPDH (T CCATCTTCCAGGAGCGAGA/ATACATTGGGG GTGGGGACA/499 bp). The PCR conditions were 95 °C for the initial denaturation, followed by 30 cycles of 45 s at 95 °C (denaturing), 45 s at 57 °C (annealing) and 60 s at 72 °C (extension), then maintained at 4 °C. The PCR was performed with 0.5 μL of cDNA, 2 μL each of forward and reverse primers, 20.5 μL of nuclease-free H_2O and 25 μL of PCR master mix (Qiagen) in a total volume of 50 μL. The PCR was performed with the same thermocycler that was used for the cDNA synthesis. The amplified PCR products were separated by electrophoresis on 2% agarose gels with 0.01% ethidium bromide, and then analysed by FluorChem® Q (Alpha Innotech, Santa Clara, CA, USA) under UV light.

Irradiation

The irradiation source in these experiments was a 6 MV x-ray beam produced by a linear accelerator (Clinac 600C, Varian Medical Systems, Palo Alto, CA, USA) at a dose rate of 2.5 Gy min^{-1} at the Cancer Treatment Unit of the Department of Veterinary Clinical Sciences, Louisiana State University. Beam dosimetry is part of routine maintenance. Cell culture flasks were irradiated at a 100 cm source-to-surface distance with a 1.5 cm of tissue equivalent bolus material to obtain maximum dose at the level of bottom of the flasks. The flasks were irradiated with a single dose of either 3 Gy or 6 Gy with a 6 MV x-ray beam at 180° of gantry of the linac. Doses at the bottom of flasks were determined using standard formulas from dose output calibrated at a 100 cm source-to-surface protocol following the American Association of Physicists in Medicine (AAPM) TG-51 protocol.

Western blot

Protein expression of aurora-A and aurora-B in canine mast cell tumours cell lines

To assess the protein expression of aurora-A and aurora-B in canine mast cell tumours, 2×10^7 CoMS, CM-MC1 and VI-MC1 cells were harvested from T-75 flasks, washed twice with phosphate-buffered saline (PBS, ATCC), and lysed with RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) according to the manufacturer's protocol. Briefly, the harvested cells were lysed with the RIPA buffer containing 10 μL of phenylmethylsulfonyl fluoride (PMSF), 10 μL of sodium orthovandate and 10 μL of protease inhibitor cocktail per milliter of buffer for 30 min on ice. The lysed cells were then disrupted with a 21-gauge needle, incubated another 30 min, and then centrifuged at 10 000 × *g* for 10 min at 4 °C. Protein concentrations were determined by the BCA protein assay according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL, USA). Western blot analysis was performed with 16% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The samples were mixed with a gel-loading buffer, heated at 95 °C for 5 min, and then 20 μg of protein was loaded into each lane of the SDS-PAGE gel. The SDS-PAGE gel was transferred to nitrocellulose

membranes by electroblotting. Five percent non-fat dry milk in PBS with 0.2% TWEEN-20 was used to block the blots. The blots were probed with anti-rabbit Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2000 dilution; monoclonal mouse antibody, Abcam, Cambridge, MA, USA), anti-human aurora-A (1:1000 dilution; polyclonal goat antibody, Tocris Bioscience, Ellisville, MO, USA) and anti-human aurora-B (1:1000 dilution; polyclonal rabbit antibody, Abcam) antibodies at room temperature for 1 h. The membranes were then incubated with the appropriate horseradish peroxidase conjugated secondary antibody (1:2000 dilution; Santa Cruz Biotechnology) for 1 h. The proteins were visualized using Amersham ECL plus substrate (GE Healthcare, Buckinghamshire, UK).

To detect caspase-3 expression for evaluation of apoptosis, canine mast cell tumour cell lines were irradiated with/without ENMD-2076, harvested and lysed as described above, but harvested at different time points (control, 0.5, 6 and 24 h). Briefly, CoMS and VI-MC1 cells were seeded into T-75 flasks, and then 5 mL of this culture, containing 1×10^7 cells, was allocated to 10 T-25 flasks. One flask served as a control (no radiation or drug), and was treated with 0.01% DMSO. The remaining flasks were exposed to 3 Gy of radiation using the 6 MV linac. Three had no drug, three were treated with the IC_{10} of ENMD-2076 and three were treated with the IC_{50} of ENMD-2076. The cells were harvested after 0.5, 6 and 24 h of incubation. The protein concentrations were determined as before, blots were made, and the blots were probed with anti-human caspase-3 (1:1000 dilution; polyclonal rabbit antibody, Cell Signaling Technology, Danvers, MA, USA), incubated at 4 °C overnight with shaking. The blots were visualized in the same manner as described above.

MTT assay

The cytotoxicity of ENMD-2076 was evaluated by MTT assay (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's protocol. Briefly, 5×10^4 cells of CoMS, CM-MC1 and VI-MC1 were counted manually with a haemocytometer, and seeded in 96 well plates. The plated cells were incubated with various concentrations of ENMD-2076 for 48 h. The ENMD-2076 concentrations ranged from 0 to up to 5 μM (0 nM, 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 1 μM, 2.5 μM and 5 μM). Ten microliters of MTT labelling reagent was applied to each well, incubated for 4 h, and then 100 μL of solubilization solution were added. The plates were incubated at least 18 h or until complete solubilization of purple formazan crystal, and then the absorbance at 570 nm was measured on a microplate reader. Cell viability was calculated as a percentage of the absorbance of the treated cells relative to the absorbance of the control cells (cell viability % = absorbance of treated cells/absorbance of control). Non-ENMD-2076 treated cells were incubated only with vehicle (0.01% DMSO), and served as controls. Cell survival fraction curves were generated, and the IC_{10} and IC_{50} were calculated for CoMS, CM-MC1 and VI-MC1 using Graph Pad Prism version 5 (Graph Pad Prism Software, La Jolla, CA, USA). The samples were run in duplicate and the experiments were repeated at least three times.

In addition, to evaluate the concurrent effect of ENMD-2076 and radiation exposure, MTT assays were performed using the same protocol described above. Only CoMS and VI-MC1 cell lines were used for this experiment because the effect of ENMD-2076 on the cell cycle was not obvious in CM-MC1 cells, so this cell line was not used. CoMS and VI-MC1 were grown in T-75 flasks, and then treated with no-drug (treated only with 0.01% DMSO), IC_{10}, or IC_{50} of ENMD-2076 with 0 Gy, 3 Gy or 6 Gy of radiation using 6 MV x-rays. After treatment, 5×10^4 cells were plated in 96 well plates, and were incubated for 48 h. Again, the survival fraction (SF) was calculated. The samples were run in duplicate and the experiments were repeated at least three times.

Cell cycle analysis

To evaluate the effect of ENMD-2076 on the cell cycle phases in canine mast cell tumours, cell cycle analysis was performed. Briefly, 2×10^5 CoMS, CM-MC1 and VI-MC1 cells were cultured in 48-well plates. The IC_{10} and IC_{50} of ENMD-2076 was added to each well and incubated for 48 h. The treated cells were washed twice with cold-PBS and then resuspended in 100 μL of cold-PBS.

Ice-cold methanol was added slowly and mixed well, then incubated at 4 °C for 30 min. The cells were then washed with cold-PBS and resuspended in 450 μL of PBS containing 25 μL Lf RNAse A (Sigma-Aldrich, St. Louis, MO, USA) and 25 μL of propidium iodide and incubated for 30 min at room temperature in the dark. The stained cells were gated and counted by flow cytometry (BD Biosciences, San Jose, CA, USA). The data was analysed using Verity Modfit LT software (Verity Software House, Topsham, ME, USA) and reported as a percentage of each cell-cycle phase, which are G1, S and G2/M. Untreated cells served as controls, and were treated with 0.01% DMSO. The samples were kept in the dark, and analysed within 3 h. The samples were run in duplicate, and the experiments were repeated at least three times.

Statistical and data analysis

For the MTT and cell cycle assay, the data were reported as the mean and standard deviation (SD). The Shapiro–Wilk test was used to analyse the normal distribution of the MTT assay, cell cycle assay and the MTT assay SF after radiation and ENMD-2076 treatments. To compare outcome in group, analysis of variance (ANOVA) was used, and then post hoc comparisons were conducted with Tukey's test for pairwise comparisons of significant overall effects. A P value < 0.05 was considered significant. The collected cell SFs and their propagated standard errors (1σ) were plotted versus dose (D) and fit to the Linear Quadratic (LQ) hypothesis:

$$\mathrm{SF} = \exp\left(-\alpha D - \beta D^2\right) \qquad (1)$$

where α and β are fitting parameters related to the dual radiation action theory. For cross-comparison purpose, the sensitization enhancement ratio was utilized at the 50% cell survival level (SER_{50}). The SER_{50} is defined as the ratio of doses required to reach 50% cell inactivation in each of the mast cell tumour cell lines without ENMD-2076 treatment to those treated with the IC_{10} and IC_{50} of ENMD-2076. The SER_{50} values were determined for CoMS and VI-MC1 cells. All data was analysed using either Graph Pad Prism version 5 (Graph Pad Prism Software) or SPSS version 18 (IBM, Armonk, NY, USA). Graph Pad Prism was used to calculate the IC_{10} and IC_{50} for each cell line.

Results

Expression of aurora kinase in malignant canine mast cells

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to detect the expression of aurora-A and aurora-B in canine mast cell tumours. The expression of the mRNA of aurora-A and aurora-B was detected in all canine mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1; Fig. 1). The intensity of the GAPDH bands was the same throughout all three cell lines and served as a positive control. Both aurora-A and aurora-B were identified and sequenced from normal canine aortic endothelial cells (CnAOECs) in a previous study[24] and they also served as a positive control (Data not shown here). RNA extracted from each canine mast cell tumour cell line was used as a template in negative control reactions. No bands were detected from the negative control (Data not shown here).

Protein expression of aurora kinases

To evaluate the protein expression of aurora-A and aurora-B kinase in canine malignant mast cell tumours, western blot analysis was performed on protein extracts from CoMS, CM-MC1 and VI-MC1 cells. The protein expression of aurora-A and aurora-B kinase was detected in all canine mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1; Fig. 2). Cross reactivity between aurora-A and aurora-B antibodies was seen in all three cell lines. The molecular weights of aurora-A and aurora-B are 48 and 39KD, respectively. GAPDH served as a loading control, and the intensity of the bands of GAPDH in all three mast cell tumour cell lines are identical. The protein expression of aurora-A and aurora-B kinase from normal canine aortic endothelial cells (CnAOECs) was identified in a previous study,[24] and it also served as a positive control in the same manner as the RT-PCR.

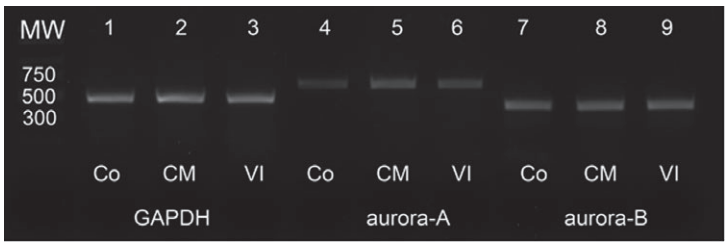

Figure 1. Results of RT-PCR of GAPDH, aurora-A and aurora-B from canine malignant mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1). GAPDH served as the positive control. Amplicons in lanes 1, 2, 3 are GAPDH, amplicons in lanes 4, 5, 6 are aurora-A, and amplicons in lanes 7, 8, 9 are aurora-B. Bands of the size of aurora-A and aurora-B were clearly detected from all canine mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1). MW, Co, CM and VI represent molecular weight, CoMS, CM-MC1 and VI-MC1, respectively. The size of amplicons for each target gene is identical to the size predicted from the primers.

Cell viability with ENMD-2076 in canine malignant mast cell tumour cells (CoMS, CM-MC1, and VI-MC1)

The cytotoxic effect of ENMD-2076 was evaluated by MTT assay after 48 h of incubation with a variety of concentrations of ENMD-2076 in the canine malignant mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1; Fig. 3). The IC_{10} (10% decrease of cell viability) and IC_{50} (50% decrease of cell viability) of ENMD-2076 were calculated. The IC_{10} and IC_{50} for CoMS are 117 and 360 nM, respectively. The IC_{10} and IC_{50} for CM-MC1 are 17.5 and 404 nM, respectively. The IC_{10} and IC_{50} for VI-MC1 are 291 and 539 nM, respectively. The cytotoxic effect of ENMD-2076 was observed as dose-dependent. These three canine mast cell tumours cell lines are susceptible to ENMD-2076 even though some variation of response was seen among those cell lines.

Cell cycle analysis and DNA content change with ENMD-2076 in canine malignant mast cell tumour cells (CoMS, CM-MC1 and VI-MC1)

We evaluated the effect of ENMD-2076 on the cell cycle phases of mast cell tumours cells because aurora kinases are involved with the regulation of the cell cycle. The cell cycle analysis was evaluated

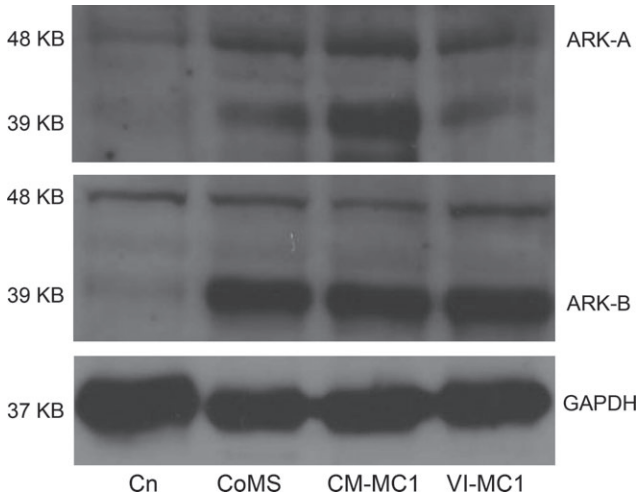

Figure 2. The protein expression of aurora-A and aurora-B kinase in canine mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1). Canine aortic endothelial cells served as a positive control. The aurora-A and aurora-B kinases were detected in all three canine malignant mast cell tumour cell lines. There is cross reactivity between the aurora-A and aurora-B antibodies. The protein size of aurora-A, aurora-B and GAPDH are approximately 48, 39 and 37 KD, respectively. GAPDH was used as a loading control, and the intensity of bands of GAPDH in all three mast cell tumour cell lines are identical. Cn, ARK-A and ARK-B represent canine aortic endothelial cells, aurora-A kinase and aurora-B kinase, respectively.

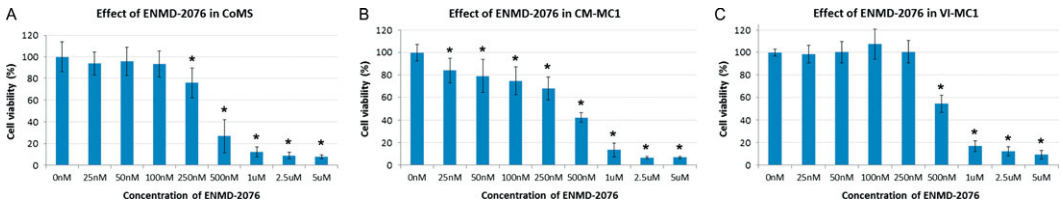

Figure 3. Cytotoxic effect and cell viability of the canine malignant mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1). CoMS, CM-MC1, VI-MC1 were cultured with various concentrations of ENMD-2076 for 48 h. The IC_{10} and IC_{50} for CoMS are 117 and 360 nM, respectively. The IC_{10} and IC_{50} for CM-MC1 are 17.5 nM and 404 nM, respectively. The IC_{10} and IC_{50} for VI-MC1 are 291 nM and 539 nM, respectively. The cytotoxic effect of ENMD-2076 was observed to be dose dependent. The three canine mast cell tumour cell lines are susceptible to ENMD-2076 even though some variation of the responses was seen among the cell lines. The mean ± SD cell viability was measured by MTT assay. The absorbance was measured at 570 nm and the cell viability was calculated as the mean absorbance of the treated cells divided by the mean absorbance of the control. The samples were run in duplicate and the experiments were repeated three times. *Values are significantly different ($p < 0.05$) for each control.

by flow cytometry after 48 h of incubation with the IC_{10} and IC_{50} of ENMD-2076 in canine malignant mast cell tumour cell lines (CoMS, CM-MC1 and VI-MC1). The percentage of G1, S and G2/M phases was represented as the mean ± SD, then the bar graphs were generated (Figs. 4A–C). The most affected cell cycle phases were the S and G2/M phases in CoMS, and VI-MC1 cells, and a significant effect was seen after 48 h of incubation with the IC_{50} ($P < 0.05$). The CoMS and VI-MC1 cells in the G2/M phase incubated with the IC_{50} for 48 h were selectively killed. The DNA content of the CoMS, CM-MC1 and VI-MC1 cells were present in the diploid (2 N), and tetraploid (4 N) state, and a histogram of cell cycle phases was generated (Fig. 4D). A dramatic decrease of the DNA content of the 4 N cells is obvious in the G2/M phase when incubated with the IC_{50} for 48 h in CoMS and VI-MC1 cells. The comparison was performed between treated and non-treated cells within each phase in each cell line.

Enhancement of radiosensitivity with ENMD-2076 in CoMS and VI-MC1

We evaluated the potential radiosensitizing effect of ENMD-2076 on canine mast cell tumours. CoMS and VI-MC1 were selected because the effect on the cell cycle distribution in CM-MC1 cells is less than with the CoMS and VI-MC1 cells. The cells were irradiated using a 6 MV linac at the dose rate of 2.5 Gy min^{-1} with 3 Gy and 6 Gy with/without the IC_{10} or the IC_{50} of ENMD-2076, and then incubated for 48 h in 96-well plates. Figs 5A,B show the radiation response of CoMS and VI-MC1 cells with and without treatment using ENMD-2076 at levels IC_{10} and IC_{50}. The LQ curve fits have root mean square (RMS) and chi-squared values of zero in all cases, except for IC_{50}. For CoMS with IC_{50} the RMS and chi-squared values are 1.7E-8 and 5.6E-10, respectively, which indicate a very good fit. For VI-MC1 with IC_{50} RMS = 3.9E-2 and $\chi^2 = 1.52$. Sensitization enhancement values from these fits at 50% survival for CoMS cells are 1.4 and 2.3 with IC_{10} and IC_{50}, respectively. In the case of VI-MC1 cells, SER_{50} (IC_{10}) = 2.1 and SER_{50} (IC_{50}) = 2.8. These results indicate significant radiosensitizing effects.

Induced apoptosis with ENMD-2076 in CoMS and VI-MC1

Caspase-3 expression

To evaluate caspase-3 expression and the mechanism of cell death induced by ENMD-2076, western blot analysis was performed in the same manner described above, but harvested at different time points (control, 0.5, 6 and 24 h; Fig. 6). Minimum detection of caspase-3 was seen at 0.5 h. However, the intensity of the caspase-3 bands gradually increased, and maximum intensity of expression was observed at the final 24-h time point following the 3 Gy exposure in the presence of ENMD-2076. The maximum expression of caspase-3 in cells that were not treated with the drug was also observed at the 24-h time point. Moreover, in the cells that received the maximum dose of ENMD-2076 (IC_{50}),

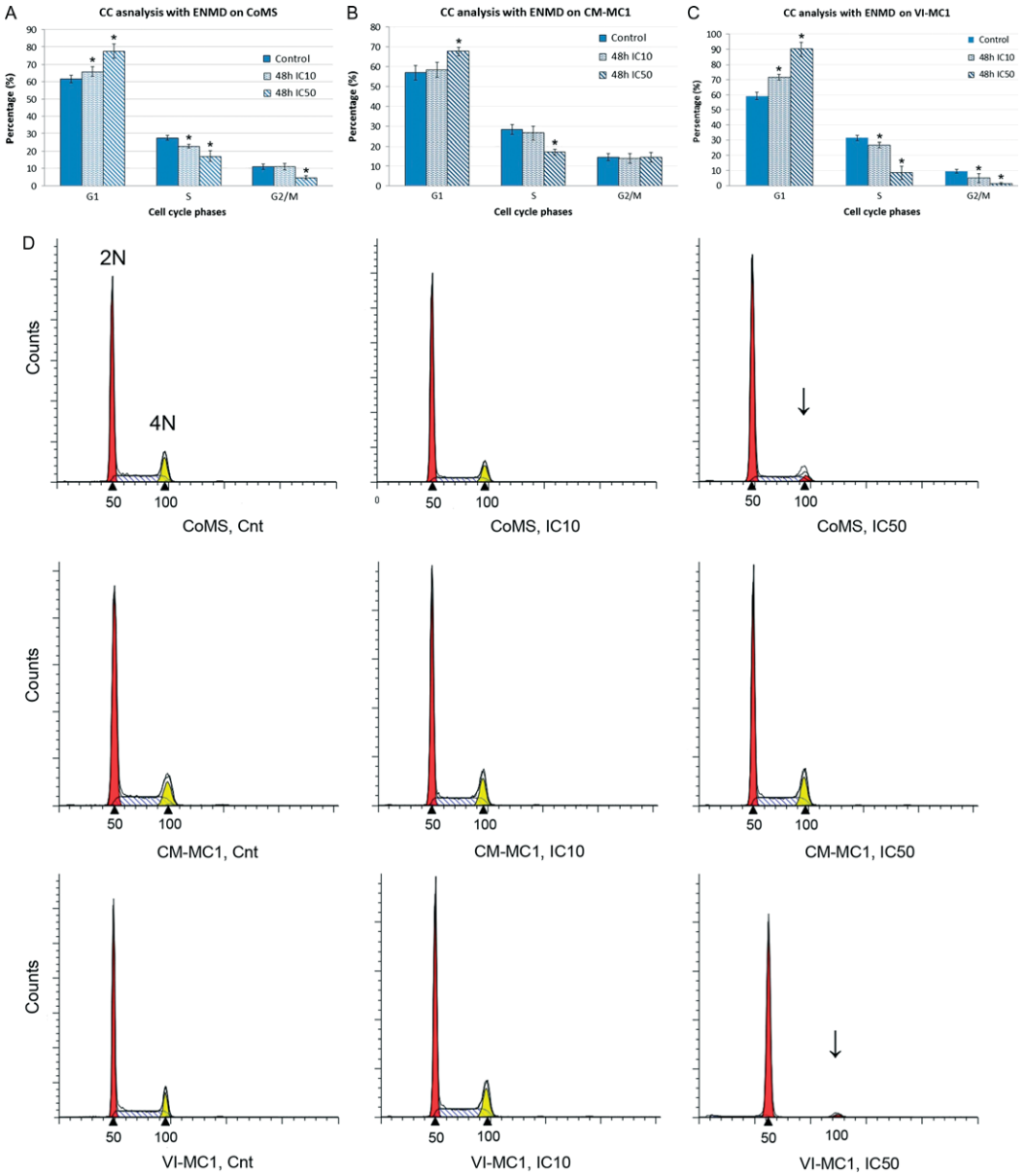

Figure 4. The cell cycle effect of ENMD-2076 in CoMS, CM-MC1, and VI-MC1 cells. The cell cycle analysis was evaluated by flow cytometry after 48 hours of incubation with the IC_{10} and IC_{50} of ENMD-2076. The percentage of G1, S, and G2/M phases were calculated as the mean ± standard deviation (SD), and then bar graphs were generated. The bar graphs show the percentage of cells in each cell cycle phase within each cell line (A, B and C). An obvious decrease was seen in the S and G2/M phases in CoMS and VI-MC1 cells incubated with the IC_{50} of ENMD-2076. The histogram of the cell cycle shows the change of DNA content in CoMS, CM-MC1, and VI-MC1 cells. The arrows point out the dramatic distribution change of the diploid (2N), and tetraploid (4N) cells (D). The comparison was done between the control and either the IC_{10} or IC_{50} of each group. The samples were run in duplicate and the experiments were repeated at least three times. *Values are significantly different ($P < 0.05$) for each control. CC and Cnt are abbreviations for the cell cycle and control.

there was lower expression of caspase-3 after 24 h of incubation than with the IC_{10} of ENMD-2076. In other words, the expression of caspase-3 in the cells treated with the IC_{50} of ENMD-2076 peaked before 24 h. All taken together, the results indicate that apoptosis was induced earlier than 24 h with 3 Gy radiation exposure in the presence of the IC_{50} of ENMD-2076.

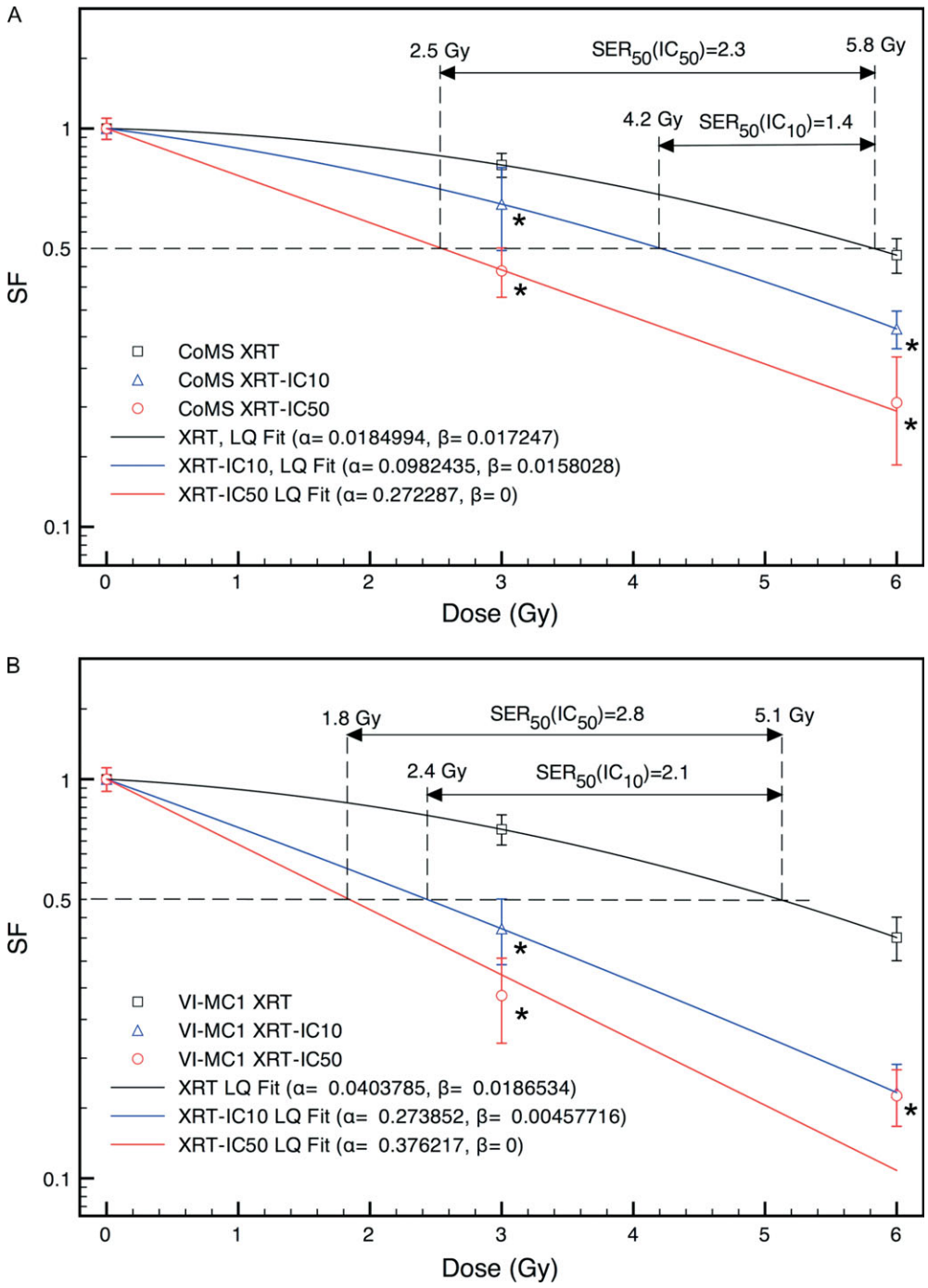

Figure 5. Cell survival curves for CoMS and VI-MC1 cells after irradiation by 6 MV x-rays. Error bars are $\pm 1\sigma$ uncertainty. Treatment with ENMD-2076 enhances the radiation sensitivity of the canine malignant mast cell tumour cell lines CoMS (A) and VI-MC1 (5B). CoMS and VI-MC1 were irradiated with 3 Gy and 6 Gy in the presence of either the IC_{10} or IC_{50} of ENMD-2076, and then incubated for 48 h. The survival fraction (SF) was fitted with linear-quadratic curves on the survival data for CoMS and VI-MC1. The samples were run in duplicate and the experiments were repeated at least three times. *Values are significantly different ($P < 0.05$) for each control. Goodness of fit: CoMS: root mean square (RMS) and χ^2 values are zero except for IC_{50} where RMS = 1.7E-8 and $\chi^2 = 5.6E\text{-}10$. VI-MC1: RMS and χ^2 values are zero except for IC_{50} where RMS = 3.9E-2 and $\chi^2 = 1.52$.

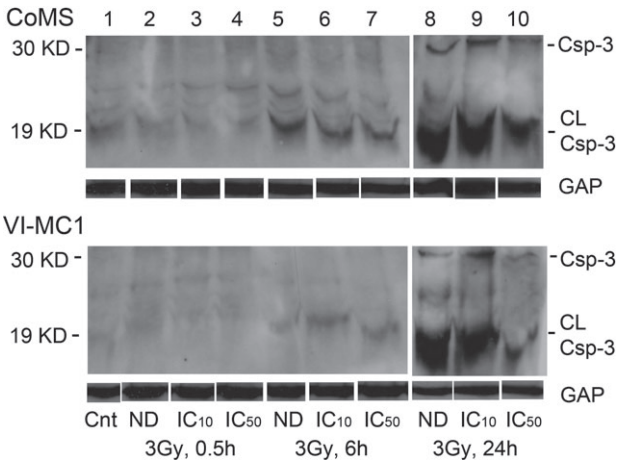

Figure 6. The caspase-3 expression at 24 h after 3 Gy exposure with concurrent treatment either with/without the IC10 or IC50 of ENMD-2076. The caspase-3 activity was evaluated at different time points (control, 0.5, 6 and 24 h). GAPDH was used as a loading control, and the intensity of bands of GAPDH in the CoMS and VI-MC1 cells are identical. The protein size of cleaved caspase-3, caspase-3, and GAPDH are approximately 19, 30 and 37 KD, respectively. GAP, Csp-3, CL-Csp-3, Cnt and ND indicate GAPDH, Caspase-3, cleaved caspase-3, Control, and Non-drug, respectively.

Discussion

One of the main functions of aurora kinases is mitotic spindle formation and cell separation.[22] Due to the fact that aurora kinases are involved with cell cycle regulation, targeting the aurora kinases in cancer therapy would be a promising approach. Scant information exists regarding aurora kinases in veterinary medicine,[24,29] but our group has shown the existence of mRNA and protein expression of aurora kinases in both normal canine endothelial cells and malignant canine lymphoid cells.[24] Our group has also shown that the aurora-A and aurora-B genes are highly conserved between humans and dogs. We have started to evaluate the mRNA and protein expression of aurora-A and aurora-B in canine malignant mast cell tumours. The expression of mRNA (Fig. 1) and protein expression (Fig. 2) of aurora-A and aurora-B were clearly detected by RT-PCR and western blots. In humans, over-expression of aurora-A and/or aurora-B has been suggested as prognostic markers in some types of malignant tumours.[30–33] Quantification of the mRNA between malignant tumours, and investigating its relationship to the treatment response and/or prognosis would be a useful next step.

We also evaluated the cytotoxic effect of ENMD-2076 in canine mast cell tumours. All three cell lines used in this study are susceptible to ENMD-2076 (Fig. 3). More importantly, the IC_{50} for CoMS, CM-MC1 and VI-MC1 ranges from 360 to 539 nM; which is much lower than in a previous study using malignant canine lymphoid cells.[24] This indicates that ENMD-2076 is effective on canine mast cell tumours. The common side effects of ENMD-2076 in humans have been reported in phase I clinical trials as hypertension, nausea, vomiting, fatigue and/or diarrhoea. The severity of these side effects appears to be dose-dependent,[34] therefore, using less of the drug may avoid these unfavourable side effects. The response curves with ENMD-2076 in canine malignant mast cell tumours are very similar to those reported in human studies using small molecules, which have very narrow therapeutic windows and steep dose-response curves.[35,36] Clinically, it might be a challenge to find the optimum dose of small molecule drugs because patients exhibit a variation of tumour sensitivity to the drug relative to normal tissues. Toceranib phosphate (Palladia, SU11654) has been approved for the treatment of canine mast cell tumours, and it is beginning to be widely used to treat a variety of tumours in dogs.[16,20,21] The current recommended dose of toceranib phosphate is $3.25\,mg\,kg^{-1}$, every other day (three times/week), and with this dosage, optimum plasma concentrations of $40\,ng\,mL^{-1}$ were attained.[37] However, in actual clinical situations, we still see some unacceptable

side effects even with the recommended dosage, and it is not rare to be forced use a lower dose of toceranib phosphate in veterinary medicine, which may also be a less effective dose.

The histograms of cell cycle analysis allow us to evaluate any change of the distribution of cell cycle phases by ENMD-2076 (Fig. 4D). The majority of aurora kinases induce polyploidy (increase to 4 N and/or 8 N) and apoptosis.[38,39] Wang *et al.*[40] showed the change of cell cycle phase distribution and increased polyploidy with ENMD-2076 using human multiple myeloma cells. However, in this study, we did not observe the same response in two canine mast cell tumour cell lines (CoMS and VI-MC1). In the cell cycle analysis, the G2/M phases were selectively depleted with ENMD-2076 after a 48-h incubation, which indicates that the cells in the G2/M phase of two canine malignant mast cell tumour cell lines were selectively killed by ENMD-2076, and/or the cells did not move into G2/M. It is unknown why no accumulations of 4 N or 8 N cells were seen with the canine mast cell tumour cells as is seen with other aurora kinases. Most of the Wang study was conducted using human cell lines, and therefore different results may be observed in the canine cells and with a different tumour type. ENMD-2076 is a multi-target aurora kinase inhibitor, which also can inhibit the activity of tyrosine kinases and/or their receptors. The multiple target mechanisms might explain why the ENMD-2076 did not cause polyploidy. It also did not appear that there was a sub G1 accumulation of cells even with evidence of undergoing apoptosis. It is known that sub G1 accumulation would be seen if apoptosis is present due to condensation of chromatin.[41] However, G1 accumulation could be obscured or invisible due to various reasons, such as DNA loss due to the washing process, the generation of bleb, debris, and necrotic cells.[41,42] Because there is the potential failure to detect a discreet peak of chromatin condensation, assessing apoptosis using G1 sub accumulation by flow cytometry analysis would not always be ideal. Because apoptosis pathways are extremely complicated processes, it has been suggested that multiple assays would best assess apoptosis pathways.

Using small molecules as radiation-sensitizers is currently getting a lot of attention in veterinary medicine. EGFR inhibitors are some of the best known of the small molecules, and have been investigated as potential radiosensitizers for head and neck tumours in human medicine. Many phase II clinical trials of chemoradiation treatment in the presence of cetuximab have been conducted, and most of the results are encouraging moving to a higher stage of clinical trial due to the improvement of clinical responses and tumour control duration even with an increased incidence of acceptable acute toxicity.[43–45] Multi-institutional clinical trials of canine nasal adenocarcinoma using radiation therapy and Palladia (tyrosine kinase inhibitors) are being conducted by the Veterinary Radiation Therapy Oncology Group(VRTOG). We evaluated the effect of ENMD-2076 with concurrent radiation exposure to 6 MV therapy x-rays and observed a radiosensitizing effect in CoMS, and VI-MC1 cells. The LQ fits of the IC_{50} data in both CoMS and VI-MC1 cells show the lack of a shoulder region in the survival curves (Figs 5A,B), indicating that at this level the effect of ENMD-2076 aurora kinase inhibitor is significant. In the case of VI-MC1, the LQ model shows a nearly pure exponential decrease even at the IC_{10} level, which suggests that this cell line is more sensitive to radiation at this level of ENMD-2076 compared to CoMS. In this study, the evaluation of the shoulder region was hindered by the low number of dose points. In the case of VI-MC1, the SF of IC_{50} at 6 Gy exhibits a pseudo-radiation hardening, which is due to overkill, and it is not well reproduced by the LQ curve. However, this is not the case with the CoMS line, which also shows a pure exponential decrease with the IC_{50} without a shoulder. Because of the general similarities between the two sets of survival curves and because of the evidently higher sensitization in VI-MC1, it is likely that the current LQ fit correctly predicts the lack of a shoulder in the VI-MC1 IC_{50}. To further evaluate the degree of sensitization, the sensitivity enhancement ratio was evaluated at the 50% SF (SER_{50}) using the fitted LQ models. The 50% endpoint was selected because the assessment at this degree of cell inactivation did not require extrapolation. Significant radiosensitization was seen with the IC_{10} and the IC_{50} in both CoMS and VI-MC1 cells (Figs 5A,B). In CoMS cells treated with the IC_{10}, the

$SER_{50} = 1.4$, whereas when treated with the IC_{50}, the $SER_{50} = 2.3$. A much greater radiosensitizing effect was seen in the VI-MC1 cells, where the SER_{50} $(IC_{10}) = 2.1$, while the SER_{50} $(IC_{50}) = 2.8$. Our results are consistent with various sensitization enhancement experiments conducted with mammalian cells. For example, using the chemical agent iododeoxyuridine (IUdR) in conjunction with 4 MV clinical beams has been shown to yield SER_{10} values ranging from 1.5 to 2.6 depending on the tissue IUdR concentration and on the uptake by the DNA.[46] Our data show a comparatively higher sensitization because in our case it is the SER_{50} that has similar values. Because of the divergent nature of the survival curves, the SER is higher at lower SFs. The IC_{10} of ENMD-2076 can decrease cell survival 30% without a concurrent 3 Gy radiation treatment in VI-MC1 cells, which suggests that an even lower dose of small molecules might be able to cause a similar response in a clinical setting. When using toceranib phosphate as a radiation sensitizer in veterinary medicine, there exists the possibility of life threatening side effects (such as gastrointestinal bleeding and/or perforation). The ability to lower the dose to acquire a raidosensitizing effect allows the clinician to more easily use the drug without worrying about causing life-threatening side effects. Using a lower dose of toceranib phosphate may result in T-regulatory cell suppression, immunomodulatory effects, and anigiogenesis inhibition, which could lead to a superior tumour control rate and/or longer survival time.[47–49]

Caspase-3 expression was also evaluated to investigate the mechanism and timing of cell death with ENMD-2076 treatment concurrent with radiation exposure. Caspase-3 expression was highest after 24 h of incubation without ENMD-2076 after a 3 Gy exposure (Fig. 6). Owing to the radiosensitizing effects of ENMD-2076 treatment with radiation exposure seen in CoMS and VI-MC1 cells, the results indicate that the cell death was induced before 24 h. The expression of caspase-3 in both CoMS and VI-MC1 cells at control and after 0.5 h is low. The intensity of expression gets stronger at 6 h and reaches a maximum at 24 h. The stronger expression of caspase-3 at 6 hours in VI-MC1 cells in the presence of ENMD-2076 rather than at 6 h with radiation treatment alone indicates that ENMD-2076 accelerates cell death through apoptosis earlier than with radiation alone.

There are some limitations to this study. We have not investigated the actual mechanism of radiosensitivity with ENMD-2076. It would be useful to know how the ENMD-2076 enhances the radiosensitivity of canine mast cell tumours. Because we do not know the importance of the expression of aurora-A and aurora-B regarding the tumour activity, treatment response, and prognosis, it would be ideal to quantitate the mRNA expression level with quantitative RT-PCR. This is an *in vitro* study; the results of this study might not be seen in an *in vivo* study. To better assess the shoulder region of the survival curve, multiple radiation exposures at low doses will be required. However, when comparing the two cell lines, we feel that our general conclusion with respect to the lack of pronounced shoulder at the IC_{50} is likely accurate. No other definitive conclusions pertaining to the shoulder of the survival curve can be drawn. In this current experiment, the more sensitive cell line was a visceral mast cell tumour, which is likely a more aggressive variant and generally not treatable with radiation therapy given the location. Also two of the three cell lines were of visceral origin and the third was of mucosal origin. These may behave differently than cutaneous mast cell tumours.

In conclusion, we have detected mRNA and protein expression of aurora-A and aurora-B in canine mast cell tumour cells lines. We also observed a dose-dependent cytotoxic effect by ENMD-2076. The cell cycle distribution and DNA content changes were seen particularly in CoMS and VI-MC1 cells. The ENMD-2076 caused a radiosensitizing effect in the tested cells yielding sensitization enhancement ratios at 50% survival ranging from 1.4 to 2.8, with VI-MC1 cells being much more sensitive than CoMS. These results suggest the general potential for treating canine mast cell tumours with aurora kinase inhibitors, and specifically warrant further *in vitro* and *in vivo* studies using ENMD-2076 treatment in conjunction with radiotherapy.

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References

1. Thamm DH and Vail DM. Mast cell tumors. In: *Withrow & MacEwen's Small Animal Clinical Oncology*. 4 edn., Withrow SJ and Vail DM Eds., Edinburgh, Elsevier Saunders, 2007: 402–424.
2. Patnaik AK, Ehler WJ and MacEwen EG. Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. *Veterinary Pathology* 1984; **21**: 469–474.
3. Preziosi R, Sarli G and Paltrinieri M. Multivariate survival analysis of histological parameters and clinical presentation in canine cutaneous mast cell tumours. *Veterinary Research Communications* 2007; **31**: 287–296.
4. Northrup NC, Harmon BG, Gieger TL, Brown CA, Carmichael KP, Garcia A, Latimer KS, Munday JS, Rakich PM, Richey LJ, Stedman NL, Cheng AL and Howerth EW. Variation among pathologists in histologic grading of canine cutaneous mast cell tumors. *Journal of Veterinary Diagnostic Investigation* 2005; **17**: 245–248.
5. Maglennon GA, Murphy S, Adams V, Miller J, Smith K, Blunden A and Scase TJ. Association of Ki67 index with prognosis for intermediate-grade canine cutaneous mast cell tumours. *Veterinary and Comparative Oncology* 2008; **6**: 268–274.
6. Thompson JJ, Yager JA, Best SJ, Pearl DL, Coomber BL, Torres RN, Kiupel M and Foster RA. Canine subcutaneous mast cell tumors: cellular proliferation and KIT expression as prognostic indices. *Veterinary Pathology* 2011; **48**: 169–181.
7. Webster JD, Yuzbasiyan-Gurkan V, Miller RA, Kaneene JB and Kiupel M. Cellular proliferation in canine cutaneous mast cell tumors: association with c-KIT and its role in prognostication. *Veterinary Pathology* 2007; **44**: 298–308.
8. Fulcher RP, Ludwig LL, Bergman PJ, Newman SJ, Simpson AM and Patnaik AK. Evaluation of a two-centimeter lateral surgical margin for excision of grade I and grade II cutaneous mast cell tumors in dogs. *Journal of the American Veterinary Medical Association* 2006; **228**: 210–215.
9. Schultheiss PC, Gardiner DW, Rao S, Olea-Popelka F and Tuohy JL. Association of histologic tumor characteristics and size of surgical margins with clinical outcome after surgical removal of cutaneous mast cell tumors in dogs. *Journal of the American Veterinary Medical Association* 2011; **238**: 1464–1469.
10. Chaffin K and Thrall DE. Results of radiation therapy in 19 dogs with cutaneous mast cell tumor and regional lymph node metastasis. *Veterinary Radiology & Ultrasound* 2002; **43**: 392–395.
11. Thamm DH, Turek MM and Vail DM. Outcome and prognostic factors following adjuvant prednisone/vinblastine chemotherapy for high-risk canine mast cell tumour: 61 cases. *The Journal of Veterinary Medical Sciences* 2006; **68**: 581–587.
12. Hume CT, Kiupel M, Rigatti L, Shofer FS, Skorupski KA and Sorenmo KU. Outcomes of dogs with grade 3 mast cell tumors: 43 cases (1997-2007). *Journal of the American Animal Hospital Association* 2011; **47**: 37–44.
13. Rassnick KM, Bailey DB, Russell DS, Flory AB, Kiselow MA, Intile JL, Malone EK, Balkman CE and Barnard SM. A phase II study to evaluate the toxicity and efficacy of alternating CCNU and high-dose vinblastine and prednisone (CVP) for treatment of dogs with high-grade, metastatic or nonresectable mast cell tumours. *Veterinary and Comparative Oncology* 2010; **8**: 138–152.
14. Cooper M, Tsai X and Bennett P. Combination CCNU and vinblastine chemotherapy for canine mast cell tumors: 57 cases. *Veterinary and Comparative Oncology* 2009; **7**: 196–206.
15. London CA, Malpas PB, Wood-Follis SL, Boucher JF, Rusk AW, Rosenberg MP, Henry CJ, Mitchener KL, Klein MK, Hintermeister JG, Bergman PJ, Couto GC, Mauldin GN and Michels GM. Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. *Clinical Cancer Research* 2009; **15**: 3856–3865.
16. Robat C, London C, Bunting L, McCartan L, Stingle N, Selting K, Kurzman I and Vail DM. Safety evaluation of combination vinblastine and toceranib phosphate (Palladia®) in dogs: a phase I dose-finding study. *Veterinary and Comparative Oncology* 2012; **10**: 174–183.
17. London C. Kinase inhibitors in cancer therapy. *Veterinary and Comparative Oncology* 2004; **2**: 177–193.
18. Fukutome M, Maebayashi K, Nasu S, Seki K and Mitsuhashi N. Enhancement of radiosensitivity by

dual inhibition of the HER family with ZD1839 (“Iressa”) and trastuzumab (“Harceptin”). *International Journal of Radiation Oncology, Biology, Physics* 2006; **66**: 528–536.
19. Wang M, Morsbach F, Sander D, Gheorghiu L, Nanda A, Benes C, Kriegs M, Krause M, Dikomey E, Baumann M, Dahm-Daphi J, Settleman J and Willers H. EGF receptor inhibition radiosensitizes NSCLC cells by inducing senescence in cells sustaining DNA double-strand breaks. *Cancer Research* 2011; **71**: 6261–6269.
20. London C, Mathie T, Stingle N, Clifford C, Haney S, Klein MK, Beaver L, Vickery K, Vail DM, Hershey B, Ettinger S, Vaughan A, Alvarez F, Hillman L, Kiselow M, Thamm D, Higginbotham ML, Gauthier M, Krick E, Phillips B, Ladue T, Jones P, Bryan J, Gill V, Novasad A, Fulton L, Carreras J, McNeill C, Henry C and Gillings S. Preliminary evidence for biologic activity of toceranib phosphate (Palladia®) in solid tumours. *Veterinary and Comparative Oncology* 2012; **10**: 194–205.
21. LaDue T, Price GS, Dodge R, Page RL and Thrall DE. Radiation therapy for incompletely resected canine mast cell tumors. *Veterinary Radiology and Ultrasound* 1998; **39**: 57–62.
22. Mountzios G, Terpos E and Dimopoulos MA. Aurora kinases as targets for cancer therapy. *Cancer Treatment Reviews* 2008; **34**: 175–182.
23. Dar AA, Goff LW, Majid S, Berlin J and El-Rifai W. Aurora kinase inhibitors–rising stars in cancer therapeutics? *Molecular Cancer Therapeutics* 2010; **9**: 268–278.
24. Shiomitsu K, Xia X, Sehgal I and Li S. Evaluation of the aurora kinase inhibitor, ZM447439, in canine malignant lymphoid cells in vitro. *Open Journal of Veterinary Medicine* 2013; **3**: 29–38.
25. Tentler JJ, Bradshaw-Pierce EL, Serkova NJ, Hasebroock KM, Pitts TM, Diamond JR, Fletcher GC, Bray MR and Eckhardt SG. Assessment of the in vivo antitumor effects of ENMD-2076, a novel multitargeted kinase inhibitor, against primary and cell line-derived human colorectal cancer xenograft models. *Clinical Cancer Research* 2010; **16**: 2989–2998.
26. Ishiguro T, Kadosawa T, Mori K, Takagi S, Okumura M and Fujinaga T. Establishment and characterization of a new canine mast cell tumor cell line. *The journal of veterinary medical science* 2001; **63**: 1031–1034.
27. Takahashi T, Kitani S, Nagase M, Mochizuki M, Nishimura R, Morita Y and Sasaki N. IgG-mediated histamine release from canine mastocytoma-derived cells. *International Archives of Allergy and Immunology* 2001; **125**: 228–235.
28. Takeuchi Y, Fujino Y, Watanabe M, Nakagawa T, Ohno K, Sasaki N, Sugano S and Tsujimoto H. Aberrant autophosphorylation of c-kit receptor in canine mast cell tumor cell lines. *Veterinary Immunology and Immunopathology* 2010; **137**: 208–216.
29. Cannon CM, Pozniak J, Scott MC, Ito D, Gorden BH, Graef AJ and Modiano JF. Canine osteosarcoma cells exhibit resistance to aurora kinase inhibitors. *Veterinary Comparative Oncology* 2013. doi: 10.1111/vco.12018.
30. Mendiola M, Barriuso J, Mariño-Enríquez A, Redondo A, Domínguez-Cáceres A, Hernández-Cortés G, Pérez-Fernández E, Sánchez-Navarro I, Vara JA, Suárez A, Espinosa E, González-Barón M, Palacios J and Hardisson D. Aurora kinases as prognostic biomarkers in ovarian carcinoma. *Human Pathology* 2009; **40**: 631–638.
31. Lassusn H, Staff S, Leminen A, Isola J and Butzow R. Aurora-A overexpression and aneuploidy predict poor outcome in serous ovarian carcinoma. *Gynecologic Oncology* 2011; **120**: 11–17.
32. García-Fernández E, De Diego JI, Collantes-Bellido E, Mendiola M, Prim MP, Pérez-Fernández E, Miguel-Martín M, Nistal M and Hardisson D. Aurora B kinase expression in laryngeal squamous cell carcinoma and its prognostic implications. *Histopathology* 2011; **58**: 368–376.
33. Nadler Y, Camp RL, Schwartz C, Rimm DL, Kluger HM and Kluger Y. Expression of Aurora A (but not Aurora B) is predictive of survival in breast cancer. *Clinical Cancer Research* 2008; **14**: 4455–4462.
34. Diamond JR, Bastos BR, Hansen RJ, Gustafson DL, Eckhardt SG, Kwak EL, Pandya SS, Fletcher GC, Pitts TM, Kulikowski GN, Morrow M, Arnott J, Bray MR, Sidor C, Messersmith W and Shapiro GI. Phase I safety, pharmacokinetic, and pharmacodynamic study of ENMD-2076, a novel angiogenic and Aurora kinase inhibitor, in patients with advanced solid tumors. *Clinical Cancer Research* 2011; **17**: 849–860.
35. Dubreuil P, Letard S, Ciufolini M, Gros L, Humbert M, Castéran N, Borge L, Hajem B, Lermet A, Sippl W, Voisset E, Arock M, Auclair C, Leventhal PS, Mansfield CD, Moussy A and Hermine O. Masitinib (AB1010), a potent and selective tyrosine kinase inhibitor targeting KIT. *PLoS One* 2009; **4**: e7258.
36. Ikezoe T, Yang Y, Nishioka C, Bandobashi K, Nakatani H, Taguchi T, Koeffler HP and Taguchi H. Effect of SU11248 on gastrointestinal stromal tumor-T1 cells: enhancement of growth inhibition via inhibition of 3-kinase/Akt/mammalian target of rapamycin signaling. *Cancer Science* 2006; **97**: 945–951.

37. Yancey MF, Merritt DA, Lesman SP, Boucher JF and Michels GM. Pharmacokinetic properties of toceranib phosphate (Palladia, SU11654), a novel tyrosine kinase inhibitor, in laboratory dogs and dogs with mast cell tumors. *Journal of Veterinary Pharmacology and Therapeutics* 2010; **33**: 162–171.
38. Yang J, Ikezoe T, Nishioka C, Tasaka T, Taniguchi A, Kuwayama Y, Komatsu N, Bandobashi K, Togitani K, Koeffler HP, Taguchi H and Yokoyama A. AZD1152, a novel and selective aurora B kinase inhibitor, induces growth arrest, apoptosis, and sensitization for tubulin depolymerizing agent or topoisomerase II inhibitor in human acute leukemia cells in vitro and in vivo. *Blood* 2007; **110**: 2034–2040.
39. Ikezoe T, Takeuchi T, Yang J, Adachi Y, Nishioka C, Furihata M, Koeffler HP and Yokoyama A. Analysis of Aurora B kinase in non-Hodgkin lymphoma. *Laboratory Investigation* 2009; **89**: 1364–1373.
40. Wang X, Sinn AL, Pollok K, Sandusky G, Zhang S, Chen L, Liang J, Crean CD, Suvannasankha A, Abonour R, Sidor C, Bray MR and Farag SS. Preclinical activity of a novel multiple tyrosine kinase and aurora kinase inhibitor, ENMD-2076, against multiple myeloma. *British Journal of Hematology* 2010; **150**: 313–325.
41. Tounekti O, Belehradek J Jr and Mir LM. Relationships between DNA fragmentation, chromatin condensation, and changes in flow cytometry profiles detected during apoptosis. *Experimental Cell Research* 1995; **217**: 506–516.
42. Darzynkiewicz Z and Bedner E. Analysis of apoptotic cells by flow and laser scanning cytometry. *Methods in Enzymology* 2000; **322**: 18–39.
43. Dewdney A, Cunningham D, Tabernero J, Capdevila J, Glimelius B, Cervantes A, Tait D, Brown G, Wotherspoon A, Gonzalez de Castro D, Chua YJ, Wong R, Barbachano Y, Oates J and Chau I. Multicenter randomized phase II clinical trial comparing neoadjuvant oxaliplatin, capecitabine, and preoperative radiotherapy with or without cetuximab followed by total mesorectal excision in patients with high-risk rectal cancer (EXPERT-C). *Journal of Clinical Oncology* 2012; **30**: 1620–1627.
44. Chen Y, Wu X, Bu S, He C, Wang W, Liu J, Guo W, Tan B, Wang Y and Wang J. Promising outcomes of definitive chemoradiation and cetuximab for patients with esophageal squamous cell carcinoma. *Cancer Science* 2012; **103**: 1979–1984.
45. Keil F, Selzer E, Berghold A, Reinisch S, Kapp KS, De Vries A, Greil R, Bachtiary B, Tinchon C, Anderhuber W, Burian M, Kasparek AK, Elsäßer W, Kainz H, Riedl R, Kopp M and Kornek G. Induction chemotherapy with docetaxel, cisplatin and 5-fluorouracil followed by radiotherapy with cetuximab for locally advanced squamous cell carcinoma of the head and neck. *European Journal of Cancer*, in press.
46. Dugas JP, Varnes ME, Sajo E, Welch CE, Ham K and Hogstrom KR. Dependence of cell survival on iododeoxyuridine concentration in 35-keV photon-activated Auger electron radiotherapy. *International Journal of Radiation Oncology, Biology, Physics* 2011; **79**: 255–261.
47. Mitchell L, Thamm DH and Biller BJ. Clinical and immunomodulatory effects of toceranib combined with low-dose cyclophosphamide in dogs with cancer. *Journal of Veterinary Internal Medicine* 2012; **26**: 355–362.
48. Desar IM, Jacobs JFM, Hulsbergen-vandeKaa CA, Oyen WJ, Mulders PF, van der Graaf WT, Adema GJ, van Herpen CM and de Vries IJ. Sorafenib reduces the percentage of tumour infiltrating regulatory T cells in renal cell carcinoma patients. *International Journal of Cancer* 2011; **129**: 507–512.
49. Murray A, Little SJ, Stanley P, Maraveyas A and Cawkwell L. Sorafenib enhances the in vitro anti-endothelial effects of low dose (metronomic) chemotherapy. *Oncology Report* 2010; **24**: 1049–1058.