

RESEARCH ARTICLE

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The long-acting COX-2 inhibitor mavacoxib (Trocoxil™) has anti-proliferative and pro-apoptotic effects on canine cancer cell lines and cancer stem cells *in vitro*

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Abstract

Background: The NSAID mavacoxib (Trocoxil™) is a recently described selective COX-2 inhibitor used for the management of inflammatory disease in dogs. It has a long plasma half-life, requiring less frequent dosing and supporting increased owner compliance in treating their dogs. Although the use of NSAIDs has been described in cancer treatment in dogs, there are no studies to date that have examined the utility of mavacoxib specifically.

Results: In this study we compared the *in vitro* activity of a short-acting non-selective COX inhibitor (carprofen) with mavacoxib, on cancer cell and cancer stem cell survival. We demonstrate that mavacoxib has a direct cell killing effect on cancer cells, increases apoptosis in cancer cells in a manner that may be independent of caspase activity, and has an inhibitory effect on cell migration. Importantly, we demonstrate that cancer stem cells derived from osteosarcoma cell lines are sensitive to the cytotoxic effect of mavacoxib.

Conclusions: Both NSAIDs can inhibit cancer cell proliferation and induce apoptosis *in vitro*. Importantly, cancer stem cells derived from an osteosarcoma cell line are sensitive to the cytotoxic effect of mavacoxib. Our results suggest that mavacoxib has anti-tumour effects and that this *in vitro* anti-cancer activity warrants further study.

Keywords: Mavacoxib, COX-2, Cancer stem cells, Osteosarcoma, Canine

Background

Cyclooxygenase (COX) is a key enzyme in the synthesis of prostaglandins from arachidonic acid. There are two isoforms of cyclooxygenase: COX-1 and COX-2. COX-1 is constitutively expressed in almost all tissues, whereas COX-2 is predominantly induced in response to certain stimuli including growth factors and pro-inflammatory cytokines [1]. Overexpression of COX-2 has been reported in diseases associated with inflammation and various cancers [2,3]. Furthermore, there has been increasing evidence that COX-2 overexpression is associated with tumourigenesis [4], angiogenesis [5], resistance to apoptosis, cancer cell proliferation and metastasis [6,7]. COX-2 protein expression has been associated with a poor prognosis in human breast cancer [8], colon cancer [9], malignant mesothelioma [10], head

and neck squamous cell carcinoma [11], and chronic myelogenous leukemia [12].

In recent years the traditional stochastic model of cancer development has been challenged by a new model, which implicates cancer stem cells (CSC) as the subpopulation of cancer cells that drives tumour growth, recurrence and metastasis [13]. CSCs share several characteristics with embryonic and somatic stem cells including self-renewal and differentiation abilities, and represent a small fraction of the cellular population of the tumour [13]. The role of CSCs was initially established in leukaemia, and more recently in solid tumours including melanomas [14,15], glioblastomas [16], and epithelial cancers [17-21]. Increasing evidence has implicated CSCs in tumourigenesis and response to treatment of many tumour types. Significantly, the resistance of these cells to conventional chemotherapeutic regimes suggests that CSCs play a major role in drug resistance and treatment failure [22]. Interestingly,

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COX-2 has been shown to be upregulated in colon [23] and breast CSCs and constituted part of an eight-gene signature that correlated with breast cancer patient survival [24].

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the COX enzyme thereby reducing the production of prostaglandins. NSAIDs are therefore widely used to reduce the clinical signs associated with inflammation. Moreover, increasing evidence from animal models has demonstrated the anti-tumoural and chemopreventative effects that NSAIDs have on several tumours, including intestinal, breast, skin, lung, and bladder tumours [25-28]. In addition, Thun *et al.* (1991) reported that low dose NSAIDs reduced relative risk of colorectal cancer [29], and subsequent studies support increasing evidence that NSAIDs significantly reduce colon polyp formation and recurrence [30,31]. Currently, there are several ongoing human clinical trials utilising NSAIDs as cancer therapeutics [32,33].

In dogs, overexpression of COX-2 and prostaglandin E2 (PGE2) have been identified in a wide variety of cancers, including transitional carcinoma of urinary bladder [34], lymphoma, mammary gland tumours and osteosarcoma [35-37]. As an example, previous studies have shown that COX-2 is not normally expressed in canine bone, but that around 77% of osteosarcomas are positive for COX-2 expression [38]. The suggestion that this makes COX-2 and the prostaglandin PGE2 promising therapeutic targets, is supported by the demonstrable therapeutic benefits of using NSAIDs in tumours that overexpress COX-2, such as prostatic carcinoma [39] and osteosarcoma [40]. Concurrently, use of the NSAID piroxicam is a standard recommendation of treatment for dogs with transitional cell carcinoma. In a pilot study, it was found that 20 percent of dogs with bladder tumours treated with piroxicam alone had a partial or complete response [41].

Mavacoxib (Trocoxil™) is a member of the coxib class of selective COX-2 inhibitors and is approved in the European Union for the treatment of pain and inflammation in canine osteoarthritis, where continuous treatment exceeding 1 month is indicated [42]. Mavacoxib is unique among NSAIDs because its combination of low clearance and relatively large apparent volume of distribution mean that it has a plasma half-life that is much longer than those of other NSAIDs, leading to a much reduced frequency of dosing. The potential benefits of using mavacoxib clinically are therefore high, but to date, there have been no studies evaluating the anti-cancer effects of this drug.

In this study we evaluated the anti-cancer effects of mavacoxib and compared this to another clinically important NSAID, carprofen (a non-selective COX inhibitor). Using a panel of canine cancer cell lines we demonstrate that both drugs can inhibit cancer cell

proliferation *in vitro* and we show that both drugs induce apoptosis in cancer cells in a manner that may be independent of caspase activity. Furthermore, mavacoxib, but not carprofen, is cytotoxic to cancer stem cells derived from osteosarcoma cell lines.

Results

Mavacoxib (Trocoxil™) inhibits cell proliferation of canine cancer cell lines

A panel of canine cell lines, including CPEK (normal epidermal keratinocyte), D17 (osteosarcoma), KTOSA5 (osteosarcoma), CSKOS (osteosarcoma), J3T (glioma), 3132 (lymphoma), C2-S (mast cell tumour) and SB (hemangiosarcoma) were used to determine the effect of NSAIDs on cell viability (Figure 1). Both carprofen and mavacoxib suppressed cancer cell proliferation effectively in a dose dependent manner, however mavacoxib showed a superior effect in the majority of the cell lines tested ($p < 0.05$), with the exception of SB cells (Figure 1). C2-S showed the greatest sensitivity to mavacoxib with an IC_{50} value of 29.3 μ M, whereas 3132 cells showed the greatest sensitivity to carprofen with an IC_{50} value of 63.46 μ M (Table 1).

Mavacoxib induces apoptosis in cancer cells and is independent of caspase activation

To determine the mechanism by which these NSAIDs reduce cell viability, we assayed treated cells for evidence of apoptosis by utilizing Annexin V staining, and explored expression of mediators of apoptosis including caspase activity and levels of Bax and Bcl2. In both KTOSA5 and CSKOS cells, we demonstrated a dose-dependent increase in the percentage of apoptotic cells when either carprofen or mavacoxib were used. Interestingly, both drugs had little effect on non-cancerous CPEK cells, even at high doses of the inhibitor. Both mavacoxib and carprofen had a significant time-dependent and dose-dependent effect on KTOSA5 and CSKOS apoptosis (Figure 2). Generally, the percentage of cells undergoing apoptosis was highest in cells treated with 100 μ M NSAIDs for 48 hours, although CSKOS cells were equally effected by 50 μ M and 100 μ M mavacoxib at 48 hours (Figure 2). To explore the effects on apoptosis further, we investigated the ability of both drugs to cause activation of caspase-2, caspase-8, caspase-9 and caspase-3 following 48 hours of treatment with either mavacoxib or carprofen. We demonstrated no significant difference between the enzyme activity of the initiator caspases -2, -8, -9 and the main effector caspase -3 in cells treated or not treated, suggesting that apoptosis in these cells may be caspase-independent (Additional file 1: Figure S1).

When we then assayed the treated cells for levels of Bax or Bcl2, we found that treatment stimulated Bcl2 but had no effect on basal Bax levels. Treatment of cells

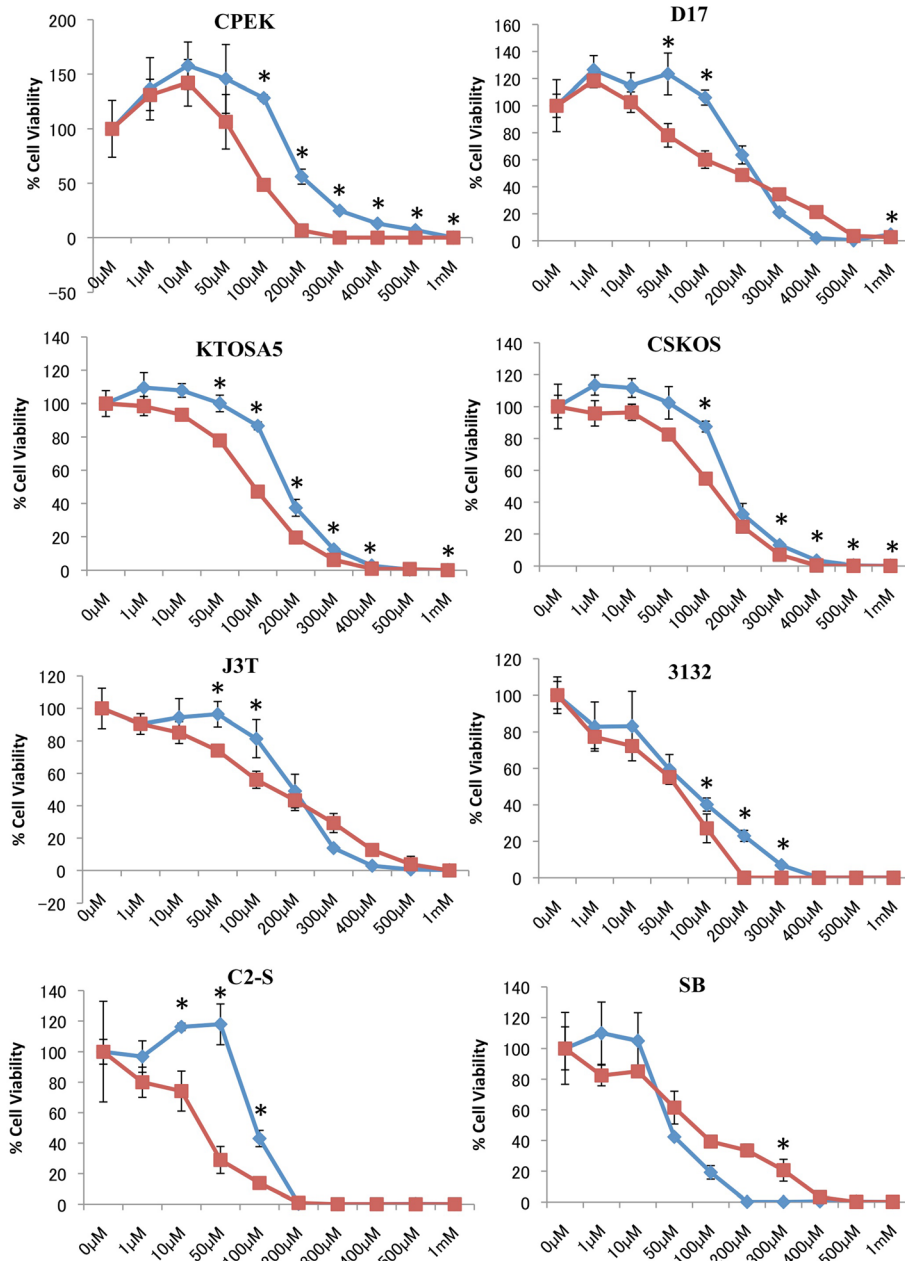


Figure 1 Mavacoxib inhibits cells proliferation of canine cancer cell lines. Normal keratinocytes (CPEK); osteosarcoma (D17, KTOSA5, CSKOS); glioma (J3T); lymphoma (3132); mast cell tumour (C2-S); and haemangiosarcoma (SB) cell lines were treated with the indicated doses of either mavacoxib or carprofen and cell viability was assayed 48 hours after treatment. Red line represents mavacoxib. Blue line represents carprofen. *p < 0.05 indicate a significant difference between mavacoxib and carprofen at the indicated concentration by a student's t-test.

with either carprofen or mavacoxib increased the level of the pro-survival Bcl2 protein, whereas the level of the pro-apoptotic Bax protein was unchanged. These results were consistent in all cell lines tested (Figure 3) and indicate that cell death induced by NSAID treatment does not involve mitochondrial apoptosis.

Mavacoxib has cell-type dependent effects on cell invasion
 The invasion index (%) was calculated for cells treated with either carprofen or mavacoxib. Migrated cells were counted using the transwell assay as described in materials and methods. In the CSKOS cell line, there was no significant difference between the control, untreated cells and

Table 1 Determination of IC₅₀ values for mavacoxib and carprofen in normal keratinocytes (CPEK); osteosarcoma (D17, KTOSA5, CSKOS); glioma (J3T); lymphoma (3132); mast cell tumour (C2-S); and haemangiosarcoma (SB) cell lines

Cell type	IC ₅₀	
	Mavacoxib	Carprofen
CPEK	87.1 μM	226.6 μM
D17	152.7 μM	225.6 μM
KTOSA5	93.4 μM	172.1 μM
CSKOS	107.8 μM	167 μM
J3T	68.4 μM	239.8 μM
3132	36.5 μM	63.5 μM
C2-S	29.3 μM	93.5 μM
SB	63.5 μM	70.4 μM

All measurements were made in triplicate.

cells treated with carprofen. However, in cells treated with mavacoxib there was significant ($p < 0.001$) inhibition of invasion that was dose dependent. KTOSA5 cells demonstrated a similar pattern to CSKOS cells, where the invasion index was unaffected at 50 μM carprofen, but significantly decreased at doses of 100 μM ($p = 0.024$). Treatment with either 50 μM or 100 μM mavacoxib, showed significant inhibition ($p < 0.02$) of invasion (Figure 4). There was no clear inhibition in J3T with either carprofen or mavacoxib treatment (Figure 4).

Mavacoxib inhibits cell survival of putative cancer stem cells

We have previously isolated cancer stem cells from canine osteosarcoma cell lines [43]. As NSAIDs are used as a palliative treatment for dogs with osteosarcoma [44], we

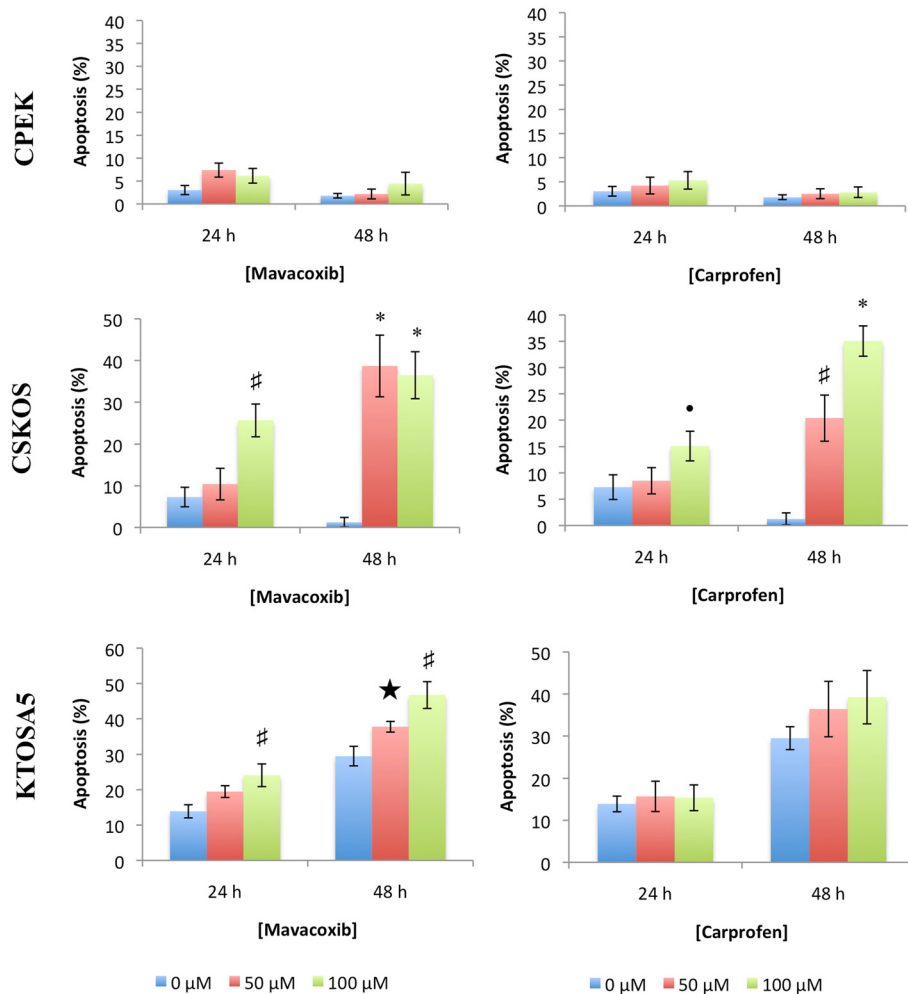
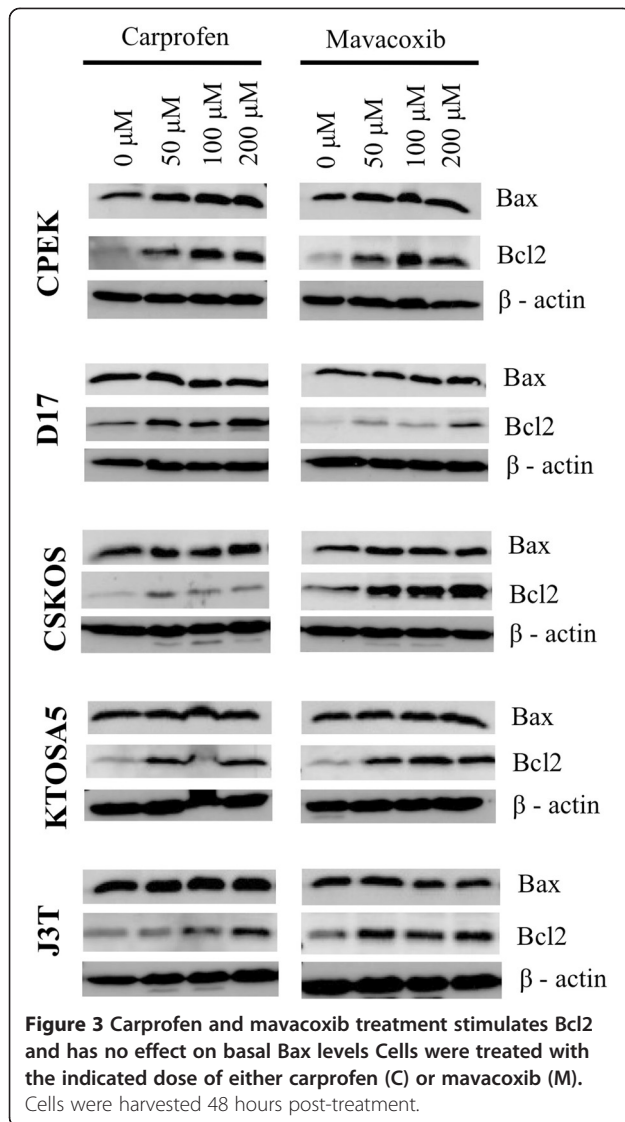


Figure 2 Effects of NSAIDs on apoptosis. CPEK, CSKOS, and KTOSA5 cells were treated with the indicated doses of either carprofen or mavacoxib. Annexin V-FITC staining was analysed by FACS 24 hours and 48 hours after treatment. The percentage of Annexin V positive cells was quantified. † $p = 0.02$, ★ $p = 0.01$, # $p = 0.002$, * $p < 0.001$ by comparing the indicated drug concentration at the indicated time point to the untreated control by a student's t-test.



wanted to determine the effect of NSAID treatment on the cancer stem cell population of KTOSA5 and CSKOS cell lines. Cells were magnetically sorted for expression of the cancer stem cell marker, CD34 (data unpublished), and assayed for cell viability after treatment with either mavacoxib or carprofen. There was no significant difference between CD34⁻ and CD34⁺ cells. As before, both mavacoxib and carprofen suppressed cancer cell proliferation effectively in a dose dependent manner, however mavacoxib was more effective at lower concentrations (Figure 5). For example, KTOSA5 CD34⁺ cells treated with mavacoxib had an IC₅₀ value of 36.03 μM, whereas the same cells treated with carprofen had an IC₅₀ value of 49.6 (Table 2).

A colony formation assay was used to determine the effect of NSAID treatment on cell reproductive death. Here, KTOSA5 and CSKOS cells were sorted for CD34 and treated with either mavacoxib or carprofen. As before,

both cell lines showed no difference between CD34⁻ and CD34⁺ cells. In KTOSA5 cells, treatment with 50 μM mavacoxib significantly ($p < 0.001$) decreased the number of colonies formed compared to untreated cells, and no colonies were formed after treatment with 100 μM mavacoxib. In contrast, treatment with 50 μM carprofen had no effect on the colony forming ability of KTOSA5 cells, and treatment with 100 μM carprofen only inhibited colony formation by approximately 20% (Figure 6A). Similar results were obtained for the CSKOS cell line (Figure 6B). This data indicates that the long-acting COX-2 inhibitor, mavacoxib is a more effective cytotoxic agent than the short-acting non-selective COX inhibitor carprofen.

Discussion

NSAIDs can be broadly divided into non-selective inhibitors of COX indicating that they inhibit both COX-1 and COX-2 or they can be COX-2 selective. Carprofen falls into the former category inhibiting both COX-1 and COX-2. In addition it is suggested that carprofen may have additional anti-inflammatory mechanisms of action such as inhibition of fatty acid amide hydrolase. In contrast mavacoxib is COX-2 selective, a characteristic of the coxib class of NSAIDs. Meloxicam, although not a coxib, is also considered to be relatively COX-2 selective [45].

When used for its anti-inflammatory properties the prolonged half-life for mavacoxib supports the approved regimen in which doses are separated by 2–4 weeks. In inflammatory conditions, compliance with this regimen is improved when compared to once-daily NSAID treatment [46], and because of the prolonged half-life, a continuous therapeutic response may be more likely in instances when there are problems with dosing, such as a delay in drug administration. Because of the relatively long half-life of mavacoxib, a regimen with a non-constant dosing interval (i.e. 2 weeks between the first two doses but with a maintenance 4 week interval) is used for rapid attainment of steady-state concentrations. With this regimen, plasma mavacoxib concentrations are relatively constant (0.52-1.11 μg/ml). In contrast carprofen has a more typical NSAID half-life in the dog supporting once daily drug administration.

In this current study we have demonstrated that mavacoxib, a COX-2 inhibitor, induced significant cell growth arrest and apoptosis in a panel of canine cancer cell lines, including D17 (osteosarcoma), KTOSA5 (osteosarcoma), CSKOS (osteosarcoma), J3T (glioma), 3132 (lymphoma), C2-S and SB (hemangiosarcoma). In comparison to carprofen, mavacoxib exhibited superior inhibition of cancer cell viability with IC₅₀ values 3.5-fold to 1.5-fold higher, depending on the cell line. Interestingly, neither drug induced apoptosis in normal cells (CPEK). Whereas, in the osteosarcoma cell lines tested, both carprofen and mavacoxib induced a dose-dependent increase in the

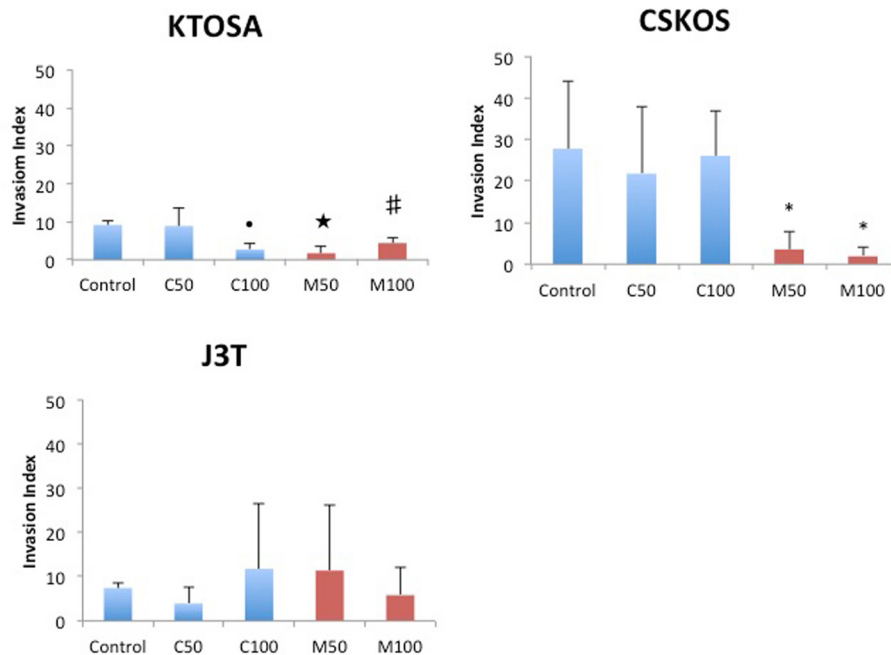


Figure 4 NSAID treatment has cell type dependent effects on cell invasion. Cells were treated with the indicated dose of either carprofen (C) or mavacoxib (M). Cells were counted 48 hours post-treatment. † $p = 0.024$ by Mann-Whitney test, ★ $p = 0.007$, # $p = 0.002$, * $p < 0.001$ by a student's t-test.

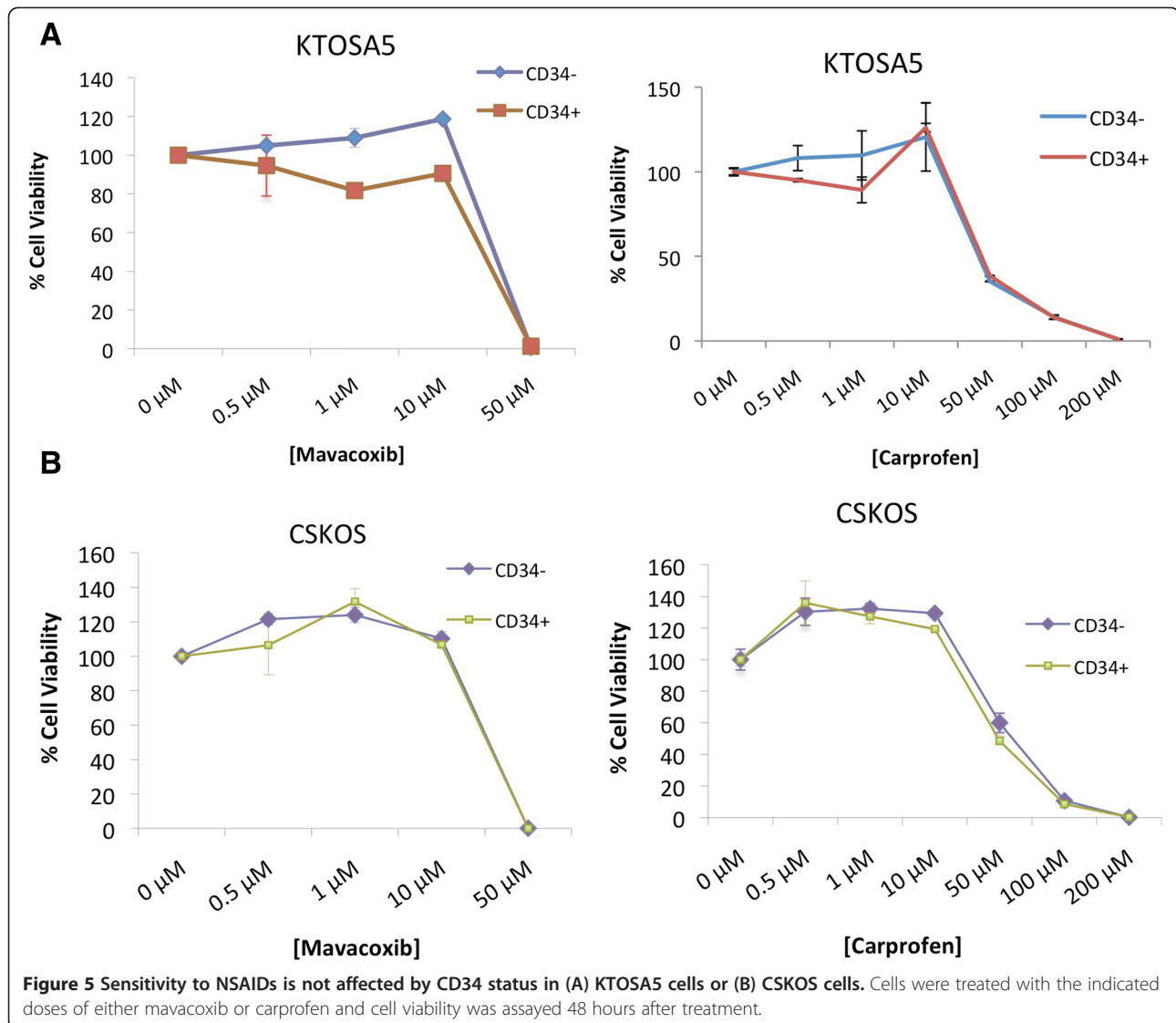
percentage of apoptotic cells after 24 and 48 hours, indicating that cancer cells may be more sensitive to the killing effect of COX inhibition compared to normal cells.

Apoptosis is a conserved cell death mechanism essential for normal development and tissue homeostasis. Classically, apoptotic cell death is characterized by caspase activation, down-regulation of Bcl2 and up-regulation of pro-apoptotic Bax [47]. Here, we show that apoptosis induced by either mavacoxib or carprofen is independent of the initiator caspases 2, 8 and 9, and the effector caspase 3. We also show that all the cell lines tested have a high basal level of Bax, which is not induced by increasing doses of either mavacoxib or carprofen. Concurrently, there is a dose-dependent increase in levels of anti-apoptotic Bcl2. Previous studies have shown that both apoptotic and anti-apoptotic factors are up-regulated in human tumours, and this leads to an impairment of the apoptotic signaling pathway and can confer resistance to apoptosis [48]. However, we observed this pattern in normal cells that have a functioning apoptotic pathway. We also confirmed that cell death was due to apoptosis and not necrosis. Caspase-independent and Bax-independent cell death has previously been characterised [49]. Further investigation is required to determine if mavacoxib induces caspase-independent cell death in canine cancer cell lines. In human studies of osteosarcoma and gastric cancer cell lines treated with meloxicam and SC236 (a potent COX-2 selective inhibitor) respectively, no induction of

Bax protein was demonstrated [35,50]. Naruse *et al.* (2006) discussed that the COX-2 selective NSAID meloxicam may affect a Bax-independent apoptotic pathway, which is yet to be elucidated [35].

We also demonstrated that in the osteosarcoma cell lines, KTOSA5 and CSKOS, high doses of mavacoxib are better at inhibiting invasiveness compared to carprofen. In the J3T glioma cell line there was no difference in invasiveness compared to control, indicating that COX-2 may have cell type specific effects. The mechanism of this inhibition requires further investigation, but it suggests that mavacoxib may have potential therapeutic benefits. Previous studies have demonstrated that COX-2 derived PGE2 activates CD44 and MMP-2, and in turn stimulates invasiveness of lung cancer cells *in vitro* [51]. In addition, COX-2 inhibition of the human osteosarcoma cell line, MG-63, by meloxicam can also inhibit invasiveness [35]. The role of COX-2 in the tumour microenvironment should also be considered; Williams *et al.* (2000) showed that the tumours grown in COX-2-null mice had decreased growth, as well as vascular density, compared to wild type mice [52], suggesting that both host cells and tumour cells are affected by COX-2 expression levels.

To fully determine the anti-proliferative effect of COX-2 inhibition on canine cancer, it is fundamental to study the effect of COX-2 inhibition on the cancer stem cell population. Cancer stem cells are a small subpopulation of cells that can influence initiation, recurrence and chemoresistance of a tumour [13]. As we have previously isolated



cancer stem cells from canine osteosarcoma cell lines [43] and NSAIDs are used as a palliative treatment for dogs with osteosarcoma, we used the canine osteosarcoma cell lines, to look at the effect of COX-2 inhibition with mavacoxib. Cancer stem cells isolated from these cell lines are

Table 2 Determination of IC₅₀ values for mavacoxib and carprofen in KTOSA5 and CSKOS cells sorted for CD34 protein expression

Cell type	Drug	IC ₅₀	
		CD34-	CD34+
KTOSA5	Mavacoxib	38.63 μM	27.37 μM
KTOSA5	Carprofen	42.94 μM	44.87 μM
CSKOS	Mavacoxib	37.62 μM	36.03 μM
CSKOS	Carprofen	55.86 μM	49.6 μM

All measurements were made in triplicate.

inherently more resistant to the cytotoxic effect of the anthracycline antibiotic, doxorubicin (data unpublished). Here, we show that there is no significant difference between cancer cells and putative cancer stem cells. This is significant as it shows that cancer stem cells are not resistant to COX-2 inhibition. As before, mavacoxib is more effective at killing cells at a lower concentration than carprofen.

We also determined the long-term effect of COX-2 inhibition on replicative cell death of both cancer and cancer stem cells. Significantly, mavacoxib dramatically decreased the colony forming ability in a dose-dependent manner in both cell populations, whereas carprofen only had a modest effect. Mavacoxib is a long-acting COX-2 inhibitor with a relatively long plasma half-life compared to carprofen, and this may account for the difference in colony forming ability. As we have shown here, mavacoxib is more effective

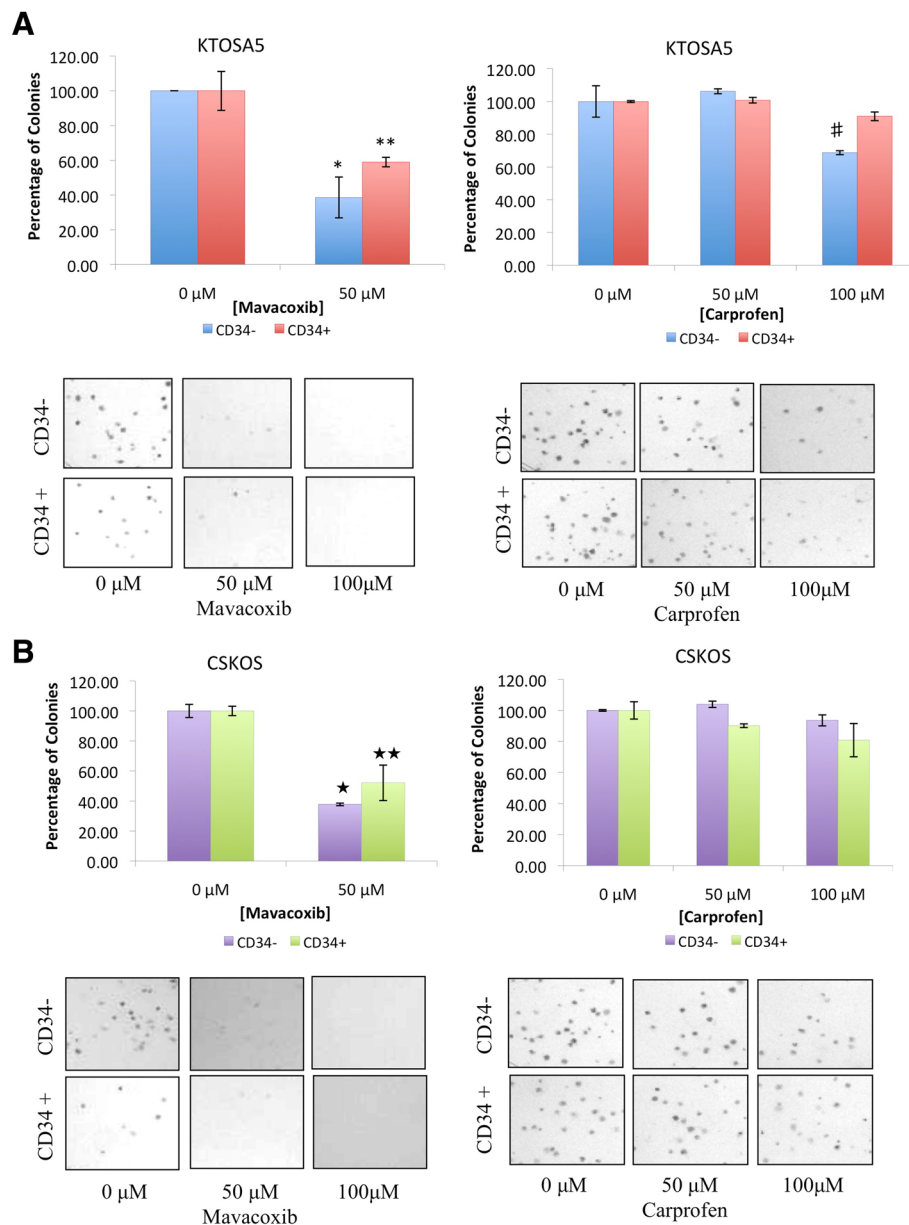


Figure 6 Mavacoxib is better than carprofen at inhibiting colony forming ability of KTOSA5 cells (A) and CSKOS cells (B) independent of CD34 status. Cells were treated with the indicated doses of either mavacoxib or carprofen and colony formation was assayed. Number of colonies per plate was counted. Representative images are shown. * $p = 0.001$, ** $p < 0.001$, # $p = 0.007$, ★ $p < 0.001$, ★★ $p = 0.002$ by a student's t-test.

than carprofen as a cytotoxic agent against both cancer cells and cancer stem cells.

Mavacoxib is closely related to the human drug, celecoxib. Previous studies have shown that putative cancer stem cells isolated from human glioma cell lines express constitutively high levels of COX-2 protein, which are positively correlated with radioresistance. Treatment with celecoxib enhanced radiosensitivity of glioma stem cells and suppressed the expression of angiogenic and stemness-related genes [53]. To date, the molecular mechanism by

which the COX-2 inhibitors, celecoxib and mavacoxib, exert their anti-tumour effect on the cancer stem cell population is yet to be elucidated. Further studies should be conducted to determine if mavacoxib also has synergistic effects with other chemotherapeutic agents. The data presented here demonstrates that the anti-proliferative effects of mavacoxib are effective on a range of canine cancer cell types and that the therapeutic potential of mavacoxib, either as a single agent or as a multimodal therapy, should be further investigated.

Conclusions

Our study provides the first evidence that mavacoxib is an effective anti-tumour agent. Mavacoxib can inhibit cell proliferation and invasiveness better than carprofen. Importantly, mavacoxib can inhibit cancer stem cell survival in an *in vitro* osteosarcoma model. Therefore, mavacoxib could be a potential candidate for the treatment of canine cancer and further studies of its clinical use are now required.

Methods

Cell culture

The KTOSA5 cell line was derived from an osteosarcoma affecting the right hind limb of an 8-year-old, chemotherapy naïve, female entire Rottweiler (approved by the University of Edinburgh Veterinary Ethical Review Committee). KTOSA5 (osteosarcoma), CSKOS (osteosarcoma), J3T (glioma) cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum and 100 µg/ml streptomycin (Invitrogen, Paisley, UK). D17 (osteosarcoma), C2-S (mast cell tumour) and SB (hemangiosarcoma) cells were grown in DMEM-GlutaMAX™ (Invitrogen) supplemented with 10% fetal bovine serum and 100 µg/ml streptomycin. 3132 (lymphoma) cells were grown in RPMI medium 1640 (Invitrogen) supplemented with 10% fetal bovine serum and 100 µg/ml streptomycin. CPEK (normal epidermal keratinocyte) were cultured in CnT-09 epidermal keratinocyte medium (CELLnTEC, Bern, Switzerland). All cell cultures were maintained at 37°C in a humidified CO₂ incubator.

Magnetic cell sorting

Cells were labelled with CD34 microbeads and sorted using the Miltenyi Biotec CD34 cell isolation kit according to the manufacturer's protocol (Miltenyi Biotec, Surrey, UK). Briefly, cells were resuspended in 300 µl PBS solution (pH 7.2, 0.5% BSA, 2 mM EDTA) per 10⁸ cells. Then blocking reagent FcR (100 µl/10⁸ cells; Miltenyi Biotec, Surrey, UK) and CD34 microbeads (100 µl/10⁸ cells) were added and mixed at 4°C for 30 minutes with rotation. Cells were washed in 20× volume with PBS solution. The pellet was resuspended in 500 µl PBS solution and added to a pre-washed magnetic separation (LS) column on the magnetic holder. The column was washed four times and the collected cells were designated as the negative fraction. The column was removed from the magnetic holder and the positive fraction was collected.

Cell viability assay

To determine cell viability, CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) was used according to manufacturer's instructions. Briefly, 500 cells were seeded per well in a 96-well plate, in triplicate. Cells were incubated for 24 h at 37°C, 5% CO₂. Drugs

were applied at a range of concentrations (0 µM – 5 mM) and incubated for 48 h. The plate was equilibrated at room temperature for 30 min and 100 µl of CellTiter-Glo® reagent was added to each well. Luminescence was recorded by a luminometer (Viktor3, PerkinElmer, Massachusetts, USA). IC₅₀ values were calculated from three replicates using GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA).

Colony formation assay

Cells were trypsinised into single cells and seeded at 500 cells/10 cm plate. The cells were incubated with appropriate doses of either mavacoxib or carprofen whilst in suspension. Plates were incubated at 37°C in a humidified CO₂ incubator until colonies were visible. Growth media was changed once a week. The colonies were fixed by incubating with ice-cold methanol for 5 minutes at room temperature. Colonies were stained with Giemsa stain (Invitrogen, Paisley, UK) according to the manufacturer's instructions and the number of colonies were counted.

Caspase profiling assay

Activation of caspase-2, caspase-8, caspase-9 and caspase-3 on indicated drug treatments was determined using a caspase profiling plate (Clontech lab., CA, USA) according to the manufacturer's instructions. Briefly, cells were grown until 60-70% confluent, then treated with either carprofen or mavacoxib (50 µM and 100 µM), and incubated for 48 h at 37°C, 5% CO₂. Untreated cells were also prepared as a control for each cell line. After treatment, 2 × 10⁵ cells were prepared per well, lysed in cold 1× Cell Lysis Buffer (50 µl/2 × 10⁵ cells) and incubated for 10 min on ice. The plates were preincubated with 50 µl of 2× Reaction buffer (mixed with 0.1 M DTT) to each well for 5 min at 37°C, 5% CO₂. Cell lysates were transferred to each well and incubated for 2 h at 37°C, 5% CO₂. Caspase activities were read using a fluorescent plate reader (excitation: 380 nm, emission: 460 nm) (Viktor3, PerkinElmer, Massachusetts, USA).

Annexin V assay

The BD FITC Annexin V Apoptosis Detection kit (BD PharMingen, San Diego, CA) was used to determine the effect of carprofen or mavacoxib treatment on the level of apoptosis. The Annexin V analysis allows monitoring of changes in necrosis, early apoptosis and late apoptosis. The assay was performed according to the manufacturer's instructions. Briefly, cells were grown until 60-70% confluent and treated with either carprofen or mavacoxib at final concentrations of 50 µM and 100 µM. Untreated cells were used as controls for each cell line. Cells were treated for 24 h and 48 h at 37°C, 5% CO₂. Subsequently, cells were washed twice with cold PBS, trypsinized and resuspended in 1 ml of 0.1% BSA containing PBS. 1 × 10⁵ cells/ml and

were then stained with Annexin V FITC and propidium iodide for 15 min in the dark. The CyAn ADP analyser (Beckman Coulter Inc. CA, USA) was used and the data was analyzed using Summit v4.3 software.

Migration and invasion assay

To evaluate the effect of carprofen and mavacoxib on the metastatic potential of the cell lines, motility and invasiveness were analysed using a transwell migration and invasion assay. CPEK, KTOSA5, CSKOS, D17 and J3T cells were grown to 60-70% confluency and then treated with carprofen or mavacoxib at concentrations of 50 μ M and 100 μ M. Cell lines were incubated for 48 h at 37°C, 5% CO₂. Subsequently, cells were detached and re-suspended in FBS-free medium. For the migration assay, 2.5 \times 10⁴ cells in 500 μ l volume were plated on the top chamber with a non-coated polycarbonate membrane with 8.0 μ m pore size (BD Bioscience, Bedford, MA, USA) in duplicate. Medium with 10% FBS was added in the lower chamber as a chemoattractant. After incubation for 24 h, the cells that migrated to the lower surface of the polycarbonate membrane were fixed with methanol for 1 min, followed by Giemsa stain for 15 min. The cells which did not migrate through the pores, were mechanically removed by a cotton swab moistened with the medium. The mean number of migrated cells was counted in 5 random fields of each membrane. The invasion assay was performed as for the previously described migration assay, except that the transwell inserts consist of Matrigel-coated polycarbonate membranes with 8.0 μ m pore size. Matrigel mimics the basement membrane *in vitro* and provides a barrier to non-invasive cells. Data is expressed as the percent invasion calculated by the following formula; % Invasion = (Mean number of cells invading/Mean number of cells migrating) \times 100.

Protein detection

Cells were lysed in urea lysis buffer (7 M urea, 0.1 M DTT, 0.05% Triton X-100, 25 mM NaCl, 20 mM Hepes pH 7.5). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis (SDS PAGE), transferred to Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) and hybridised to an appropriate primary antibody and HRP-conjugated secondary antibody for subsequent detection by ECL. Primary antibody against β -actin was purchased from Abcam (Cambridge, UK). Antibodies against Bax and Bcl2 were purchased from Santa Cruz Biotechnology (CA, USA). The secondary antibodies, HRP-conjugated rabbit anti-mouse IgG and swine anti-rabbit IgG, were obtained from DakoCytomation (Glostrup, Denmark).

Statistical analysis

Data were expressed as mean \pm SD. Statistical analysis was performed with Minitab® statistical software (PA, USA) using analysis of variance and student's t test or Mann-Whitney test. The criterion for significance was $p < 0.05$ for all comparisons.

Additional file

Additional file 1: Figure S1. NSAID treatment has cell-type dependent effects on caspase activity. Cells were treated with either 50 μ M or 100 μ M carprofen (C) or mavacoxib (M) for 48 hours. Caspase activity was determined using the apoalert caspase assay kit (Calbiochem). Blue bars represents carprofen treatment. Red bar represents mavacoxib treatment.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

LYP carried out cancer stem cell studies, statistical analysis, designed the study and drafted the manuscript. SAA contributed to the drafting of the manuscript. AK carried out cell viability assays, apoptotic assays and statistical analysis. KOM contributed to cancer stem cell studies. DJA conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

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