Research Article

The Effects of Piroxicam and Deracoxib on Canine Mammary Tumour Cell Line

Fulya Üstün Alkan,1 Oya Üstünler,1 Tülay Bakırel,1 Suzan Çınar,2 Gaye Erten,2 and Günner Deniz2

1 Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Istanbul University, Avcılar, 34320 Istanbul, Turkey
2 Department of Immunology, Institute of Experimental Medicine, Istanbul University, Çapa, 34093 Istanbul, Turkey

Correspondence should be addressed to Fulya Üstün Alkan, fustun@istanbul.edu.tr

Received 14 August 2012; Accepted 17 October 2012

Academic Editors: D. Endoh, M. F. Landoni, I. Valpotic, and B. I. Yoon

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Cyclooxygenase (COX) inhibitors, already widely used for the treatment of pain and inflammation, are considered as promising compounds for the prevention and treatment of neoplasia. The aim of our study was to determine the direct antiproliferative effects of nonsteroidal anti-inflammatory drugs (NSAIDs), piroxicam and deracoxib, at a variety of concentrations as both single and combined treatments on canine mammary carcinoma cell line CMT-U27 and to understand the mechanisms of cell death. MTT assay was performed to determine cell viability, and flow cytometric analyses were performed to evaluate apoptosis and cell cycle alterations. Significant decrease in cell viability was observed at high concentrations of piroxicam and deracoxib in both single and combined treatments after 72 h incubation. Combined treatment produced a significantly greater inhibition than that caused by either agent alone. Also apoptotic cell number was increased by both drugs at the cytotoxic concentrations. However, concomitant treatment of cells with piroxicam and deracoxib resulted in significant induction of apoptosis at lower concentrations and accumulation of cells in the G0/G1 phase. Significant cytotoxic effects exhibited by the combination of piroxicam and deracoxib against canine mammary carcinoma cells in vitro suggest an attractive approach for the treatment of canine mammary carcinoma.

1. Introduction

Canine mammary tumors are the most common malignant neoplasm constituting up to 52% of all tumors in female dogs [1]. Canine mammary carcinomas have epidemiologic, clinical, morphologic, and prognostic features similar to those of human breast cancer and therefore represent a comparative model to understand the underlying molecular mechanisms of carcinogenesis in both species [2]. Treatment for cancer of the mammary includes surgical excision, radiation therapy, and chemotherapy or a combination [3]. However, development of resistance to antitumour treatments has resulted in clinical failures, therefore; new treatments are needed for those animals who fail to respond to standard therapy or who initially present with metastatic disease [4].

Cyclooxygenase (COX) is an important enzyme that catalyzes the conversion of arachidonic acid to prostaglandin. Many studies have shown that a variety of tumors (breast, colon, lung, and bladder) produce greater amounts of prostaglandins than the normal tissue from which they were derived [5]. COX-1 is constitutively expressed in most tissues and regulates multiple physiologic processes, whereas COX-2 is induced by proinflammatory or mitogenic stimuli and overexpressed in a variety of cancers [6, 7]. In dogs, COX-2 is overexpressed in several mammary, rectal, bladder, cutaneous, and oral and ocular melanocytic tumors [8], and this expression has been associated with uncontrolled cell proliferation and differentiation, inhibition of apoptosis, increased angiogenesis and metastasis [9].

Nonsteroidal anti-inflammatory drugs (NSAIDs) that can block the activity of COXs are reported to have chemopreventive effects in several experimental studies and clinical trials [10–14]. Accordingly, the suppression of COX-2 has been proposed to underlie the chemopreventive effects of NSAIDs. Recent reports suggest that the anticancer effects of NSAIDs and selective COX-2 inhibitors can occur through
COX-independent pathways [15, 16]. However, evidence for the use of COX inhibitors in cancer prevention and the mechanism by which NSAIDs cause protective and anti-carcinogenic effects are still to be determined.

Piroxicam is an NSAID used for treatment of osteoarthritis and also found to be effective in the treatment of transitional cell carcinoma of the bladder [17], in the treatment of inflammatory mammary carcinoma [18] and oral squamous cell carcinoma in dogs [19]. Also, it was shown to have antitumor activity against naturally acquired tumors in dogs in phase I and phase II clinical trials. In addition, high concentrations of piroxicam have been shown to inhibit cellular proliferation of canine osteosarcoma [20] and canine mammary carcinoma in vitro [21]. However, no significant reduction in cell growth occurred at concentrations that could be achieved in vivo without a significant risk of severe toxic side effects [21]. Deracoxib is a selective COX-2 inhibitor licensed for the treatment of pain and inflammation associated with osteoarthritis and orthopedic surgery in canines [22]. Deracoxib was shown to have in vivo antitumor activity and in vitro cytotoxic properties. Also, deracoxib was found to reduce the growth of canine mammary cancer xenografts in mice [14] and was found to be cytotoxic in osteosarcoma cell lines [20]. The present study, therefore, was designed to determine the antineoplastic mechanism of piroxicam and deracoxib especially to determine the efficiency of the combination of these drugs on canine mammary carcinoma cells.

2. Materials and Methods

2.1. Cell Line. The canine mammary carcinoma cell line CMT-U27 (a generous gift from Assoc Professor Eva Hellmén) was obtained from the Uppsala University, Sweden. CMT-U27 cell line was derived from a primary tumor (infiltrating ductal carcinoma) and when inoculated in the fat mammary pad of female nude mice, it metastasized to the lymph nodes, lungs, liver, and heart [23].

2.2. Cell Culture and Treatment. Mammary carcinoma cells, at passage 134, were cultured in Dulbecco’s modified Eagle’s medium-F12 (Sigma Chemicals, St. Louis, USA), supplemented with 2 mM L-glutamine (Sigma, St. Louis, USA), 10% fetal bovine serum (Biological Industries, Israel), 100 IU mL⁻¹ penicillin G, 100 µg mL⁻¹ streptomycin, and 2.5 µg mL⁻¹ amphotericin B (Sigma, St. Louis, USA), and maintained in monolayer culture at 37°C in a humidified 5% CO₂ atmosphere. Culture media was changed every 2-3 days for maintaining the exponential growth of the cells, and cells were subcultured as they reached 80–90% confluence. Subconfluent cells were passaged with 0.25% trypsin and 0.02% EDTA solution (Sigma, St. Louis, USA). Nonspecific COX inhibitor piroxicam (Sigma, St. Louis, USA) and selective COX-2 inhibitor deracoxib (Novartis, Pharmaceuticals Inc.) were dissolved in sterile dimethylsulfoxide (DMSO) to create a stock solution, filtered through 0.2 µm filter, and stored at −20°C. The stock solution was diluted with the medium, and the cells were treated with 50, 100, 250, 500, and 1000 µM concentrations of each compound for 72 h. Control group was cultured without piroxicam and deracoxib, and corresponding amount of DMSO was added to the medium.

2.3. Cell Viability Assay. Cells at passage 138 were cultured at a density of 1 × 10⁴ cells/100 µL in 96-well flat-bottom microtiter plates (Jet Biofil, Canada) and allowed to attach for 24 h. Thereafter, medium was removed and replaced with 100 µL of medium containing 50, 100, 250, 500, and 1000 µM concentrations of piroxicam and deracoxib in triplicate wells. After 72 h incubation, cell viability was assessed using cell proliferation kit (MTT, Roche, Germany), according to the manufacturer’s instructions. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) test is based on the enzymatic reduction of the tetrazolium salt MTT to a formazan (1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan) by mitochondria of living cells [24]. Briefly, 10 µL of MTT solution 5 mg/mL in phosphate buffered saline (PBS) was added to each well and incubated for 4 h at 37°C in CO₂ incubator. The purple water insoluble formazan salt was then dissolved with 10% SDS in 0.01 M HCl and incubated overnight in a humidified 5% CO₂ atmosphere. The optical densities (OD) of the wells were measured at 550 nm by microplate reader (ELX800, Biotek Instruments, USA). The effect of each compound on growth inhibition was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable. The dose-response curves were plotted for each drug, and the concentration of drug required for 50% inhibition of cell viability (IC₅₀) was determined graphically.

2.4. Apoptosis Assay. Flow cytometric analyses of phosphatidylserine exposure were quantitatively detected using Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Bioscience, San Jose, CA). The method is based on the binding of Annexin V to phosphatidylserine that is translocated from the inner membrane leaflet to the outer layer in cells undergoing apoptosis [25]. The cells were cultured at a density of 1 × 10⁵/mL in 24-well flat-bottom microtiter plates (Jet Biofil, Canada) and cultivated in medium as described above. After 24 h, medium was replaced with fresh medium containing 50–1000 µM concentrations of piroxicam and deracoxib. The cells were trypsinized 72 h after the treatment, washed twice each with ice cold PBS, and then resuspended in binding buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl₂), supplemented with 5 µL of FITC-Annexin V and 5 µL of propidium iodide (PI). The cell suspension was gently vortexed and incubated for 15 min at room temperature in the dark. Following the incubation, 400 µL of binding buffer was added to each tube and then analyzed within 1 h on a FACScan flow cytometer (BD Biosciences) using the standard optics for detecting FL1 (FITC) and FL2 (PI). Data were analyzed with CellQuest WinMDI software (BD Biosciences, San Jose, CA).

2.5. Cell Cycle Analyses. Cell cycle alterations were detected using Coulter DNA Prep Reagents Kit (Beckman Coulter, UK) by flow cytometry. The cells were cultured at a density of 1 × 10⁵/mL in 24-well flat-bottom microtiter plates (Jet Biofil, Canada) and cultivated and treated in medium as
of the control level, respectively, whereas 1000 µM incubation, significant reductions were seen at 250, 500, and 1000 µM doses of deracoxib by 16.49%, 16.64%, and 40.69% of the control level, respectively, whereas 1000 µM incubation, significant reductions were seen at 250, 500, and 1000 µM concentrations (36.5%, 34.94%, 43.84%, and 50.66%, resp.) (Figure 2). The combination of both COX inhibitors exhibited a synergistic effect in CMT-U27 cells.

3.2. Apoptosis Assay. The apoptotic cell number of CMT-U27 cells after 72 h incubation in the presence or absence of piroxicam and deracoxib at various concentrations (50–1000 µM) was shown in Figure 3. Piroxicam at 1000 µM concentration (P < 0.05), deracoxib at 250 µM, and higher concentrations (P < 0.01, P < 0.001) decreased the number of viable cells and increased the number of apoptotic cells as a sum of early and late apoptotic cells significantly.

Whereas, again, the combination of piroxicam and deracoxib (100 µM and higher) exhibited a significant increase in the apoptotic activity. The percentages of apoptotic cells were 5.56% (control), and 5.32% (piroxicam 50 µM + deracoxib 50 µM), 23.47% (piroxicam 100 µM + deracoxib 100 µM), 24.14% (piroxicam 250 µM + deracoxib 250 µM), 28.86% (piroxicam 500 µM + deracoxib 500 µM), 53.62% (piroxicam 1000 µM + deracoxib 1000 µM). Figure 4 shows representative results of flow cytometry for the apoptosis of CMT-U27 cells after incubation in the presence of piroxicam and deracoxib.

3.3. Cell Cycle Analyses. Cell cycle phase distribution was assessed which cell cycle changes contribute to CMT-U27 cell number reduction by piroxicam and deracoxib, and cellular DNA contents were examined by flow cytometry. The results showed that treating cells with piroxicam and deracoxib at 500 and 1000 µM concentrations caused a significant inhibition of cell cycle progression in CMT-U27 cells at 72 h (Table 1) resulting in a clear increase of the percentage of cells in the G0/G1 phase when compared...
with the control. The cell cycle evaluation in CMT-U27 cell line showed an average: 45.23%, 35.62%, and 19.16% of cells in G0/G1, S, and G2/M phases, respectively.

Treatment with the combination of 1000 µM concentrations of piroxicam and deracoxib resulted in an increase in the number of cells in the G0/G1 phase and a decrease in the number of cells in the S and G2/M phase indicating the cell cycle arrest at G0/G1 phase correlating with the induction of apoptosis (Table 2). At the highest dose combination of two drugs, the percentage of cells in the G0/G1 phase was increased from 63.8 to 89.95% when compared with control. Conversely, the percentage of cells in S phase decreased from 22.48% to 8.27%, and G2/M phase cells decreased from 13.72% to 1.78%.
**Figure 4:** Flow cytometric analysis of apoptosis of coadministration of piroxicam and deracoxib on canine mammary carcinoma cell line after 72 h incubation. The lower left quadrant of the histogram shows the viable cells, which exclude PI, and are negative for FITC-Annexin-V-binding FITC−/PI− (LL). The lower right compartment represents the early apoptotic cells, which are PI negative and Annexin-V positive, indicating the translocation of phosphatidylserine to the external cell surface FITC+/PI− (LR). The upper right quadrant represents the late-stage apoptotic cells which are PI and Annexin-V positive FITC+/PI+ (UR), and the upper left compartment shows the necrotic cells which are only PI positive FITC−/PI+ (UL). The numbers written on histograms represent the sum of early and late apoptotic cells (%).

**Table 1:** Effects of piroxicam and deracoxib on cell cycle phase distribution of CMT-U27 cells. Data are expressed as mean values ± standard error of means shown with *P < 0.05 versus control group.

| Drug concentrations | % G0/G1 | % S | % G2/M |
|---------------------|---------|-----|--------|
| Control             | 45.23 ± 1.17 | 35.62 ± 2.57 | 19.16 ± 1.81 |
| Piroxicam (50 μM)   | 55.93 ± 2.79 | 35.49 ± 1.92 | 8.58 ± 0.83 |
| Piroxicam (100 μM)  | 58.44 ± 2.48 | 34.63 ± 2.25 | 6.93 ± 1.03 |
| Piroxicam (250 μM)  | 70.31 ± 1.32 | 24.13 ± 2.06 | 5.56 ± 0.39 |
| Piroxicam (500 μM)  | 80.22 ± 1.67* | 8.46 ± 0.77* | 11.32 ± 1.78 |
| Piroxicam (1000 μM) | 79.05 ± 2.19* | 11.93 ± 0.84* | 9.02 ± 0.51 |
| Deracoxib (50 μM)   | 50.21 ± 2.55 | 41.35 ± 1.92 | 8.44 ± 1.10 |
| Deracoxib (100 μM)  | 53.74 ± 2.12 | 42.51 ± 1.77 | 3.75 ± 0.29 |
| Deracoxib (250 μM)  | 65.57 ± 1.45 | 15.04 ± 1.55 | 19.39 ± 1.29 |
| Deracoxib (500 μM)  | 79.94 ± 2.14* | 14.03 ± 2.37 | 6.03 ± 0.53 |
| Deracoxib (1000 μM)| 71.34 ± 2.36* | 4.72 ± 0.85* | 23.94 ± 2.26 |

**4. Discussion**

In recent years, experimental, epidemiological, and clinical studies have identified COX inhibitors as promising compounds in anticancer therapy. There is an ample evidence of the involvement of COX-1 and COX-2 in carcinogenesis for many different types of malignant tumor, and results of numerous studies indicate that various NSAIDS exert anti-proliferative and antineoplastic effects on several canine cancer cell lines [20, 21, 26]. These findings have raised the possibility that COX inhibitors might also act as tumor suppressors. Deracoxib, a selective COX-2 inhibitor, and piroxicam, a nonselective COX inhibitor, and also frequently used drugs in veterinary medicine were evaluated to determine the cytotoxic effects on canine mammary carcinoma cells.

In the present study, we investigated firstly the *in vitro* effects of piroxicam and deracoxib on canine mammary carcinoma cell line CMT-U27. As a result, both drugs suppressed proliferation of canine mammary tumor cells in a concentration-dependent manner. No significant difference in cell viability was seen after incubating cells with piroxicam
was seen at much lower concentrations as above 100 µM. The increase in both early and late apoptotic activity reported in several studies [20, 21]. Authors suggested that the significant inhibition of proliferation and induction of apoptosis could only occur when drug concentrations were in excess that can be achieved in vivo following maximum recommended dose rate of piroxicam [21]. Royals and colleagues [20] reported that piroxicam and deracoxib significantly decreased the cell proliferation of highly metastatic canine osteosarcoma cells at ≥500 µM and ≥100 µM concentrations, respectively. This effect was similar to the effect reported by Wollesberger and colleagues [27] that high concentrations of meloxicam (400 and 600 µM) showed a clear antiproliferative and pro-apoptotic effect on canine osteosarcoma cells in vitro. Also a high IC50 (615 µM) was reported for piroxicam against canine squamous cell carcinoma cell line [26], and although this concentration can never be achieved in the serum of dogs, reduction on tumour volumes was seen in a xenograft model of canine mammary tumours in piroxicam-treated mice [14]. In contrast to these findings, deracoxib at a dose of 6 mg/kg had no effect on the growth of canine mammary tumour xenografts in nude mice [14]. Our findings correspond to previous studies that highly significant inhibition of cell proliferation was obtained only at higher concentrations of piroxicam and deracoxib. The observed cytotoxic effects of piroxicam and deracoxib at various concentrations can be cell-type specific as demonstrated by the variation in responses by different tumour cell lines reported in several studies [20, 21].

The mechanism responsible for piroxicam and deracoxib-mediated cell proliferation inhibition in the present study is not known. To elucidate the growth inhibition of CMT-U27 cells by piroxicam and deracoxib, apoptosis assay and cell cycle analyses were performed. Increases in the number of apoptotic cells reached significance (P < 0.05) at doses of piroxicam of 1000 µM and at doses of deracoxib 250 µM above for CMT-U27 cells. However, concentrations required for induction of apoptosis as single treatments of both drugs were high. In combination experiments, the apoptotic effect was seen at much lower concentrations as above 100 µM than that seen with piroxicam and deracoxib as single treatment. The increase in both early and late apoptotic activity seen in CMT-U27 cells with combination treatment indicated that regulation in apoptosis is at least partially responsible for their effect. An increase in early apoptotic activity indicates that the cells are in a static, nonproliferative state, while increases in late apoptotic activity suggest that the cells are in the final stages of the apoptotic, cycle and the cell death is imminent [28]. Experimental studies indicated that apoptosis induction is one of the proposed effects of NSAIDs used in chemoprevention studies [29, 30]. Several mechanisms are described as playing a role in the induction of apoptosis and the mitochondrial pathway seems to be the most common apoptotic mechanism. Cytotoxic stress leads to expression of the proteins of the Bcl family that acts independently or in complexes on the mitochondrial membrane. The degree of apoptosis was shown to correlate with the levels of the expression of Bcl-2, main antiapoptotic protein, and its overexpression in cancer cells ensures their resistance to chemotherapy [31]. As reported in a previous study, Bcl-2 expression was high in CMT-U27 cells [32], thus correlating with the results of our study that high doses of piroxicam and deracoxib were required for the induction of apoptosis in response to apoptotic stimulus. The positive association between apoptosis and proliferation suggests that apoptosis might reflect not only cell loss but also the proliferative activity.

More recent data with non-COX inhibiting NSAIDs and the effectiveness of NSAIDs in COX-deficient cell lines indicated that NSAID-induced growth inhibition and apoptosis may be occurring through COX-independent mechanisms such as cell cycle arrest and inhibition of angiogenesis [33, 34]. In our study, although piroxicam inhibited the cell proliferation dose-dependently, the inhibition was associated with cell death only at the higher dose, suggesting that the drug may inhibit the cell growth by retarding cell cycle progression. Our flow cytometric analyses showed that both drugs at 500 and 1000 µM induced G0/G1 arrest with no significant effect on G2/M transition. Conversely, the percentage of cells in S phase were significantly decreased. However, when these two agents were combined, greater effects were seen. A significant increase of cells in the G0/G1 phase and a significant decrease of cells entering the S phase and G2/M phase were observed showing that the cells were at rest. The cell cycle alterations were achieved at both drug concentrations that repressed CMT-U27 cells growth, indicating that cell cycle arrest is one of the primary mechanisms responsible for the antiproliferative action of deracoxib and

| Drug concentrations | % G0/G1 | % S | % G2/M |
|---------------------|---------|----|--------|
| Control             | 63.8 ± 2.11 | 22.48 ± 1.52 | 13.72 ± 1.50 |
| Piroxicam + deracoxib (50 + 50 µM) | 67.4 ± 3.04 | 19.97 ± 1.63 | 12.63 ± 1.26 |
| Piroxicam + deracoxib (100 + 100 µM) | 64.53 ± 2.51 | 22.27 ± 1.22 | 13.2 ± 1.10 |
| Piroxicam + deracoxib (250 + 250 µM) | 69.58 ± 3.50 | 17.75 ± 1.45 | 12.67 ± 0.76 |
| Piroxicam + deracoxib (500 + 500 µM) | 72.2 ± 2.17 | 19.95 ± 1.12 | 7.85 ± 1.51 |
| Piroxicam + deracoxib (1000 + 1000 µM) | 89.95 ± 3.35* | 8.27 ± 1.81* | 1.78 ± 0.68* |

(50–500 µM) and deracoxib (50–100 µM), but highly significant cell proliferation inhibition was seen after 72 h incubation with 1000 µM piroxicam and 250, 500, and 1000 µM deracoxib. In a previous investigation, it was shown that piroxicam inhibited cellular proliferation of canine mammary carcinoma in vitro at concentrations above 1 µg/mL and induced apoptosis in a dose-dependent manner, and the authors suggested that the significant inhibition of proliferation and induction of apoptosis could only occur when drug concentrations were in excess that can be achieved in vivo following maximum recommended dose rate of piroxicam [21].
piroxicam. Krol et al. [32] reported that growth rate (short cell cycle) and antiapoptotic potential of CMT-U27 cell line were high and spontaneous and induced apoptosis was low. The authors observed that the high growth rate and antiapoptotic potential in CMT-U27 cells were associated with enhanced expression of genes-involved Ca$^{2+}$ signaling pathway (Calmodulin 1, 2, 3, and SPSB2) and growth hormone cellular pathway. The cell cycle length of CMT-U27 line was reported as 53.4 hour, and the distribution of cells was G0/G1 64%, S phase 15%, and G2/M 20%. In the present study, similar to those reported results we found the percentage of cells 63.8%, 22.48%, and 13.72% in G0/G1, S, and G2/M phase, respectively.

Although antiproliferative and apoptotic effects were seen with both drugs, the concentrations we used to obtain significant effects appear to be too high to be achieved in vivo, therefore our results cannot be directly extrapolated to dogs but can provide insight into potential mechanisms of NSAID action in mammary cancer cells. However, intralesional or topical therapy may be appropriate in some types of tumours, and local administration of the drug could increase the concentration to which the tumour is exposed and minimise side effects.

In the present study, the combination of the two inhibitors significantly increased the response over that observed with single agents, suggesting that combined treatment with COX inhibitors may be an attractive approach for the treatment of canine mammary carcinoma. Further, in vivo clinical studies should be considered before recommending their clinical use either alone or in combination with other agents for treatment of mammary cancer in dogs.

Conflict of Interests

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgment

The authors would like to thank Assoc Professor Eva Hellmén of the Department of Anatomy and Physiology, Uppsala University for kind donation of the CMT-U27 cell line. This study was supported by the Research Fund of Istanbul University (project no. 459/27122005).

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