

Effect of selected nonsteroidal anti-inflammatory drugs on the viability of canine osteosarcoma cells of the D-17 line: *in vitro* studies

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Abstract

Introduction: Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in veterinary medicine. They are used in pain control and in anti-inflammatory and antipyretic therapies. Some NSAIDs, *e.g.* piroxicam, also have a documented anticancer effect. The objective of this study was to evaluate which of the commonly used NSAIDs (etodolac, flunixin, tolfenamic acid, carprofen, and ketoprofen) are cytotoxic to the D-17 cell line of canine osteosarcoma. **Material and Methods:** The viability of the cells was evaluated using the MTT assay. Four independent repetitions were performed and the results are given as the average of these values; EC₅₀ values (half maximal effective concentration) were also calculated. **Results:** The analysis of results showed that carprofen and tolfenamic acid displayed the highest cytotoxicity. Other drugs either did not provide such effects or they were very poor. For carprofen, it was possible to determine an EC₅₀ which fell within the limits of concentrations obtainable in canine serum after the administration of routinely used doses. **Conclusion:** The results are promising but further studies should be conducted to confirm them, since this study is only preliminary. The possibility of introducing carprofen and tolfenamic acid into the routine treatment of osteosarcoma in dogs should be considered.

Keywords: dogs, non-steroidal anti-inflammatory drugs, canine osteosarcoma, cytotoxicity assay.

Introduction

The mechanism of non-steroidal anti-inflammatory drugs' (NSAIDs) action is based on the preferential or non-preferential inhibition of the activity of cyclooxygenase-2 (COX-2, the inducible form). This enzyme takes part in the pathway of arachidonic acid transformations which result in the formation of prostanoids. These compounds are involved in the inflammation process and contribute to the development of pain, swelling, or fever. By reducing COX-2 action with the use of NSAIDs, it is possible to reduce prostanoid synthesis, and thereby the activity, extent, and intensity of inflammatory processes. NSAIDs are commonly used in veterinary medicine not only because of their anti-inflammatory effect but also due to their analgaesic and antipyretic properties. However, their most interesting feature is their potential anticancer effect.

Osteosarcoma is a primary malignant bone tumour of mesenchymal origin with a very diverse histological structure. Osteosarcoma is diagnosed in approximately 80%–85% of dogs with bone cancer, making it the most common lesion in this group of tumours. Seen in the context of all cancer types diagnosed in dogs, however, the frequency of osteosarcoma is considered moderate. Osteosarcoma usually occurs in adult individuals in the age range of 2–15 years. Large and giant breeds, such as the German Shepherd, St. Bernard, Irish Setter, Great Dane, Bernese Mountain Dog, Boxer, Golden Retriever, Rottweiler, and Doberman Pinscher (17, 21), are most predisposed to this disease. The area prone to develop this type of cancer in dogs are the long bones which are most exposed to overloads and related microtraumata, which explains their markedly more frequent occurrence in large and giant breeds (15). Osteosarcoma is less commonly encountered in flat bones (the maxilla, mandibula, skull, scapulae, ribs, and pelvis). This cancer

belongs to the group of particularly aggressive proliferative lesions causing bone damage visible in radiographic images. It spreads quickly to the surrounding soft tissues and also often metastasises to nearby (regional) lymph nodes and lungs as well as other bones, the skin and the liver (8, 11, 19).

The treatment consists primarily in the amputation of the tumour-affected extremity and adjuvant chemotherapy. The most commonly used drugs include: platinum derivatives (cisplatin and carboplatin) and anthracycline antibiotics (doxorubicin). To alleviate pain symptoms associated with osteosarcoma, NSAIDs are used very often, while opioid receptor agonists are administered less frequently.

The objective of this experiment was to assess how NSAIDs from different groups varying in terms of their chemical structure affect cell viability in a specific cell line (D-17) of canine osteosarcoma, and whether they show any evidence of antitumour activity.

Material and Methods

Active substances and cell line. The active substances were all acid derivatives and were all procured from Sigma-Aldrich (Germany). The pyrazinoic acid derivative used was etodolac, the anthranilic acid derivatives were flunixin and tolfenamic acid, and the propionic acid derivatives were carprofen and ketoprofen. The canine osteosarcoma cell line (D-17) was acquired from the American Type Culture Collection (ATCC, USA).

Culture conditions and viability assessment. Cell cultures were grown at 37°C in culture flasks with an area of 25 cm² in an MCO-18AIC incubator with a constant 5% flow of CO₂ (Sanyo, Japan). Commercial Eagle's Minimum Essential Medium (EMEM) culture medium (ATCC) was supplemented with 10% foetal bovine serum (Sigma-Aldrich, USA), 4 nM of L-glutamine (Sigma-Aldrich, UK), 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma-Aldrich, Germany).

Etodolac, flunixin, and tolfenamic acid were dissolved in 70% ethyl alcohol (Stanlab, Poland). Carprofen and ketoprofen were dissolved in a 1:1 mixture of 70% ethyl alcohol and double distilled water.

Etodolac, flunixin, and tolfenamic acid were tested at concentrations of 0.05, 0.1, 0.5, 1, 5, 10, and 20 µg/ml, while carprofen and ketoprofen assay concentrations were the same and additionally 40 µg/ml. The concentrations in which these compounds were tested were established based on those in the literature (20), the maximum concentrations they reach in canine serum, and the permissible maximum non-cytotoxic solvent concentration. As a positive control, non-treated cells were used, and as a negative control, cells were challenged with doxorubicin at a concentration of 0.5 µg/ml.

The cells treated with indicated concentrations of the tested drugs were incubated for 72 h, and then their viability was assessed with the MTT assay. Each well on the culture plate was supplied with 20 µl of MTT solution (Sigma-Aldrich, USA), and the cells were incubated for an additional 4 h. Then 80 µl of lysis buffer was added to the samples. After 24 h, the optical density of the samples was measured with an ELx800 plate reader (BioTek, USA). Four independent repetitions were performed for each of the test drugs, and their results are provided as the average. In addition to a viability assessment, an attempt was made to determine the EC₅₀ (*i.e.* half maximal effective concentration), or the drug concentration where the cell viability in the tested samples was 50%.

Statistical analysis. The statistical analysis was carried out using the Statistica 12.0 programme (StatSoft, now Tibco, USA). Data normality was checked using the Shapiro–Wilk test. The comparison of relationships was made using the Kruskal–Wallis test, and comparison of compounds with the positive and negative control was arrived at using the Mann–Whitney U test. The level of significance was assumed to be $P < 0.05$.

Results

Cell viability assay. Cell viability in the positive control was $96.08 \pm 2.05\%$ and in the negative control it was $12.28 \pm 1.15\%$. Figs 1 and 2 show the mean viability percentages of the treated cells and the standard deviations.

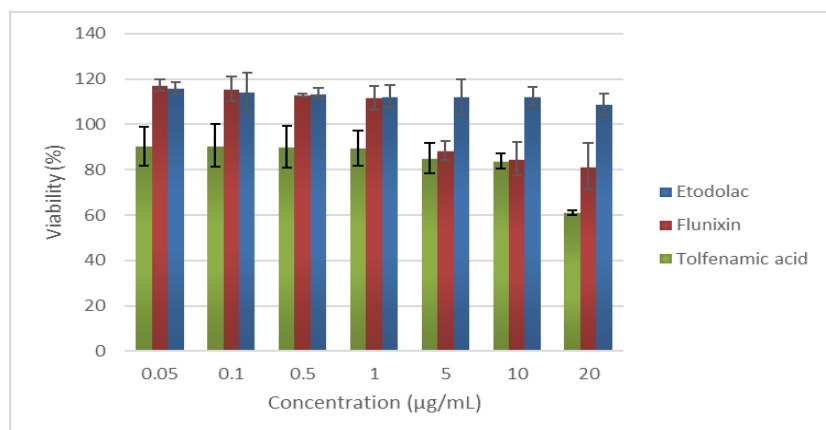


Fig. 1. Effect of etodolac, flunixin, and tolfenamic acid on the viability of D-17 canine osteosarcoma cell line

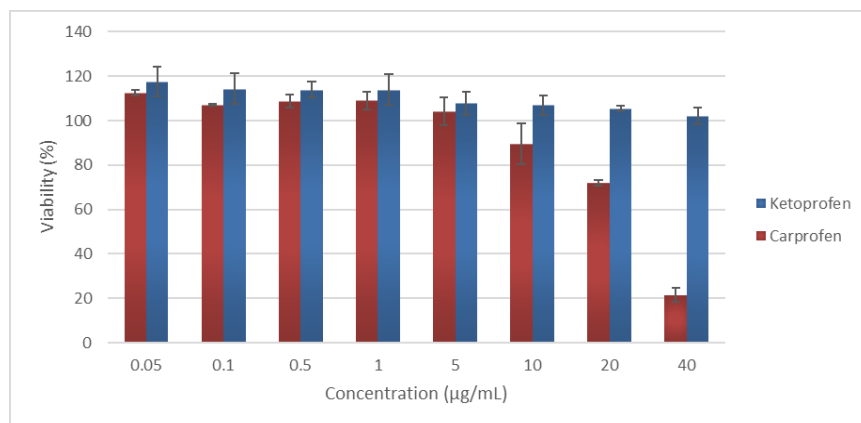


Fig. 2. Effect of carprofen and ketoprofen on the cell viability of D-17 canine osteosarcoma cell line

Carprofen displayed concentration-dependent cytotoxicity within the tested concentration ranges (Fig. 2). The strongest cytotoxic effect of the test drug was observed in the samples treated with concentrations of 20 and 40 µg/ml, where the cell viability was $71.98 \pm 1.16\%$ and $21.56 \pm 3.33\%$, respectively.

Ketoprofen showed no cytotoxic activity in any but the highest concentration tested, which was only used for experimental purposes, because it is not possible to achieve such a concentration in the patients' serum (Fig. 2). The maximum obtainable concentration was approximately 2 µg/ml.

Statistical analysis showed significantly different values for the tested compounds compared to the positive and negative controls ($P = 0.03$ for each compound). From the graph of Fig. 2, we can see that carprofen possessed the strongest cytotoxic activity against canine osteosarcoma cells, however, due to the low number of repetitions made during this study, the statistical picture only demonstrates that it works more strongly than etodolac ($P = 0.02$).

EC₅₀ values. The values of EC₅₀ are shown in Table 1. Only with carprofen was the EC₅₀ within the range of concentrations used for testing and it was 28.71 ± 2.31 µg/ml. For other drugs, the EC₅₀ was above the concentration range used and the maximum concentration they can have in the patient's serum.

Table 1. EC₅₀ values for the drugs tested

Drug	EC ₅₀
Etodolac	> 20 µg/mL
Flunixin	> 20 µg/mL
Tolfenamic acid	> 20 µg/mL
Carprofen	28.71 ± 2.31 µg/mL
Ketoprofen	> 40 µg/mL

Discussion

The NSAIDs we used in the experiment are applied with lower or higher frequency in anti-inflammatory and analgesic therapies in dogs. The most widely used NSAID is carprofen, which turned out to have the

strongest cytotoxic activity, as the analysis of results and their comparison with the results across the complement of drugs proved. It is interesting and promising that cytotoxic concentrations are easily achieved in the serum of patients through the administration of a standard anti-inflammatory and analgesic dose, *i.e.* approximately 4 mg/kg. Furthermore, it is worth noting that carprofen is well tolerated by patients and can be used in long-term therapies, which in combination with the results we obtained, could represent a starting point for further *in vitro* and subsequent *in vivo* studies on the use of this drug in the treatment of canine osteosarcoma. Moreover, carprofen is the only drug tested of which the EC₅₀ could be determined, and this is also a valuable piece of information. Pang *et al.* (13) obtained similar results in their studies with different cancer cell lines, including D-17 osteosarcoma. They demonstrated the cytotoxic effect of carprofen and determined an EC₅₀ that was similar to that obtained in our experiments. However, it is worth noting that in their study, they used concentrations exceeding the ones obtainable in the patients' serum, and that the EC₅₀ was also slightly higher than the said values (≈ 61.8 µg/ml). They also used a different cell incubation time for the test drug. A similar effect, *i.e.* a suppressed viability of tumour cells was observed by Khwaja *et al.* (9) in their experiments on human prostate cancer cell lines. It should be noted, however, that carprofen-containing drugs for humans have not been available on the Polish or global markets for several years.

It is worth highlighting that carprofen is generally very well tolerated by patients, which may be an additional argument in favour of using this drug in the treatment of osteosarcoma in dogs. However, to make an unequivocal statement that carprofen can be introduced for routine use in the treatment of this type of tumours a series of additional studies would be required. The results obtained in this experiment may be a starting point for further research. It should be emphasised that there is little information available in the literature on the effect of carprofen on animal cancer cells.

Tolfenamic acid showed cytotoxic activity at a concentration greater than its obtainable level in canine

serum after the application of a standard dose. However, Wilson *et al.* (20) used similar drug doses in their studies, recording the inhibition of cell growth in two canine osteosarcoma lines (UWOS1 and UWOS2). Based on the research literature, they concluded that double the standard dose of 4 mg/kg of tolfenamic acid was safe for the patient (7, 10, 16), thereby allowing a higher concentration of tolfenamic acid in serum, and the emergence of a cytotoxic effect. Similarly, in the studies on cell lines of human lung cancer, neuroblastoma, pancreatic cancer, oesophageal cancer, ovarian cancer, and prostate cancer (1, 2, 4, 5, 6, 14), a significant reduction in the viability of test cells was observed after the application of tolfenamic acid. This information is important since it may offer an additional argument for considering the possibility of introducing tolfenamic acid into anti-neoplastic therapy regimens. However, due to the very limited information on the long-term use of tolfenamic acid in dogs and its effect on cancer cells, a series of additional studies both *in vitro* and *in vivo* is required to confirm the results obtained.

The investigation showed that at obtainable concentrations in canine serum, flunixin does not produce a satisfactory cytotoxic effect. There are also no reports on studies conducted on animal cancer cell lines with this drug. The results we obtained are not promising and raise questions as to the desirability of further testing with flunixin and possibility of its use in the treatment of osteosarcoma in dogs. Testing higher concentrations of flunixin is pointless, because higher serum concentrations with potentially cytotoxic effects might cause an overdose. Unfortunately, there are no clinical studies showing the symptoms of flunixin overdose. The situation with ketoprofen is similar. The maximum obtainable concentration is approximately 2 µg/ml. It does not manifest any cytotoxic action, unlike carprofen which comes from the same chemical group. There are also few data on *in vitro* studies with ketoprofen.

In this study, no cytotoxic effect of etodolac on canine osteosarcoma cells from the D-17 line was recorded. Research literature contains several reports confirming its inhibitory action on cancer cells but it must be noted that these reports regard human and not animal cells. There is no information available on trials with animal cells. For human cell lines, satisfactory results were obtained in studies using prostate cancer or bladder cancer cells, among others (12, 18). Chen *et al.* (3) demonstrated in their *in vitro* study with Matrigel that etodolac was capable of reducing the invasiveness and liver metastasis of human colon cancer. That there was no effect of etodolac on osteosarcoma cells can be explained by its insufficient concentration or the resistance of the D-17 osteosarcoma cell line to its action. The concentration of 20 µg/ml is the highest one obtainable in canine serum after oral administration of a standard dose of this drug with anti-inflammatory and analgaesic effects; that standard dose is 12–17 mg/kg. A serum concentration exceeding 20 µg/ml may result in an etodolac overdose, which may cause extensive and

possibly lethal ulceration of gastrointestinal mucosa. However, to definitively determine what the real cause is of etodolac's ineffectiveness, it would be necessary to carry out further studies.

In summary, the studies we completed are only preliminary and limited to the assessment of any potential cytotoxic action of the test drugs. Among all the NSAIDs used in the experiment, the most effective and promising are carprofen and tolfenamic acid. The possibility of their introduction into the routine treatment of osteosarcoma in dogs should be considered. However, further *in vitro* and *in vivo* studies are required beforehand.

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