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ORIGINAL ARTICLE

Selective inhibition of cyclooxygenase-2 by enflicoxib, its enantiomers and its main metabolites in vitro in canine blood

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Abstract

Enflicoxib is approved for the treatment of pain and inflammation in canine osteoarthritis. The objective of this work was to assess the mechanistic basis of enflicoxib therapy investigating the COX inhibitory activity of enflicoxib (racemate), its enantiomers and its main metabolites using the canine whole blood assay. The (R)-(+)-Enflicoxib enantiomer and metabolite M8 (hydroxylated pyrazoline) did not induce significant COX inhibition. Enflicoxib and its (S)-(-)-Enflicoxib enantiomer inhibited COX-1 and COX-2 with variable degree of preferential isoform inhibition, but no significant therapeutic effect is anticipated in vivo. The pyrazol metabolite showed the highest COX-2 inhibition and was the most selective (IC_{50} COX-1/ COX-2 ratio: 19.45). As the pyrazol metabolite shows saturable binding to red blood cells, its in vivo concentrations in plasma are lower than in whole blood. Accordingly, when applying the red blood cell partitioning, the respective IC_{50} and IC_{80} for COX-2 inhibition decreased from 2.8 μM (1129 ng/ml) and 13.4 μM (5404 ng/ml) to 0.2 μM (80.7 ng/ml) and 1.2 μM (484 ng/ml) and the selectivity ratio increased to close to 55. The corrected pyrazol metabolite IC_{50} and IC_{80} are well within the plasma levels described in treated dogs.

KEYWORDS
blood, COX-2, dog, enflicoxib, metabolites, pyrazol

1 | INTRODUCTION

Prostanoids constitute a group of lipid mediators involved in key physiological processes, among them, the promotion of inflammatory responses. They are derived from arachidonic acid through the cyclooxygenase pathway, the enzyme that primarily catalyses its transformation to the endoperoxide common intermediate PGG_2 (Smith., 1992; Smith & Marnett, 1990). Two isozymes of cyclooxygenase co-exist. The COX-1 has a widespread constitutive expression, and it is mainly involved in the formation of mediators of homeostatic body processes. The COX-2 is mostly undetectable in tissues in normal non-pathological conditions. Although there is evidence of constitutive expression in specific organs such as kidney, where it has been postulated to potentially cause harmful effects in case of inhibition (Harris, 2013), COX-2 expression is mostly induced and it is synthetized in response to pro-inflammatory stimuli and in pathologic conditions such as cancer or neurodegeneration (Simmons et al., 2004; Vane et al., 1998; Xie et al., 1991).

Non-steroidal anti-inflammatory drugs (NSAIDs) belong to a wide drug group of cyclooxygenase inhibitors. It is accepted that most side effects of NSAIDS are related to their anti-COX-1 activity affecting predominantly the gastrointestinal tract (GI), while the

therapeutic anti-inflammatory effects are mainly driven by COX-2 inhibition (Hawkey, 2001; Verburg et al., 2001). To overcome the side effects on the GI tract associated with COX-1 inhibition, a group of selective COX-2 inhibitors, also known as coxib drug class of NSAIDs, have been developed during the last decades (Bergh & Budsberg, 2005; FitzGerald & Patrono, 2001). While the therapeutic benefit of selective COX-2 inhibitors *vs*. traditional non-selective NSAIDs lies in their ability to reduce GI ulceration and bleeding, their adverse reactions cannot be overlooked. Some studies suggest that users of COX-2 selective drugs may be at increased risk for cardiovascular (CV) events, but this is a controversial matter and more recent studies suggest that not all COX-2 selective inhibitors are the same in terms of CV safety (Walker, 2018), that COX-2 selectivity may not play a role in the CV risk of NSAIDs (Gunter et al., 2017) and that both COX-2 inhibitors and traditional NSAIDs can cause side effects related to heart and blood pressure (Katz, 2013). Accordingly, the Food and Drug Administration has since grouped all NSAIDs, whether COX-2 selective or non-selective, into one class with similar warnings regarding GI, renal, CV and skin side effects. Still, NSAID-induced adverse effects in companion animals are related not only to the safety profile of the drug itself, but also to the administered dose, the dose interval and the route of administration (McLean & Khan, 2018; Monteiro & Steagall, 2019).

Enflicoxib is a pyrazoline drug of the coxib class that shows selective COX-2 inhibition in vitro and in vivo (Wagemakers et al., 2009). It has been recently approved for the treatment of pain and inflammation associated with osteoarthritis in dogs with a proposed therapeutic dosage schedule consisting of an 8 mg/kg loading dose followed by once-a-week dose of 4 mg/kg (European Commission, 2021). It is a racemic mixture of two enantiomers: (S)-(-)-Enflicoxib and (R)-(+)-Enflicoxib (Calvet et al., 2002). In in vitro and in vivo studies, (S)-(-)-Enflicoxib has been demonstrated to be the active enantiomer while the pharmacological activity of (R)-(+)-Enflicoxib is negligible (Cendrós et al., 2021; Iñiguez et al., 2010).

Following oral absorption in dogs, the pyrazoline ring of enflicoxib is transformed into two main phase I metabolites: a major hydroxylated pyrazoline metabolite (namely M8) and a pyrazol metabolite (See Figure 1). The results from in vitro studies using isolated cyclooxygenase enzymes and in vivo in inflammation models in rats have proven that pyrazol metabolite is largely more active and COX-2 selective than the parent compound (Wagemakers et al., 2009). In addition, the pyrazol metabolite shows marked, saturable binding to red blood cells, plasma concentrations at therapeutic levels being considerably lower than those at whole blood, and the opposite expected at very high circulating concentrations (Homedes et al., 2021). Moreover, the pyrazol metabolite shows slow rates of formation and elimination together with a high volume of distribution in vivo. Consequently, the circulating levels of the pyrazol metabolite become sustained over time after administration and are considered the basis for the long-lasting therapeutic efficacy of enflicoxib (Homedes et al., 2021).

Both the hydroxylated pyrazoline and the pyrazol metabolites are effectively formed from both enflicoxib enantiomers through the action of cytochrome P450 enzyme system (Pretel et al., 2001). The latter displays no chirality, while the hydroxylated pyrazoline metabolite contains two chiral centres and is considered as the major enflicoxib metabolite accounting for a relevant fraction of the AUC of the drug-related material in vivo (more than half of the enflicoxib's AUC with very similar elimination slope, Homedes et al., 2021). However, neither the isomeric nature of M8 formed in vivo, nor its pharmacological activity have been studied to date.

In the present work, the inhibitory effect of enflicoxib, its two separate enantiomers, its major metabolite M8 and its active pyrazol metabolite on COX-1 and COX-2 have been investigated in vitro using whole blood from healthy adult Beagle dogs, with the aim to provide relevant information to support the mechanistic basis of enflicoxib therapeutic efficacy. COX-1 activity was measured by determining the formation of thromboxane B_2 (TXB_2) arising from the active and short-lived thromboxane A_2 (TXA_2) mainly in blood platelets during the clotting process (Alessandrini et al., 1985). For COX-2 inhibition, the formation of prostaglandin E_2 (PGE_2) in whole blood after stimulation with lipopolysaccharide (LPS) was determined (Brideau et al., 1996). Prior to the inhibition experiments, the isomeric characterization of the hydroxylated pyrazoline metabolite, M8, formed in vivo in dogs following enflicoxib administration was assessed in plasma by liquid chromatography coupled to mass spectrometry (LC-MS).

2 | MATERIALS AND METHODS

2.1 | Chemicals

Racemic enflicoxib and its separate enantiomers (S)-(-)-Enflicoxib and (R)-(+)-Enflicoxib [molecular weight (MW 405.34; purity >99.5%) were synthesized by Esteve (Barcelona, Spain); the pyrazol metabolite (MW 403.33; purity 99.03%) and the hydroxylated pyrazoline metabolite (M8, racemic -anti and -syn diastereoisomers, MW 421.05; purity 98.9% and 92.3% respectively) were synthesized by GalChimia (A Coruña, Spain). TXB_2 and PGE_2 ELISA kits (96 Solid Wells, Refs. 501020 and 514010 respectively) were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). All reagents including LPS (from *E. coli* 0111:B4) and the solvents for LC-MS analysis were purchased from Merck Sigma-Aldrich (St. Louis, MO) except where indicated otherwise.

2.2 | Dog whole blood samples

Whole blood was freshly obtained from Beagle dogs at Isoquimen S. L. (Sant Feliu de Codines, Barcelona, Spain). The animal facilities are managed under UNE-EN ISO 9001:2015 regulations and accredited for Good Laboratory Practices (GLP, OECD, 1997 adopted on Spanish Royal Decree 1369/2000). The study was reviewed and approved by the study site Institutional Animal Care and Use Committee and it followed current animal welfare recommendations.

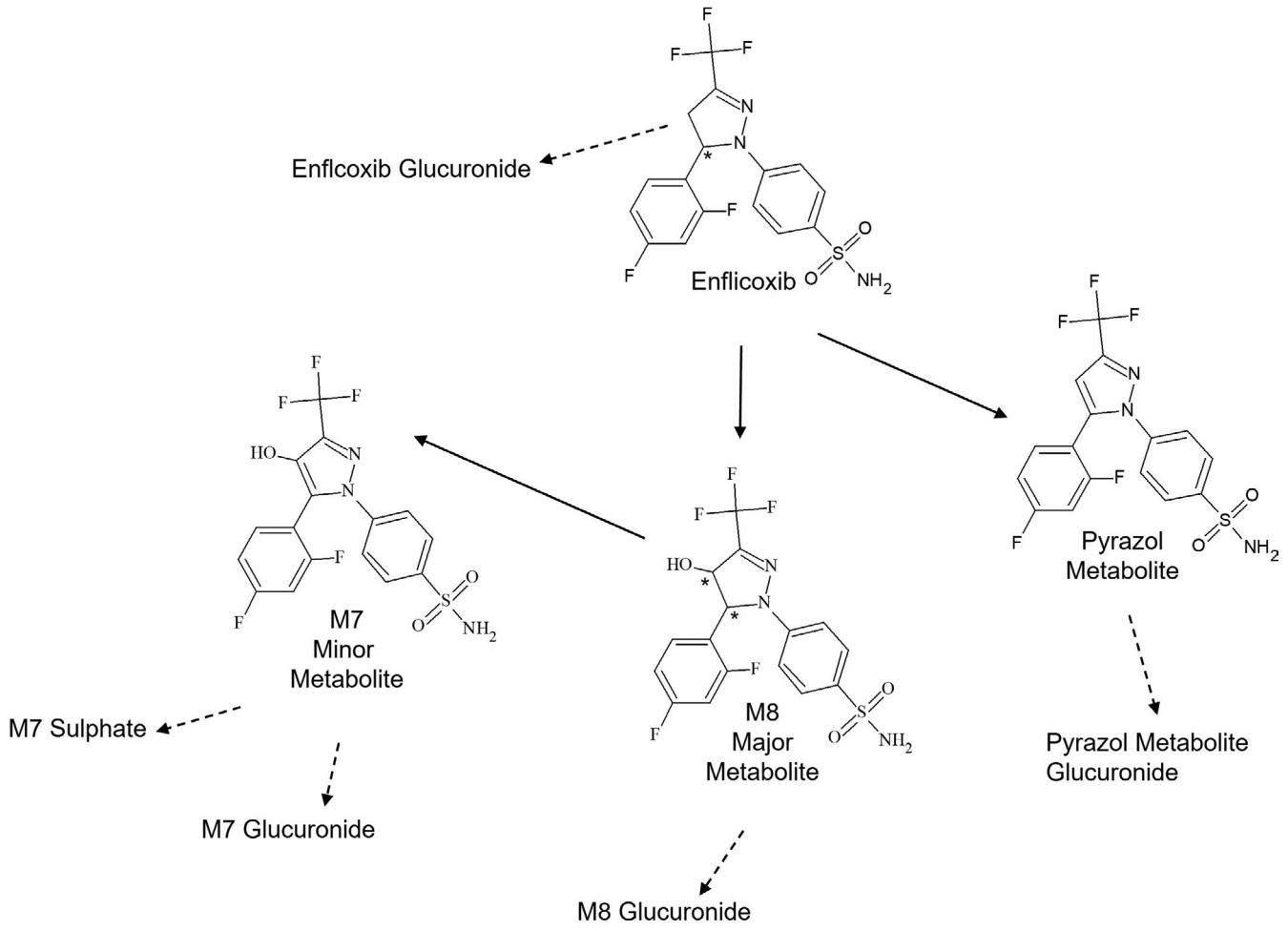

FIGURE 1 Metabolic pathway of enflicoxib showing the chemical structures of enflicoxib, its active pyrazol metabolite and its hydroxylated pyrazoline major metabolite M8. Asterisks indicate chiral centres. (Extracted from Solà et al., 2021)

Twelve non-naive Beagle dogs (nine males and three females of 9 months to 5 years old) were used to prepare two sets (batches) of incubations. Each batch consisted of whole blood from six separate donors. Batch 1 was used for COX-1 inhibition, and batch 2 was used for COX-2 inhibition. Animals were subjected to physical examination before being considered suitable for the study and were non-pregnant and non-lactating. In addition, they had not received any drug treatment at least within the month preceding the study.

Blood from batch No. 1 was extracted from six individual males in tubes without anticoagulant; blood from batch No. 2 was extracted separately from three males and three females in tubes containing sodium citrate as anticoagulant and was used after 4–6 h storage at *ca* 2–8°C.

The whole blood assays were performed without anticoagulant for TBX_2 formation, as it is mainly produced during clotting as a response of the platelets to thrombin stimulation. For PGE_2 formation the assays were performed with anti-coagulated blood under 24 h LPS stimulation in order to assess the long-term induction of COX-2 enzyme mainly in leukocytes.

2.3 | Stock and working solutions

Stock and working solutions of enflicoxib, (R)-(+)-Enflicoxib, (S)-(-)-Enflicoxib, M8 metabolite and pyrazol metabolite were prepared freshly at 125 mM concentration in dimethylsulfoxide (DMSO). Working solutions were also prepared freshly in DMSO at concentrations 250-fold higher than the final ones in the blood incubates, which were 500, 300, 100, 30, 10, 3, 1 and 0 μM for COX-1 inhibition and 100, 30, 10, 3, 1, 0.3, 0.1 and 0 μM for COX-2 inhibition assessment. For (R)-(+)-Enflicoxib in the COX-2 experiments, the maximum concentration assayed was 30 μM.

A stock solution of LPS was prepared at 500 μg/ml concentration in phosphate-buffered saline (PBS, Corning, Manassas, Virginia, USA) and was stored at *ca* −20°C in aliquots until use.

2.4 | Incubations for COX inhibition

Incubations were performed at *ca* 37°C in duplicate in commercially available plastic low binding tubes specially designed to reduce

protein binding (Eppendorf International, Hamburg, Germany). For each incubate, the volume of blood was 0.5 ml. Incubations were started by spiking the blood samples with 2 µl of the corresponding test compound working solution (alternatively DMSO vehicle for concentration = 0). The concentration of DMSO in all the incubates was the same (0.4% v/v).

For COX-1 activity assessment, blood from the individual dogs of batch 1 was used immediately after extraction. Two microliters of PBS was immediately added after addition of test compound. The samples were gently stirred and placed in a Thermomixer incubator (Eppendorf International) at *ca* 37°C and were incubated for 1 h with gentle shacking. For COX-2 assessment, blood from the individual dogs of batch 2 was used. Two microliters of 500 µg/ml LPS in PBS was immediately added after addition of inhibitor (1 µg/ml LPS final concentration). The samples were gently stirred were placed in an orbital tube agitator inside and oven (JP Selecta, Abrera, Barcelona, Spain) set at *ca* 37°C and were incubated for 24 h. After incubation, blood samples were immediately placed in ice–water bath to stop reactions until were centrifuged at 1300 ×g for 15 min at 4°C to obtain serum for TXB_2 determination, or at 2000 ×g for 10 min at 4°C to obtain plasma for PGE_2 determination. The serum or plasma from each of the two individual replicates for each dog and test compound concentration was subsequently pooled and immediately stored frozen at *ca* −80°C until analysis.

2.5 | Sample analysis for TXB_2 and PGE_2 determination

The samples were analysed using specific ELISA kits for TXB_2 and PGE_2 following the instructions supplied by the manufacturer. The quantification standards for both analytes are included in each ELISA kit. For TXB_2 determination, the calibration curves were prepared at the following concentration levels using ELISA buffer as diluent: 1000 (duplicate), 400, 160, 64, 25.6, 10.2, 4.1 and 1.6 ng/ml (duplicate). For PGE_2 determination, the calibration curves were prepared at the following concentration levels also using ELISA buffer as diluent: 1000 (duplicate), 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 ng/ml (duplicate). Quality control samples (QC) were included in each analytical batch at three concentration levels in duplicate: 600, 300 and 100 ng/ml for TXB_2 and 700, 300 and 50 ng/ml for PGE_2. The test samples were serially diluted with ELISA buffer to final dilution factors of 1:3000 for TXB_2 and 1:1000 for PGE_2. The sample dilution factors for each analyte were the same for all study samples and showed no plasma/serum matrix effect due to the high concentrations of TXB_2 and PGE_2 reached after stimulation/induction. The amount of matrix in each assay was of 0.03% serum for TXB_2 determination and of 0.1% plasma for PGE_2 determination. Due to that, the analyte levels in the samples could be analysed without any previous extraction procedure.

2.6 | Analytical runs and quantification

The absorbance at λ: 412 nm of the 96-well plates obtained after ELISA chromophore development was measured in a plate reader (Synergy HTX, Biotek-Agilent, Vermont, USA). After blank and non-specific binding subtraction, the absorbance in each sample was normalized by the maximum binding value (B0, from wells containing non-standard or test samples) giving the response value. The response values in the calibration samples were fitted to a four-parameter logistic curve using the on-line software www.myassays.com. At least six calibration points were used to construct the calibration curves. The individual accuracy of back-calculated values was within 20% of the nominal value (25% for the lower limit of quantification). Analytical runs were accepted if at least 67% of the QC samples (50% at each concentration level) were within 20% of the nominal value. Acceptance criteria for calibration curve and QC samples were adopted from FDA and EMA guidelines on bioanalytical method validation and test sample analysis (European Medicines Agency, 2012; U.S. Department of Health and Human Services, Food and Drug Administration (FDA), 2018).

2.7 | Calculations and statistical analysis

For each separate dog and test compound concentration, TXB_2 or PGE_2 concentration data were fitted to the 4-parameter Hill equation (sigmoid model, see below) by means of least squares non-linear fitting using GraphPad Prism V8 Software. The curve parameters values were estimated.

$$\text{Hill Equation: } E = E_0 \times (1 - (C^\gamma / C^\gamma + IC_{50}^\gamma))$$

where

E is the enzymatic activity (as total TXB_2 or PGE_2 formation during the incubation period), E_0 is the enzymatic activity in the absence of test compound (basal TXB_2 or PGE_2 formed after stimulation/induction), C is the concentration of test compound, IC_{50} is the test compound concentration that causes 50% inhibition of E_0, and is the sigmoidicity factor.

IC_{20} and IC_{80} values were calculated from the IC_{50} and the sigmoidicity factor of the Hill Equation, using the on-line tool QuickCalcs GraphPad (www.graphpad.com/quickcalcs/).

Separate one-way ANOVA tests were used to compare IC_{50}, IC_{20} and IC_{80} parameters among each test compound. Differences between the mean value of each parameter (mean from 6 individual dogs) were assessed using Tukey test.

For COX-2 selectivity, a paired t test was carried out to assess the significance of IC_{50} ratios COX-2 *vs* COX-1, respectively, for each test compound respectively.

Statistic comparisons were performed using GraphPad Prism V8 Software, and the significance level was set at $p < .05$.

3 | RESULTS

3.1 | Inhibition of COX-1 and COX-2 in vitro in dog blood

Two analytical runs were performed, respectively, for TXB_2 and PGE_2 determination. The mean accuracy for the back-calculated values of the calibration curves ranged from 90% to 110% (TXB_2) and 93% to 110% (PGE_2) of their nominal concentrations. The overall accuracy of the QC samples at the three concentration levels ranged from 96% to 109% (TXB_2) and from 99% to 106% (PGE_2). The four runs complied the accuracy acceptance criteria for the calibration curve and QC samples described in the materials and methods, and therefore, they were accepted.

Table 1 shows the results from the serum determinations of TXB_2 in the incubations of canine blood used for COX-1 activity assessment. The inhibition plots for TXB_2 formation in the presence of the test items are shown in Figure 2. Metabolite M8 and the (R)-(+)-Enflicoxib enantiomer did not induce a decrease of the TXB_2 levels. Therefore, IC_{50} and IC_{80} values were above the highest concentration tested of 500 μM. On the contrary, enflicoxib, the (S)-(-)-Enflicoxib enantiomer and the pyrazol metabolite effectively inhibited COX-1. The estimated respective COX-1 inhibition parameters were 37.5, 23.9 and 50.8 μM (for IC_{50}); 17.6, 10.6 and 22.6 μM (for IC_{20}); and 100.3, 169.3 and 132.8 μM (for IC_{80}). Due to experimental variability (Table 1), no statistically significant differences between compounds were found other than between enflicoxib and (S)-(-)-Enflicoxib enantiomerfor IC_{80} parameter.

Table 2 shows the results from the plasma determinations of PGE_2 in the incubations of whole blood from dogs used for COX-2 activity assessment. Enflicoxib, (S)-(-)-Enflicoxib enantiomer and the pyrazol metabolite effectively inhibited PGE_2 formation. The COX-2 inhibition parameters estimated for all three compounds are shown in Table 2 and were, respectively, 11.7, 6.5 and 2.8 μM (for IC_{50}); 4.0, 2.4 and 0.8 μM (for IC_{20}); and 64.6, 38.3 and 13.4 μM (for IC_{80}). All three compounds showed statistically significant lower IC_{50} values for COX-2 than for COX-1 (Table 3). Figure 2 show the plots corresponding to the percentages of inhibition of PGE_2 formation. Metabolite M8 and (R)-(+)-Enflicoxib enantiomer did not induce decrease of the PGE_2 levels (see Table 2 and Figure 2). For M8, IC_{50} and IC_{80} were above 100 μM, while for E-6231 were above 30 μM which were the highest concentrations tested.

Table 3 show the selectivity values for COX-2 inhibition (IC_{50} ratio for TXB_2 *vs* PGE_2 formation). The selectivity rank order for these compounds was as follows: enflicoxib (3.2) ≈ E-6232 (3.8) < E-6132 (19.5). As expected, the selectivity ratio between enflicoxib and its active enantiomer (S)-(-)-Enflicoxib showed no differences. Importantly, the COX-2 selectivity was largely higher for the active pyrazol metabolite than for enflicoxib or (S)-(-)-Enflicoxib.

4 | DISCUSSION

The long-lasting pharmacodynamic activity of enflicoxib is attributed to its active pyrazol metabolite. However, another phase I metabolite, M8, is thought to account for most of the fraction metabolized of enflicoxib accounting for more than 50% of enflicoxib AUC in vivo and about 7% of the total drug-related material in vitro in liver microsomes (Solà et al., 2021). While the pyrazol metabolite is a non-chiral molecule, the chemical structure of M8 contains two chiral centres, and therefore, two diastereoisomers are chemically possible. The first step of the present work was the isomeric characterization of M8 formed in vivo. This was performed by means of LC-MS characterization in plasma samples from dogs orally dosed with 8 mg/kg enflicoxib (Homedes et al., 2021). The results of these analyses are shown as supporting information to this manuscript and demonstrate that M8-anti is the major M8 component, meaning that the transformation of enflicoxib to M8 in dogs is stereo-selective.

The data from the assays of inhibition of COX-1 in the absence of inhibitor showed mean basal-stimulated TXB_2 levels that ranged

TABLE 1 In vitro inhibition of COX-1-mediated TXB_2 formation in dog blood by enflicoxib, its enantiomers and its main phase I metabolites

	IC_{50} (μM)		IC_{20} (μM)		IC_{80} (μM)		Gamma	
Compound	**Mean**	***SEM***	**Mean**	***SEM***	**Mean**	***SEM***	**Mean**	***SEM***
Enflicoxib	37.5	3.8	17.6	2.0	100.3†	9.5	1.7	0.1
(S)-(-)-Enflicoxib	23.9	1.9	10.6	0.6	169.3†	10.3	1.8	0.2
(R)-(+)-Enflicoxib	>500	–	na	–	>500	–	na	–
Pyrazol Metabolite	50.8	12.5	22.6	8.0	132.8	18.6	1.7	0.4
M8	>500	–	na	–	>500	–	na	–

Note: Estimates of IC_{50}, IC_{20}, IC_{80} and sigmoidicity factor (gamma). Parameters were estimated after non-linear fitting TXB_2 formation vs. compound concentration data to the four-parameter Hill equation. Results are expressed as mean ± standard error (*SEM*) of blood incubates from six individual dogs.

Statistical analysis was performed by means of one-way ANOVA and Tukey test for mean comparisons. Significance: $p < .05$.

Abbreviation: na, not applicable.

†Statistically significant differences between the highlighted treatments

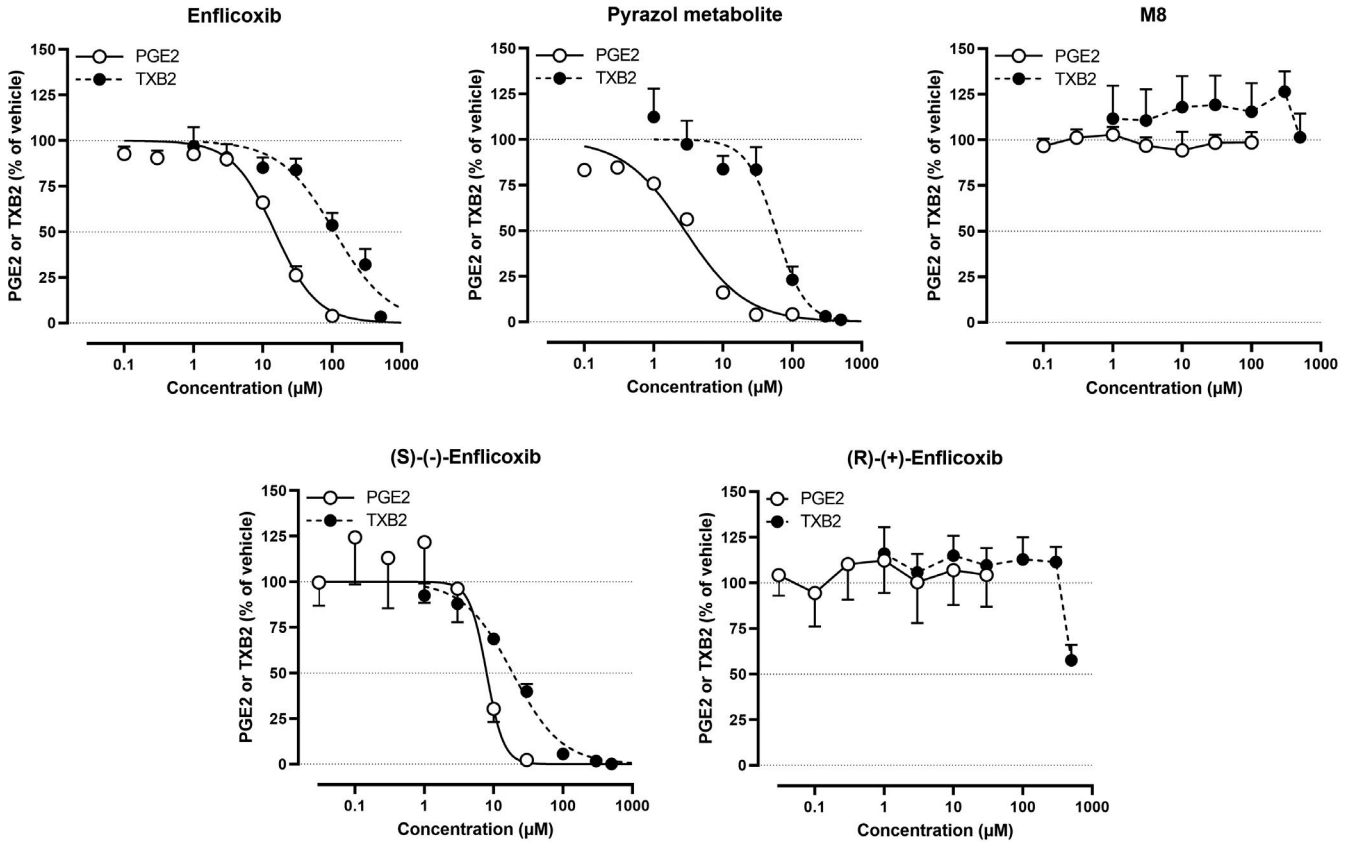

FIGURE 2 Inhibition curves for TXB_2 formation (COX-1, filled circle symbols) and PGE_2 formation (COX-2, circle empty symbols) in canine blood by enflicoxib, the pyrazol metabolite, the hydroxy pyrazoline (M8) metabolite, (S)-(-)-Enflicoxib enantiomer and (R)-(+)-Enflicoxib enantiomer. Results are expressed as mean ± *SEM* of blood incubates from six individual dogs and fitted percentages of inhibition from vehicle control vs. concentration of inhibitor (logarithmic scale)

TABLE 2 In vitro inhibition of COX-2-mediated PGE_2 formation in LPS-stimulated dog blood by enflicoxib, its enantiomers and its main phase I metabolites

	IC_{50} (μM)		IC_{20} (μM)		IC_{80} (μM)		Gamma	
Compound	Mean	*SEM*	Mean	*SEM*	Mean	*SEM*	Mean	*SEM*
Enflicoxib	11.7†	0.6	4.0†	0.4	64.6†	2.0	4.3	0.9
(S)-(-)-Enflicoxib	6.5†,‡	0.4	2.4†,‡	0.2	38.3†,‡	2.4	2.7	0.3
(R)-(+)-Enflicoxib	>30	–	na	–	na	–	na	–
Pyrazol metabolite	2.8†,‡	0.1	0.8†,‡	0.2	13.4‡	2.3	1.4	0.2
M8	>100	–	na	–	na	–	na	–

Note: Estimates of IC_{50}, IC_{20}, IC_{80} and sigmoidicity factor (gamma). Parameters were estimated after non-linear fitting PGE_2 formation vs. compound concentration data to the four-parameter Hill equation. Results are expressed as mean ± *SEM* of blood incubates from six individual dogs.

Statistical analysis was performed by means of one-way ANOVA and Tukey test for mean comparisons. Significance: $p < .05$.

Abbreviation: na, not applicable.

†,‡: statistically significant differences between/among the highlighted treatments

from 1016.6 to 1353.5 ng/ml after curve fitting (E_0 parameter). These values agree with those described elsewhere in dog under the same experimental conditions of incubation and serum generation (Gilmer et al., 2003; Jerin et al., 2009). Metabolite M8 and the (R)-(+)-Enflicoxib enantiomer did not induce a decrease of the TXB_2 levels (the data could not be fitted to any inhibitory Equation), while enflicoxib, the (S)-(-)-Enflicoxib enantiomer and the pyrazol metabolite effectively inhibited COX-1. For enflicoxib, (R)-(+)-Enflicoxib enantiomer and pyrazol metabolite, the inhibition parameters could be estimated and were very similar. A trend to lower IC_{50} was observed for (S)-(-)-Enflicoxib enantiomer as compared with enflicoxib. This was expected considering the racemic nature of enflicoxib and the

lack of inhibitory activity of (S)-(-)-Enflicoxib enantiomer. The data obtained by Iñiguez et al. (2010) in vivo in the rat carrageenan model confirmed these results with inhibition values for PGE_2 formation in gastric mucosa of 10% for enflicoxib and 18% for (S)-(-)-Enflicoxib at a dose of 40 mg/kg. The pyrazol metabolite inhibited COX-1 with similar potency to enflicoxib.

The results of the COX-2 inhibition assay showed mean E_0 levels of PGE_2 that agreed previously published works (King et al., 2010) and ranged from 188.8 to 232.1.3 ng/ml after LPS stimulation. For COX-2 blood incubations, some authors use aspirin to selectively inhibit any potential contribution of COX-1 on the generation of prostanoids from white cells (Giraudel et al., 2009). In the present work, aspirinated blood was not used in the COX-2 assays because the basal PGE_2 levels in dog plasma are described to be around 1.2 ng/ml and increase 275-fold after LPS treatment (Brideau et al., 2001). In addition, platelet COX-1 enzyme is not stimulated under incubation with LPS. In our study, the plasma samples were diluted 1000-fold for PGE_2 determination. Therefore, the potential contribution of COX-1 derived PGE_2 is negligible (about 0.0012 ng/ml) and of potential lesser impact on the study results than a potential fraction of COX-2 activity to be inhibited by aspirin.

TABLE 3 Selectivity for COX-2 inhibition by enflicoxib, its enantiomers and its main phase I metabolites

	Ratio IC_{50} (COX-1/COX-2)		
Compound	**Mean**	***SEM***	***p***
Enflicoxib	3.21†	0.26	.0007
(S)-(-)-Enflicoxib	3.77‡	0.44	.0003
(R)-(+)-Enflicoxib	na	na	na
Pyrazol metabolite	19.46†,‡	5.76	.0125
M8	na	na	na

Note: Selectivity for each test item was expressed as the respective quotient of the average IC_{50} parameters for COX-1 *vs.* COX-2. Results are expressed as mean ± *SEM* of blood incubates from 6 individual dogs. One-way ANOVA and Tukey test for mean comparisons were applied to compare COX-2 selectivity for each compound. Significance: $p < .05$.

Abbreviation: na, not applicable.

†,‡: statistically significant differences between the highlighted treatments.

For COX-2, again, metabolite M8 and (R)-(+)-Enflicoxib enantiomer did not show enzyme inhibition. Both IC_{50} and IC_{80} were above 100 and 30 μM, respectively, for M8 and (R)-(+)-Enflicoxib. These values corresponded to blood levels higher than 42100 and 12000 ng/ml that are far above the circulating concentrations after therapeutic doses of enflicoxib (Cendrós et al., 2021; Homedes et al., 2021).

Enflicoxib, (S)-(-)-Enflicoxib and the pyrazol metabolite effectively inhibited PGE_2 formation. As expected, (S)-(-)-Enflicoxib enantiomer showed approximately half value for the three estimated inhibition parameters as compared to racemic enflicoxib (statistically significant differences), supporting the previous data indicating that (S)-(-)-Enflicoxib is the active enantiomer (Iñiguez et al., 2010; Wagemarkers et al., 2009). Importantly, the pyrazol metabolite showed statistically higher COX-2 inhibitory potency as compared to the parent compound (4.2-fold higher for IC_{50}) or to its active enantiomer (S)-(-)-Enflicoxib (2.3-fold higher IC_{50}). The COX-2 selectivity ratio for the pyrazol metabolite was calculated to be 19.45, similar to the ratios obtained for other selective COX-2 inhibitors like mavacoxib or cimicoxib (Lees et al., 2015; Chegaev et al. 2007, in humans), and higher than non-coxib drugs that show preferential COX-2 inhibition like carprofen (ratio close to 6.5, Briedeau et al.,

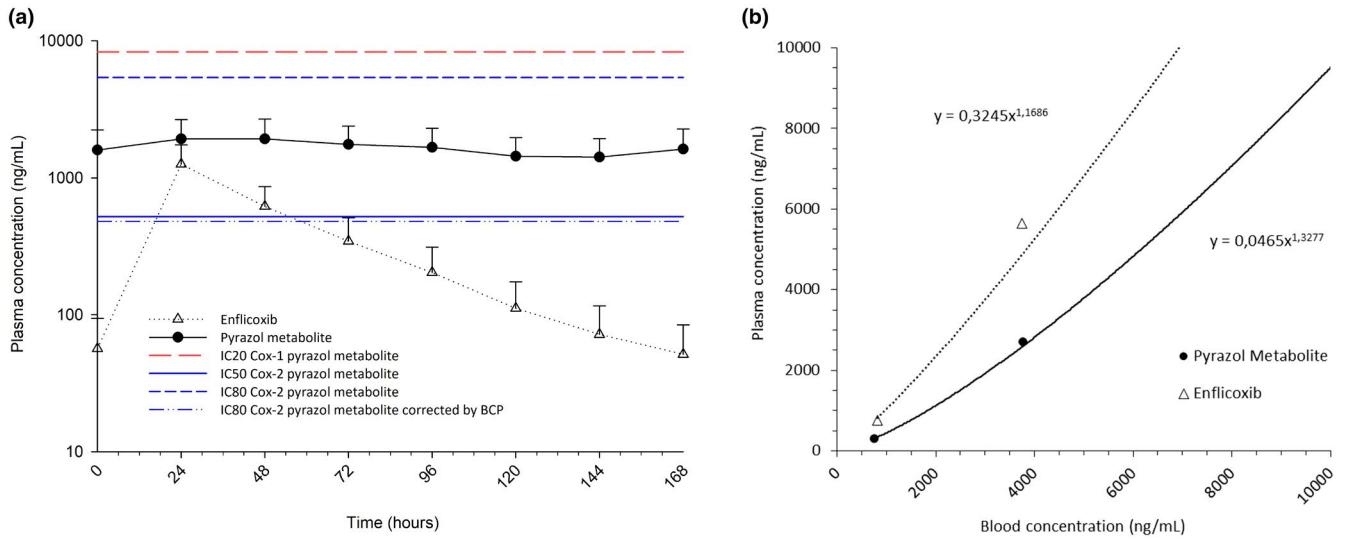

FIGURE 3 (a) Plasma levels of enflicoxib and its pyrazol metabolite in beagle dogs treated with enflicoxib orally at a loading dose and once-a-week nominal doses of 8 + 4 mg/kg for 31 weeks. Colour lines indicate concentrations of pyrazol metabolite (ng/ml) at the COX-2 IC_{50} and IC_{80} and of COX-1 IC_{20} concentration levels. IC_{80} concentration is depicted with and without correction by the blood cell partitioning. (b) Partitioning of enflicoxib and its pyrazol metabolite in dog blood and plasma at increasing concentrations. (Graphs a and b are extracted from data published by Homedes et al., 2021)

2001) or meloxicam (ratio 7 to 10, Briedeau et al., 2001; King et al., 2010). This is in contrast with other coxibs with higher selectivity ratios such as firocoxib (384), robenacoxib (129) and deracoxib (48.5), with IC_{50} values for COX-2 inhibition at the nano-molar range (King et al., 2010; McCann et al., 2004).

Enflicoxib and its pyrazol metabolite show non-linear and saturable blood cell partitioning (BCP) (Homedes et al., 2021). This means that, at low/therapeutic concentrations, the plasma levels are considerably lower than those in whole blood. Figure 3a shows the plasma profile of enflicoxib and its pyrazol metabolite obtained after treating dogs with the recommended dose for 31 weeks (extracted from the work of Homedes et al., 2021). The IC_{20} for COX-1 and IC_{50} and IC_{80} for COX-2 for the pyrazol metaboltie (for IC_{80} both non-corrected and BCP-corrected data) are introduced as lines in the graph as markers of the temporal evolution of efficacy. The magnitude of this effect is depicted graphically in Figure 3b (data generated from information in Homedes et al., 2021). The actual concentrations in plasma or serum were not determined in the same samples used for TXB_2 or PGE_2 determination in the present work, but the effect of BCP on COX-1 and COX-2 was estimated. The BCP effect on COX-2 inhibition by enflicoxib would decrease the IC_{50} and IC_{80} to values close to 7.6 and 23.9 μM, respectively, which represent plasma concentrations of approximately 3000 and 9700 ng/ml respectively. The plasma concentrations of parent enflicoxib at the dose regime recommended for treating osteoarthritis in dogs (Homedes et al., 2021) do not reach these values at any time point (which is consistent with the findings by Cendrós et al. (2021) reporting lack of efficacy of the parent compound in an arthritis induction model. By contrast, the BCP effect would be clinically meaningful in the case of the pyrazol metabolite. The COX-2 inhibition parameters for the pyrazol metabolite would decrease to 0.18 μM (IC_{50}) and 1.2 μM (IC_{80}) when BCP was considered. Both values represent, respectively, 80.7 and 484 ng/ml and are well below the plasma concentration of the pyrazol metabolite after the recommended dosage (Figure 3a). Interestingly, the BCP-corrected IC_{80} is very similar to the minimum effective concentration (MEC) determined by Cendrós et al. (2021) using a population PK/PD approach in the arthritis induction model. It is also worth noting that the corrected IC_{50} of the pyrazol metabolite for COX-2 is in the range of some of the above mentioned most potent COX-2 inhibitors (the COX selectivity ratio for the pyrazol metabolite was ≥55 based on the predictive equation of BCP; Figure 3b).

Importantly, the efficacy of such concentrations was demonstrated in clinical studies at the recommended dose and even at half the recommended dose (Salichs et al., 2021). It is also worth noting that the pharmacological effect of enflicoxib treatment could be based not only on a direct effect on enzymatic COX-2 activity but also on COX-independent immunosuppressant and anti-inflammatory actions shared by parent and main metabolite. These include the inhibition of the activation of key pro-inflammatory transcription factors including nuclear factor (NF)-κB and nuclear factor of activated T cells (NFAT), leading ultimately to the down-regulation of proliferation and production of cytokines as IL-2, IFN-γ and TNF-α (Iñiguez et al., 2010). In addition, we cannot disregard the possibility of a time-dependent COX-2 inhibition as a contributor to the activity of the compound. Indeed, celecoxib and other COX inhibitors are known to induce time-dependent structural changes in the COX-2 enzyme by binding and forming an enzyme-inhibitor complex that is slowly reversible/virtually irreversible (Dong et al., 2019; Walker et al., 2001). As it regards to selectivity, the concentrations of enflicoxib and its pyrazol metabolite are some ninefold lower than the IC_{20} for COX-1, which indicates a good selectivity at the concentrations reached during treatment, which in turn is highly relevant for the safety of the product.

In conclusion, the results of the present study demonstrated the COX inhibitory potential and COX-2 selectivity of enflicoxib and its active enantiomer (S)-(-)-Enflicoxib in the target (dog) species, supporting previous in vitro data obtained using isolated enzymes and in vivo data in rodents and in dogs. (R)-(+)-Enflicoxib was confirmed as the inactive enflicoxib enantiomer showing no effect in the canine blood model. In addition, the major enflicoxib metabolite M8 showed negative results for both COX isozymes. Finally, the active pyrazol metabolite appeared as the most potent and selective COX-2 inhibitor, consistent with the mode of action proposed for enflicoxib therapy that is based on the long-term exposure and efficacy of the pyrazol metabolite.

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CONFLICT OF INTEREST

J. Homedes and M. Salichs are employees of Ecuphar Veterinaria S.L.U. (Animalcare group), who funded this project. J. Solà and A. Menargues conducted the research funded by Ecuphar Veterinaria S.L.U. (Animalcare group). I. Álvarez, J-M. Vela and L. Romero declare no conflict of interest.

AUTHOR CONTRIBUTION

JS, IA and JH and MS participated in the research design and in the experimental activities. JS and LR performed or contributed to the analytical part. All authors wrote or contributed to the writing of the manuscript, contributed to data interpretation, drafted parts of the manuscript and approved the final manuscript.

ANIMAL WELFARE AND ETHICS STATEMENT

Animal subjects were only used once to obtain fresh blood samples, but all the experiments described in the manuscript were performed in vitro.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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