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In vitro enantioselective pharmacodynamics of Carprofen and Flunixin-meglumine in feedlot cattle

M. MICILETTA*[†] B. CUNIBERTI* R. BARBERO* & G. RE*

*Division of Pharmacology and Toxicology, Department of Animal Pathology, University of Torino, Grugliasco, Torino, Italy;

[†]Pfizer Animal Health Italia, Roma, Italy

Abstract

The activity of the anti-inflammatory agents Flunixin-meglumine (FLU), RS (–) Carprofen (CPF) and S (+) CPF on bovine cyclooxygenases (COXs) has been characterized in feedlot calves using an *in vitro* whole blood model. The drugs showed equivalent efficacy in their inhibitory activity on COXs, and the rank order of potency for both COX-1 and COX-2 inhibition was FLU > S (+) CPF > RS (–) CPF. Our results indicated that FLU is a nonselective inhibitor of bovine COXs, whereas RS (–) CPF and S (+) CPF exhibited different degrees of preferential inhibition of COX-2 isoenzyme. The rank order of IC₅₀ COX-1: IC₅₀ COX-2 potency ratios was in fact S (+) CPF (51.882) > RS (–) CPF (13.964) > FLU (0.606), and the calculated percentage inhibition of COX-1 corresponding to COX-2 inhibition values comprised between 80% and 95% was comprised between 57.697 and 79.865 for FLU, 33.373 and 51.319 for RS (–) CPF, and 0.230 and 4.622 for S (+) CPF, respectively. These findings are discussed in relation to the prediction of the clinical relevance of COX inhibition by the test drugs in cattle.

Introduction

Prostaglandin H₂ synthase-1 and -2 [referred to colloquially as cyclooxygenase-1 and -2; (COX-1 and COX-2)] are extensively studied mammalian oxygenases that oxidize arachidonic acid [(5Z,8Z,11Z,14Z)-5,8,11,14-Eicosatetraenoic acid; AA], a poly-unsaturated fatty acid that is an integral component of biological membranes, to prostaglandin (PG) H₂ (Smith et al., 2000). This reaction represents the rate-limiting step in the biosynthesis of prostanoids, bioactive lipid mediators that play a primary role in supporting specific homeostatic processes (e.g. cytoprotection of gastrointestinal mucosa, renal, cardiovascular and reproductive function) and pathophysiological states as the inflammatory response and tumorigenesis (Vane et al., 1998; Funk, 2001; Medzhitov, 2008; Smyth et al., 2009; Wang & DuBois, 2010). It has been known for a long time that COXs are the main pharmacological targets of nonsteroidal anti-inflammatory drugs (NSAIDs), a chemically heterogeneous class of COX-inhibiting compounds that share therapeutic and side-effects (Vane, 1971; Vane, 2000; Flower, 2003). NSAIDs are widely used in human and veterinary medicine for their anti-inflammatory, antipyretic and non narcotic analgesic activity. In the bovine species, they are extensively employed as ancillary therapy for the control of the inflammatory response in a series of disease states including mastitis, metritis, pneumonia, and enteritis of the newborn calves. In beef cattle medicine, NSAIDs are largely used in the treatment of respiratory tract and musculoskeletal system diseases (e.g. bovine respiratory disease complex, interdigital phlegmon, toe abscesses, arthritis) and for the symptomatic control of fever and/or pain associated with various clinical conditions and surgical procedures (Apley, 1997; Apley & Fajt, 1998; Smith et al., 2008; Coetzee, 2011). There are remarkable differences amongst NSAIDs in the selectivity of action toward the two COX isoforms. Based on the evidence that COX-1-mediated prostanoid synthesis largely underlies housekeeping functions whereas COX-2-derived prostanoids play a predominant role during pathological conditions, COX-1 and COX-2 inhibition was originally considered responsible for the undesired and therapeutic effects of NSAIDs, respectively (DeWitt et al., 1993; Mitchell et al., 1993; Vane et al., 1998). It is now well appreciated that to strictly follow this para-

digim is an oversimplification (Mitchell & Warner, 2006), but it still remains a core pharmacological interest to characterize and compare the activity of NSAIDs on COX isoforms in species of interest, to achieve pharmacodynamic data useful for the preclinical and clinical development of such drugs. Allowing for possible differences in potency and selectivity of NSAIDs amongst animal species despite the high degree of inter-species homology of COX amino acid sequences, it is unsuitable to transpose the results of NSAIDs activity obtained from one species to another and it is therefore necessary to assess the pharmacodynamic properties of NSAIDs in target species (Brideau et al., 2001; Lees et al., 2004; Giraudel et al., 2005). Moreover, within the same species, inter-animal differences related to breed and physiological condition may influence NSAIDs pharmacokinetics and pharmacodynamics (Lees, 2009). Several in vitro assays have been established for testing the potency and relative inhibitory activities of NSAIDs against COX isoenzymes, ranging from models employing COX proteins of native or recombinant origin used as purified enzymes or in microsomal and whole cell preparations; to blood cells (isolated or in whole blood assays); to specific cell lines and, finally, to cells that are targets for the therapeutic or adverse effects of NSAIDs (Pairet et al., 1998). The great difference in the experimental conditions amongst these bioassays accounts for the variability in the results obtained by testing the same drug in different models, but also can allow to investigate different aspects of the COX inhibition, and points out the need of selecting an appropriate test system depending on the purposes of the investigation (Pairet & van Ryn, 1998). The whole blood assay is a well-established and convenient experimental model utilized in human and animals to evaluate the in vitro activity of NSAIDs on COX isoforms separately (Patrignani et al., 1994; Brideau et al., 2001). Flunixin-meglumine (2-[[2-Methyl-3-(trifluoromethyl)phenyl]amino]nicotinic acid - 1-deoxy-1-(methylamino)-D-glucitol (1:1); FLU), an N-aryl-anthranilic acid derivative, and Carprofen (2-(6-chloro-9H-carbazol-2-yl)propanoic acid; CPF), a 2-aryl-propionic acid derivative, are two NSAIDs commonly used in beef cattle medicine. Pharmacodynamic studies investigating the inhibitory activity of FLU on bovine COXs have been already carried out using in vitro, ex vivo, and in vivo models of acute inflammation (Lees et al., 1991; Landoni et al., 1995a,b; Myers et al., 2010; Donalisio et al., 2013). Concerning CPF, at the time when our experiment was performed it was believed that the drug could mainly exert its pharmacological activity via COX-independent mechanisms of action in cattle, as the activity of this drug on bovine COXs was considered weak or inconsistent (Delatour et al., 1996; Lees et al., 1996; EMEA, 1999). It is only very recently that a study aimed to explore CPF in vitro activity on bovine COXs has been conducted in calves (Brentnall et al., 2012a). However, pharmacodynamic data on efficacy, affinity, potency, and selectivity of both FLU and CPF toward bovine COX isoenzymes in beef cattle are totally lacking. CPF possesses an asymmetrical carbon atom within its propionic acid side-chain moiety, and therefore this drug exists as two non superimposable mirror image forms, R (–) and S (+) enantiomers. In this study, we tested CPF as both the equimolar mixture of R (–) and S (+) enantiomers licensed for veterinary use and the single S (+) enantiomer, that plays a predominant role in the in vitro inhibition of prostaglandin synthesis in human and several animal species (Hayball, 1996; Landoni et al., 1997; Lees, 2009). Therefore, the aims of the present study were: (i) to describe whole blood in vitro assays for testing COX isoenzymes inhibition in cattle; (ii) to investigate in a whole blood model the in vitro pharmacodynamics of FLU and CPF in feedlot beef calves, further characterizing the activity of CPF on bovine COXs, that has been little explored. To our knowledge, this is the first study describing NSAIDs activity on COX isoenzymes in feedlot cattle.

Materials&Methods

Animals and samples collection

For this study, 14 male Limousine beef calves with body-weights and ages ranging from 240 to 385

kg and from 5 to 9 months, respectively, were selected as test animals. The calves enrolled in this study, did not show overt signs of diseases or physical injuries or trauma, their rectal temperatures ranged from 38.9 to 39.4 °C and they were considered healthy after individual clinical examination. None of the subjects received any pharmacological treatment during the 15 days before the day of the experiment, with the exception of the feedlot routine immunization programme 1 week before the day of the experiment, consisting in the administration of a single dose of modified-live viral vaccines licensed for use in cattle (Imuresp R-A-P : intranasal route of administration; Rispoval RS-BVD : intramuscular route of administration; Pfizer Animal Health, Rome, Italy). Fresh whole blood samples were collected via jugular venipuncture both into sterile anticoagulant-free and into sterile sodium-heparine containing vacuum tubes (Venosafe Plastic Tubes; Terumo, Rome, Italy). The blood samples were then readily transported to the laboratories of the Division of Pharmacology and Toxicology, Department of Animal Pathology, University of Torino. The time between blood collection and testing did not exceed two hours for any of the samples tested, during which they were kept at room temperature.

Chemicals and test compounds

All chemical reagents and test articles utilized were commercially purchased (Sigma-Aldrich Chemical Co., Milan, Italy), except for S (+) CPF that was generously given from Pfizer Animal Health R&D (Sandwich, Kent, UK). Stock solutions of test compounds were prepared dissolving FLU in phosphate buffer solution (NaCl 0.14 M, KCl 2.7 mM, KH₂PO₄ 1.5 mM, Na₂HPO₄ 9.0 mM, pH 7.4; PBS), and RS (–) and S (+) CPF in dimethylsulphoxide; subsequent 1:10 dilutions were prepared up in the same vehicles. Final vehicle concentration in test tubes during the COX-1 and COX-2 assays did not exceed 1% (v/v) for PBS and 0,1% (v/v) for dimethylsulphoxide.

COX-1 assay

COX-1 activity in whole blood was assessed by the measurement of thromboxane (TX) B₂ synthesis as a specific biomarker of platelets COX-1 activity stimulated during the spontaneous clotting process. Aliquots of 1 mL of anticoagulant-free fresh blood were transferred immediately after collection to glass tubes containing either the drug vehicle (positive controls) or an equal volume of a test compound. Aliquots of 1 mL of heparinized fresh blood were added to glass tubes preloaded with the drug vehicle, providing the negative controls (baseline TXB₂ production in the unstimulated blood). Following the method described by Brideau et al. (2001) with some minor modifications, test compounds were assayed at final concentrations of 0.0001; 0.001; 0.01; 0.1; 1; 10; 100; 1000 IM. A commercial enzyme immunoassay (EIA) kit (Thromboxane B₂ ELISA; DRG Diagnostics, Marburg, Germany) was used to determine in triplicate TXB₂ concentration in the samples diluted 1:10 in EIA buffer, following the manufacturer's instructions.

COX-2 assay

COX-2 activity in whole blood was assessed by measurement of PGE₂ synthesis in response to bacterial lipopolysaccharide (LPS) stimulation as a specific biomarker of monocytes COX-2 activity. Aliquots of 1 mL of heparinized fresh blood were transferred to glass tubes containing either the drug vehicle (positive and negative controls) or an equal volume of a test compound. Blood was preincubated for 15 min at 37 °C in a shaking waterbath, then LPS of *Escherichia coli* O111:B4 (L4130; Sigma-Aldrich Co.) solubilized in PBS was added to all tubes at a final concentration of 10 lg/mL, with the exception of the negative controls tubes that were added with an equal volume of PBS (baseline production of PGE₂ in the unstimulated blood). Following the method described by Brideau et al. (2001) with some minor modifications, test compounds were assayed at final concentrations of 0.0001; 0.001; 0.01; 0.1; 1; 10; 100; 1000 IM. The PGE₂ concentration in the samples diluted 1:50 in EIA buffer was determined in triplicate by using a commercial EIA kit

(DetectX Prostaglandin E2 High Sensitivity Immunoassay Kit; Arbor Assays, Ann Arbor, MI, USA) following the manufacturer's instructions.

Data analysis

Background prostanoid production values measured in the negative control tubes were subtracted from the prostanoid concentrations measured in all the remaining test tubes, then inhibition of prostanoid production was expressed as a percent-age of the positive control values.

Data were analyzed with a software program (Prism v. 5.00; GraphPad Software Inc., San Diego, CA, USA), utilizing a four parameter logistic nonlinear regression model to describe bio-logical responses as functions of tested drug concentrations, according to the following Hill equation:

$$\% \text{ Inhibition} = \frac{I_{\max} - I_0}{I_0} \frac{1}{1 + 10^{\delta \log(C - IC_{50}) / nH}}$$

where % Inhibition is the inhibition of prostanoid production expressed as percentage of the positive control value, C is the logarithmic value of the test compound concentration, I₀ is the baseline inhibition, I_{max} is the maximal response achieved by the test compound (drug efficacy), IC₅₀ (drug potency) is the test compound concentration giving 50% of maximal inhibition, and nH is the Hill coefficient (drug affinity) determining the slope of the concentration-response curve. The iterative procedure performed by the software program utilized the Levenberg–Marquardt method to identify the best fit values and the corresponding 95% lower and upper confidence intervals for the above-mentioned parameters.

The native pooled data approach was utilized to describe COX isoenzymes inhibition by test compounds; mean dose-response curves for COX-1 and COX-2 were determined by fitting simultaneously the data sets of the individual experimental subjects to the same Hill equation.

ICX values for COX isoenzymes inhibition by the test compounds were calculated solving the following equation:

$$\log IC_X = \log IC_{50} - \frac{\delta}{nH} \log \left(\frac{100 - X}{X} \right)$$

where IC_X is the test compound concentration giving a COX-1 or COX-2 fractional inhibition of X percent of the maximal inhibition, and IC₅₀ and nH parameters belong to the Hill equations describing COX isoenzymes inhibition by test compounds. For each of the test drugs, the percentage inhibition values of COX-1 corresponding to selected percentage values of COX-2 inhibition were calculated by rescaling the Hill equations for COX-1 and COX-2 on a 0–100% scale, and incorporating in the Hill equation for COX-1 the test compound concentrations leading to the given percentage values of COX-2 inhibition. Differences amongst test drugs ICX values were evaluated after log-transformation of data by using Student's t-test for unpaired data. Other statistical analyses of differences between drugs were performed using Student's t-test for unpaired data or one-way analysis of variance (ANOVA) followed by the New-man–Keuls multiple-comparison post test. Differences were accepted as significant at P < 0.05.

RESULTS

COX-1 assay

Intra-assay and inter-assay coefficient of variance (CV) of COX-1 assay were 8.32% and 6.85%, respectively. The overall background (negative control samples) and induced (positive control samples) TXB2 concentrations were 17.25 ± 2.57 and 110.90 ± 15.65 ng/mL, respectively (mean \pm SEM; n = 14), indicating that COX-1 activity was present and quantifiable. Induced TXB2 concentration was significantly different from unstimulated control ($P < 0.001$).

COX-2 assay

Intra-assay and inter-assay CV of COX-2 assay were 12.92% and 8.40%. The overall background (negative control samples) and induced (positive control samples) PGE2 concentrations were 169.06 ± 31.51 and 995.30 ± 169.15 pg/mL, respectively (mean \pm SEM; n = 14), indicating that COX-2 activity was present and quantifiable. Induced PGE2 concentration was significantly different from unstimulated control ($P < 0.001$).

Inhibitory effects on cyclooxygenases of test compounds

COX-1 and COX-2 activity in bovine whole blood was inhibited in a concentration-dependent manner by FLU and RS (–) CPF; the two drugs produced a complete inhibition of the COX-1 and COX-2 mediated prostanoid synthesis (Figs 1 & 2). By contrast, S (+) CPF showed a concentration-dependent inhibitory effect reaching the complete inhibition in the COX-2 assay, but displayed no inhibitory activity against COX-1 over nearly all of the range of tested concentrations. A COX-1 inhibition significantly different from negative control was only observed at the highest S (+) CPF concentration assayed ($P < 0.001$), that nevertheless failed to produce a complete inhibition of COX-1 isoenzyme activity (Fig. 3). In Table 1, the measures of potency, efficacy, and affinity of test compounds for bovine COXs are shown. We observed no significant differences amongst the three drugs in the efficacy for COX-2 inhibition, and in the efficacy for COX-1 inhibition between FLU and RS (–) CPF. Comparison of IC₅₀, IC₈₀ values showed that the rank order of potency of tested NSAIDs for both COX-1 and COX-2 inhibition was FLU > S (+) CPF > RS (–) CPF. RS (–) CPF/S (+) CPF relative potency values for COX-1 inhibition varied, depending on the level of inhibition considered, and were 3.34 and 12.22 based on IC₅₀ and IC₈₀, respectively. Similarly, RS (–) CPF/S (+) CPF relative potency values for COX-2 inhibition varied from 12.42 to 3.90 based on IC₅₀ and IC₈₀, respectively. A series of indices describing COX isoform selectivity for the test drugs is shown in Table 2. Classically, IC₅₀ COX-1: IC₅₀ COX-2 and IC₈₀ COX-1: IC₈₀ COX-2 potency ratios (values >1 reflect selectivity for COX-2) were calculated. Dissimilarities in slopes of the inhibition curves for COX-1 and COX-2, even if not statistically significant, led to the differences in the estimates of potency and potency ratios depending on the drug concentration considered. To achieve a better description of test drugs selectivity toward COX isoforms, we reported in Table 2 the calculated percentage inhibition values of COX-1 corresponding to selected percentage values of COX-2 inhibition. shown, which allowed an immediate visualization of the selectivity of test compounds for bovine COX isoenzymes and an estimate of its clinical relevance.

DISCUSSION

The whole blood assay is an experimental model that, studying the *in vitro* inhibition of COX isoforms separately, can be preferable to other models mainly because: (i) it is a method that represents the drug-protein interactions that occur *in vivo*, taking into account the binding of the test drug to plasma proteins at a physiological protein concentration and composition (this is especially relevant for NSAIDs, including CPF and FLU in bovine species, which are extensively bound to plasma proteins); (ii) the blood used for both COX-1 and COX-2 assays, is collected from the same subject at the same time, thus allowing a direct comparison of the inhibition of the two isoforms;

(iii) intact cells that are targets for the pharmacological effects of NSAIDs (platelets and monocytes) are used in the assay; (iv) COX-mediated prostanoid synthesis arise from AA released from endogenous stores; (v) the necessary time required for time-dependent NSAIDs to bind productively the COX active site is allowed (Odensvik & Johansson, 1995; Pairet & van Ryn, 1998; EMEA, 1999; Brideau et al., 2001; Simmons et al., 2004; Lees, 2009; Giraudel et al., 2005). As a result, pharma-codynamic data obtained in whole blood assay are suitable to predict the clinical relevance of COX isoenzymes inhibition (Pairet & van Ryn, 1998; Lees et al., 2004; Huntjens et al., 2005).

In this study, we classically used TXB2 and PGE2 production, elicited by the spontaneous clotting process and by the addition of LPS, respectively, as specific markers of COX-1 (TXB2) and COX-2 (PGE2) activity in whole blood (Patrignani et al., 1994, 1999; Brideau et al., 1996; Capone et al., 2007). Some authors have proposed TXB2 production as common readout for the assessment of both COX-1 and COX-2 induced activity in whole blood assay, as an alternative approach to the quantification of TXB2 and PGE2 (Glaser et al., 1995; Young et al., 1996; Giraudel et al., 2005). These authors have used acetyl-salicylic acid (ASA) in the COX-2 assay to irreversibly inhibit platelets and monocytes COX-1, with the aim of avoiding the contribution of COX-1 to TXB2 synthesis without affecting the activity of the LPS-induced COX-2. The used concentration of ASA, however, was not able to provide a complete inhibition of COX-1 induced activity in whole blood COX-2 assay (Giraudel et al., 2005). Additionally, even if salicylic acid exhibited low potency for COX-2 inhibition in LPS-stimulated human whole blood (IC₅₀: 1481 IM; Patrignani et al., 1997), it has to be considered as the interference exerted in vitro by salicylate on intracellular pro-inflammatory signaling pathways (Amann & Peskar, 2002) and COX-2 activity in isolated cells (Mitchell et al., 1997; Xu et al., 1999; Warner et al., 2006); these effects have been also observed at salicylate concentrations that are obtained by the above-mentioned authors with the addition of ASA to whole blood in the COX-2 assay. Moreover, the binding of test drug to proteins might be unpredictably altered by the presence of salicylic acid during incubation. Taken as a whole, these aspects may represent a perturbation of the assay. Therefore, we preferred to use the quantification of TXB2 and PGE2 production as endpoints in the evaluation of whole blood COXs activity, providing in both our COX-1 and COX-2 assay a negative control that allowed us to take into account the baseline prostanoid production. This reasonably counteracted an eventual undesired induction of COXs activity merely due to test conditions and not evoked by the specific experimental stimulus. Moreover, the in vitro characterization of the inhibition of TXB2 and PGE2 synthesis in whole blood has been successfully used to predict in vivo pharmacological effects of NSAIDs and has been proposed as an efficient approach to select effective doses of such drugs in humans (Cryer & Feldman, 1998; Huntjens et al., 2005).

Results from our study, show that FLU is a potent inhibitor of bovine COXs: a concentration of 0.1 IM FLU inhibited >90% of both COX-1 and COX-2 activity. S (+) CPF and RS (–) CPF are weaker COXs inhibitors than FLU, especially considering COX-1 isoenzyme. To achieve the same level of COX-2 inhibition, S (+) CPF and RS (–) CPF concentration was in fact increased to 10 and 100 IM, respectively, and only at the concentration of 1000 IM RS (–) CPF inhibited >90% of COX-1 activity. S (+) CPF did not reach such level of COX-1 inhibition even at the highest tested concentration, producing a 76.50 ± 10.68% inhibition of COX-1 (mean ± SEM) at 100 IM. We did not test S (+) CPF at the 1000 IM concentration, but it is reasonable to assume that 1000 IM S(+) CPF may be able to inhibit almost completely bovine whole blood COX-1 activity at our test conditions, similarly to RS (–) CPF, given that: (i) in a separate whole blood in vitro study S (+) CPF produced an inhibition of bovine COX-1 comprised between 90% and 100% at the 1000 IM concentration (Brentnall et al., 2012a); (ii) the S (+) enantiomers of the 2-aryl propionate derivatives, CPF included, have a predominant role in the COX inhibitory properties showed by the racemates of this NSAIDs subgroup, and in our study RS (–) CPF produced a 55.84 ± 5.7% inhibition of COX-1 (mean ± SEM) at 100 IM and completely inhibited COX-1 at 1000 IM concentration. As a further

consequence, to correctly describe the measured COX-1 inhibition produced by S (+) CPF by the Hill equation, the I_{max} parameter of the COX-1 curve for S (+) CPF has been assigned to be equal to 100. The difference between the RS (–) CPF and S (+) CPF concentrations required to completely inhibit COX-1 and COX-2, and the finding that at the concentration producing a complete COX-2 inhibition RS (–) CPF and S (+) CPF inhibited COX-1 activity only by the 55.84 – 5.7% and 3.78 – 15.12% (mean – SEM), respectively, suggest that a selectivity toward COX-2 exists for these two drugs. On the basis of the results from our study, FLU appeared to be a nonselective inhibitor of bovine COXs, RS (–) CPF resulted a moderately preferential COX-2 inhibitor, whereas S (+) CPF exhibited a preferential inhibition of COX-2 isoenzyme. Considering the 50% and 80% level of COX inhibition, COX-1 inhibition by FLU was actually estimated to be only 1.66- and 3.12-fold greater than COX-2 inhibition. Moreover, the COX-1 and COX-2 inhibition curves basically overlapped, so no significant differences were observed between $IC_{20,50,80}$ COX-1 vs. corresponding $IC_{20,50,80}$ COX-2 (Table 1). Concomitantly, high levels of COX-1 inhibition (>50%) were calculated for COX-2 inhibition values >80% (Table 2). These findings clearly indicate the lack of FLU selectivity for bovine COXs over the entire range of tested concentrations. Our data are in agreement with mean potency and selectivity measures obtained for FLU in an *in vitro* whole blood assay conducted in Holstein-Friesian cows using similar test conditions (Donalisio et al., 2013), where mean COX-1 IC_{50} value was 0.0108 IM (our study: 0.0177 IM), mean COX-2 IC_{50} value was 0.0406 IM (our study: 0.0292 IM), and IC_{50} COX-1: IC_{50} COX-2 potency ratio was 0.260 (our study: 0.606). Concerning RS (–) CPF, the COX-1 inhibition curve was shifted toward the right of the COX-2 curve, indicating a slight preference for COX-2 inhibition that may be reflected by the potency ratios estimating a 13.96- and 40.46-fold greater inhibition of COX-2 compared to COX-1 at 50% and 80% inhibition levels, respectively. However, the wide confidence intervals for RS (–) CPF $IC_{50,80}$ COX-1 and the lack of significant differences between $IC_{20,80}$ COX-1 vs. corresponding $IC_{20,80}$ COX-2 (Table 1) suggest to be cautious when considering this drug as a COX-2 preferential NSAID in cattle at high levels of COX inhibition. By contrast, COX-1 inhibition curve for S (+) CPF was markedly shifted toward the right of the COX-2 curve, and significant differences were observed between $IC_{20,50,80}$ COX-1 vs. corresponding $IC_{20,50,80}$ COX-2 ($P < 0.01$; Table 1). At the 50% and 80% inhibition levels, COX-2 inhibition by S (+) CPF was estimated to be 51.88- and 12.90-fold greater than COX-1, and a very low extent of COX-1 inhibition corresponding to COX-2 inhibition values ranging from 80% to 95% was calculated (Table 2). On the whole, these data clearly indicate the preferential inhibition of bovine COX-2 by S (+) CPF. The present results regarding S (+) CPF are not in complete agreement with the data resulting from the study of Brentnall et al. (2012a). We fundamentally measured a weaker COX-1 inhibition produced by 0.01–10 IM S (+) CPF compared to this study. This finding explains the 5-fold greater $IC_{20,50,80}$ COX-1 values, the 8-fold greater IC_{50} COX-1: IC_{50} COX-2 potency ratio, and the calculated lesser inhibition of COX-1 for COX-2 inhibition values >80% of our study compared to the Brentnall's study. This may be due to the different test conditions of the two experiments. However, no measures of variability between subjects or confidence intervals of the estimated means are provided in the above-mentioned study, making a further comparison of results not possible.

In our research, we examined the inhibitory activity of both RS (–) CPF and the single S (+) enantiomer on COXs, to investigate if an interaction between enantiomer pairs could occur and it could influence the activity on COXs of the S (+) enantiomer. A slight degree of enantioselectivity characterizes in fact CPF distribution in cattle, so after administration of CPF racemate both R (–) and S (+) enantiomers are present in biological fluids at quite similar concentrations. After administration of a single dose of CPF racemate to calves at 0.7 mg/kg (half of the approved dose), the enantiomeric ratio, R: S, ranged from about 55: 45 at 2 h after administration to 58: 42 at 48 h and 61: 39 at 72 h (Delatour et al., 1996). Very similar results were obtained in the study of Lees et al. (1996), which furthermore showed that no enantioselectivity occurs in the processes governing the distribution of CPF enantiomers from plasma to transudate and inflammatory exudate, because

the percentage enantiomer concentrations were almost identical in the three biological fluids at each sampling time. Similar results indicating an only slight degree of enantioselectivity in CPF distribution were obtained administering once the approved dose of 1.4 mg/kg CPF racemate to calves (Brentnall et al., 2012a,b).

Examining the RS (–) CPF/S (+) CPF relative potency values for COX inhibition obtained in our study, data are suggestive of an interaction between CPF enantiomers. If the inhibitory activity of a given NSAID racemate is measured and is found to be about half that of the S (+) enantiomer, it is suggestive that the activity of the racemate is due solely to the S (+) enantiomer and that a lack of interaction between enantiomers occurs (Evans, 1992; Hayball, 1996). In the COX-1 assay, CPF racemate possessed 3.34 and 12.22 times lesser potency than S (+) enantiomer, considering the 50% and 80% level of inhibition, respectively. In the COX-2 assay, CPF racemate possessed 12.42 and 3.90 times lesser potency than S (+) enantiomer, considering the 50% and 80% level of inhibition, respectively. As a consequence, our data suggest that in cattle, depending on the level of inhibition considered, the inhibitory activity of S (+) CPF against COXs may be reduced to a varying degree in the presence of R (–) CPF, at an enantiomeric ratio of 50: 50 (R:S). Pharmacodynamic studies on separate enantiomers are the necessary steps to perform a sound pharmacokinetic/pharmacodynamic approach of selected optically active NSAIDs in

target species. We suggest that the subsequent evaluation of interactions between enantiomer pairs can be useful from the perspective of predicting the clinical relevance of the *in vitro* COXs inhibition, if the test drug is utilized as a racemate in target species. Ideally, such evaluation should be performed at enantiomeric ratios representative of the drug enantiomers *in vivo* concentrations in biological fluids/target tissues at selected time points.

Concerning the clinical significance of COXs inhibition, it is widely accepted that a level of COX-2 inhibition >80% is likely required to achieve an *in vivo* significant therapeutic effect (Warner et al., 1999; Lees et al., 2004); and according to Giraudel et al. (2005), we considered 20% of COX-1 inhibition as a safety cut-off value to estimate an increase in the likelihood to observe NSAIDs-related adverse effects, mainly attributable to the COX-1 inhibition. We agree with Giraudel et al. (2005, 2009) that a useful approach in the estimate of the clinical relevance of *in vitro* COX selectivity by test compounds can be the calculation of the percentage inhibition of COX-1 corresponding to fixed percentage inhibition values of COX-2. In our study, for COX-2 inhibition values comprised between the 80% and 90%, FLU inhibited COX-1 by values comprised between the 50% and 70%; an inhibition of COX-1 >90% was calculated for COX-2 inhibition values of 99% (Table 2 and Fig. 4a). Our results indicate that for FLU there may be a high risk of COX-1 related side-effects at levels of COX-2 inhibition that are expected to lead to a therapeutic effect *in vivo*. FLU and CPF, are generally well tolerated therapeutic agents in cattle, but prolonged therapies with FLU for more than three days have led to the development of hematochezia and hematuria in cattle (Veterinary Medicine Expert Committee on Drug Information, United States Pharmacopeia, 2004). Our data suggest a better safety margin for RS (–) CPF compared to FLU: for COX-2 inhibition values comprised between the 80% and 90%, the drug inhibited COX-1 by values comprised between the 20% and 50%, and considering the 99% of COX-2 inhibition, COX-1 was inhibited by about the 70%. Hence, a COX-1 inhibition level potentially responsible of undesired effects is attained by RS (–) CPF at therapeutically significant levels of COX-2 inhibition, but the magnitude of risk of developing toxic effects in bovine, also depending on the drug dosage administered *in vivo*, may be lesser for RS (–) CPF compared to FLU. This aspect may be especially relevant when instauring an anti-inflammatory therapy of long duration in cattle. The selectivity for COX-2 exhibited by S (+) CPF in this study, reflected in an estimated large safety margin for this agent

when utilized in clinical settings: a low probability of observing COX-1 related side-effects in cattle is expected at clinically significant COX-2 inhibition levels; only at very high COX-2 inhibitory values (>95%) the S (+) CPF selectivity is lost. Moreover, little inter-individual variability in such data has been observed for S (+) CPF compared with RS (–) CPF and FLU, leading to a good uniformity between subjects in the prediction of the clinical relevance of COXs selectivity for S (+) CPF, that is not achieved by the other two experimental drugs (Fig. 4b). In summary, the data of this study in feedlot beef calves indicate that FLU is a potent nonselective inhibitor of bovine COXs. RS (–) CPF and S (+) CPF attain the same level of efficacy of FLU in inhibiting COXs, although they possess less potency especially against the COX-1 isoform when compared with the COX-2 isoform. This is responsible of the varying degrees of COX-2 preferential inhibition exhibited by RS (–) CPF and S (+) CPF in beef cattle, that lead to a predicted better safety margin for these two drugs compared with FLU when used in clinical settings.

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FIGURES & TABLES

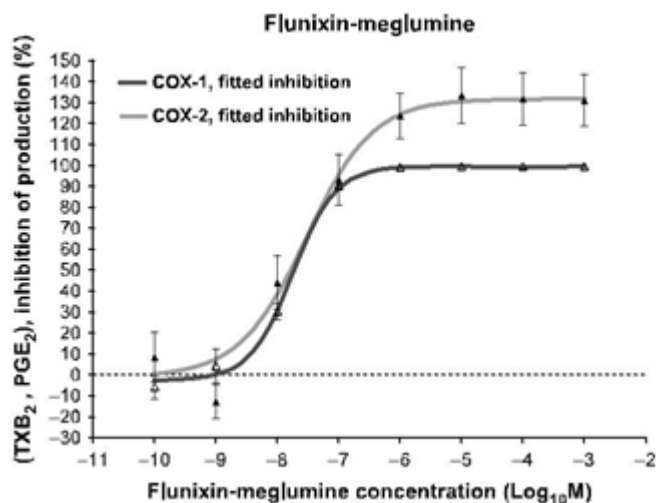


Fig. 1. Dose-response relationship for inhibition of bovine cyclooxygenase (COX)-1 and COX-2 by Flunixin-meglumine in whole blood assays. COX-1 (Δ) and COX-2 (\blacktriangle) percentage inhibition values for each drug concentration were calculated by averaging the individual percentage inhibition values of 8 calves and are expressed as mean \pm SEM. Mean inhibition curves were obtained by simultaneously fitting the individual percentage inhibition values of the test subjects using an Hill equation (see Materials and methods section).

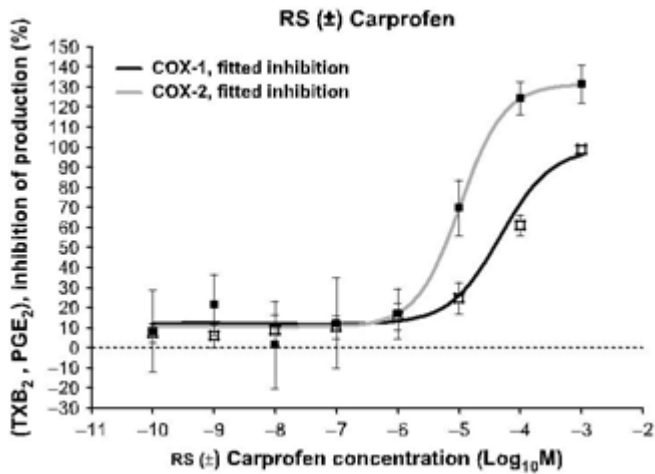


Fig. 2. Dose-response relationship for inhibition of bovine cyclooxygenase (COX)-1 and COX-2 by RS (□) Carprofen in whole blood assays. COX-1 (□) and COX-2 (■) percentage inhibition values for each drug concentration were calculated by averaging the individual percentage inhibition values of 14 calves and are expressed as mean \pm SEM. Mean inhibition curves were obtained by simultaneously fitting the individual percentage inhibition values of the test subjects using an Hill equation (see Materials and methods section).

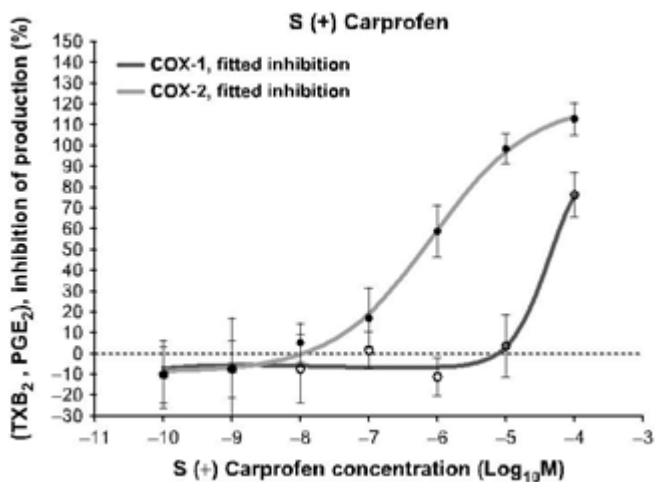


Fig. 3. Dose-response relationship for inhibition of bovine cyclooxygenase (COX)-1 and COX-2 by S (+) Carprofen in whole blood assays. COX-1 (○) and COX-2 (●) percentage inhibition values for each drug concentration were calculated by averaging the individual percentage inhibition values of 14 calves in the COX-1 assay and 8 calves in the COX-2 assay, and are expressed as mean \pm SEM. Mean inhibition curves were obtained by simultaneously fitting the individual percent-age inhibition values of the test subjects using an Hill equation (see Materials and methods section).

Parameters	COX-1			COX-2		
	L 95% CI	Mean	U 95% CI	L 95% CI	Mean	U 95% CI
Flunixin-meglumine						
I ₀ (%)	_8.197	_2.330	3.542	_24.43	_0.60	23.23
I _{max} (%)	96.08	99.87	103.70	118.20	132.0	145.80
n _H	0.85	1.26	1.66	0.25	0.80	1.35
IC ₂₀ (lM)	0.0039	0.0059	0.0088	0.0009	0.0051	0.0288
IC ₅₀ (lM)	0.0132	0.0177	0.0207	0.0105	0.0292	0.0813
IC ₈₀ (lM)	0.0321	0.0533	0.0885	0.0405	0.1660	0.6808
RS (–) Carprofen						
I ₀ (%)	1.641	8.12	14.59	_5.94	10.58	27.10
I _{max} (%)	49.90	126.50	203.10	91.38	131.80	172.20
n _H	0.1466	0.6281	1.110	_0.87	1.21	3.29
IC ₂₀ (lM)	4.3152	15.4528	59.0201	0.5070	3.3113	21.5774
IC ₅₀ (lM)	12.7057	145.2112	1659.5869	3.4277	10.3992*	31.5500
IC ₈₀ (lM)	24.4906	1318.256	71121.351	6.7764	32.5837	156.6751
S (+) Carprofen						
I ₀ (%)	_18.47	– ‡	4.89	–	8.72	12.35
I _{max} (%)	NA	~100	NA	77.98	120.70	163.50
n _H	0.09	1.53	2.96	0.02	0.60	1.19
IC ₂₀ (lM)	3.8019	17.4985	80.7235	0.0092	0.0839**	0.7656
IC ₅₀ (lM)	16.8267	43.4510	112.2019	0.1429	0.8375**	4.9091
IC ₈₀ (lM)	41.4000	107.8434	281.8382	1.7258	8.3560**	40.4576

Table 1. Mean values for the parameters of the Hill equation describing bovine COX isoenzymes inhibition by test compounds in the in vitro whole blood assays

I₀, baseline inhibition; I_{max}, maximal inhibition (efficacy); n_H, Hill coefficient (affinity); IC_X, test compound concentration giving a COX-1 or COX-2 inhibition of X percent of the maximal inhibition (IC₅₀: potency) (see Materials and methods section); COX, cyclooxygenase; NA = not applicable. Data are expressed as mean and related lower (L) and upper (U) 95% confidence intervals (CI). Whole blood samples from 14 calves were tested in RS (–) Carprofen COX-1, RS (–) Carprofen COX-2 and S (+) Carprofen COX-2 assays; whole blood samples from eight calves were tested in Flunixin-meglumine COX-1, Flunixin-meglumine COX-2 and S (+) Carprofen COX-1 assays. ‡Assigned parameter (see Discussion section). *Significantly (P < 0.05) different from the corresponding IC_X COX-1 value determined for the same compound. **Significantly (P < 0.01) different from the corresponding IC_X COX-1 value determined for the same compound

Table 2. Indices describing the in vitro selectivity of test compounds for bovine COX isoenzymes in whole blood assays

Selectivity indices*	Test compounds		
	Flunixin-meglumine	RS (-) Carprofen	S (+) Carprofen
IC ₅₀ COX-1: IC ₅₀ COX-2	0.606	13.964	51.882
IC ₈₀ COX-1: IC ₈₀ COX-2	0.321	40.458	12.906
% inhibition of COX-1 for 80% inhibition of COX-2 (0–100% scale)	57.697	33.373	0.230
% inhibition of COX-1 for 90% inhibition of COX-2 (0–100% scale)	70.390	42.456	1.113
% inhibition of COX-1 for 95% inhibition of COX-2 (0–100% scale)	79.865	51.319	4.622
% inhibition of COX-1 for 99% inhibition of COX-2 (0–100% scale)	92.476	69.868	54.907

*IC_X COX-1: IC_X COX-2, ratio of the test compound concentrations giving X percentage of the maximal inhibition of COX-1 and COX-2; COX cyclooxygenase. The Hill equations describing COX isoenzymes inhibition by test drugs, from which IC_X values were calculated (see Table 1), were re-scaled on a 0–100% scale to compute the percentage inhibition of COX-1 corresponding to a given percentage value of COX-2 inhibition.

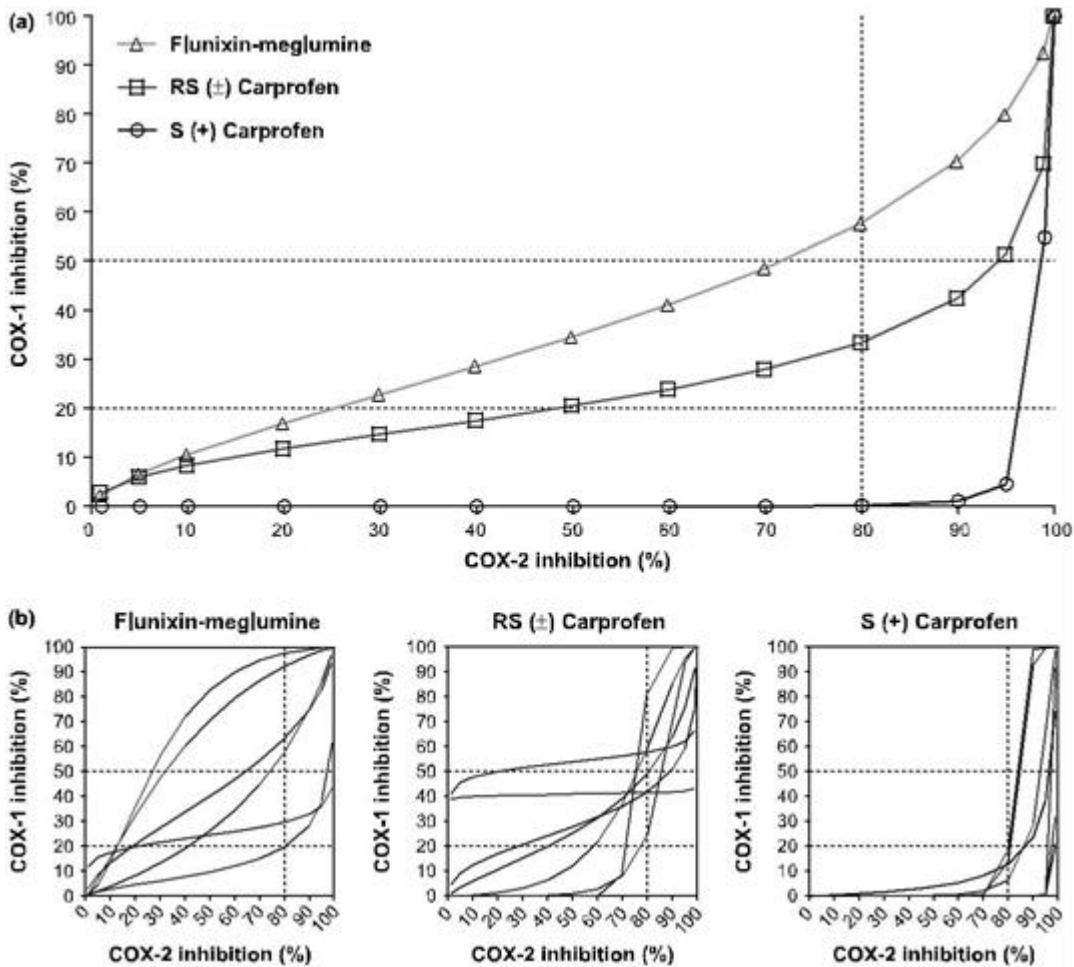


Fig. 4. (a) Plot of percentage inhibition of cyclooxygenase (COX)-1 vs. the corresponding percentage inhibition of COX-2 by Flunixin-meglumine, RS (±) Carprofen and S (+) Carprofen in bovine whole blood assays. (b) Graphs illustrating inter-animal variability of the relationship between percentage inhibition of COX-1 and the corresponding percentage inhibition of COX-2 by Flunixin-meglumine, RS (±) Carprofen and S (+) Carprofen in bovine whole blood assay. Representative subjects are showed; each line depict a single experimental subject. The Hill equations describing COX isoenzymes inhibition by test drugs were re-scaled on a 0% to 100% scale to calculate the percentage inhibition of COX-1 corresponding to a given percentage value of COX-2 inhibition. Dotted lines indicate proposed cut-off values for COX-1 inhibition which might be correlated to an increased risk of adverse effects in vivo, and the proposed cut-off value for COX-2 inhibition above which a significant in vivo therapeutic effect is expected.

