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In vitro and ex vivo pharmacodynamics of selected non-steroidal anti-inflammatory drugs in equine whole blood

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ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenases (COX), and the inhibition of COX-2 rather than COX-1 can limit the onset of NSAID-related adverse effects. The pharmacodynamics properties of eltenac, naproxen, tepoxalin, SC-560 and NS 398 in healthy horses were investigated using an in vitro whole blood assay. To predict COX selectivity in clinical use, eltenac and naproxen were also studied ex vivo after intravenous administration. SC-560 acted as a selective COX-1 inhibitor, tepoxalin as a dual inhibitor with potent activity against COX-1, and NS 398 as a preferential COX-2 inhibitor. Eltenac was a preferential COX-2 inhibitor in vitro but un-selective in the ex vivo study. Naproxen maintained its non-selectivity both in vitro and ex vivo. These findings have demonstrated that in vitro studies may not accurately predict in vivo NSAID selectivity for COX and should be confirmed using an ex vivo whole blood assay.

Keywords: Non-steroidal anti-inflammatory drugs, Pharmacodynamics, Horse, Whole blood assay

INTRODUCTION

The most important mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs) is to inhibit the cyclooxygenase (COX) enzyme which catalyses the conversion of arachidonic acid (AA) into prostanoids and thromboxane (Moses and Bertone, 2002). Two different isoforms, COX-1 and COX-2, have been cloned and sequenced in several mammalian species, including humans (Jones et al., 1993), dogs (Lascelles et al., 2009), cats (Sayasith et al., 2009), cows (Asselin et al., 1997) and horses (Giantin et al., 2006).

The two isoforms are structurally distinct proteins with only 60% and 69% homology in humans (Jones et al., 1993) and horses (Giantin et al., 2006), respectively. As COX-1 is considered the constitutive isoform implicated in housekeeping functions and COX-2 the inducible form, over-expressed during inflammation (Moses and Bertone, 2002), it is reasonable to assume that the use of COX-2 selective inhibitors will enhance the therapeutic gain with minimal adverse effects (Toutain et al., 2001). However, remaining AA not metabolised to prostaglandins by COX enzymes may enter the lipoxygenase (LO) pathway, so the use of COX inhibitors has been suggested to enhance the pro-inflammatory effects of leukotrienes (Rainsford, 1993). For this reason, 'dual inhibitor' compounds have been introduced into clinical practice (Charlier and Michaux, 2003). COX expression and activity differ among animal species and can be heterogeneous within the same tissue; therefore, transposing the results for NSAIDs potency and selectivity from one species to another is inappropriate (Giraudel et al., 2005). In equine medicine, the data on NSAIDs pharmacodynamics from studies using whole blood assays are limited. To date, only carprofen, phenylbutazone, flunixin meglumine, indomethacin, tepoxalin, SC-560 and NS 398 have been studied (Brideau et al., 2001; Beretta et al., 2005; Caruso et al., 2009; Giorgi et al., 2010). The advantages of whole blood assays are that (1) they can be used in vitro in the pre-clinical assessment of COX inhibitors as well as ex vivo during phase I/II studies; (2) they are accurate enough to estimate potency and selectivity for time-dependent COX inhibitors; (3) they compare clinically relevant target cells (i.e., platelets and monocytes); and (4) they take into account the drug to plasma protein binding that occurs in vivo (Mitchell et al., 1993). The aim of the present study was to investigate the efficacy, potency and selectivity of some NSAIDs deficient in pharmacodynamic data, namely, naproxen, eltenac and the dual inhibitor tepoxalin in vitro in equine whole blood, by comparing them to two well-known selective COX-1 and COX-2 inhibitors, SC-560 (Bolego et al., 2009) and NS 398 (Panara et al., 1995). An ex vivo experiment for eltenac and naproxen was also performed to determine whether the data obtained with the whole blood in vitro assay provided an accurate estimation of COX selectivity of NSAIDs in clinical use.

MATERIALS AND METHODS

Test compounds

Eltenac, Tepoxalin and Telzenac (eltenac, injectable solution) were kindly supplied by Schering-Plough. Naproxen sodium, NS 398 (N-(2-cyclohexyloxy-4-nitrophenyl) methane sulphonamide) and SC-560 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-rifluoromethylpyrazole) were purchased from Sigma. Equiproxen 10% (naproxen, injectable solution) was purchased from Fort Dodge Animal Health. Stock solutions of eltenac, naproxen, tepoxalin and NS 398 were made up with ethanol, and subsequent dilutions were prepared in phosphate buffer saline (PBS). SC-560 was dissolved in dimethylsulfoxide (DMSO) to give stock solutions which were further diluted with PBS. The range of the final test compound concentration was between 10 pM and 1 mM, depending on drug, and the vehicle maximum concentration in the incubation did not exceed 0.1% (v/v).

In vitro COX-1/COX-2 and 5-LO assays

Fresh blood samples were drawn from healthy female Standardbred trotters with a mean (±SEM) bodyweight (BW) of 562 ± 17 kg and a mean age of 4.0 ± 0.4 years. Blood samples were collected to test the following drugs: eltenac (n = 5); naproxen (n = 8); tepoxalin (n = 5); SC-560 (n = 6), and NS 398 (n = 9). The blood samples were placed into siliconised glass tubes (Venoject, Terumo) to measure COX-1 activity, into sodium-heparinised tubes (Venoject) to measure COX-2 activity, and into lithium-heparinised tubes (Venoject) to measure 5-LO activity. COX-1/COX-2 and 5-LO activities were determined using the method previously described by Brideau et al. (2001) and by Giorgi et al. (2010), respectively, with some minor modifications. Thromboxane (TX) B2 and prostaglandin (PG) E2 were assayed using a commercial radioimmunoassay kit (Thromboxane B2 [^{125}I] Biotrak Assay System, Amersham Biosciences; Prostaglandin E2 [125I] Biotrak Assay System) as described by Lees and Landoni (2002). Leukotriene (LT) B4 was assayed using a commercial colorimetric EIA (Leukotriene Enzyme Immunoassay Kit, Assay Designs) as described by Giorgi et al. (2010).

Animals and drug administration

The experimental protocol was approved by the Local Ethical Committee (2003071153-002). Eight healthy Standardbred female trotters weighing 389 ± 18 kg (mean ± SEM) and aged 5.5 ± 0.4 years for the ex vivo study were randomly divided into two groups of four horses each. The first group received naproxen (Equiproxen 10%, 10 mg/kg bodyweight(BW) IV); the second group received eltenac (Telzenac, 0.5 mg/kg BW IV). None of the animals in the experimental protocol had received any pharmacological treatment during the 30 days before the start of the experiment.

Ex vivo COX-1/COX-2 and 5-LO assays

Blood samples (30 mL) were drawn by venepuncture from the jugular vein. Blood was collected before the administration of eltenac and naproxen, and at 5 min the 4, 12, 24, 36 h after drug administration. In order to construct a sigmoidal dose–response curve, a blood sample was also collected 48 h after naproxen administration. The blood was collected and treated as described for the in vitro assay. A parallel set of aliquots (10 mL) was collected in siliconized glass tubes and centrifuged at 400 g for 10 min. Serum samples were used to assess naproxen and eltenac levels by HPLC methods as described by Suh et al. (1995) and Dyke et al. (1998), respectively, and fully validated by the Dipartimento di Scienze e Tecnologie Veterinarie per la Sicurezza Alimentare of the University of Milan. (Cagnardi et al., 2006, 2011). The limit of quantification for both naproxen and eltenac was 0.05 lg/mL and the limit of detection was 0.01 µg/mL and 0.0007 µg/mL, respectively.

Statistical data analysis

The results are expressed as means ± SEM. The sigmoidal dose–response curves were analysed using GraphPad Prism vers. 4.00 (GraphPad Software) for plotting data in a nonlinear curve fitting model according to the four parameter logistic equation:

$$\% \text{ Inhibition} = I_0 + (I_{max} - I_0) / \{1 + 10^{\wedge[(LogIC50C)*nH]}\}$$

where % inhibition is the inhibition of eicosanoid (TXB2, PGE2 or LTB4) production expressed as a percentage of the control value; C is the logarithmic value of the test

compound concentration; IC50 (drug potency) is the test compound concentration that resulted in 50% of maximal inhibition (see later) of COX-1 (IC50 COX-1), COX-2 (IC50 COX-2) and 5-LO (IC50 5-LO); I_{max} is the maximum inhibition (drug efficacy); I_0 is the baseline inhibition; n_H (drug affinity) is the Hill coefficient equal to the slope of the concentration–response curve. Selectivity for eltenac, naproxen, NS 398 and SC-560 was determined by calculating the COX-1:COX-2 ratio; selectivity for the dual inhibitor tepoxalin was determined by calculating the COX-1:COX-2:5-LO ratio. The other clinically relevant IC_x and IC_y ratios were calculated similarly. The IC50, Imax and n_H values were compared using Student's t test for unpaired data or one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple-comparison post test. Differences were accepted as significant at $P < 0.05$.

RESULTS

In vitro efficacy and selectivity of test compounds The induced value of TXB2 and the basal value of PGE2 and LTB4 were 40.6 ± 8.5 ng/mL, 40.0 ± 7.0 pg/mL and 165.5 ± 32.8 pg/mL, respectively. The PGE2 and LTB4 concentrations in the positive controls increased by a minimum of 23.5- and 12.4-fold, respectively, compared to the negative controls. Fig. 1 shows the concentration-dependent inhibitory effect of the NSAIDs on TXB2, PGE2 and LTB4 production in the in vitro study. A 100% inhibition of TXB2, PGE2 and LTB4 production was nearly achieved by all drugs (Table 1). Table 1 reports the IC50 and IC80 values and ratios, and the Hill coefficient (nH). In order to predict the clinical relevance of COX selectivity and the safety factor above which unacceptable gastrointestinal adverse effects occur over a concentration producing maximal effect or therapeutic efficacy, the percentage inhibition of COX-2 was plotted against the corresponding inhibition of COX-1 (Fig. 2). The selectivity and safety index were also estimated (Table 2), where the safety index expressed as a ratio of a cut-off concentration above which unacceptable adverse effects occur over a concentration producing maximal effect or therapeutic efficacy. Comparison of IC50 values showed that the order of potency of NSAIDs as inhibitors of COX-1 and COX-2 was: SC-560 > tepoxalin > eltenac > NS 398 > naproxen and eltenac > NS 398PSC- 560 > tepoxalin > naproxen. However, the rank of order of isoform selectivity (COX-1:COX-2 ratio) was: NS 398 > eltenac > naproxen > tepoxalin > SC-560, which suggested that NS 398 was the most selective inhibitor of COX-2 isoform in this study. A statistically significant difference emerged between IC50 COX-1 and IC50 COX-2 for eltenac ($P = 0.0014$), SC-560 ($P = 0.0002$) and NS 398 ($P < 0.0001$), and between IC50

COX-1 and IC50 5-LO for tepoxalin ($P < 0.05$). A statistically significant difference also resulted between IC80 COX-1 and IC80 COX-2 for eltenac ($P = 0.0021$), SC-560 ($P = 0.0003$) and NS 398 ($P < 0.0001$), and between IC80 COX- 1/COX-2 and IC80 5-LO for tepoxalin ($P < 0.05$). There was no significant difference in the efficacy (Imax) or the Hill coefficient (nH) for COX-1, COX-2 and 5-LO inhibition of the test drugs. Ex vivo efficacy and selectivity of eltenac and naproxen In the ex vivo study, the stimulated values of TXB2 and the basal values of PGE2 were 51.6 ± 17.0 ng/mL and 316.6 ± 56.2 pg/mL, respectively. The PGE2 concentration in the positive controls increased 3.4-fold over the negative controls. Intravenous injection of naproxen and eltenac led to a time-dependent decrease in TXB2 and PGE2 synthesis during the experimental times. This decreased inhibitory effect was associated with a reduction in serum drug levels (Table 3). The dose–response curve calculated for eltenac and naproxen (Fig. 3) shows that the eltenac IC50 COX-1 (1.34 μM) was similar to the IC50 COX-2 (1.06 μM; $P = 0.2012$) (ratio 1.26). The IC80 values were slightly higher than the corresponding IC50, but still similar (IC80 COX-1 = 1.81 μM; IC80 COX-2 = 1.23 μM; $P = 0.0505$). Naproxen inhibited COX-1 (IC50 = 36.56 μM; IC80 = 62.34 μM) and COX-2 (IC50 = 28.12 μM; IC80 = 40.21 μM) isoforms at similar concentrations (ratio 1.30) without significant differences ($P = 0.2715$ and $P = 0.0901$, respectively). The inhibition curves against COX-1 and COX-2 by both naproxen and eltenac overlapped. There was a statistically significant difference in the efficacy (Imax) of eltenac in COX-1 vs. COX-2 inhibition ($P = 0.0352$).

DISCUSSION

The present study demonstrated that PGE2 and LTB4 concentrations increased after 10 μg/mL LPS and 60 μM calcium ionophore stimulation, respectively, which suggested that in vitro whole blood assay revealed measurable activity of COX-2 and 5-LO enzymes in the horse. The natural clotting process is known to stimulate platelets to aggregate and activate COX-1. Platelets are a rich source of COX-1 but normally do not express COX-2 (Funk et al., 1991), except in the case of clinical conditions associated with high platelet regeneration, where newly released thrombocytes express COX-2 (Rocca et al., 2002). Platelet activation by clotting induces the synthesis of the unstable metabolite TXA2 via the COX-1 pathway, which is rapidly hydrolyzed non-enzymatically to TXB2 (Hamberg et al., 1975). LPS-activated blood monocytes induce COX-2 to convert AA to PGH2, subsequently converted to PGE2 via PGE synthase (Brideau et al., 2001). Similarly,

circulating neutrophils activated by calcium ionophore induce 5-LO to convert AA to LTB4 (Cunningham et al., 1997). Therefore, determination of TXB2, PGE2 and LTB4 levels in ex vivo and in vitro whole blood assays can be used as markers for COX-1, COX-2 and 5-LO activity, respectively. The basal PGE2 levels in equine whole blood ex vivo were significantly higher than those measured in the ex vivo study ($P < 0.0001$), which may reflect the variation of COX activity among individuals. Prostanoid production also differed from values measured in humans (Warner et al., 1999), rats (Huntjens et al., 2006), dogs and cats (Brideau et al., 2001; Giraudel et al., 2005), confirming a consistent variation among species. However, we used the same radioimmunoassay for PGE2, but with different batches of reagents, as the experiment was performed at different times. This could have affected the difference of PGE2 measurement between the in vitro and the ex vivo studies. Due to their potent activity against COX-1 and COX-2, the experimental compounds SC-560 and NS 398 are usually employed as reference drugs for COX-1 and COX-2 selective inhibition, respectively. In our study, SC-560 acted as a potent and selective COX-1 inhibitor against the equine isoenzyme. It inhibited COX-1 and COX-2 (IC50 of 0.002 μM and 0.04 μM, respectively), and the difference between the two values was significant ($P = 0.0002$). Variable IC50 values for inhibition of COX activity by SC-560 in vitro have been found with the same assay in previous studies performed on whole blood from humans (IC50 COX-1 = 0.013 μM; IC50 COX-2 = 0.93 μM; Bolego et al., 2009) and horses (IC50 COX-1 = 0.0006 μM; IC50 COX-2 = 0.0009 μM; Caruso et al., 2009). Our study showed that the COX-1:COX-2 ratio in the horse (0.05) was similar to that reported in humans (0.01), which confirmed the selectivity of SC-560 against the COX-1 isoform, while it differs from that found in the equine study (0.73), where SC-560 acted as a potent, but poorly selective COX-1 and COX-2 inhibitor. The reason for this discrepancy is unclear. The lack of data on sample analysis (e.g., LPS strain, incubation time) renders a comparison of the results difficult. NS 398 inhibited COX-1 and COX-2 (IC50 1.51 μM and 0.04 μM, respectively; ratio 39.74) and there was a significant difference between the two isoforms ($P < 0.0001$), suggesting that the drug apparently acts as a selective COX-2 inhibitor. However, Fig. 2 shows that for a given percentage inhibition of COX-2 > 50%, the corresponding inhibition of COX-1 is >20%, leading NS 398 to act as a preferential rather than a selective COX-2 inhibitor. Similar results were reported from whole blood studies in humans (IC50 COX-1 = 6.9 μM; IC50 COX-2 = 0.35 μM [Warner et al., 1999]; IC50 COX-1 = 9.7 μM; IC50 COX-2 = 0.35 μM [Berg et al., 2000]) and horses (IC50 COX-1 = 0.41 μM; IC50 COX-2 = 0.013 μM [Caruso et al., 2009]). In a human study, however, Panara et al.

(1995) obtained a ratio of 163. The difference may be due to the use of different anti-PGE2 and anti-TXB2 sera, which were obtained from an internal laboratory in our study and from commercial kits in the other studies. From this evidence it would appear that use of SC-560 for comparing NSAIDs-related activity was a valid method for equine whole blood assay, whereas NS 398 was not the best drug for this purpose. Eltenac ([4-(2,6-dichlorophenyl)amino]-3-thiophene acetic acid) is an acetic acid-derived compound. In equine medicine, only pharmacokinetic (Dyke et al., 1998) and clinical studies (Prügner et al., 1991; Hamm et al., 1997; MacKay et al., 2000) with this drug are available, but not pharmacodynamic data. In the current study, although eltenac was the most potent inhibitor of the COX-2 isoform (IC50 = 0.02 μM), notwithstanding the presence of a significant difference between the two COX isoforms (P = 0.0014) and a ratio of 4.80, it acted more as a preferential than a selective COX- 2 inhibitor. This was demonstrated by the low concentration of drug necessary to inhibit COX-1 (0.10 μM) and the high percentage inhibition of COX-1 (>50%) when COX-2 was inhibited by >80%. Different results were reported in an in vitro human study (Klein et al., 2008), in which eltenac acted as an unselective COX-1 and COX-2 inhibitor (IC50 COX-1 = 0.03 μM; IC50 COX- 2 = 0.03 μM). However, the preferential COX-2 inhibition of eltenac was lost in the ex vivo study, where the drug acted as a non-selective NSAID. Moreover, the IC50 was higher than that of the in vitro study and the drug had sub-maximal inhibition of COX-2, which, from a clinical point of view, would imply a weak anti-inflammatory effect. This finding may reflect the fact that inhibition of COX isoforms depends on NSAIDs pharmacokinetics, as the drugs can accumulate in the target cells, can be degraded variably between individuals or may act through their metabolites (Pairet, 1998). In the case of eltenac, this is a limitation to the in vitro whole blood assay for predicting COX selectivity in the clinical use of this drug. Furthermore, for a normal haematocrit value of 36% in the horse, the multiplying factor to transform whole blood into plasma concentrations was approximately 1.6. However, when the expected whole blood concentration of eltenac and the concentration actually measured by HPLC in plasma were compared, the correcting factor was 10 and 300 for COX-1 and COX-2 inhibition, respectively, which further suggested that eltenac did not enter into whole blood cells. Naproxen or (S)-6-methoxy-a-methyl-2-naphthaleneacetic acid, a propionic acid derivative widely used to treat equine ‘tying up’ syndrome, and is particularly effective in soft tissue inflammatory conditions (Lees and Higgins, 1985). In the in vitro study, it was the least potent inhibitor of both COX isoforms, and it was less potent than eltenac in the ex vivo study. No significant difference was

observed between the in vitro IC50 values for TXB2 and PGE2 inhibition, indicating that naproxen acts as a non-selective COX inhibitor, as confirmed by the overlapping of its dose–response curves (Fig. 1b) and the middle position occupied by the graph of percentage inhibition of COX-2 for the given inhibition of COX-1 (Fig. 2). Naproxen acted as a non-selective NSAID also in the ex vivo study: at 48 h after treatment, it produced a sustained inhibition of both COX-1 and COX-2, leading to a prolonged anti-inflammatory activity. Moreover, the corresponding IC50 values for the in vitro and ex vivo studies differed by less than a log unit range, which suggested a possibility for predicting the drug's activity in horses. It has been demonstrated that naproxen is a non-selective NSAID in humans (Huntjens et al., 2006; Capone et al., 2007) and rats (Huntjens et al., 2006). Unlike eltenac, the corrected multiplying factor for naproxen to transform whole blood into plasma concentrations was <1.6 (0.16 for COX-1 and 0.26 for COX-2), which suggested that naproxen will enter, at least in part, into whole blood cells. This finding was recently demonstrated by Manrique-Moreno et al. (2010) through the observation of naproxen interaction with the outer and inner moiety of the erythrocyte membrane. Tepoxalin (5-(4-chlorophenyl)n-hydroxy-1-(4-methoxyphenyl)-N-methyl-1H-pyrazole-propamide) is a dual inhibitor approved for use in dogs in Europe (Zubrin, EPARs/02/10/08). The in vitro study confirmed this compound's dual activity in equine whole blood, although tepoxalin is 9.3- and 5.5-fold more potent against COX-1 and COX-2, respectively, than 5-LO. More clinically relevant than potency is its greater efficacy towards COX-1 than COX-2, as shown in the dose–response curve. In addition, when the percentage inhibition of COX-2 and 5-LO was plotted against that of COX-1, the results showed that for 80% inhibition of COX-2 and 5-LO (considered the value above which a therapeutic effect is expected), the percentage inhibition of COX-1 was very high (99.17% and 99.26%, respectively), indicating that tepoxalin may potentially induce collateral effects with poor benefits. Other in vitro studies conducted in both humans (Argentieri et al., 1994) and horses (Caruso et al., 2009) have demonstrated the dual inhibition of tepoxalin and its preferential potent activity against the COX-1 isoform. In addition, the ex vivo study performed by Giorgi et al. (2010) established the weak inhibitory activity of tepoxalin against COX-2 and 5-LO isoform and the strong and long-lasting activity against COX-1, mainly due to its metabolite RWJ-142. Conversely, the potencies of tepoxalin as a LO and COX inhibitor in dogs seemed to be in the same range (Zubrin, EPARs/02/10/08). In general, the Hill coefficients (nH) for the test compounds and the COX isoforms of the drugs were similar, which

indicated that the enzymes have analogous binding properties, although the COX isoforms showed about 69% homology.

CONCLUSIONS

This study evaluated the potency and the selectivity of different NSAIDs in vitro and ex vivo in equine whole blood. SC-560 acted as a selective COX-1 inhibitor, tepoxalin as a dual inhibitor but with a potent activity against COX-1, and NS 398 as a preferential COX-2 inhibitor. Eltenac was a preferential COX-2 inhibitor in vitro but non-selective ex vivo. Naproxen maintained its non-selectivity both in vitro and ex vivo. The method accounted for the variable degree of protein binding of NSAIDs and clinical relevant target cells; even so, it is not always predictive of accurate estimation of the COX selectivity of NSAIDs in clinical use, as was demonstrated for eltenac. In addition, variable IC50 values showed that COX-1 and COX-2 activity and inhibitor selectivity were species dependent, as in the case of NS 398.

Conflict of interest statement

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

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FIGURES AND TABLES

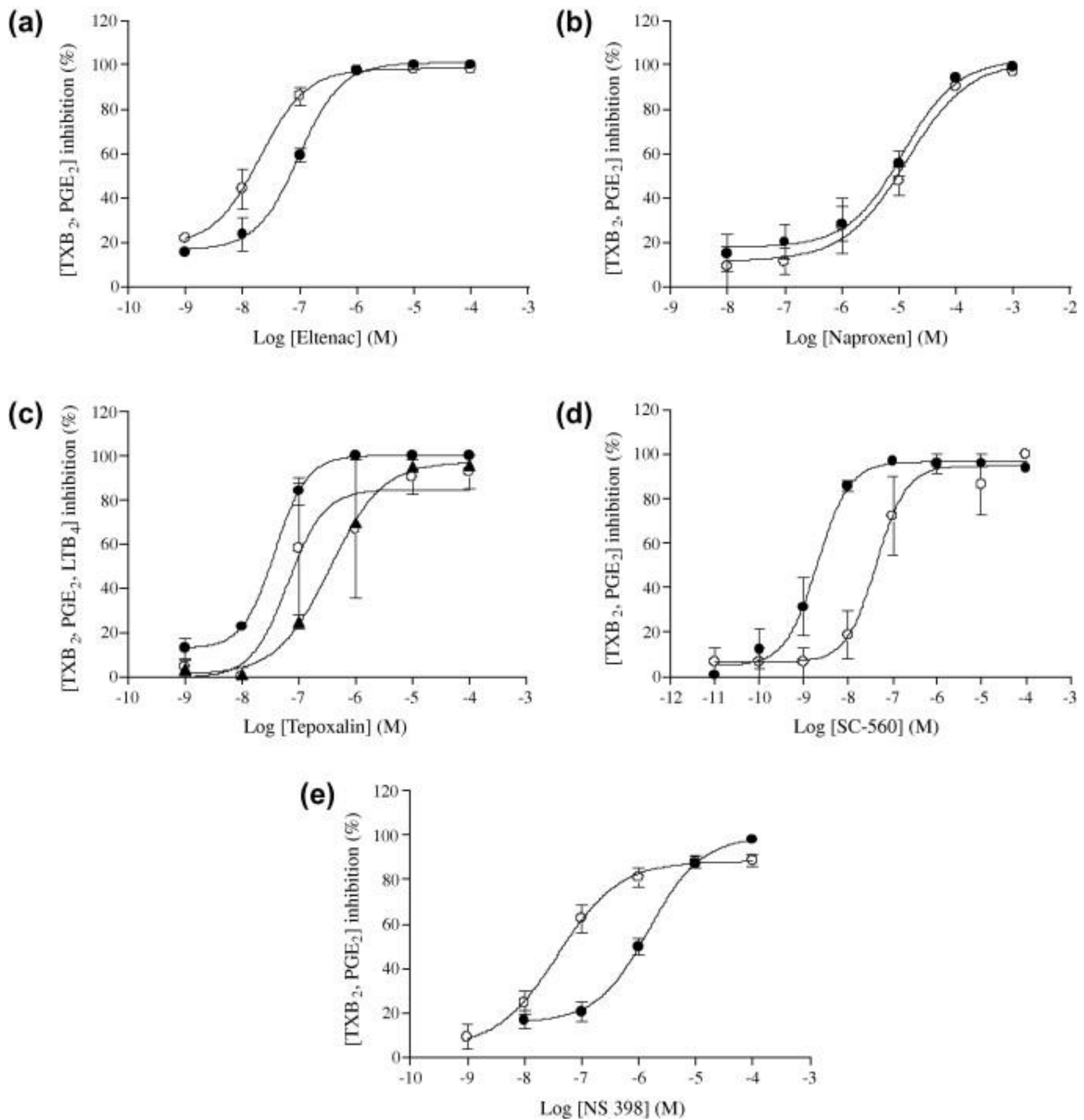

Fig. 1. Mean ± SEM percentage inhibition of TXB2 (closed circles), PGE2 (open circles) and LTB4 (closed triangles) production in equine whole blood (in vitro study) vs. concentrations of eltenac (a; n = 5), naproxen (b; n = 8), tepoxalin (c; n = 5), SC-560 (d; n = 6) and NS 398 (e; n = 9).

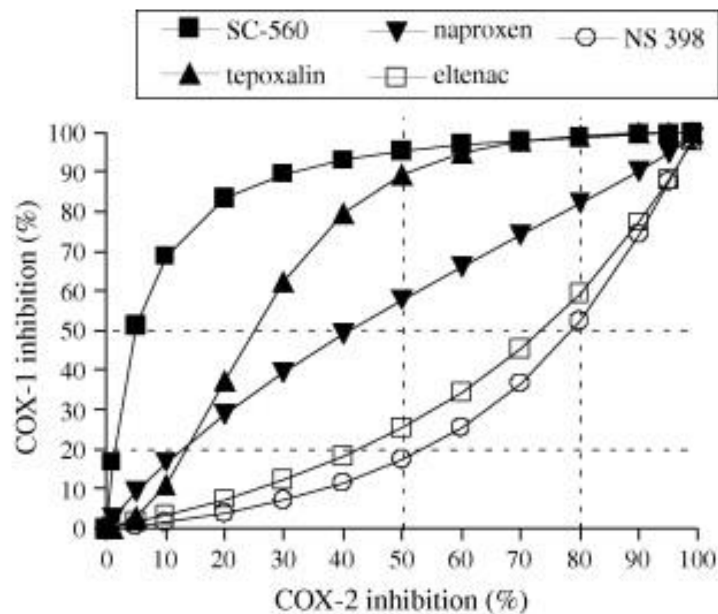

Fig. 2. Percentage inhibition of COX-2 and the corresponding percentage inhibition of COX-1 by eltenac, naproxen, tepoxalin, SC-560, and NS 398 measured in equine whole blood (in vitro study). Dotted lines indicate cut-off values for inhibition of both isoforms (i.e., 50% inhibition of COX-1 and COX-2 represents the percent value of NSAIDs selectivity; 20% and 80% inhibition are considered the percent values above which there may be a risk of adverse effects and a good therapeutic effect, respectively).

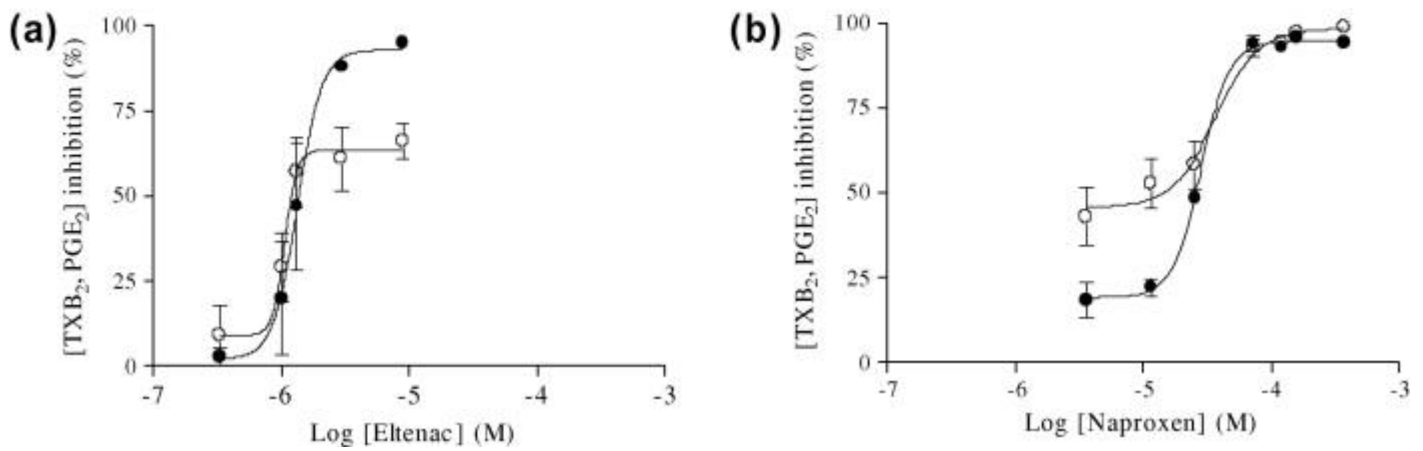

Fig. 3. Mean ± SEM (n = 4) percentage inhibition of TXB2 (closed circles) and PGE2 (open circles) production in equine whole blood (ex vivo study) vs. concentrations of eltenac (a) and naproxen (b).

Table 1. Mean parameters derived from the Hill equation by fitting the individual percentage inhibition values from equine whole blood in the in vitro study (IC_X = test compound concentration producing a COX-1 or COX-2 inhibition of X percent of the maximum inhibition. I0 = baseline inhibition. Imax = maximum inhibition. n_H = Hillslope).

Test compound parameters	Assay			IC_x ratio	
	COX-1	COX-2	5-LO	COX-1:COX-2	COX-1:COX-2:5-LO
Eltenac ($n = 5$)					
I_0 (%)	16.60 ± 3.65	18.70 ± 6.22			
I_{max} (%)	100.80 ± 2.38	98.28 ± 2.50			
IC_{50} (μM)	0.10	$0.02^{\dagger}$		4.80	
IC_{80} (μM)	0.31	$0.07^{\dagger\dagger}$		4.26	
n_H	1.17 ± 0.29	1.07 ± 0.12			
Naproxen ($n = 8$)					
I_0 (%)	17.81 ± 4.73	11.49 ± 6.71			
I_{max} (%)	102.20 ± 7.20	101.80 ± 9.36			
IC_{50} μM)	11.57	13.57		0.85	
IC_{80} μM)	53.50	78.26		0.68	
n_H	0.90 ± 0.33	0.79 ± 0.30			
Tepoxalin ($n = 5$)					
I_0 (%)	12.69 ± 3.31	-0.34 ± 19.12	1.06		
I_{max} (%)	100.20 ± 1.83	84.75 ± 11.15	97.44		
IC_{50} (μM)	0.04	0.06	0.35^{*}	0.59	1.67
IC_{80} (μM)	0.09	0.18	1.55^{**}	0.53	0.34
n_H	1.53 ± 0.23	1.36 ± 1.71	0.94		
SC-560 ($n = 6$)					
I_0 (%)	4.84 ± 4.57	6.41 ± 7.05			
I_{max} (%)	96.84 ± 3.58	94.96 ± 6.61			
IC_{50} (μM)	0.002	$0.04^{\ddagger}$		0.05	
IC_{80} (μM)	0.006	$0.13^{\ddagger\ddagger}$		0.05	
n_H	1.21 ± 0.28	1.28 ± 0.56			
NS 398 ($n = 9$)					
I_0 (%)	15.59 ± 3.09	4.13 ± 11.39			
I_{max} (%)	99.42 ± 3.95	88.30 ± 3.58			
IC_{50} (μM)	1.51	$0.04^{\S}$		39.74	
IC_{80} (μM)	6.54	$0.21^{\S\S}$		30.70	
n_H	0.95 ± 0.19	0.81 ± 0.24			

* Significantly ($P < 0.05$) different from IC50 COX-1 determined for the same compound.

** Significantly ($P < 0.05$) different from IC80 COX-1 and IC80 COX-2 determined for the same compound.

† Significantly ($P = 0.0014$) different from IC50 COX-1 determined for the same compound.

†† Significantly ($P = 0.0021$) different from IC80 COX-1 determined for the same compound.

‡ Significantly ($P = 0.0002$) different from IC50 COX-1 determined for the same compound.

‡‡ Significantly ($P = 0.0003$) different from IC80 COX-1 determined for the same compound.

§ Significantly ($P < 0.0001$) different from IC50 COX-1 determined for the same compound.

§§ Significantly ($P < 0.0001$) different from IC80 COX-1 determined for the same compound.

Table 2 Indices used to describe the selectivity of eltenac, naproxen, tepoxalin, SC-560 and NS 398 by fitting individual percentage inhibition values in equine whole blood in the in vitro study.

Indices	Test compounds				
	SC-560	Naproxen	Tepoxalin	Eltenac	NS 398
IC_X:IC_Y of COX-1:COX-2[a]					
IC_1:IC_{99}	0.000029	0.000016	0.0010	0.0013	0.001
IC_5:IC_{95}	0.000416	0.000796	0.0099	0.0243	0.047
IC_{10}:IC_{90}	0.001384	0.004636	0.0279	0.0924	0.259
IC_{20}:IC_{80}	0.005103	0.031928	0.0861	0.3957	1.643
% Inhibition of COX-1 (0–100%)					
For 50% inhibition of COX-2	95.22	55.77	89.36	25.35	17.42
For 80% inhibition of COX-2	98.76	82.10	99.17	59.22	52.36
For 95% inhibition of COX-2	99.74	94.70	99.96	88.15	87.54
For 99% inhibition of COX-2	99.95	98.69	100.00	97.67	98.04

[a] Selectivity can be defined as safety index, which is usually expressed as a ratio of a cut-off concentration above which unacceptable adverse effects occur over a concentration producing maximal effect or therapeutic efficacy.

Table 3 Ex vivo study: percentage of TXB2 and PGE2 inhibition at different experimental time points after IV administration of naproxen (10 mg/kg BW) or eltenac (5 mg/kg BW) injection in four horses and corresponding serum levels. Results are expressed as means ± SEM.

Experimental time points ($n = 4$)	Naproxen			Eltenac		
	% Inhibition		Serum levels (μg/mL)	% Inhibition		Serum levels (μg/mL)
	TXB_2	PGE_2		TXB_2	PGE_2	
T5 min	98.5 ± 1.4	96.1 ± 0.8	94.1 ± 4.1	95.0 ± 0.7	66.0 ± 5.3	2.7 ± 0.6
T4 h	97.0 ± 0.5	95.6 ± 1.1	39.9 ± 2.8	88.0 ± 1.3	61.0 ± 9.4	0.9 ± 0.1
T12 h	92.6 ± 2.5	93.7 ± 2.4	18.4 ± 2.5	47.0 ± 18.7	57.0 ± 10.2	0.4 ± 0.1
T24 h	58.3 ± 7.0	48.6 ± 0.7	6.4 ± 1.7	20.0 ± 16.8	29.0 ± 9.9	0.3 ± 0.1
T36 h	52.6 ± 7.0	22.3 ± 2.6	2.9 ± 0.9	2.7 ± 2.7	9.0 ± 9.0	0.1 ± 0.1
T48 h	42.8 ± 8.6	18.5 ± 5.4	0.9 ± 0.5			