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AUTHORS: Pelligand, L and King, J N and Hormazabal, V and Toutain, P L and Elliott, J and Lees, P

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Differential pharmacokinetics and pharmacokinetic/pharmacodynamic modelling of robenacoxib and ketoprofen in a feline model of inflammation

L. PELLIGAND*

J.N. KING†

V. HORMAZABAL‡

P.L. TOUTAIN§

J. ELLIOTT*

P. LEES*

**Department of Comparative and Basic Sciences, Royal Veterinary College, Hawkshead Campus, Hatfield, Hertfordshire, UK; †Novartis Animal Health Inc., Clinical Development, Basel, Switzerland (J.N.K.); ‡Department of Food Safety and Infection Biology, The Norwegian School of Veterinary Science, Oslo, Norway (V.H.); §UMR 1331 Toxalim INRA/INP/UPS. Ecole Nationale Vétérinaire de Toulouse, Toulouse, France (P.L.T.)*

Corresponding author: Ludovic Pelligand, Department of Comparative and Basic Sciences, Royal Veterinary College, Hawkshead Campus, Hatfield, Hertfordshire, UK. Email: lpelligand@rvc.ac.uk

Short title: robenacoxib and ketoprofen in the cat

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ABSTRACT

Robenacoxib and ketoprofen are acidic non-steroidal anti-inflammatory drugs (NSAIDs). Both are licensed for once daily administration in the cat, despite having short blood half-lives. This study reports the pharmacokinetic/pharmacodynamic (PK/PD) modelling of each drug in a feline model of inflammation. Eight cats were enrolled in a randomised, controlled, three period cross-over study. In each period, sterile inflammation was induced by injection of carrageenan into a subcutaneously implanted tissue cage, immediately before the subcutaneous injection of robenacoxib (2 mg/kg), ketoprofen (2 mg/kg) or placebo. Blood samples were taken for determination of drug and serum thromboxane (Tx)B_2 concentrations (measuring COX-1 activity). Tissue cage exudate samples were obtained for drug and prostaglandin(PG)E_2 concentrations (measuring COX-2 activity). Individual animal pharmacokinetic and pharmacodynamic parameters for COX-1 and COX-2 inhibition, were generated by PK/PD modelling. S(+) ketoprofen clearance scaled by bioavailability (CL/F) was 0.114 L/kg/h (elimination half-life =1.62 h). For robenacoxib, blood CL/Fwas 0.684 L/kg/h (elimination half-life =1.13 h). Exudate elimination half-lives were 25.9 and 41.5 h for S(+) ketoprofen and robenacoxib, respectively. Both drugs reduced exudate PGE_2 concentration significantly between 6 and 36 h. Ketoprofen significantly suppressed (>97%) serum TxB_2 between 4 min and 24 h, whereas suppression was mild and transient with robenacoxib. *In vivo* IC_{50}COX-1/IC_{50}COX-2 ratios were 66.9:1 for robenacoxib and 1:107 for S(+) ketoprofen. The carboxylic acid nature of both drugs may contribute to the prolonged COX-2 inhibition in exudate, despite short half-lives in blood.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase (COX) and have been used for many decades to alleviate inflammation-related pain in human and veterinary medicine. COXibs belong to a class of NSAIDs that selectively inhibit the COX isoform up-regulated in inflammation (COX-2), with much less inhibition of the constitutively expressed COX isoform (COX-1) responsible for the production of so-called "housekeeping" eicosanoids. Several COXibs were associated with an increased risk of myocardial infarction or stroke in man when evaluated against non-selective NSAID comparators and administered at recommended dose rates (Bombardier *et al.*, 2000; Silverstein *et al.*, 2000). However, lumiracoxib contrasted with other COXibs by displaying favourable cardiovascular and gastrointestinal safety profiles in a study incorporating more than 18,000 human patients (Farkouh *et al.*, 2004).

Despite a short plasma half-life (2 to 6.5 h) in man, lumiracoxib was authorised for once daily administration (Mysler, 2004), whereas rofecoxib and celecoxib were both administered twice daily, despite their long respective half-lives of 17 and 19 to 32 h (Vasquez-Bahena *et al.*, 2010). The persistent clinical efficacy of lumiracoxib may be related to a prolonged residence of the drug in inflamed joints (Scott *et al.*, 2004; Brune & Furst, 2007).

Robenacoxib is a structural analogue of lumiracoxib, licensed for use in cats (and dogs). Both drugs are structurally related to diclofenac (Fig. 1), containing a carboxylic acidic function instead of the methylsulphone (as for rofecoxib) or the sulphonamide (as for celecoxib) groups characteristic of first generation COXibs. Robenacoxib has a short blood half-life of 1.9 h after

subcutaneous administration in the cat (Pelligand *et al.*, 2012b) but is as effective as an analgesic as meloxicam (half-life 37 h) for at least 22 h post-operatively (Kamata *et al.*, 2012).

Ketoprofen is a COX-1 selective NSAID (Fig. 1), licensed for once daily administration in man and cats (Warner *et al.*, 1999; Schmid *et al.*, 2010) despite short elimination half-lives for the eutomer S(+) ketoprofen of 1.2 h in the cat and 2 to 3 h in man (Rudy *et al.*, 1998; Lees *et al.*, 2003).

We hypothesised that robenacoxib and ketoprofen would be similarly effective as anti-inflammatory drugs in a feline model of inflammation, despite the differences in COX selectivity, because of similar concentration-time profiles and potency for COX-2 inhibition. Several models of inflammation have been developed in the cat (Giraudel *et al.*, 2005b; Pelligand *et al.*, 2012a) but only the carrageenan-based tissue cage model of Pelligand *et al.* (2012a) allows serial measurement of NSAID concentrations in blood and at the site of inflammation, as well as determination of the magnitude and time-profiles of COX-1 and COX-2 inhibition. We have previously described exudate sampling from these cages for serial measurement of NSAID and Prostaglandin(PG)E_2 concentrations, the latter inflammatory mediator as a surrogate of COX-2 activity, together with blood sampling for measurement of serum thromboxane(Tx)B_2 as a surrogate of COX-1 activity (Pelligand *et al.*, 2012a; Pelligand *et al.*, 2012b). The model further enables calculation of pharmacodynamic parameters of NSAIDs, namely I_{max} (efficacy), IC_{50} (potency) and n (slope of the concentration-effect relationship). These parameters are used to calculate dosage regimens for clinical use (Lees *et al.*, 2004).

The aims of this study were to: (i) compare the pharmacokinetic and the pharmacodynamic profiles of robenacoxib and ketoprofen in feline blood and exudate; (ii) compare *in vivo* IC_{50} COX-2, *ex vivo* IC_{50} COX-1 and COX inhibition selectivity for each drug; and (iii) compare and contrast the pharmacokinetic and pharmacodynamic profiles of two carboxylic acid sub-groups of NSAIDs, the profens and COXibs, in the cat.

MATERIALS AND METHODS

Animals

Eight domestic short hair cats (all neutered, 5 males and 3 females, aged 1 to 3 years), weighing 4.0±0.39 kg, were enrolled into the study after an acclimatisation period of one month. Health checks were performed before the start of each sampling period. Between study periods, the cats were grouped housed altogether. They were fed a dry commercial diet (RF23, Royal Canin, Aimargues, France) in two equal portions daily, based on their metabolic requirements. Drinking water was available *ad libitum*. The room was lit from 7.00 am to 7.00 pm.

The study complied with United Kingdom Home Office regulations (Project License Number 70/6132). The protocol was approved by the Royal Veterinary College Ethics and Welfare Committee. All tissue cages were electively removed under general anaesthesia 6 months after implantation, before the cats were re-homed, as no long lasting sequelae resulted from the protocol.

Animal preparation and induction of inflammation

Four tissue cages were implanted surgically in each cat, as previously described (Pelligand *et al.*, 2012b). Briefly, medical grade silicone cylindrical tissue cages (SF Medical, Fresno, CA, USA) were prepared to the following dimensions: 70 mm length, 15.9 mm external diameter, 12.7 mm internal diameter (6.7 cm^3 internal volume) with 12 holes at each pole providing a total surface exchange area of 3.0 cm^2 per cage. They were sterilised and surgically inserted subcutaneously, under isoflurane (IsoFlo, Abbott Animal Health, Maidenhead, UK) general anaesthesia, parallel to the vertebral column in the flank and thorax areas. Analgesia was provided intra- and post-operatively as described by Pelligand et al. (2012a). The tissue cages were flushed with sterile saline under general anaesthesia 7 weeks after implantation to remove any remaining cellular debris and subsequently used experimentally, not earlier than 10 weeks after implantation. On the day before dosing, the fur over the cages was clipped and a double-lumen catheter (CS15402E, Arrow International Ltd, Uxbridge, UK) was inserted in a jugular vein under general anaesthesia (Pelligand et al .2012a).

On day 1 of each period, 1 mL of a 2% sterile carrageenan solution (Viscarin, FMC biopolymers, Philadelphia, PA, USA) was injected into a naïve tissue cage (Pelligand *et al.*, 2012b) and this tissue cage was used to harvest exudate in that period. A different tissue cage was stimulated for each subsequent period.

Experimental design

The experiment was conducted as a three-period, three-sequence, cross-over with 28 day washout intervals. The treatment for the first period was allocated following a randomised blocked design and the sequence of treatments for the subsequent periods followed an

incomplete latin square design. All cats received each of the treatments. The three treatments, administered subcutaneously in the neck area, were: robenacoxib 2 mg/kg (Onsior 2.0% solution, Novartis Animal Health, Basel, Switzerland); racemic ketoprofen 2 mg/kg (Ketofen 1% solution, Merial Animal Health, Harlow, Essex, UK); and 0.9% saline (0.1 mL/kg) as placebo. On each test day, the cats were fed 2 h before dosing and again after the final blood sample of the day (12 h after dosing). The carrageenan-stimulated cages were sampled (1.0 to 1.3 mL of exudate on each occasion) before and at 3, 6, 9, 12, 24, 34, 48, 72, 96 and 120 h after carrageenan injection (11 serial samples from the same cage). Samples were transferred immediately to 1.5 mL Eppendorf tubes containing 10 µg indomethacin (Sigma Aldrich, Poole, Dorset, UK) to prevent artefactual *ex vivo* eicosanoid generation. The tube was mixed by gentle inversion and placed on ice until centrifugation at 1000 *g*, 4° C for 10 min. The supernatant was aliquoted and frozen at -80°C prior to measurement of exudate PGE_2 and concentrations of ketoprofen or robenacoxib.

Blood samples (maximum 1.5 mL per sample) were taken from the distal port jugular catheter before dosing and at 4, 15, 30 min, then 1, 1.5, 2, 3, 4, 6, 9, 12, 24 and 48 h after dosing. An aliquot of each blood sample (0.2 mL) was allowed to clot in a glass tube (Chromacol, Welwyn Garden City, Herts., UK) whilst incubated in a water bath at 37º C for 1 h then centrifuged (1500 *g*, 4º C, 10 min) and the supernatant stored at -80º C prior to measurement of serum TxB_2. The remainder of the sample (1.3 mL) was transferred into an EDTA tube (International Scientific Supplies Ltd., Bradford, Yorks., UK) for blood robenacoxib measurement or a heparin tube for plasma ketoprofen measurement and stored at -80º C.

To ensure accuracy of pharmacokinetic calculations, cats were weighed on the day of catheter placement, actual injected doses were calculated by weighing syringes before and after injection and actual rather than nominal times of blood sampling were used.

Measurement of NSAID concentrations

Plasma (ketoprofen), blood (robenacoxib) and exudate (both drugs) were spiked with known drug concentrations to establish standard curves, and quality controls (QCs) were prepared and dispersed over the sequence of unknown samples, to monitor the overall performance of each analytical method. The percentage of back-calculated concentrations of standards within ±15% of their nominal value and the percentage of QCs within ±15% of their theoretical value were calculated. Imprecision (indicator of between day repeatability) was expressed as the coefficient of variation (CV%) between standard concentrations run on different days. Inaccuracy was expressed as the deviation of the mean (% Relative Error) from the theoretical concentration spiked into blank matrix.

Robenacoxib concentrations in feline blood were measured using a sensitive analytical method, as described by Jung *et al*. (2009). Briefly, the method involved an initial analysis by HPLC-UV, covering the range of 500-20,000 ng/mL and, if required, a subsequent analysis by LC-MS, covering the range of 3-100 ng/mL for blood. Depending on the results obtained by UV analysis, samples were diluted if necessary in order not to exceed a concentration of 100 ng/mL in the MS method. The same method was used for exudate, except that 250 µL of sample were extracted and diluted two-fold with water, instead of using 500 µL of blood. For blood with the MS method, the lower and upper limits of quantification were 3 and 100 ng/mL, respectively. Since

the exudate was diluted two fold, the MS method had a range of 6-200 ng/mL for exudate in the initial method validation, but this was extended subsequently to 3.5 – 200 ng/mL, as it was established during the analysis that reliable results were obtained at the lower end of the range. For robenacoxib, inaccuracy was less than 10.4% and imprecision was less than 9.3%.

R(-) ketoprofen and S(+) ketoprofen concentrations were measured in exudate and plasma by liquid chromatography mass-spectrometry (API 2000 LC/MS/MS system, Applied Biosystems, Ontario, Canada). The method, previously validated for cat and piglet plasma (Fosse *et al.*, 2011 and Hormazábal, unpublished data), had lower and upper limits of quantification of 10 and 8,000 ng/mL for both matrices. After extraction, filtration and centrifugation of 0.5 mL plasma/exudate, 50 µL of the supernatant was separated on a 100 x 4.6 mm Chirobiotic R column packed with 5 µm ristocetin A particles (Chirobiotic R, Astec, NJ, USA) at 18°C. The mobile phase comprised 46% of 10 nM ammonium acetate (containing 0.3% formic acid) and 54% methanol. Flow rate was 0.6 mL/min. A guard column with similar sorbent was used (20 x 4.0mm). Average retention times were 4.9 and 5.4 min for S(+) and R(-) ketoprofen, respectively. The detector operated in multiple reactions monitoring (MRM) mode and collected ion data in positive mode. The protonated molecular ion was m/z 255. The product ion m/z 209.1 was used for screening and quantification, while the ratios with the product ion m/z 105.2 were used for confirmation of the identity. For ketoprofen enantiomers, inaccuracy was less than 1.3% and imprecision was less than 0.7%.

Pharmacodynamic measurements

In vivo generation of exudate PGE_2 and *ex vivo* generation of serum TxB_2 were used as surrogates for COX-2 and COX-1 activities, respectively. Although the main source of TxB_2 in serum is platelet COX-1, a minor contribution from COX-2 or from other cells cannot be completely excluded. Additionally, COX-1 may contribute to the synthesis of PGE_2 in exudate (Nantel *et al.*, 1999; Wallace *et al.*, 1999), but the magnitude of this production is likely to be negligible, based on the fact that COX-1 is not induced in carrageenan inflammatory models (Tomlinson *et al.*, 1994).

Serum TxB_2 and exudate PGE_2 concentrations were measured with competitive radio-immunoassays, adapted from Higgins *et al.* (1984) as described in a previous validation paper (Pelligand *et al.*, 2012a). Two concentrations of pooled samples were aliquoted, and used as quality controls, dispersed over the sequences of unknown samples to calculate inter- and intra-assay variability. Exudate PGE_2 intra-assay variability was 3% for the high control concentration (2.9 ng/mL) and 23.9% for the low control concentration (0.13 ng/mL). Inter-assay variability was 2.1% for the high control and 31% for the low control concentrations. Serum TxB_2 intra-assay variability was 7.2% for the high control (235.1 ng/mL) and 13.2% for the low control (56.5 ng/mL) concentrations. Inter-assay variability was 2.3% and 11.3% for the high and low control concentrations, respectively. All validation data complied with analytical recommendations guidelines (Kelley & DeSilva, 2007; Viswanathan *et al.*, 2007) except for PGE_2 inter-assay variability. Therefore, all samples from the same cats were always analysed in the same batch.

Pharmacokinetic data analysis

Pharmacokinetics and PK/PD modelling were performed by the least-squares regression method, using commercial software (WinNonlin version 5.2, Pharsight Corporation, Mountain View, CA, USA). Goodness of fit and selection of the appropriate model were evaluated using the Akaike Information Criterion estimate (Yamaoka *et al.*, 1978) and by visual inspection of the fitted curves and residuals.

Blood robenacoxib and plasma ketoprofen enantiomer concentrations C(t) were fitted for each cat using an equation corresponding to drug disposition in a two-compartmental model with absorption phase (subcutaneous administration, Eq. 1):

$$C(t) = -(Y_1 + Y_2).e^{-k_a.(t-tl_{ag})} + Y_1.e^{-\lambda_1.(t-t_{lag})} + Y_2.e^{-\lambda_2.(t-t_{lag})} \qquad \text{(Eq. 1)}$$

where λ_1 and λ_2 are the initial and terminal slopes (/h), Y_1 and Y_2 the intercepts on the Y axis (ng/mL), when *C(t)* is plotted on a semi-logarithmic scale, k_a is the first-order absorption rate constant (/h) and t_{lag} the absorption lag time after subcutaneous administration. Data were weighted by the reciprocal of the estimated value for blood or plasma concentration when necessary.

Exudate concentrations of robenacoxib or ketoprofen enantiomers $C_e(t)$ were fitted for the data from each cat using an equation corresponding to drug disposition in a bicompartmental model with an absorption phase after dose normalisation (Eq. 2):

$$C_e(t) = -(Y_{e1} + Y_{e2}).e^{-k_{ea}.t} + Y_{e1}.e^{-\lambda_{e1}.t} + Y_{e2}.e^{-\lambda_{e2}.t} \qquad \text{(Eq. 2)}$$

where λ_{e1} and λ_{e2} are the initial and terminal slopes (/h), Y_{e1} and Y_{e2} the intercepts on the Y axis (ng/mL) when $C_e(t)$ is plotted on a semi-logarithmic scale, and k_{ea} is the first-order invasion rate constant in exudate (/h). No weighting was applied to the data for fitting. It was assumed that only a negligible amount of each NSAID gained access to the tissue cage and that the

pharmacokinetics in exudate had no effect on the time-course of drug disposition in the rest of the body.

Pharmacokinetic parameters were generated for robenacoxib (in blood and exudate) and S(+) and R(-) ketoprofen (in plasma and exudate) by non-compartmental analysis for individual cats, as follows: Maximum NSAID concentration, C_{max}, Time of maximum NSAID concentration, T_{max}, Area under NSAID concentration-time curve, $AUC_{0\text{-}inf}$, Area under first the Moment Curve, $AUMC_{0\text{-}inf}$, NSAID Mean Residence Time (MRT) = $AUMC_{0\text{-}inf}$ /$AUC_{0\text{-}inf}$, NSAID terminal half-life, $t_{½}=\ln(2)/\lambda_z$, where λ_z is the slope of the drug elimination phase, computed by linear regression of the logarithmic concentration versus time curve during the elimination phase, NSAID clearance scaled by bioavailability (F), CL/F= dose/(F x $AUC_{0\text{-}inf}$), where F is the bioavailability for extravascular administration, Apparent volume of distribution of NSAID during the elimination phase, $V_{area}/F = (dose/F)/(AUC_{0\text{-}inf} \times \lambda_z)$.

Pharmacodynamic data analysis and PK/PD modelling

A user program was purposely written in WinNonlin for PK/PD modelling. The equations of robenacoxib and S(+) ketoprofen enantiomer disposition in blood/plasma C(t) or in exudate $C_e(t)$ were obtained by compartmental pharmacokinetic analysis by fitting equations (1) or (2), respectively, to the observed data. Individual pharmacokinetic parameters were entered as constants to solve the PK/PD models in a 2 stages analysis (Giraudel *et al.*, 2005a).

In vivo generation of exudate PGE_2 was used as a surrogate for COX-2 activity in order to carry out PK/PD modelling of the NSAIDs in exudate (Lees *et al.*, 2004). An indirect response model described by Pelligand *et al.* (2012b) was used to model the effect of robenacoxib and S(+)

ketoprofen on exudate PGE_2 production. The model did not include the R(-) enantiomer, as it was considered to be devoid of activity on cyclooxygenase at the concentrations achieved. Indeed, S(+) ketoprofen is the eutomer of the S(+)/R(-) ketoprofen enantiomeric pair (Lees *et al.*, 2003). The response is indirect because it is the consequence of a dynamic physiologic equilibrium between PGE_2 production after carrageenan injection, the natural clearance of PGE_2 from exudate and the reversible inhibition of COX-2 by NSAIDs, preventing the build-up of PGE_2 in exudate as in Equation 3 (Dayneka *et al.*, 1993):

$$\frac{dPGE_2}{dt} = K_{in}(t) - K_{out} \times PGE_2 \qquad \text{(Eq. 3)}$$

where $dPGE_2/dt$ (ng/mL/h) is the rate of change of PGE_2 concentration in exudate, K_{out} (/h) is a first order parameter expressing PGE_2 disappearance rate and $K_{in}(t)$ (ng/mL/h) is a zero-order time-function expressing PGE_2 production rate. K_{in} is considered as a time-dependent parameter, influenced by carrageenan administration and NSAID concentration (in the periods when administered). To express the action of carrageenan on K_{in}, a stimulation function (named $stimul_{PLACEBO}$ and $stimul_{NSAIDs}$ was selected as Eq. 4 and Eq. 5 for the placebo and NSAID periods, respectively:

$$stimul_{PLACEBO} = carrag \times \left(e^{-k_1 \times (t - tlag1)} - e^{-k_2 \times (t - tlag1)} \right) \qquad \text{(Eq. 4)}$$

$$stimul_{NSAIDs} = carrag \times \left(e^{-k_1 \times (t - tlag2)} - e^{-k_2 \times (t - tlag2)} \right) \qquad \text{(Eq. 5)}$$

where k_1 and k_2 are the first-order rate constants (/h) describing the time-development of the carrageenan stimulation, *carrag* is a scalar factor, and *tlag1* and *tlag2* represent the delays in the onset of inflammation for the placebo and NSAID periods, respectively. Consequenlty, t*lag* is the only difference between $stimul_{PLACEBO}$ and $stimul_{NSAIDs}$ function. Equation 4 and Eq. 5

assume that the effect of carrageenan stimulation of COX builds up progressively (as reflected by k_2) after injection, then steadily decreases (as reflected by k_1) (Lepist & Jusko, 2004).

It was assumed that robenacoxib and ketoprofen suppressed the carrageenan action in exudate through an I_{max} function (Lees *et al.*, 2004) of the form (Eq. 6):

$$I(t) = 1 - \frac{I_{max} \times C_e(t)^n}{IC_{50}{}^n + C_e(t)^n} \qquad \text{(Eq. 6)}$$

I(t) is a time-dependant scalar. I_{max} is a scalar fixed to 1, expressing the fact that robenacoxib can totally inhibit carrageenan pro-inflammatory effect. IC_{50} expresses the NSAID potency against carrageenan effect; n is the Hill exponent expressing the steepness of the NSAID concentration versus effect curve. Finally, incorporating Eq. 4 (placebo) or Eq. 5 and Eq. 6 (NSAID) in the general Eq. 3, the time development of PGE_2 concentration in exudate was described by Eq. 7 (placebo) and Eq. 8 (NSAID):

$$\frac{dPGE_2}{dt} = K_{in}(t) - K_{out} \times PGE_2 = K_{in} \times stimul_{PLACEBO} - K_{out} \times PGE_2 \text{ (Eq. 7)}$$

$$\frac{dPGE_2}{dt} = K_{in} \times stimul_{NSAIDs} \times \left[1 - \frac{C_e(t)^n}{IC_{50}{}^n + C_e(t)^n}\right] - K_{out}.PGE_2 \qquad \text{(Eq. 8)}$$

The time-courses of exudate PGE_2 were modelled simultaneously for placebo and robenacoxib, then placebo and S(+) ketoprofen, as the equations for placebo and NSAIDs share several common parameters in the same cat (K_{in}, K_{out} and *carrag*, k_1 and k_2). Nine parameters were estimated by the model, namely k_{in}, carrag, k_1, k_2, tlag1, tlag2, k_{out} IC_{50} and n.

Ex vivo generation of serum TxB_2 was used as a surrogate marker of COX-1 activity for PK/PD modelling. The NSAID concentration in the central compartment produced an inhibition of

serum TxB_2 synthesis according to the following sigmoid I_{max} model selected to fit the serum TxB_2 data (Eq.9):

$$I(C(t)) = I_0(t) - \frac{(I_0 - I_{max}) \times C(t)^n}{IC_{50}{}^n + C(t)^n} \quad \text{(Eq. 9)}$$

where I_0 (t)is the baseline serum TxB_2 concentration (ng/mL) for an individual cat, I_{max} (%) is the percentage of maximal TxB_2 suppression (corresponding to the lower limit of quantification of the assay) relative to I_0(t), IC_{50} (ng/mL) is the concentration that achieves half of the maximal TxB_2 suppression and n is the slope of the NSAID concentration-effect curve. In most cats, the serum TxB_2 concentration had drifted below baseline by the end of the period when placebo was administered, as also reported in a previous study (Pelligand *et al.*, 2012b). This drift of baseline throughout the course of the experiment was modelled as (Eq. 10):

$$I_0(t) = I_0 - d \times t \quad \text{(Eq. 10)}$$

where d represents the slope of the baseline function for an individual cat and I_0 the initial TxB_2 concentration during the treatment period (Ollerstam *et al.*, 2006). The slope was calculated for each cat by linear regression of the serum TxB_2 concentration after placebo administration. As blood samples were collected for 48 h during the ketoprofen period but only for 24 h after placebo and robenacoxib dosing, the drift was not applied between 24 h and 48 h (Eq. 11):

$$I_0(t \geq 24h) = I_0 - d \times 24 \quad \text{(Eq. 11)}$$

Calculation of potency indices and estimation of extent of COX-2 blockade centrally

Individual concentration-effect curves for (i) *in vivo* inhibition of COX-2 and (ii) *ex vivo* inhibition of COX-1 were simulated using calculated pharmacodynamic parameters expressing the maximal effect (I_{max}), potency (IC_{50}) and steepness of the NSAIDs concentration-effect

relationship (n). An average curve for COX-1 and COX-2 was fitted to the individual curves previously simulated (naïve pooled approach) using the same Hill equation (Giraudel *et al.*, 2005b; Pelligand *et al.*, 2012b). The corresponding average parameter values ($_aIC_{50}$ and $_an$) and 95% confidence intervals were derived to calculate the selectivity indicesto describe the relative *in vivo* selectivity.. Finally, the predicted percentage of COX-1 inhibition was calculated for 50, 80, 95 and 99% inhibition of COX-2.

Statistical analysis

Figures and potency curve fitting were computed using Prism version 5 (GraphPad, La Jolla, CA, USA). Statistics were performed with PASW Statistics (version 17, IBM, New York, USA) using a linear mixed model for PGE_2 and TxB_2. Treatment, time and treatment-time interaction were entered as fixed effects and cat was entered as a random effect. Time was nested within treatment and cat, a first order autoregressive covariance structure (AR1) was used (Littell *et al.*, 1998; Kristensen & Hansen, 2004). The normality assumption of the residuals was assessed by visual inspection and was verified after a log transformation of exudate PGE_2 and serum TxB_2 concentrations. All reported P values are two-tailed, with statistical significance defined as $P < 0.05$. In the *post hoc* tests, multiple analyses were corrected using the Bonferroni method. Arithmetic, geometric and harmonic means are presented (in tables only) as mean ± SD, mean [95% Confidence Interval] and mean ± pseudoSD (obtained by the Jackknife method), respectively (Lam *et al.*, 1985).

RESULTS

Pharmacokinetics

Pharmacokinetic parameters for plasma ketoprofen and blood robenacoxib concentrations are summarised in Table 1. The plasma concentration-time curve of ketoprofen was best described by a bicompartmental model with first order absorption for the S(+) enantiomer and a monocompartmental model with first order absorption for the R(-) enantiomer (Fig. 2). Peak plasma concentrations were 4,306 ng/mL for S(+) ketoprofen (T_{max}= 0.53 h) and 3,787 ng/mL for R(-) ketoprofen (T_{max}= 0.25 h). Apparent clearances (CL/F) were 0.114 L/kg/h for S(+) ketoprofen and 0.325 L/kg/h for R(-) ketoprofen. Terminal elimination half-life was longer for S(+) ketoprofen (t½=1.62 h, MRT=1.7 h) than for R(-) ketoprofen (t½=0.44 h, MRT=0.7 h).

The blood concentration-time curve of robenacoxib was best described by a bicompartmental model with first order absorption (Fig. 3). Peak plasma concentration of 1,313 ng/mL was reached after 0.9 h and the mean absorption t_{lag} was 0.05 h. Apparent blood robenacoxib clearance was moderate (0.684 L/kg/h) (Toutain & Bousquet-Melou, 2004) and elimination half-life was 1.13 h.

Pharmacokinetic parameters for exudate are summarised in Table 2. The exudate ketoprofen enantiomer concentrations followed a bi-exponential decay (Fig. 2). Harmonic mean penetration half-lives of S(+) and R(-) ketoprofen in exudate were 2.93 h and 2.06 h, respectively. Maximum exudate concentrations were reached at 7.9 h and 6.0 h after injection for S(+) and R(-) ketoprofen, respectively. The mean peak exudate concentration of S(+) ketoprofen was 169 ng/mL, and that of R(-) ketoprofen was 44 ng/mL. Elimination half-lives from tissue cages were

25.9 h for S(+) and 22.5 h for R(-) ketoprofen, accounting for correspondingly long MRTs of 35.9 h and 36.2 h.

Exudate robenacoxib concentration followed a bi-exponential decay (Fig. 3 and 4). One cat (D2) had peak robenacoxib exudate concentration (351 ng/mL) that was approximately 4-fold higher than the average C_{max} value observed in the other seven cats. However, its exudate concentrations were similar to those observed in the other seven cats by the 12^{th} hour postdose. Since a similar inconsistency was not observed when this cat was administered ketoprofen and because this cat did not behave as an outlier during the pharmacodynamics or blood level PK component of this investigation, it was assumed that these high initial robenacoxib concentrations were a function of experimental error. Accordingly, cat D2 was excluded from the robenacoxib exudate evaluations. However, it should be noted that in the absence of a confirmed source of this error, it is impossible to exclude the possibility that the exudate profiles associated with cat D2 reflect an idiosyncrasy that may exist in a subpopulation of cats. That said, the maximal robenacoxib concentration for the seven other cats was 85.2 ng/mL, attained at 8.1 h after dosing. Harmonic mean penetration half-life of robenacoxib in inflammatory exudate was 4.9 h. Exudate elimination half-life and MRT were 41.5 h and 45.7 h, respectively.

Pharmacodynamics

Both ketoprofen and robenacoxib reduced exudate PGE_2 concentrations significantly between 6 and 36 h (Fig. 5). Maximum PGE_2 inhibition, at 9 h, was 92.1% for robenacoxib and 90.9% for ketoprofen.

Maximal TxB_2 suppression with robenacoxib was 51.2 % at 2 h and this was the only time when the effect of robenacoxib was significantly different from placebo (Fig. 6). TxB_2 had returned to placebo level at 3 h. With ketoprofen, serum TxB_2 inhibition occurred rapidly, commencing 4 min after injection (97.1%) and suppression was maximal (97.9%) at 1 h (Fig. 6). Compared to placebo, ketoprofen significantly suppressed serum TxB_2 between 4 min and 24 h. Serum TxB_2 was 11.8% and 58.2% of the placebo concentration at 24 and 48 h, respectively.

PK/PD analysis

For COX-2 inhibition, the PK/PD model for estimation of pharmacodynamic parameters gave good results in 6 of 8 cats for both S(+) ketoprofen and robenacoxib. In two cats, the model did not converge, because exudate PGE_2 concentrations were reduced below the limit of quantification of the assay or did not recover to the levels in the placebo group within 120 h. Means of individual estimates of the pharmacodynamic COX-2 parameters for the carrageenan model, and after administration of ketoprofen and robenacoxib, are presented in Table 3. The geometric mean COX-2 IC_{50} was 44.7 ng/mL (0.14 µM) for robenacoxib and 45.0 ng/mL (0.18 µM) for S(+) ketoprofen.

PK/PD modelling for COX-1 was successful in all animals with robenacoxib and in 6 of 8 cats with S(+) ketoprofen. For the latter, in two cases, the number of blood samples was too low to allow bi-compartmental fitting of plasma concentrations and thus prevented PK/PD modelling. Individual geometric mean IC_{50}COX-1 was 2,951 ng/mL (1.31 µM) for robenacoxib and 0.17

ng/mL (0.67 nM) for S(+) ketoprofen (Table 4). I_{max} was 97.3 % for S(+) ketoprofen and 96.8 % for robenacoxib.

Individual concentration-effect curves were simulated using the pharmacodynamic parameters aforementioned. Average pharmacodynamic parameters ($_aImax$, $_aIC_{50}$ and $_an$) for S(+) ketoprofen and robenacoxib for inhibition of COX-1 in serum and COX-2 in exudate were calculated by naïve pooled data analysis (Table 5 and Fig. 7). The concentration-effect curves for COX-1 required re-scaling to a maximal effect of 100%. The $_aIC_{50}$ values for COX-1 were 0.45 and 2,56 ng/mL for S(+) ketoprofen and robenacoxib and the slopes ($_an$) were 0.66 and 0.87, respectively. Corresponding $_aIC50$ values for COX-2 were 48.5 and 38.2 ng/mL for S(+) ketoprofen and robenacoxib, respectively, and corresponding slopes were 1.04 and 1.46.

Three categories of indices were used to describe the selectivity of robenacoxib, determined by simultaneous fitting of individual percentage inhibition values from COX-1 and COX-2 assays (Table 6). The IC_{50}COX-1/IC_{50}COX-2 ratio was 1:107 for S(+) ketoprofen and 66.9:1 for robenacoxib. The selectivity of robenacoxib for COX-2 was confirmed at virtually maximal inhibition, as IC_{99}COX-1/IC_{99} COX-2 was 585:1. The IC_{20} COX-1/IC_{80} COX-2 ratio was 1:3,260 for S(+) ketoprofen and 1.4:1 for robenacoxib. Predicted percentage inhibitions of COX-1 versus COX-2 are illustrated in Fig. 8; the inhibition of COX-1 by S(+) ketoprofen would be almost maximal for all COX-2 inhibition percentages between 50 and 99%, whereas only 28.2% of COX-1 activity would be inhibited by robenacoxib at 99% COX-2 inhibition.

DISCUSSION

S(+) ketoprofen was the predominant enantiomer in the cat, as previously reported for the dog, rat and horse (Foster & Jamali, 1988; Delatour *et al.*, 1993; Landoni & Lees, 1995a). Chiral inversion of R(-) to S(+) ketoprofen occurs in the liver, so that the R(-) enantiomer, although itself of very low potency, is a pro-drug. Therefore, the apparent clearance of R(-) ketoprofen incorporates both elimination and inversion to the S(+) eutomer. Consequently, the drug input for S(+) ketoprofen comprises both the administered drug and S(+) ketoprofen formed by chiral inversion. The inversion rate has been calculated in the cat by separate administration of each enantiomer (Castro *et al.*, 2000; Lees *et al.*, 2003). Simultaneous enantiomer pharmacokinetic modelling was not possible, as the inversion rate could not be identified from the data of the present study. This study confirmed the short half-life of both ketoprofen enantiomers in the cat. The pharmacokinetics of robenacoxib after subcutaneous administration was also consistent with the findings from previous studies (Pelligand *et al.*, 2012b; King *et al.*, 2013), with a short elimination half-life (1.1 h).

Despite having short elimination half-lives in blood, ketoprofen and robenacoxib demonstrated marked negative hysteresis. Both drugs suppressed exudate PGE_2 significantly for up to 36 h. The likely explanation is accumulation of drugs in and slow clearance from the tissue cage. It would have been relevant to test this hypothesis by directly injecting the test article into the tissue cages. The IC_{50} COX-2 for robenacoxib was somewhat higher at 38.2 ng/mL (0.117 µM) in the present study compared with 14.1 ng/mL (0.043 µM) reported in Pelligand *et al*. (2012b). For COX-1, the difference for robenacoxib between the two studies was minimal, with IC_{50}

COX-1 of 2,557 ng/mL (7.81 µM) in the present study and 2,416 ng/mL (7.38 µM) in the previous investigation.

The persistence and duration of effect in exudate of ketoprofen enantiomers were similarly long as for robenacoxib. For 2-arylpropionates in general, and for ketoprofen in particular, COX inhibition activity resides almost exclusively with the S(+) enantiomer (Hayball *et al.*, 1992; Suesa *et al.*, 1993; Landoni *et al.*, 1996) in several species including the cat. It is, indeed, probable that COX inhibition in the cat after R(-) ketoprofen administration is attributable solely to the S(+) enantiomer formed *in vivo* by chiral inversion (Lees *et al.*, 2003). It was therefore justified, in this study, to conduct PK/PD modelling solely on S(+) ketoprofen concentration.

The IC_{50} COX-2 for S(+) ketoprofen of 48.5 ng/mL (0.191 µM) was very similar to the IC_{50} for robenacoxib in the present study (38.2 ng/mL) but was lower than the IC_{50} reported by Schmid *et al*. (2010) in *in vitro* whole blood assays: 119.9 ng/mL (0.472 µM). In serum, the *ex vivo* IC_{50} for COX-1 in the present study was 0.454 ng/mL (0.0018 µM) which was lower than the IC_{50} of 5.92 ng/mL (0.023 µM) reported by Schmid *et al.* (2010) in *in vitro* assays. Inter-laboratory differences in experimental methodology (*ex vivo* versus *in vitro*) and differences in modelling techniques are well recognised as the basis for differing results, even of this relatively high magnitude (Warner *et al.*, 1999). In consequence, we report an IC_{50} COX-1 / IC_{50} COX-2 ratio of 1:107, which is lower than that obtained by Schmid *et al.* of 1:20. Despite these numerical differences, both studies confirm that ketoprofen is COX-1 selective in the cat. The time-course of inhibition of TxB_2 with ketoprofen was similar to that reported after intravenous administration of 2 mg/kg racemic ketoprofen (Lees *et al.*, 2003).

The present data indicate that ketoprofen and robenacoxib exhibit similar pattern for distribution to a site of acute inflammation, whilst possessing opposite selectivities for inhibition of COX isoforms, ketoprofen for COX-1 and robenacoxib for COX-2. As discussed by (Brune & Furst, 2007), the first generation selective COX-2 inhibitors (sulphonamides and methylsulphones) combined reduced gastrointestinal toxicity with prolonged inhibition of constitutively-expressed COX-2 in the vascular wall and kidney. This may explain, at least partially, the reported toxicities of these COXibs with long terminal half-lives and large volumes of distribution. It is therefore likely that tissue selectivity is a potential advantage of second generation COXibs (carboxylic acids) with shorter elimination half-lives. If these drugs exert only a short duration of action on constitutively expressed COX-2 in the central pharmacokinetic compartment, this might provide a higher safety profile, for example for cardiovascular and renal side-effects. As developed in our laboratory, the tissue cage model has allowed investigation of the distribution of robenacoxib and ketoprofen (selected for this study for both their differing COX inhibition profiles and long durations of action despite short half-lives in the central pharmacokinetic compartment) to a site of acute inflammation. It may be regarded as an appropriate model to further our understanding of other carboxylic acid NSAIDs, with similar chemical structures and pharmacokinetic profiles, such as lumiracoxib (COX-2 selective) and diclofenac (COX non-selective) (Fig. 8) (Brune & Furst, 2007).

It should, however, be noted that all tissue cage models are “model dependent”, in that drug diffusion into and from exudate in the cage is influenced by tissue cage geometry (including surface area), a lack of physiological drainage as for the synovial fluid lymph drainage, as well

513 as drug molecule properties, including protein binding, pKa and lipid solubility. Therefore, tissue
514 cage models cannot mimic either accurately or quantitatively all clinical circumstances.
515 Nevertheless, it is of interest to note that lumiracoxib accumulated in inflamed joints in humans
516 and its concentration was maintained in excess of plasma concentrations for up to 18 h after
517 dosing (Scott *et al.*, 2004). Similarly, ketoprofen penetrated readily into acutely inflamed joints
518 of the horse. At one h after dosing the concentration in synovial fluid was six times higher in
519 inflamed compared to non-inflamed joints (Owens *et al.*, 1994). On the other hand,
520 concentrations of etoricoxib (a coxib of the sulphonamide group) in wound fluids did not exceed
521 plasma concentrations after pre-emptive administration before hip surgery (Renner *et al.*, 2010;
522 Renner *et al.*, 2012).
523
524 It is unlikely that the slow clearance of ketoprofen and robenacoxib from tissue cages was
525 limited by passive diffusion. This is suggested by serum and exudate clearance data. For
526 creatinine, an endogenous, non-protein bound small molecule, a $MRT_{exudate}/MRT_{serum}$ ratio of
527 3.6:1 was obtained by Pelligand *et al.* (2012a). In contrast, robenacoxib and S(+) ketoprofen
528 $MRT_{exudate}/MRT_{blood}$ ratios in the present study were substantially higher, 24.9:1 and 20.4:1
529 respectively. These high ratios are explained by two factors, slow drug clearance from the tissue
530 cages and short half-lives in plasma. Despite the use of tissue cages of different geometry
531 (spherical polypropylene cages instead of silicon cylinders similar to the feline tissue cages),
532 previous workers showed that the ketoprofen MRT ratio was also high (11.5:1) in the goat
533 (Arifah *et al.*, 2003) and in the calf (10.6:1) (Landoni & Lees, 1995b) though not in the horse
534 (2.9:1). Moreover, other tissue cage investigations demonstrated that not all COXibs are tissue

535 selective; the $MRT_{exudate}/MRT_{blood}$ ratio for firocoxib in the dog (a methylsulphone related to
536 rofecoxib) was 1.06:1 and similar to meloxicam 1.08:1 (P. Lees, unpublished data).
537
538 The binding of drugs to and slow release from a component of the inflammatory process, such as
539 protein or a specific cell population, could account for these differing results for NSAIDs
540 (Pelligand *et al.*, 2012b). For example, the search for the ideal radiolabelled marker for imaging
541 COX-2 expression revealed that a radioiodinated derivative of lumiracoxib had a higher affinity
542 and *in vitro* cell uptake for COX-2 induced macrophages than normal macrophages (Kuge *et al.*,
543 2009). A similar mechanism might explain the slow clearance of robenacoxib from exudate.
544 The prolonged plasma half-life of the sulphonamide COXibs (celecoxib, etoricoxib and
545 valdecoxib) is explained both by slow clearance and relatively high volume of distribution. In
546 contrast, the volume of distribution of carboxylic acid COXibs is very small compared to other
547 classes of COXibs. Thus, lumiracoxib steady state volume of distribution was 9 L in humans
548 (0.13L/kg for a 70kg person, Mysler, 2004) and the distribution volume for robenacoxib was
549 likewise low, 0.19 L/kg in the cat and 0.24 L/kg in the dog (Jung *et al.*, 2009; Pelligand *et al.*,
550 2012b).
551
552 The sulphonamide moiety of a radioiodinated derivative of celecoxib had a high affinity for
553 carbonic anhydrase and this could explain both the preferential distribution into rat erythrocytes
554 (88%) and slow clearance from blood (Boddy *et al.*, 1989; Kuge *et al.*, 2006). Substitution of the
555 sulphonamide moiety to a methylsulphone moiety decreased erythrocyte binding to 18% and
556 increased blood clearance (Kuge *et al.*, 2006).
557

In conclusion, the present data suggest that, despite a short blood half-life, NSAIDs can have a long-lasting local action, as a consequence of high inflammatory tissue selectivity. However, drug distribution into tissue cage fluid is model dependant and cannot be a precise predictor of penetration time course to other anatomical sites. This will indeed depend on a wide range of factors, including specific tissue blood flow and possibly the degree of acute inflammation. Tissue selectivity might be advantageous for carboxylic acids COXibs and some older NSAIDs such as ketoprofen and diclofenac, as systemic side-effects related to COX-1 and COX-2 inhibition could be reduced, whilst efficacy in experimental inflammation persists for 24 h or longer after a single dose.

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DECARATIONS OF INTEREST

L. Pelligand received a CASE award from BBSRC and Novartis Animal Health

J.N. King is an employee of Novartis Animal Health

P. Lees has acted as a consultant to Novartis Animal Health

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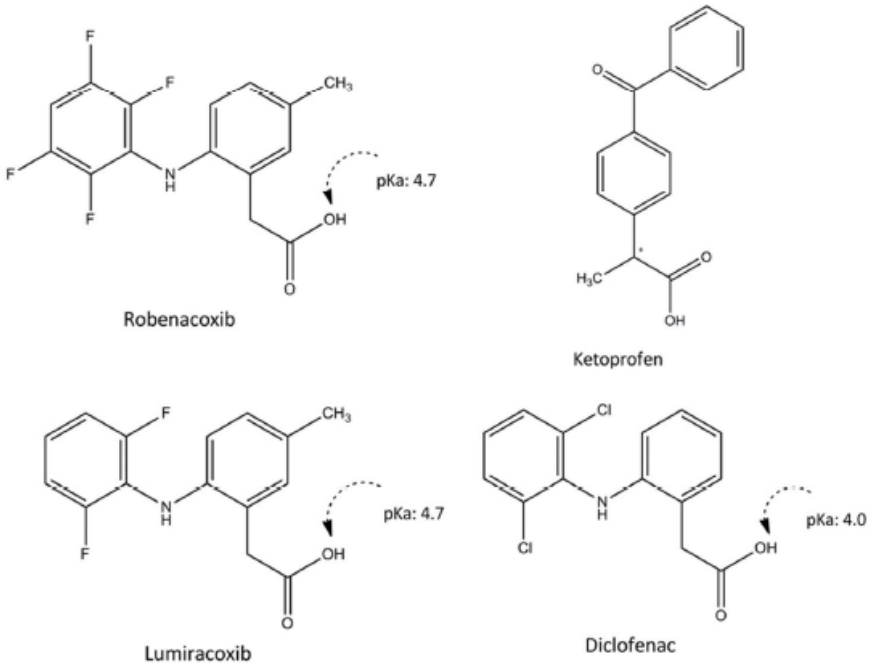
Figure legends:

Figure 1: Chemical formulae for ketoprofen and diclofenac related COXibs: lumiracoxib and robenacoxib



254x190mm (96 x 96 DPI)

Figure 2: Observed plasma S(+) ketoprofen (●), R(-) ketoprofen (■), exudate S(+) ketoprofen (○) and exudate R(-) ketoprofen (□) concentrations (ng/mL) versus time (h) profiles after subcutaneous administration of racemic ketoprofen at a total dose of 2 mg/kg. Results from eight cats are presented as mean ±SD.

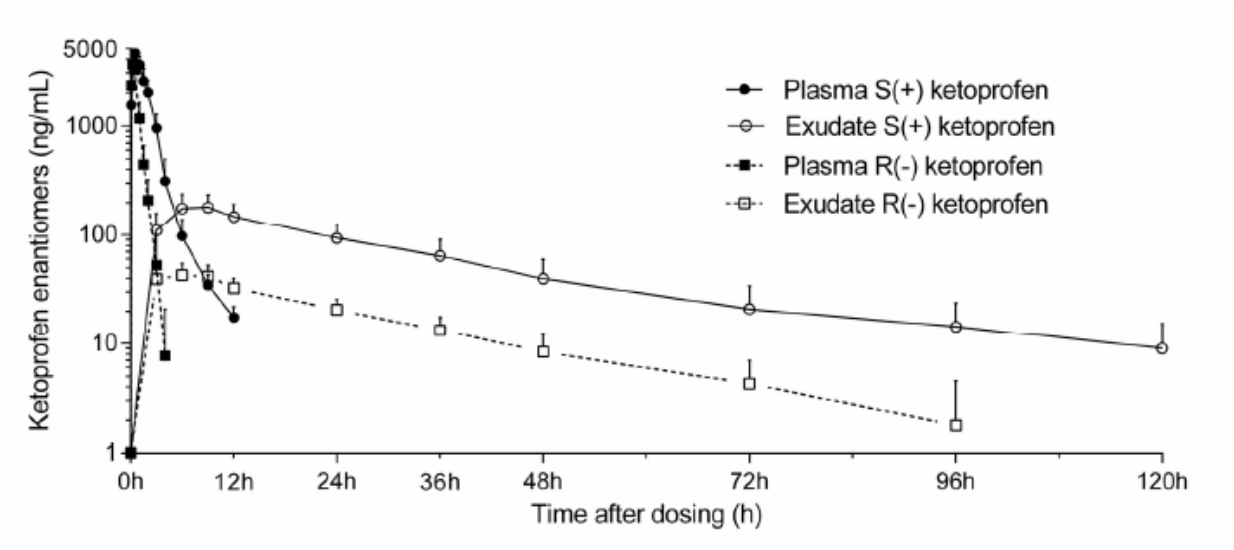


Figure 3: Observed blood (●) and exudate (○) robenacoxib concentration (ng/mL) versus time (h) profiles after subcutaneous administration of a 2 mg/kg dose. Results from 8 cats are presented as mean +SD.

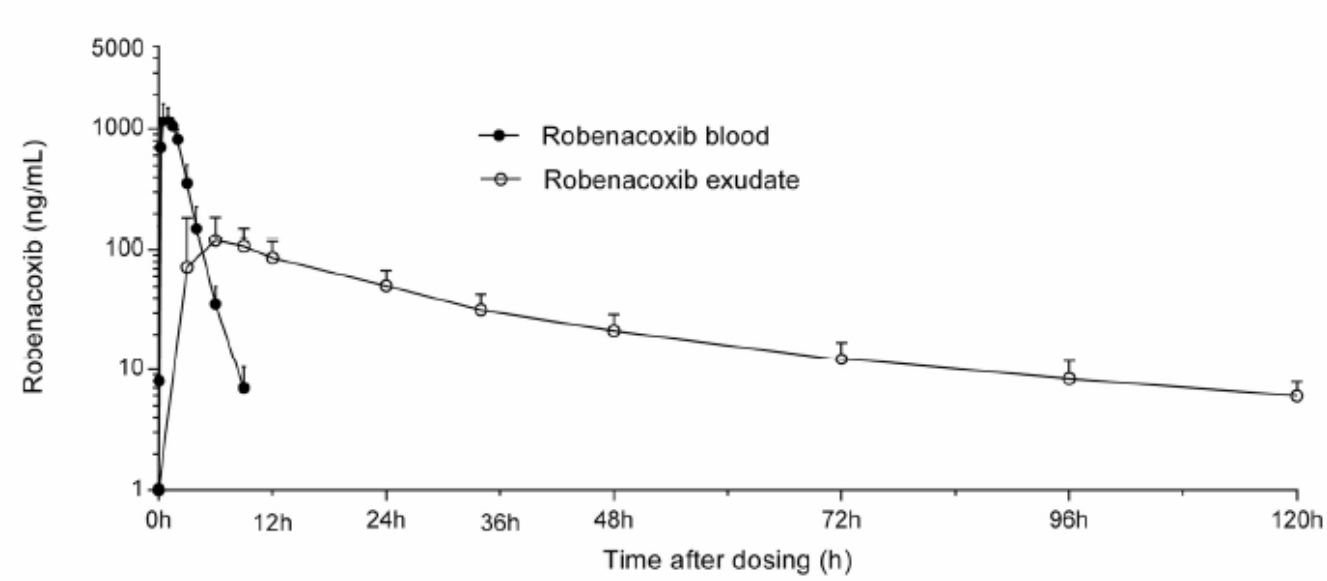


Figure 4: Individual exudate robenacoxib concentration (ng/mL) versus time (h) profiles after subcutaneous administration of a 2 mg/kg dose.

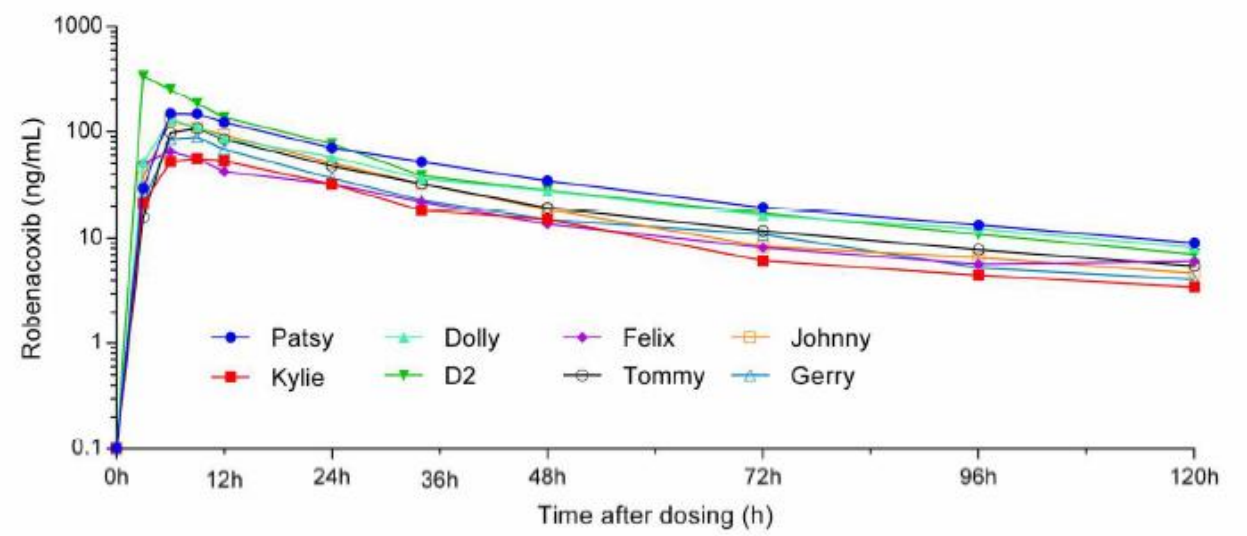
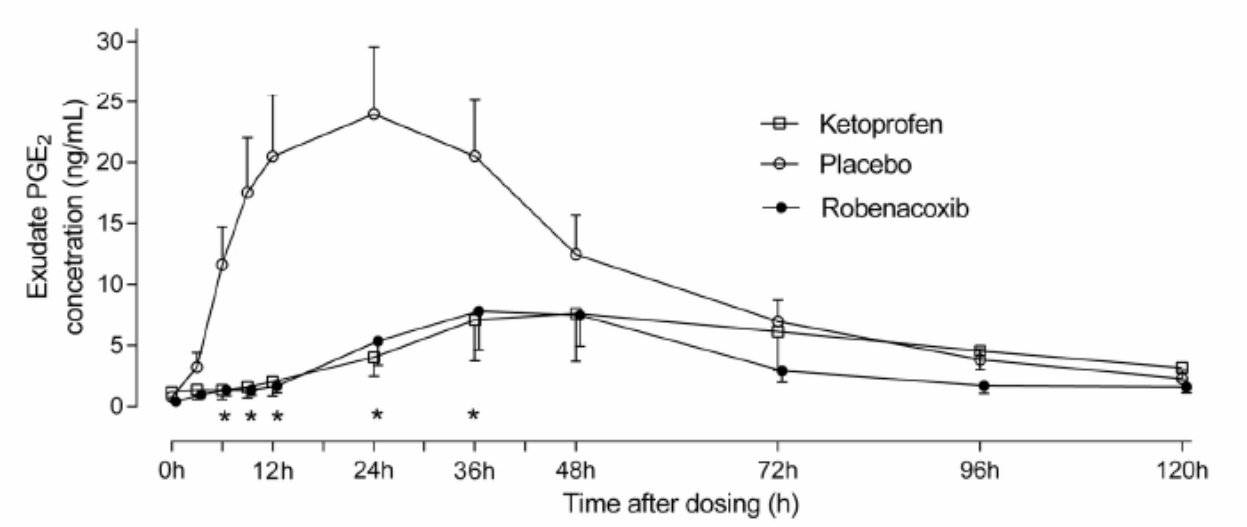
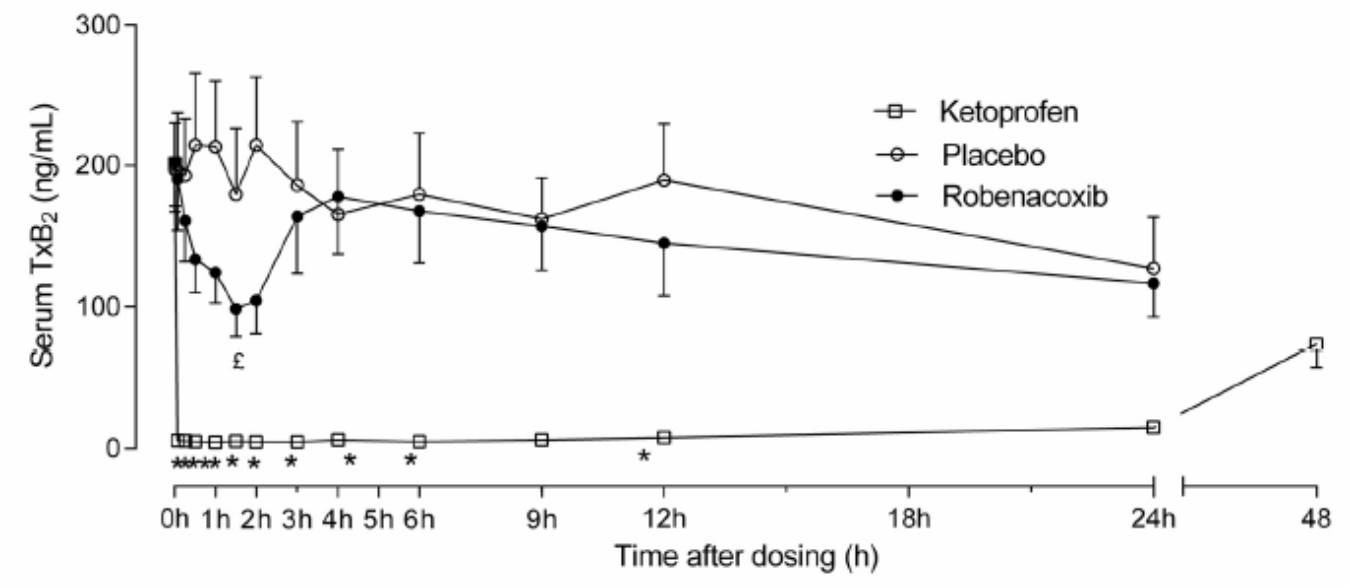


Figure 5: Exudate PGE_2 concentration (ng/mL) versus time (h) profiles after carrageenan injection and placebo, racemic ketoprofen (2mg/kg total dose) and robenacoxib (2 mg/kg) subcutaneous administration. PK/PD modelling is relevant to the time-response profile as a whole rather than to the response at sampling times taken separately and therefore values are presented as mean ±SEM. Statistical comparison of effect of treatment versus placebo (* = P <0.05) at different times (linear mixed effect model).



765 Figure 6: Serum TxB_2 concentration (ng/mL) versus time (h) profile after placebo, racemic
766 ketoprofen (2 mg/kg total dose) and robenacoxib (2 mg/kg) subcutaneous administration. PK/PD
767 modelling is relevant to the time-response profile as a whole rather than to the response at
768 sampling times taken separately and therefore values are presented as mean ±SEM. Statistical
769 comparison of effect of ketoprofen versus placebo (* = P <0.05) and robenacoxib versus placebo
770 (£ = P <0.05) at different times (linear mixed effect model).



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772

Figure 7: Observed and fitted COX inhibition (%) versus S(+) ketoprofen (red) and robenacoxib (blue) concentrations (ng/mL). Open and closed symbols represent COX-2 and COX-1, respectively. COX-1 data were rescaled for 100% I_{max}. In a naïve pooled data analysis, average regression curves for COX-1 and COX-2 were fitted with a sigmoid I_{max} model to all individual curves (n= 6 to 8 cats for each regression curve).

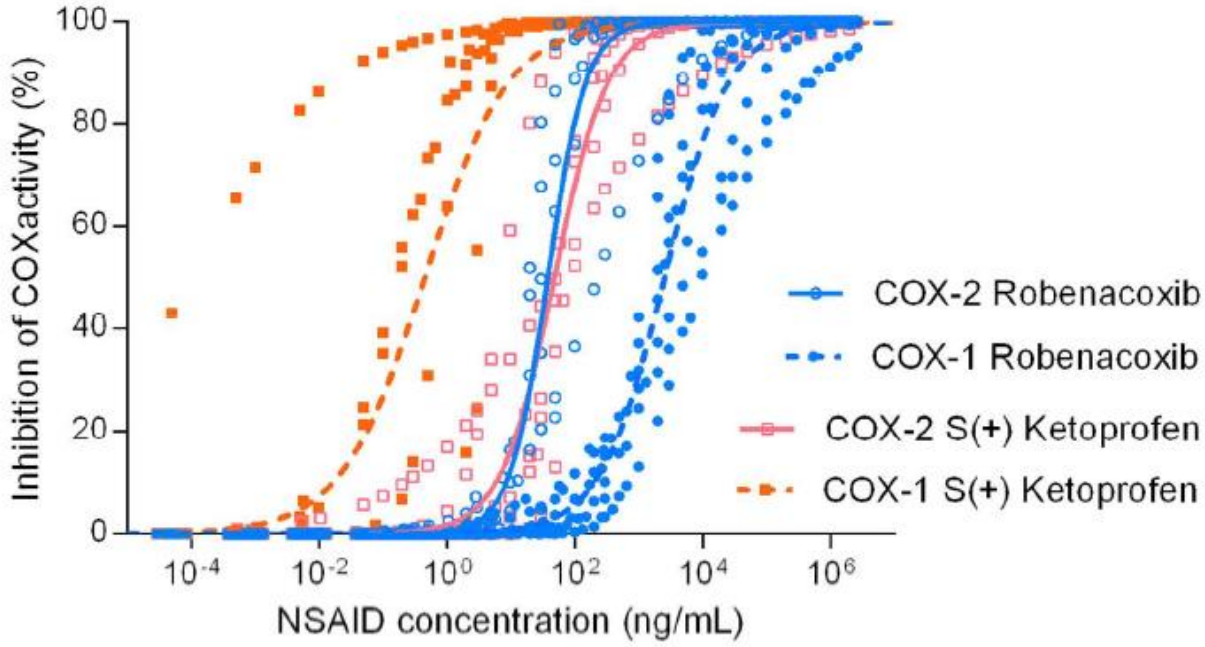

Figure 8: Inhibition percentage of COX-2 and corresponding inhibition percentages of COX-1 for a range of concentration of S(+) ketoprofen and robenacoxib. Mean inhibition curves were computed by non-linear regression, fitting an average Hill equation (I_{max} model) to individual concentration-effect profiles (ranging from 0 to I_{max} and rescaled on 0-100% scales), previously obtained by solving PK/PD models for serum TxB_2 inhibition (COX-1 activity) and exudate PGE_2 inhibition (COX-2 activity). Average NSAID concentrations for given inhibition percentages of COX-2 were used to determine corresponding COX-1 inhibition percentage. Dotted lines indicate cut off values for inhibition of COX-1 (above 20% inhibition of COX-1 increased risk of side-effects) and COX-2 (above 80% inhibition of COX-2 correlates with good clinical efficacy).

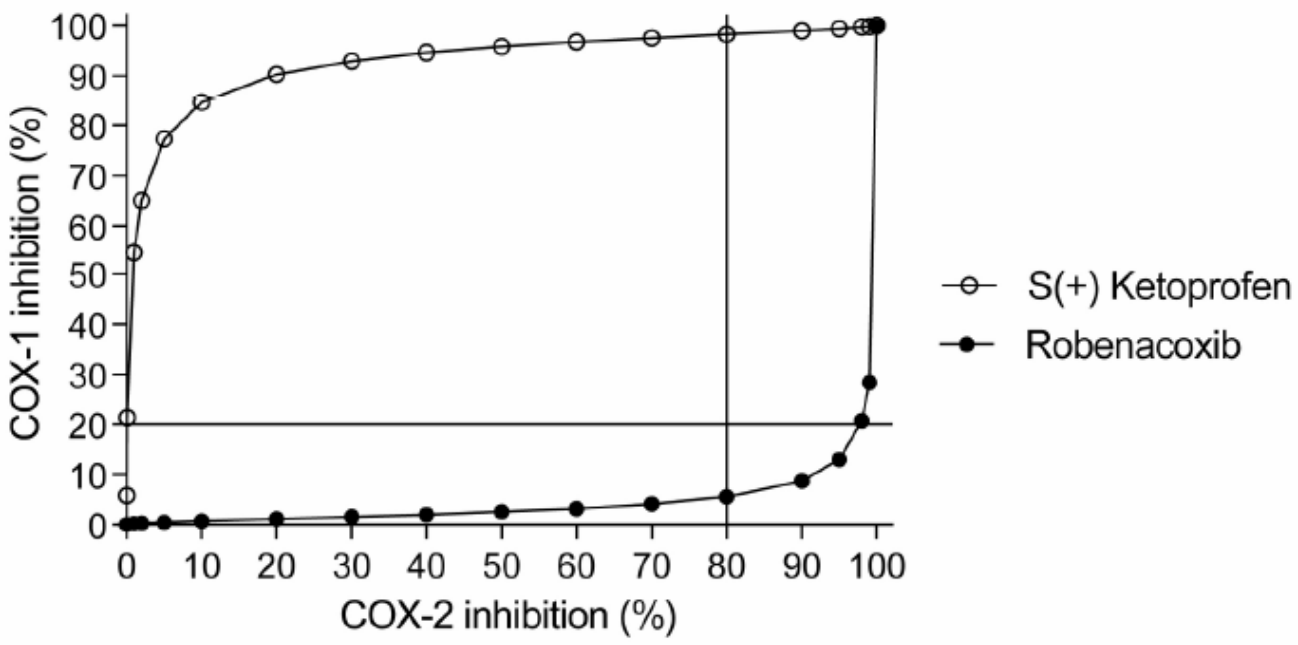

Table 1. Mean pharmacokinetic parameters for plasma S(+) ketoprofen and R(-) ketoprofen and blood robenacoxib concentrations after single subcutaneous administration of racemic ketoprofen (total nominal dose of 2 mg/kg) or robenacoxib (nominal dose 2 mg/kg) in 8 healthy cats

		S(+) Ketoprofen		R(-) Ketoprofen		Robenacoxib	
Parameter	Unit	Mean*	SD or [95%CI]	Mean*	SD or [95%CI]	Mean*	SD or [95%CI]
T_{max}	h	0.53	0.12	0.25	0.04	0.9	0.24
C_{max}	ng/mL	4,306	[3,566-5,198]	3,787	[3,015-4,757]	1,313	[1,033-1,668]
$AUC_{0\text{-}inf}$	ng.h/mL	8,778	[7,043-10,939]	3,082	[2,338-4,062]	3,043	[2,782-3,329]
MRT	h	1.73	0.27	0.68	0.16	1.85	0.38
$t_{½}$	h	1.62	1.14	0.44	0.19	1.13	0.18
V_z_F	L/kg	0.308	[0.192-0.495]	0.222	[0.148-0.331]	1.130	[0.949-1.344]
CL_F	L/h/kg	0.114	[0.092-0.142]	0.325	[0.246-0.428]	0.684	[0.622-0.753]

* T_{max} and MRT are presented as arithmetic mean ± SD, half lives presented as harmonic means with pseudo-SD estimated by the jackknife method. All other parameters are presented as geometric mean [95% CI of the mean], Calculation methods are given in the text. T_{max} : time of maximal concentration, C_{max} : maximal concentration, $AUC_{0\text{-}inf}$: area under concentration versus time curve extrapolated to infinity, λz slope of the drug elimination phase and $t_{½}$ corresponding elimination half life, V_z_F : volume of the central compartment scaled by bioavailability F, CL_F : body clearance scaled by bioavailability.

Table 2. Mean pharmacokinetic parameters for exudate S(+) ketoprofen and R(-) ketoprofen and robenacoxib concentrations after single subcutaneous administration of racemic ketoprofen (total nominal dose of 2 mg/kg) or robenacoxib (nominal dose 2 mg/kg) in 8 healthy cats

		S(+) Ketoprofen		R(-) Ketoprofen		Robenacoxib*	
	Unit	Mean	SD or [95%CI]	Mean	SD or [95%CI]	Mean	SD or [95%CI]
T_{max}	h	7.9	2.35	6.0	2.55	8.1	2.79
C_{max}	ng/mL	169.1	[133.5-214.1]	43.9	[34.9-55.2]	85.2	[63.1-115.1]
$t_{½}$ K_{ea}	h	2.93	2.29	2.06	1.52	4.86	2.99
MRT	h	35.89	7.41	36.22	14.85	45.68	6.07
$t_{½}$	h	25.88	3.67	22.54	12.29	41.48	8.85

T_{max} and MRT are presented as arithmetic mean ±SD, half-lives are presented as harmonic mean with pseudo-SD estimated by the jackknife method. All other parameters are presented as geometric mean [95% CI of the mean]. See text for calculation methods. T_{max} is the time of maximal concentration C_{max} is maximum concentration; $t_{½}$ K_{ea} and $t_{½}$: half-life of penetration in exudate and elimination from exudate respectively; MRT: mean residence time in exudate calculated by non-compartmental analysis.

* One cat had exceptionally high exudate robenacoxib concentration and was not included in the calculations.

Table 3. Individual pharmacodynamic parameters describing the inhibitory effect of robenacoxib (2 mg/kg) and ketoprofen (2 mg/kg racemate) on exudate PGE_2 production after subcutaneous administration

		Robenacoxib*		Ketoprofen*	
Parameter	Unit	Mean	95% CI or [range]	Mean	95% CI or [range]
K_{in}	ng/mL/h	0.28	0.01-0.79	0.42	0.10-1.74
Carrag	no unit	33.3	11.0-100.9	10.5	4.6-24.2
k_1	/h	0.21	0.08-0.54	0.52	0.15-1.83
k_2	/h	0.05	0.03-0.10	0.03	0.02-0.05
N	no unit	2.0	1.09-3.78	1.39	0.67-2.79
IC_{50}	ng/mL	44.7	16.9-118.2	45.9	17.8-118.7
K_{out}	/h	0.12	0.04-0.39	0.16	0.09-0.29
$t_{lag1\ (placebo)}$	h	0.5	[0-2.9]	2.9	[0.5-7.2]
$t_{lag2\ (NSAID)}$	h	5.4	[0-16.5]	9.8	[0-22.0]

An indirect response model including 9 estimated parameters was computed.

* The results form 2 of 8 cats were excluded from the calculation of the mean because the inhibition of exudate PGE_2 never recovered below 50% of placebo PGE_2. k_1 and k_2: first order time dependent variables for growth and dissipation of carrageenan stimulation on COX (respectively). Carrag is a scalar, K_{in} is a zero-order constant for basal PGE_2 production, K_{out} is a first-order rate constant for removal of PGE_2 from exudate; t_{lag1} and t_{lag2}: lag time between injection of carrageenan and beginning of the carrageenan stimulation for the placebo function (t_{lag1}) and for the NSAIDs function (t_{lag2}). Data are reported as geometric mean with 95% CI of the mean except t_{lags} (arithmetic mean, [range])

Table 4. Individual pharmacodynamic parameters describing the inhibitory effect of robenacoxib (2 mg/kg) and ketoprofen (2 mg/kg racemate) on serum TxB_2 production (COX-1 activity) after subcutaneous administration.

		Robenacoxib		Ketoprofen*	
Parameter	Unit	Mean	95% CI	Mean	95% CI
I_0	ng/mL	174	115-262	201	134-301
IC_{50}	ng/mL	2,951	1,498-5,815	0.168	0.003-9.732
N	no unit	1.01	0.65-1.58	1.67	0.57-4.91

*The results from two of eight cats receiving ketoprofen were excluded, as the number of samples was too low to fit a biexponential model to the blood concentration time profile. Data were fitted using a sigmoid I_{max} model for robenacoxib (8 cats) and ketoprofen (6 cats). I_{max} is the percentage of maximal suppression of TxB_2 (corresponding to the lower limit of quantification of the assay) relative to $I_0(t)$. I_0 is the fitted value of intercept at time 0 taking into account baseline drift in TxB_2 concentrations observed after placebo dosing. Data are presented as geometric mean and 95% CI of the mean for I_0, IC_{50} and n.

Table 5. Average maximal effect ($_aI_{max}$), potency ($_aIC_{50}$) and slope ($_an$) of S(+) ketoprofen and robenacoxib for *ex vivo* inhibition of COX-1 in serum and *in vivo* inhibition of COX-2 in exudate

PD parameters	$_aI_{max}$	$_aIC_{50}$	[95%CI]	$_an$	[95%CI]
Units	%	ng/mL		(no unit)	
COX-1					
Ketoprofen	97.3	0.45	[0.32 - 0.65]	0.66	[0.51 - 0.81]
Robenacoxib	96.8	2557	[2291 – 2818]	0.87	[0.80 - 0.94]
COX-2					
Ketoprofen	100.0	48.5	[41.6 - 56.6]	1.04	[0.89 - 1.19]
Robenacoxib	100.0	38.2	[33.9 - 42.7]	1.46	[1.22 - 1.70]

Reported parameters and bounds of the 95% confidence interval [95%CI] were calculated by naïve pooled data analysis (Giraudel *et al*., 2005). An average curve was fitted with a sigmoid I_{max} model to all simulated curves (n=8 cats for robenacoxib COX-1 and 6 cats otherwise) as if they were data from a single individual.

843 **Table 6**. Three categories of indices describing the *in vivo* selectivity of robenacoxib
844 determined by simultaneous fitting of individual percentage inhibition values from COX-
845 1 and COX-2 assays

	Ketoprofen	Robenacoxib
Classical selectivity ratios:		
IC_{50} / IC_{50}	1 : 107	66.9 : 1
IC_{80} / IC_{80}	1 : 50.4	129 : 1
IC_{95} / IC_{95}	1 : 21.6	268 : 1
IC_{99} / IC_{99}	1 : 8.8	585 : 1
IC_{20} / IC_{80}	1 : 3260	1.4 : 1
COX-1 inhibition for a given IC_x COX-2::		
% Inhibition of COX-1 at IC_{50} COX-2	95.7 %	2.5 %
% Inhibition of COX-1 at IC_{80} COX-2	98.2 %	5.6 %
% Inhibition of COX-1 at IC_{95} COX-2	99.3 %	12.9 %
% Inhibition of COX-1 at IC_{99} COX-2	99.7 %	28.2 %

846 The data were obtained by the naïve pooled approach are reported