Beyond relief : biomarkers of the anti-inflammatory effect and dose selection of COX inhibitors in early drug development

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Chapter 6

Differences in the *in vitro* and *in vivo* concentration-effect relationship of selective and non-selective COX inhibitors: role of translational pharmacology in pain research

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ABSTRACT

Background and purpose. In this manuscript we assess the concentration-effect relationships of three COX inhibitors varying in selectivity (i.e., rofecoxib, diclofenac and ketolorac) by modelling their inhibitory effect on PGE_2 and TXB_2 in vitro and *in vivo* in rats and compare their predictive value for the treatment effect in humans.

Experimental approach. PGE_2 inhibition was measured by whole blood LPS-stimulation whilst TXB_2 inhibition was assessed by blood clotting. For the evaluation of drug effects *in vitro*, pre-defined concentrations were added to blood samples. For the evaluation of the effects *in vivo*, rats were given an intraperitoneal dose of each compound. Subsequently, serial samples were collected for analysis of concentrations and drug effect on PGE_2 and TXB_2. *In vivo* human data from previous publications was used for comparison. PKPD analysis was performed using nonlinear mixed effects modelling.

Key results. Inhibition of PGE_2 and TXB_2 was characterised by a sigmoid E_{max} model. The IC_{80} values for PGE_2 and TXB_2 inhibition were used as parameter of interest for the prediction of the analgesic effect *in vivo*. All three COX inhibitors showed significant differences *in vitro* and *in vivo* ($p>0.05$) as well as between species. *In vitro-in vivo* potencies showed a correlation in rats and in humans.

Conclusions and implications. The assessment the effect of COX inhibitors *in vitro* enables evaluation of *in vitro-in vivo* correlations. *In vitro* data also provided better estimates of the selectivity and potency of different compounds within and between species. These findings strongly suggest that the use of *in vitro* human data, instead of rodent models of pain as basis for determining the effective exposure for analgesia in patients.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be effective in rheumatic and non-rheumatic diseases and have been extensively used as analgesic during the past decades. However, selection of the therapeutic dose range for novel compounds, ensuring optimal risk-benefit ratio, remains one of the major challenges in early drug development.

The recent withdrawal of rofecoxib from the market and the pressure upon which regulatory agencies have to evaluate drug safety demands basic pharmacology concepts to be reviewed in the light of methodological developments for the assessment drug effects, instead of relying solely on empirical observations or on media speculation. Despite the vast number of publications arising from the concern about cardiovascular safety of COX-2 inhibitors, very few investigators have questioned the issue of dose selection and exposure or resorted to quantitative, translational tools to mechanistically explain clinical findings. This is particularly worrying considering that evidence exists that most critical safety findings for approved drugs can be considered avoidable risk and often relate to intrinsic pharmacological or pharmacokinetic properties (1). Moreover, despite increased pharmaceutical investment and improved understanding of pain mechanisms, only very limited new chemical entities (NCE) have entered the clinic. Indeed, virtually all new analgesics approved over the past 25 years are derivatives or reformulations of opioids or aspirin-like drugs, existing drugs given for a new indication or older drugs given by a different route of administration.

Whilst scaling of pharmacokinetics between species has been recognised as essential to understand drug properties (2-4), such as clearance and volume of distribution, only a few examples show the application of this concept to extrapolate pharmacodynamics (5-9). Two of the main flaws in the current approach to drug screening are the assumption that the physiological cascades underlying inflammatory processes are comparable across species and that behavioural endpoints for the assessment of analgesia quantitatively reflect the pharmacological effects in humans. Animal models of pain may not be always appropriate (10-14). If animal models are to be used, then a quantitative description of the link between behavioural measure and pharmacology must be established, i.e., the construct validity of these models must be warranted. Moreover, the consequences of supra-optimal exposure cannot be assessed by efficacy measures in clinical trials, since the correlation between mode of action and response is not incorporated into clinical endpoints.

Rational drug therapy is based on the assumption that there is a causal relationship between dosing regimen or drug exposure and the observed therapeutic response as well as adverse effects. Hence, an important question that remains to be answered is how much and how long COX-2 and COX-1 should be inhibited to allow for sustained analgesic response and appropriate safety margin. The type and complexity of the interaction between various factors that determine the analgesic response to COX inhibitors require biomarkers to explain and understand variability in the treatment effect. In fact, biomarkers may shed light on the differences between pre-clinical findings *in vitro* and *in vivo* and contribute to better prediction of the dose range in humans. Given the mechanism of action of COX inhibitors, a number of mediators could be used as an intermediate step between

pharmacokinetics and analgesia. Primary candidates for such a role are PG and TXB. In conjunction with non-linear mixed effects modelling, it is possible to establish the relationship between these biological markers, pain measurement and safety. This approach has been successfully applied in other diseases such as diabetes mellitus, where drugs with different mechanisms of action are used. However, accurate prediction of the anti-hyperglycaemic effect can be achieved only if the complex glucose-insulin homeostasis is taken into account and insulin sensitivity is factored in the analysis (15). In fact, we have recently developed a pharmacokinetic-pharmacodynamic (PKPD) model to quantitatively analyse the inhibition of serum thromboxane B2 (TXB_2) and plasma prostaglandin E2 (PGE_2) by naproxen *in vitro* and *in vivo* in rats and healthy volunteers (16). Drug effect on biomarkers was parameterised in terms of IC_{80} (drug concentration corresponding to 80% of maximum inhibition).

In the current study, we assess the PKPD relationships of three COX inhibitors varying in selectivity by modelling their inhibitory effect on PGE_2 and TXB_2 as biomarkers of the *in vitro* and *in vivo* pharmacological effects in rats and in humans. Investigational compounds were selected that showed specific *in vitro* selectivity for COX-2 (rofecoxib), COX-1 (ketorolac) and non-selectivity for either isozyme (diclofenac) (17). *In vivo* human data from previous publications was used for comparison.

MATERIALS AND METHODS

This manuscript comprises the results from a pharmacokinetic study in cannulated animals receiving ketorolac (study 1) and a PKPD study in non-cannulated animals (study 2) with three treatment arms, in which rofecoxib, diclofenac and ketorolac were administered. Human *in vivo* data was retrieved from previous publications.

Animals. Experiments were performed on male Sprague-Dawley (SD) rats (Charles River B.V., Maastricht, The Netherlands) weighting 250 ± 2 g (Mean ± SEM, n=48) and were approved by the Ethical Committee on Animal Experimentation of the University of Leiden. The animals were housed in standard plastic cages (six per cage before surgery and individually after surgery) with a normal 12-hour day/night schedule (lights on 7 AM) and a temperature of 21°C. The animals had access to standard laboratory chow (RMH-TM; Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

Surgical procedures. For study 1, three days before the start of the experiment, indwelling pyrogen-free cannulae (Polythene, 14 cm, 0.52 mm i.d., 0.96 mm o.d.) were implanted into the right jugular vein and right femoral artery (Polythene, 4 cm, 0.28 mm i.d., 0.61 mm o.d. + 20 cm 0.58 mm i.d., 0.96 mm o.d.) for drug administration and serial blood sampling, respectively. The arterial cannula was filled with heparinised 25% (w/v) polyvinylpyrrolidone (PVP) (Brocacef, Maarssen, The Netherlands) in saline (0.9 %NaCl). Rats receiving an intraperitoneal dose were implanted with an arterial cannula only. Cannulae were tunnelled subcutanously to the back of the neck, exteriorised and fixed with a rubber ring. Prior to the experiment, the PVP solution was removed and the

cannulae were flushed with saline containing 20 I.U. ml^{-1} heparin. All surgical procedures were performed under anaesthesia with 0.1 mg kg^{-1} i.m. of medetomidine hydrochloride (Domitor, Pfizer, Capelle a/d Ijssel, The Netherlands) and 1 mg kg^{-1} s.c. of ketamine base (Ketalar, Parke-Davis, Hoofddorp, The Netherlands).

Drug administration. Ketorolac and diclofenac were purchased from Sigma Aldrich BV (Zwijndrecht, The Netherlands). Ketorolac was administered as an i.v. infusion at a dose of 5 mg kg^{-1} or as an i.p. bolus at a dose of 5 mg kg^{-1}. Diclofenac was administered as an i.p. bolus at a dose of 2 mg kg^{-1}. Rofecoxib was extracted from Vioxx® tablets (12.5 mg Vioxx® Merck, Kirkland Quebec, Canada). Vioxx® tablets were crushed to fine power, suspended in 50 ml of HPLC grade ethyl acetate, shaken for 5 minutes, and filtered. Residues were crystallised from acetonitrile after evaporating the solvent in vacuum. During the extraction process, rofecoxib was protected from direct light to prevent degradation. The purity (97%) of rofecoxib was compared in triplicate to a pure sample of rofecoxib by LC-MSMS. Rofecoxib was dissolved in dimethylsulfoxide (DMSO) at concentrations of 10 mg ml^{-1} for i.p. administration. All doses used for the *in vivo* PK/PD experiments were based on effective analgesic doses in rats (18-20).

Experimental design. Pharmacokinetic study. All experiments were started between 08.30 and 09.30 a.m. to exclude the influence of the circadian rhythms. Ketorolac (5 mg kg^{-1}) was administered intravenously at a rate of 20 µl min-1 over 5 min using an infusion pump (Bioanalytical Systems Inc., Indiana, USA) or given as an i.p. injection (5 mg kg^{-1}) to conscious and freely moving rats. Serial arterial blood samples (100 µl) were taken at pre-defined time points (0, 5, 7.5, 10, 12.5, 17.5, 25, 30, 45, 60, 90, 120, 180, 240, 360, 480 and 1440 min after i.v. administration and 0, 5, 10, 15, 20, 25, 30, 40, 45, 50, 60, 90, 120, 180, 240, 360, 480, 1440 min after i.p. administration). The total volume of blood samples was kept to 2.0 ml during each experiment. Blood samples were immediately heparinised and centrifuged at 5000 rpm for 10 min for plasma collection and stored at -20°C until analysis. The same volume of collected blood was reconstituted with physiological saline solution.

PKPD study. Drugs were administered as a single intraperitoneal dose in a volume equal to 1 ml kg-1, except for rofecoxib which was diluted to a volume 0.5 ml kg^{-1}. Blood samples of 250 µl were taken from the tail vein at pre-defined time points for the determination of drug, TXB_2 and PGE_2 concentrations. Sampling from the tail vein was limited up to a total of 7 blood samples per animal. In addition to six post-dose samples, a blood sample for the determination of the baseline PGE_2 and TXB_2 concentrations was taken between 15 and 45 min prior to drug administration to obtain accurate information on baseline concentrations. In order to characterise the entire time course of the PD effects, experiments were started in the morning (08.00 a.m.) or in the evening (06.00 p.m.). Six post-dose samples were taken for the experiments started in the morning at the following time points after administration 0.03-0.1 h (sample 1), 2.8-4.5 h (sample 2), 5.8-6.2h (sample 3), 7.2-11.3 h (sample 4), 13.0-13.4h (sample 5) and 23.8-24.2 h (sample 6). Six post-dose samples were

taken for the experiments started in the evening at the following time points after administration 0.03-0.05h (sample 1), 14.3-15.3h (sample 2), 16.0-17.1h (sample 3), 18.0-19.4 (sample 4), 21.3-22.2, (sample 5) and 23.5-24.0 h (sample 6). Blood samples were split into aliquots of 100 µl (for PK and PGE_2) and 50 µl (for TXB_2). Blood samples for PK were placed into heparinised tubes and centrifuged at 5000 rpm for 10 min. Plasma was stored at -20°C until analysis. Blood samples for TXB_2 analysis were placed into tubes and allowed to clot for 1 hour at 370C in a stirring water bath. Serum was collected after centrifugation and stored at -20°C until analysis. Tubes for the analysis of PGE_2 were prepared by evaporating aspirin (10 µg ml^{-1}) in methanol and heparin (10 I.U.). Blood samples were placed in tubes and 10 µg ml^{-1} lipopolysaccharide (LPS) was added. Samples were incubated and stirred for 24 hours at 37°C in a water bath. Plasma was separated by centrifugation and stored at -20°C until analysis.

In vitro experiments. For the *in vitro* experiments in rats, blood from four to six male SD rats was collected via the right jugular vein. The surgical procedure was performed under anaesthesia with 0.1 mg kg^{-1} intramuscular dose of medetomidine hydrochloride (Domitor, Pfizer, Capelle a/d Ijssel, The Netherlands) and 1 mg kg^{-1} s.c. dose of ketamine base (Ketalar, Parke-Davis, Hoofddorp, The Netherlands). Samples were separated into aliquots of 100 µl for PGE_2 and 50 µl for TXB_2 determination. Before the experiment, tubes were prepared by evaporating fixed amounts of each COX inhibitor (0, $4x10^{-4}$ - $3.14x10^{6}$ ng ml^{-1} or 0, $1x10^{-6}$ - $1x10^{4}$ µM) in methanol or in 10 µl DMSO (only rofecoxib). Evaporated heparin (10 I.U.) and aspirin (10 µg ml^{-1}) in methanol was added in the PGE_2 tubes. Blood samples for TXB_2 analysis were placed into tubes and allowed to clot for 1 hour at 37°C in a stirring water bath. Serum was collected after centrifugation and stored at -20°C until analysis. Blood samples for the PGE_2 analysis were placed in tubes and 10 µg ml^{-1} LPS was added. Samples were incubated for 24 hours at 37°C in a stirring water bath. Plasma was separated by centrifugation and stored at -20°C until analysis.

For the *in vitro* experiments with human blood, peripheral venous blood samples were collected from healthy volunteers (n=7) by venous puncture of the cubital vein. Informed consent was obtained from the seven subjects enrolled in the study. The subjects were between 23 and 30 years of age and had a weight range within 30% of their ideal body weight. The subjects had an unremarkable medical history and showed normal values in all haematology and biochemistry parameters. Smokers and subjects with a bleeding disorder, an allergy to aspirin or any other NSAIDs, or a history of any gastrointestinal disease were excluded. Subjects abstained from the use of aspirin and other NSAIDs for at least two weeks before enrolment. Samples were separated into aliquots of 1 ml for PGE_2 and 1 ml for TXB_2 quantification. Experimental assay and analytical procedures were performed as described above.

Additional data in humans. For a thorough analysis of the *in vitro-in vivo* correlations within and between species, potency estimates for the *in vitro* and *in vivo* inhibition of PGE_2 and TXB_2 in humans were retrieved from various publications (21;21;22). Although IC_{80} values have been shown to better reflect relevant therapeutic concentrations, these could not be extracted from the

literature data since information on the Hill coefficient was not available. Altogether data from data for celecoxib, rofecoxib, naproxen, ketorolac, diclofenac, acetaminophen, dipyrone, and meloxicam were included in the analysis.

Drug analysis. LC-MSMS analysis of rofecoxib. Concentrations of rofecoxib were determined by reverse phase HPLC/MS/MS using a heat assisted electrospray interface in negative ion mode and described in a previous investigation (23).

HPLC analysis of diclofenac. Concentrations of diclofenac were determined by HPLC with UV detection described by (24) with slight modifications and described in a previous investigation (23).

LC-MSMS analysis of ketorolac. Ketorolac plasma samples (50 µl) were extracted using protein precipitation with acetonitrile: 10 mM ammonium acetate (80:20) containing an internal standard (250 µl, 0.2 µg ml^{-1}). An aliquot of the supernatant was analysed by reverse phase HPLC/MS/MS using a heat assisted electrospray interface in positive ion mode. Nominal MRM transitions for ketorolac and internal standard (indomethacin) were 256 to 105 and 358 to 139, respectively. Samples (2 µl) were injected using a CTC Analytics HTS Pal autosampler (Presearch, Hitchin, UK) onto a Hypersil ODS-2 4.6 x 50 mm, 3-µm column (Thermo, Runcorn, Cheshire, UK) operated at 40°C and at an eluent flow rate of 1 ml min^{-1}. Analytes were eluted isocratically by means of an HP1100 binary HPLC system (Agilent, Stockport, Cheshire, UK), using 60% 1 mM ammonium acetate containing 0.1% formic acid (v/v) and 40% acetonitrile. The cycle time was 2.5 min per sample. The eluent was injected into an API4000 tandem mass spectrometer (Applied Biosystems, Ontario). Rat plasma samples were assayed in the range 0.010 to 20.0 µg ml^{-1} and the lower limit of quantification was 0.010 µg ml^{-1}. Bias and precision of the plasma calibration curves were assessed with quality control samples (n = 6) at concentrations of 0.02, 0.2, 5.0 and 20.0 µg ml^{-1}. Analytical results were accepted based on these quality controls being within 20% of the nominal concentration value. Samples showing plasma concentrations above the upper limit of quantification were re-assayed by appropriate dilution in plasma to within the range of the calibration curve.

Plasma protein binding. The methodology employed in this study was a modification of that reported by Kalvass & Maurer, 2002 (40). Briefly, a 96-well equilibrium dialysis apparatus was used to determine the free-fraction in plasma for each drug (HT Dialysis LLC, Gales Ferry, CT, USA). Membranes (3kDa cut-off) were conditioned in deionised water for 40 min, followed by conditioning in 80:20 deionised water:ethanol for 20 min, and then rinsed in deionised water before use. Freshly obtained rat or human plasma was spiked with rofecoxib, diclofenac or ketorolac (<0.5% organic solvent content) and 100-µl aliquots (n = 6 replicate determinations) were loaded into the 96-well equilibrium dialysis plate. Dialysis versus PBS (100 µl) was carried out for 6h in a temperature-controlled incubator at 37°C (Stuart Scientific, Watford, UK) using an orbital microplate shaker at 125 rev min^{-1} (Stuart Scientific, Watford, UK). At the end of the incubation period, aliquots plasma or PBS were transferred to Matrix ScreenMate tubes (Matrix Technologies,

Hudson, NH, USA) and the composition in each tube was balanced with control fluid such that the volume of PBS to plasma was the same. Sample extraction was performed by the addition of 200 µl of acetonitrile containing an internal standard. Samples were allowed to mix for 15 minutes and then centrifuged at 2465 g in 96-well blocks for 20 min (Eppendorf 5810R, VWR International, Poole, Dorset, UK). All samples were analysed by means of HPLC/MS/MS on a Sciex API-4000 tandem quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada), employing a Turbo V Ionspray operated at a source temperature of 700°C, (80 psi of nitrogen). Samples (3-10 µl) were injected using a CTC Analytics HTS Pal autosampler (Presearch, Hitchin, UK) onto a Hypersil Aquastar 3.0 x 30 mm, 3-µm column (Thermo, Runcorn, Cheshire, UK) operated at 40°C with an eluent flow rate of 1 ml min^{-1}. Analytes were eluted using a high-pressure linear gradient program, by means of an HP1100 binary HPLC system (Agilent, Stockport, Cheshire, UK) using acetonitrile as solvent B. For HPLC/MS/MS analysis in positive ion mode solvent A comprised 1 mM ammonium acetate containing 0.1% (v/v) formic acid, while in negative ion mode solvent A comprised 1 mM ammonium acetate. The gradient was held at 5% solvent B for 2 min, before increasing to 90% at 1.2 min, remaining at 90% until 1.6 min before returning to the starting conditions. The cycle time was 2.5 min per sample. Relative peak areas between the PBS and tissue half-wells were used to determine the respective free fractions.

Data analysis. The pharmacokinetics and pharmacodynamic effects of rofecoxib,diclofenac and ketorolac were assessed by non-linear mixed effects modelling, as implemented in NONMEM version V, level 1.1 (Globomax, Ellicott City, USA). Final model parameters were estimated by the first order conditional estimation method with η-ε interaction (FOCE interaction). This approach allows better estimation of inter- and intraindividual variability in model parameters. All fitting procedures were performed on a computer (AMD-Athlon XP-M 3000+) running under Windows XP with the Fortran compiler Compaq Visual Fortran version 6.1. An in-house interface for S-Plus 6.0 (Insightful Corp., Seattle, WA, USA) - NONMEM was used for data processing, management and graphical display.

Pharmacokinetic analysis. The pharmacokinetics of rofecoxib was previously described by a model that accounts for enterohepatic recirculation (EHC) (23). Briefly, a mixture model was used to discriminate between different population profiles in the data. The pharmacokinetic model included a conversion compartment describing the recycling of the parent drug. In the current investigation, the impact of EHC on concentration-time course profile was not evident due to the limited number of samples (study 2). Attempts to simultaneously analyse sparse and dense data was unsuccessful, yielding inaccurate predictions of structural and stochastic parameters. To overcome model parameter identifiably problems, simulations were performed for each population of the mixture model. These simulations were based on the population prediction of the model described by (23). (model 1.b). As input for the simulations, the average empirical bayes estimates (AEBEs) obtained for the group of cannulated rats which received i.p. dosing of rofecoxib at the same dose (10 mg kg^{-1}) as the sparse data rats were used (model 1.b, Huntjens *et al.* 2008). As the AEBEs for the 10 mg kg^{-1} i.p. dose group showed a smaller relative prediction error (Σ(DV-PRED/PRED, rPE=0.61) compared

to the population predictions based on model 1.b., the AEBE values for CL, F and $K_{recycling}$ rather than the population values as the model for the simulations. The values used for the simulations were 2.6 ml min^{-1} for Cl, 60% for F and 0.0026 min^{-1} for $K_{recycling}$. The value for the duration of recycling was 76 min.

Diclofenac also shows enterohepatic recirculation in rats (25-29). Therefore, diclofenac concentration-time profiles were analysed using an oscillatory EHC model, which was first proposed by Wajima *et al.* (30) and described in a previous investigation (details can be found in (23).

The pharmacokinetics of ketorolac was characterised by compartmental modelling. One-, two -and three compartment models with non-linear or Michaelis-Menten elimination were tested. Model selection was based on the likelihood ratio test, parameter point estimates and their respective 95% confidence intervals, parameter correlations and goodness-of-fit plots. For the likelihood ratio test, the significance level was set at 0.01, which corresponds with a decrease of 6.6 points after the inclusion of one parameter in the minimum value of the objective function (MVOF), under the assumption that the difference in MVOF between two nested models is χ^2-distributed. The following goodness-of-fit plots were subjected to visual inspection to detect systemic deviations from the model fits: individual observed vs. population or individual predicted values and weighted residuals vs. time or population predicted values. The pharmacokinetic analysis was performed by use of the ADVAN6 routine in NONMEM. Based on model selection criteria, a three compartment model was identified that best describes the pharmacokinetics of ketorolac. Model parameters included clearance, inter-compartmental clearance, and the volumes of distribution of the central and peripheral compartments.

Variability in pharmacokinetic parameters was assumed to be log-normally distributed in the population. Therefore an exponential distribution model was used to account for inter-individual variability:

$$P_i = \theta_i \cdot \exp(\eta_i) \tag{1}$$

where θ is the population estimate for parameter P, P_i is the individual estimate and η_i is the normally distributed between-subject random variable with mean zero and variance ω^2. The coefficient of variation (CV %) of the structural model parameters is expressed as percentage of the root mean square of the interindividual variance term. Selection of an appropriate residual error model was based on inspection of the goodness-of-fit plots. Hence, a proportional error model was proposed to describe residual error in plasma drug concentration of diclofenac and for ketorolac:

$$C_{obs,ij} = C_{pred,ij} \cdot (1 + \varepsilon_{ij,1}) \tag{2}$$

where $C_{obs,ij}$ is the j^{th} observed concentration in the i^{th} individual, $C_{pred,ij}$ is the predicted concentration, and ε_{ij} is the normally distributed residual random variable with mean zero and

variance σ^2. The residual error term contains all the error terms that cannot be explained by other fixed effects including experimental error (e.g. error in recording sampling times) and structural model misspecification.

During model building, the relevance of potential correlations between pharmacokinetic parameter estimates was tested by conducting covariance matrix analysis (OMEGA BLOCK option). A significant correlation between two parameters was assumed when the drop in MVOF was more than 6.6 points ($p < 0.01$). In addition, exploratory graphical analysis was performed to exclude differences between venous blood sampling via tail vein versus arterial blood sampling via cannulae and pharmacokinetic parameters.

Pharmacodynamic analysis. In this study, PGE_2 and TXB_2 concentrations were used as a measure of drug response. The sigmoid I_{max} model (equation 3) was used to relate plasma concentrations (C) to drug response by the equation:

$$Effect = I_0 - (I_0 - I_{\max}) * (C^n / (C^n + IC_{50}^n)) \quad (3)$$

where I_{max} represents the maximum inhibitory response to rofecoxib, ketorolac or diclofenac, I_0 the baseline production of PGE_2 or TXB_2 and n the Hill factor. This equation is an adaptation from the E_{max} model to obtain the inhibition level in percentage directly from the absolute values for I_0 and I_{max}. The sigmoid I_{max} model was used for the analysis of the data in rats and healthy volunteers. All three COX inhibitors were simultaneously analysed in a single run, separated by species and biomarker.

Statistical test. Unpaired student's test was used for comparison between groups. $p<0.05$ was considered statically significant.

RESULTS

Pharmacokinetics

Rofecoxib. Simulations were conducted based on the AEBE and population estimates as described on the methods. Model performance was subsequently evaluated by the relative prediction error (rPE was 0.61 versus 8.17) and visual inspection. Individual data fitting was best described by this model. The predicted plasma concentration and corresponding individual sparse data are presented in figure 1.

Diclofenac. For diclofenac, a two-compartment model that includes EHC was required to describe the profiles of sparse and dense plasma samples simultaneously (figure 1, middle panel). A summary of the estimates for structural and random effect parameters is presented in table 1. All fixed effect parameters were estimated with good coefficient of variation (less than 14%). Inter-individual variability could only be estimated for k_{45} and k_{20}, resulting in 48 and 44%. No covariates were found to significantly affect model parameters.

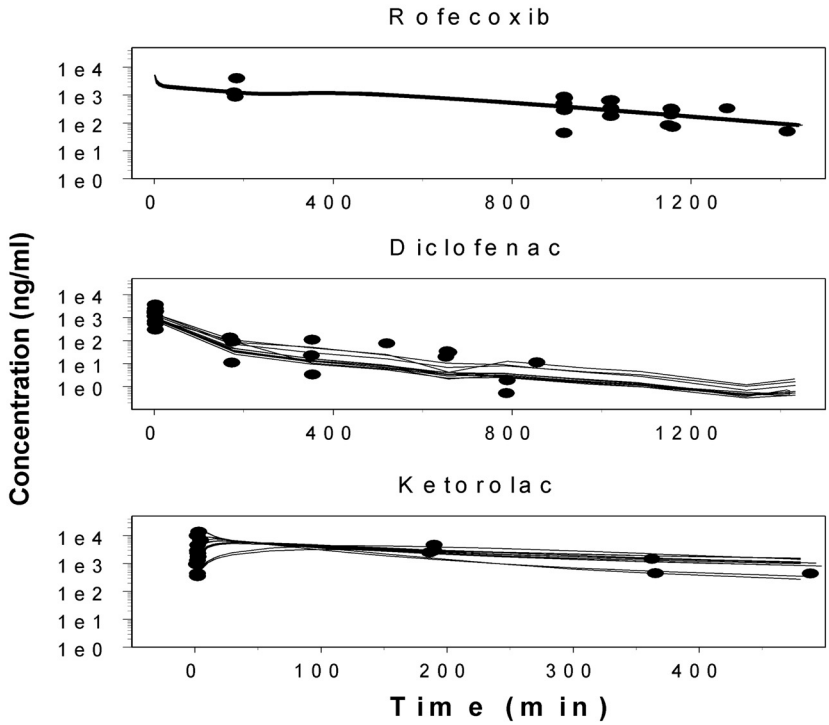

Figure 1. PK profiles for animals in study 2. Black lines indicate the individual predictions and black dots indicate the individual data points. Upper panel; rofecoxib, middle panel; diclofenac and lower panel; ketorolac.

Table 1. Population pharmacokinetic parameters and inter- and intraindividual variability of ketorolac after i.v. and i.p. administration. Values in parentheses are relative standard errors (in percent) of the estimates. IIV % is inter-individual variability in percent.

	Final Model Estimates Ketorolac	
	Fixed effects	*Random effects*
Cl (mL/min)		
$\theta_{Intercept}$	0.76 (10)	
θ_{Slope}	9.79 (27)	46 (40)
V_1 (mL)	53.80 (13)	32 (36)
V_2 (mL)	61.70 (11)	23 (64)
V_3 (mL)	109.00 (10)	n.d.
Q (mL/min)	3.83 (30)	90 (67)
Q_2 (mL/min)	0.39 (19)	68 (53)
K_a (min^{-1})	0.08 (27)	95 (41)
F (%)	80 (10)	n.d.
Residual variability		
Proportional error (%)	10.05 (13)	
Additive error (ng/mL)	79.44 (79)	

Ketorolac. A three-compartment model with first order absorption with combined proportional and additive errors accurately described the pharmacokinetics of ketorolac after i.v. and i.p. administration (figure 2). The observed and predicted concentration-time course for rats in study 2 is depicted in figure 1 (lower panel). Except for bodyweight, which was found to affect clearance, other potential covariates did not show any statistically significant effect on model parameters. Clearance was estimated for the population mean at 0.76 ml min^{-1} and bioavailability was estimated

at 80% after oral dosing. All fixed effect parameters were estimated with good coefficient of variation (less than 30%). Inter-individual variability could be estimated for most of the parameters except bioavailability, V_3 and the intercept of clearance. A summary of the estimates for structural and random effect parameters is presented in table 2.

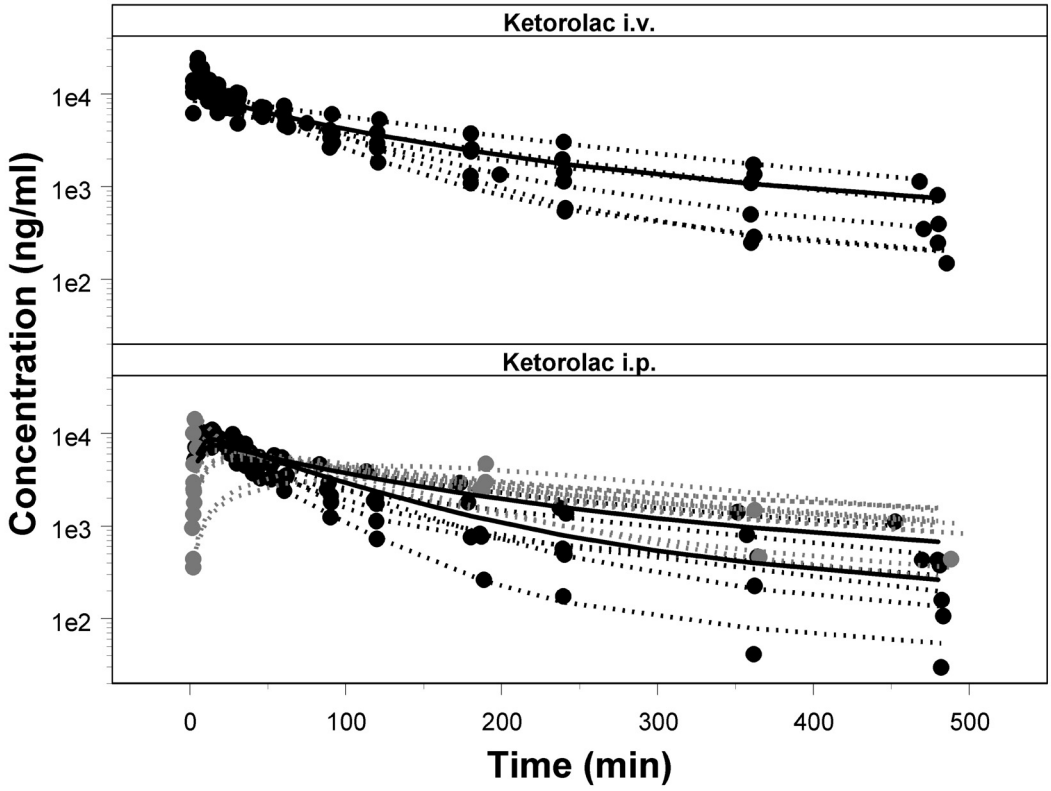

Figure 2. Population pharmacokinetic profiles of ketorolac after i.v. and i.p. administration. Solid black line shows population prediction of ketorolac, dashed black lines indicate the individual prediction in cannulated animals and grey lines indicate the individual prediction of ketorolac after tail vein sampling in uncannulated animals.
cannulated For the population predictions, median bodyweight of 0.259 kg was used.

Protein binding in vitro. There were statistically significant differences between rats an humans for the *in vitro* protein binding of rofecoxib (25.07 ± 4.89% vs. 13.43 ± 3.91%) and ketorolac (7.54 ± 2.27% vs. 2.36 ± 1.48%). In contrast, no differences for diclofenac were observed between species (2.74 ± 1.91% vs. 2.51 ± 2.03%).

PKPD analysis of COX inhibitors in vitro. Under baseline conditions, LPS-induced PGE_2 production averaged 79 ± 24 ng ml^{-1} (Mean ± SD n=15) in rats and 45 ± 20 ng ml^{-1} (Mean ± SD n=16) in healthy subjects. Whole blood TXB_2 production averaged 323 ± 165 ng ml^{-1} (n=15) in rats and 266 ± 140 ng ml^{-1} (n=16) in healthy subjects. The inhibition of PGE_2 and TXB_2 *in vitro* was modelled by an inhibitory I_{max} model (Figure 3) in both species. All fixed and random effect parameters are presented in Table 3. The reported IC_{80} values were derived from the primary pharmacodynamic parameters. As shown in table 5, the potency (IC_{50}) and IC_{80} values for PGE_2 inhibition yielded slightly different rankings in rats and in humans, i.e., rofecoxib < ketorolac < diclofenac, and diclofenac < ketorolac < rofecoxib, respectively.

Table 2. Population pharmacokinetic parameters and inter- and intraindividual variability of diclofenac after i.p., p.o. and i.v. administration. Values in parentheses are relative standard errors (in percent) of the estimates. IIV % is inter-individual variability in percent.

	Final Model Estimates Diclofenac	
	Fixed effects	*Random effects*
k_{20}^{*} (min^{-1})	0.61 (34)	44 (55)
V_2 (ml)	8.8 (48)	
k_{32}^{**} (min^{-1})	0.0486 (26)	
k_{23} (min^{-1})	0.723 (24)	
Freq (min)	390 (1)	
F (%) p.o.	60 (34)	
k_{43} (min^{-1})	4.51 (45)	
k_{45} (min^{-1})	3.09 (43)	48 (53)
k_{54} (min^{-1})	0.0061 (31)	
k_{27} (min^{-1})	0.225 (124)	
k_{72} (min^{-1})	0.011 (50)	
Residual error		
Proportional parent (%)	40 (29)	
Proportional metabolite (%)	22 (50)	

For TXB_2 a different ranking was observed in selectivity of the compounds, relative to their inhibitory effect on PGE_2. These findings are also summarised in table 5. The potency (IC_{50}) and IC_{80} values for TXB_2 inhibition did not reveal differences in ranking between rats and humans, with ketorolac < diclofenac < rofecoxib.

For PGE_2 inhibition in rats, a correlation was found between I_0 and blank PGE_2 production in rats. This correlation was implemented as covariate in the model, resulting in a significant decrease in MVOF ($p<0.001$). The population IC_{80} values for TXB_2 inhibition *in vitro* showed a stronger correlation across species ($r2=0.98$) than for PGE_2 inhibition ($r2=0.02$) (plots not shown). However, no definitive conclusions can be drawn from this observation based on three compounds.

PKPD analysis of COX inhibitors in vivo. Prior to drug administration, LPS-induced PGE_2 production averaged 86 ± 27 ng ml^{-1} (n=36), whilst whole blood TXB_2 formation averaged 407 ± 271 ng ml^{-1} (n=36). To ensure accurate estimation of system-dependent parameters, data from all three compounds were analysed simultaneously. The concentration-effect relationships of rofecoxib, ketorolac and diclofenac were characterised by a sigmoid I_{max} model. Hill factor and baseline production I_0 were estimated as a single population parameter to reflect the intrinsic properties of the experimental system, whereas potency IC_{50} and maximal inhibition I_{max} were estimated as separate parameters for each drug (table 4). The concentration-effect relationships for PGE_2 and TXB_2 inhibition are depicted in Figure 4.

Due to numerical constraints during estimation, maximum PGE_2 inhibition was fixed for rofecoxib and diclofenac. The same constraints were required for the maximum TXB_2 inhibition following administration of ketorolac and diclofenac. In these cases, parameter values were fixed to the values

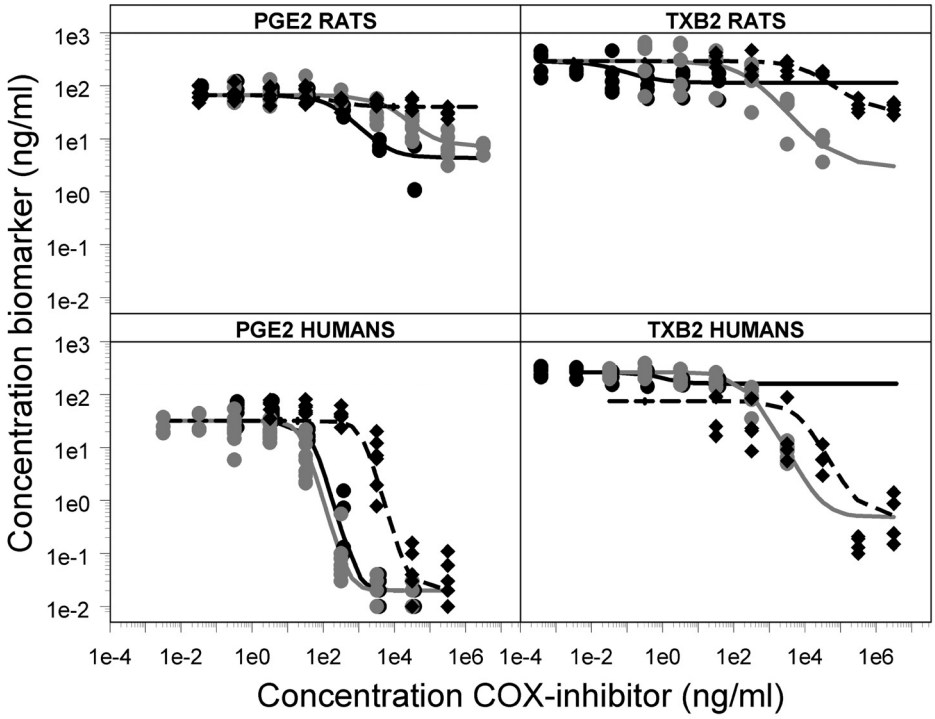

Figure 3. Rofecoxib, diclofenac and ketorolac effects in vitro. Open symbols indicate individual data points. Solid black line shows population prediction of ketorolac and black dashed line shows the population prediction of rofecoxib and grey line shows the population prediction of diclofenac. Upper panel: drug exposure versus PGE_2 concentrations. Lower panel: drug exposure versus TXB_2 concentrations.

Table 3. Population estimates of the final pharmacodynamic models for PGE_2 and TXB_2 inhibition by diclofenac, rofecoxib and ketorolac *in vitro* in rats and humans (HV). Values in parentheses are relative standard errors (in percent) of the estimates.

	Compound parameter estimates	**Diclofenac**		**Rofecoxib**		**Ketorolac**	
PGE2 inhibition	*Fixed effects*	Rats	HV	Rats	HV	Rats	HV
	I_0 (ng/mL)						
	$\theta_{Intercept}$	67.2 (3)	32.2 (15)	67.2 (3)	32.2 (15)	67.2 (3)	32.2 (15)
	θ_{Slope}	0.74 (10)		0.74 (10)		0.74 (10)	
	Hill coefficient	1.01 (31)	2.32 (11)	1.01 (31)	2.32 (11)	1.01 (31)	2.32 (11)
	IC_{50} (ng/ml)	8047 (83)	22 (13)	151 (33)	1122 (15)	258 (38)	41 (36)
	IC_{80}^* (ng/mL)	31749 (-)	40 (-)	596 (-)	2039 (-)	1018 (-)	75 (-)
	I_{max} (ng/mL)	7.10 (15)	0.02 (11)	40.2 (6)	0.02 (11)	4.37 (29)	0.02 (11)
	Random effects						
	ω I_0 (%)		25 (35)		78 (79)		61 (95)
	ω IC_{50} (%)				129 (86)		
	ω Hill (%)		52 (115)		52 (115)		52 (115)
	ω I_{max} (%)				191 (83)		
TXB2 inhibition	*Fixed effects*	Rats	HV	Rats	HV	Rats	HV
	I_0 (ng/mL)	297 (9)	264 (6)	297 (9)	76 (38)	297 (9)	264 (6)
	Hill coefficient	0.85 (6)	1.28 (26)	0.85 (6)	1.28 (26)	0.85 (6)	1.28 (26)
	IC_{50} (ng/ml)	227 (13)	219 (20)	18642 (14)	6412 (21)	0.05 (36)	0.76 (150)
	IC_{80}^* (ng/mL)	1160 (-)	647 (-)	95235 (-)	18939 (-)	0.25 (-)	2.25 (-)
	I_{max} (ng/mL)	2.99 (35)	0.49 (30)	30.3 (9)	0.49 (30)	115 (9)	162 (41)
	Random effects						
	ω I_0 (%)	68 (53)			59 (41)		

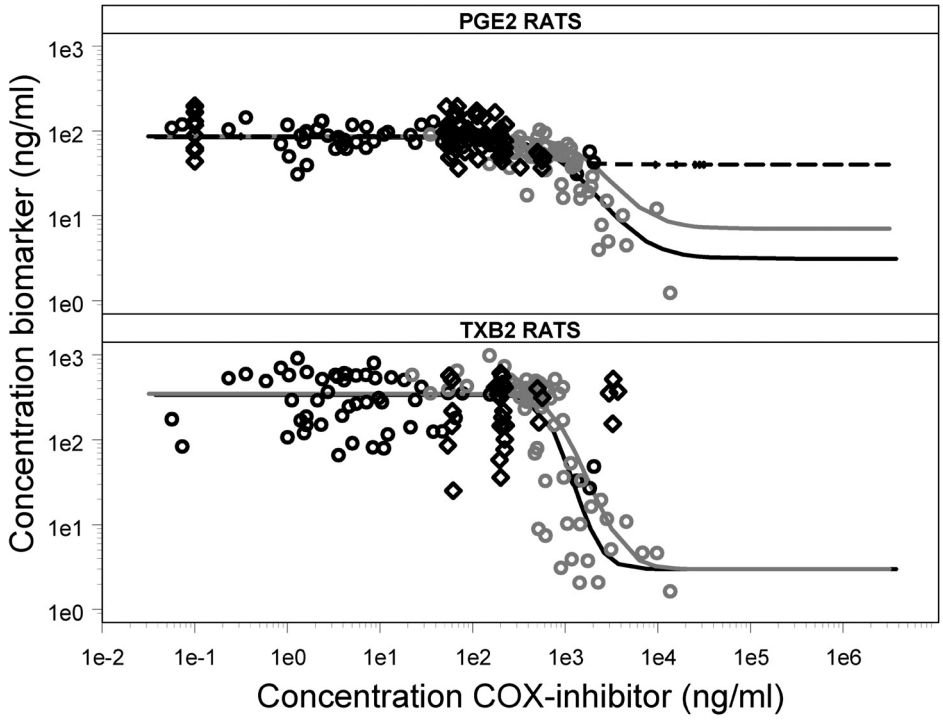

Figure 4. Rofecoxib, diclofenac and ketorolac effects *in vivo*. Upper panel: drug exposure versus PGE_2 concentrations. Lower panel: drug exposure versus TXB_2 concentrations. Open symbols represent individual data points. Solid black line indicates the population prediction of ketorolac, dashed lines represent population prediction of rofecoxib and grey line indicates the population prediction of diclofenac.

Table 4. Population pharmacodynamic models for the *in vivo* inhibitory effects of diclofenac, rofecoxib and ketorolac on PGE_2 and TXB_2 production in rats. Values in parentheses are relative standard errors (in percent) of the estimates.

	Compound	Diclofenac	Rofecoxib	Ketorolac
PGE2 inhibition		*Fixed effects*	*Fixed effects*	*Fixed effects*
	I_0 (ng/mL)	84.9 (5)	84.9 (5)	84.9 (5)
	Hill coefficient	1.76 (22)	1.76 (22)	1.76 (22)
	IC_{50} (ng/mL)	896 (10)	306 (21)	894 (10)
	IC_{80}^{*} (ng/mL)	1970 (-)	673 (1)	1965 (-)
	I_{max} (ng/mL)	7.1 (-)	40.2 (-)	3.13 (107)
TXB2 inhibition		*Fixed effects*	*Fixed effects*	*Fixed effects*
	I_0 (ng/mL)	340 (8)	n.d.	340 (8)
	Hill coefficient	3.77 (36)	n.d.	3.77 (36)
	IC_{50} (ng/mL)	647 (21)	n.d.	840 (13)
	IC_{80}^{*} (ng/mL)	935 (-)	n.d.	1213 (-)
	I_{max} (ng/mL)	2.99 (-)	n.d.	4.12 (50)

obtained *in vitro*. As shown in table 5 the potency (IC_{50}) and IC_{80} values for PGE_2 inhibition yielded similar rankings in rats and in humans, i.e., diclofenac < ketorolac < rofecoxib. For TXB_2 inhibition, the IC_{50} and IC_{80} values were relatively close to each other, with diclofenac slightly more potent than ketorolac, reflecting a very shallow slope for the concentration-effect curve for both compounds. Rofecoxib did not inhibit TXB_2 following i.p. administration of a 10 mg kg^{-1} dose.

In vitro-in vivo correlations. Initially an evaluation of the *in vitro-in vivo* correlation was performed within species, with separate analysis of rat and human data. Naproxen data from Huntjens *et al.* were included in this analysis (16). Potency estimates for PGE_2 and TXB_2 inhibition *in vitro* were graphically displayed against values *in vivo* (figure 5). Interestingly, in either species ketorolac appears to be more potent (>100 times) *in vitro* than *in vivo* with regard to its effect on TXB_2.
Since an *in vitro-in vivo* correlation was identified in rats and humans, *in vitro* rat data were subsequently plotted against *in vivo* data in humans (figure 6).

DISCUSSION AND CONCLUSIONS

Despite the widespread use of COX inhibitors and the ongoing efforts to identify and develop new compounds with high selectivity for COX-2, no formal attempt has been made to establish whether *in vitro* selectivity reflects a drug's positive efficacy: safety ratio *in vivo*, nor it is known how such a ratio is affected by variation in the extent and duration of COX-2 and/or COX-1 inhibition. Moreover, very few data is available showing in a quantitative manner how COX-inhibition correlates with analgesia. This is particularly important in the early stages of drug development, as the dose selected for clinical investigations ought to be based on a systematic evaluation of a compounds' effect on cyclo-oxygenase, rather then solely based on behavioural measures of pain.

Table 5. Comparison of the IC_{80} values obtained in rats and human data for PGE_2 and TXB_2 inhibition for diclofenac, rofecoxib and ketorolac.

	IC_{80} (nL/ml) in vitro HV	IC_{80} (nL/ml) in vitro RAT	IC_{80} (nL/ml) in vivo RAT	IC_{80} (nL/ml) in vitro HV	IC_{80} (nL/ml) in vitro RAT	IC_{80} (nL/ml) in vivo RAT
COMPOUND	PGE_2	PGE_2	PGE_2	TXB_2	TXB_2	TXB_2
Diclofenac	40	31749	1970	647	1160	935
Rofecoxib	2039	596	673	18939	95235	
Ketorolac	75	1018	1965	2.25	0.25	1213

In the current investigation, we have assessed the PKPD relationships of rofecoxib, ketorolac and diclofenac by modelling their inhibitory effect on PGE_2 and TXB_2 *in vitro* and *in vivo* in rats. To this purpose, we have used an experimental setting that takes into account the process that is in place for the evaluation of a drug's pharmacokinetic and pharmacodynamic properties at the early stage of development, i.e., by exploring whether rat data can be used to predict drug effects in humans. We have also attempted to separate the pharmacokinetic confounders often observed in *in vivo* experiments, so that pharmacodynamic properties can be parameterised and evaluated independently of dose, route of administration and time of sampling. Such an approach ensures that selectivity to the isozymes and drug concentration at the site of action remained as primary variables. This was also the basis for the evaluation of *in vitro-in vivo* correlations.
Pharmacokinetics and dose selection. One of the difficulties in exploring PKPD relationships of drugs with analgesic and anti-inflammatory properties are the potential confounding effects of pharmacokinetic sampling in an experiment aimed at the assessment of drug efficacy. Nonlinear

mixed effects modelling offers an opportunity to address this problem, as simulations can be used to derive drug exposure profiles even after sparse, limited sampling of the tail vein. In addition, the possibility to generate individual predicted concentration time profiles also overcomes the potential effects of arterial cannulation on pharmacodynamics, in particular the reduction in plasma albumin due to an acute phase reaction (31).

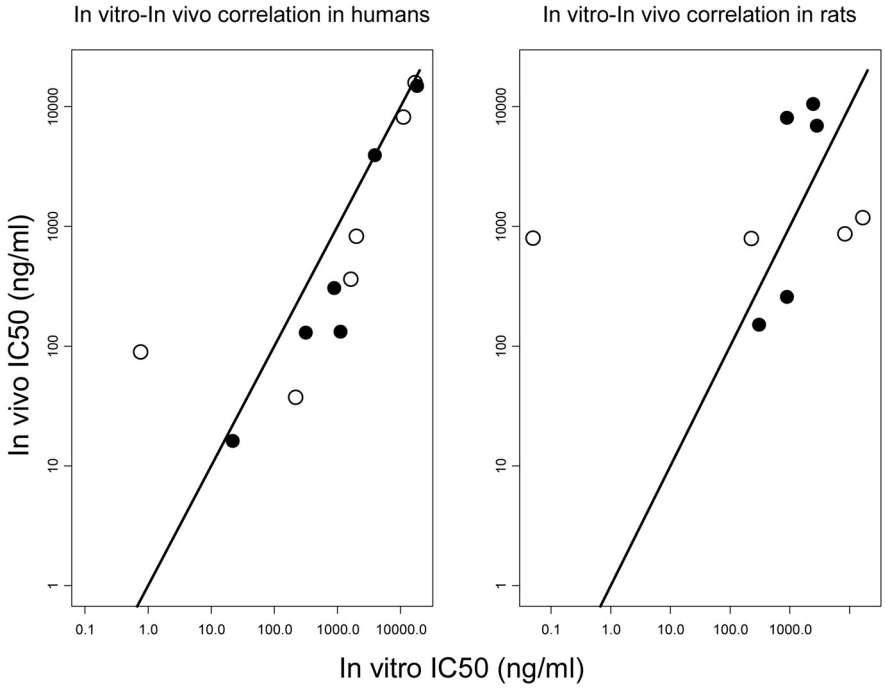

Figure 5. *In vitro-in vivo* correlations for COX-inhibitors. Left panel: *In vitro* IC_{50} (ng/ml) in humans versus *in vivo* IC_{50} (ng/ml) in humans. Filled circles represent PGE_2 inhibition, open circles represent TXB_2 inhibition. Right panel: *In vitro* IC_{50} (ng/ml) in rats versus *in vivo* IC_{50} (ng/ml) in rats. Filled circles represent PGE_2 inhibition, open circles represent TXB_2 inhibition.

It is important to mention that with regard to ketorolac, we have decided to use total plasma concentration for our data analysis, despite evidence for chiral pharmacokinetics with two enantiomers, (+)R and (-)S ketorolac. Our decision is justified by the fact that the (-)S enantioner is the active substance and is >100 times more potent than (+)R (32). In addition, ketorolac undergoes *in vivo* interconversion in rats. At a dose of 1 mg/kg i.v. 7% from (+)R to (-)S and 4% from (-)S to (+)R, in contrast to a dose of 100 mg/kg 6% from (+)R to (-)S and 55% from (-)S to (+)R. The dose used in this study (5 mg kg^{-1} i.p.) is equivalent to a dose of 4 mg/kg i.v., based on bioavailability of 80%. The marginal contribution of the (+)R enantiomer means that interconversion would be of minor influence on pharmacodynamics. Considerations were also made about the implication of differences in protein binding, which were observed for ketorolac. Ketorolac shows strong protein binding in humans whereas moderate binding in rats which could influence allometric scaling from rats to humans. These findings are in agreement with the results obtained by Mroszczak *et al.* (33). As indicated previously, the doses selected for all three compounds were aimed at achieving efficacious concentrations in rats, even if the analgesic effect was not measured in these experiments.

Based on previous evidence that exposure yielding at least 80% inhibition PGE_2 does correlate with analgesia in humans (35), we have compared the three compounds with respect to the circulating concentrations (i.e., above IC_{80}). It was found that this represents a span of 30-120 min post dose for rofecoxib and ketorolac, whereas it represents no more than 30 min for diclofenac. These rather short intervals above 80% inhibition may suggest that the doses used for these experiments in rats were less than optimal for a sustained analgesic effect. Literature data on hyperalgesia in the carrageenan model show that the ED_{50} for rofecoxib is 0.5 mg kg^{-1} for diclofenac, 0.43 mg kg^{-1} and 0.29 mg kg^{-1} for ketorolac (20;34). These data indicates that our used doses are well within the analgesic range, making it an interesting finding, given that this contrasts with the correlation between IC_{80} values from the hWBA and analgesia in humans (35).

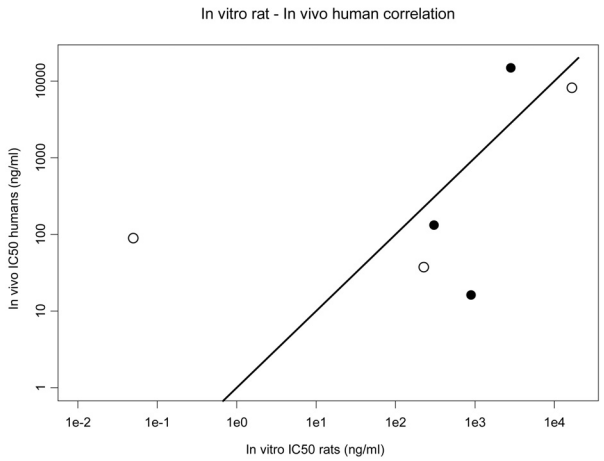

Figure 6. *In vitro-in vivo* correlations for COX-inhibitors. *In vitro* IC_{50} (ng/ml) in rats versus *in vivo* IC_{50} (ng/ml) in humans. Filled circles represent PGE_2 inhibition, open circles represent TXB_2 inhibition.

In vitro PKPD relationship. Baseline production levels of PGE_2 *in vitro* (79 ± 24 and 45 ± 20 ng ml^{-1}, for rats and humans) and TXB_2 (323 ± 165 and 266 ± 140 ng ml^{-1}, for rats and humans) are in agreement with previous results and literature data (16;36;37).
In rats, ketorolac showed to be more selective for COX-1 (IC_{50} 101 ng ml^{-1}) than COX-2 (IC_{50} 774 ng ml^{-1}) (20). The IC_{50} for TXB_2 inhibition obtained in our experiments suggest, however, that ketorolac is less potent than previously reported. Differences in potency could be explained by variation in assay systems. Jett obtained results from a purified enzyme system. Yet, smilar degrees of variability and potency has been reported by others such as Warner *et al.* (21).

Discrepancies between rat and human data were also found for diclofenac, which causes a different degree of PGE_2 and TXB_2 inhibition in rat and human blood. Species differences in binding to COX-2 could introduce such differences in potency in rats and humans, however, no such concerns have been reported in literature. These differences contrast with the findings for ketorolac, rofecoxib and naproxen, all with comparable potency across species. Yet, these differences seem to be mitigated if one considers compound properties in terms of IC_{80} values after correction for protein binding. The estimates are within a log-unit for rofecoxib and ketorolac, and >300 fold for diclofenac.

In vivo PKPD relationship. In experimental models of pain and in most published articles on the anti-

inflammatory properties of COX inhibitors, exploration of the efficacious doses of COX inhibitors is performed in non-cannulated animals or without quantitative evaluation of drug effect on markers of inflammation. Such an experimental set-up is a major limitation to understanding the relationship between dose, exposure and pharmacological activity. This also hampers any attempt to use pre-clinical findings to accurately identify efficacious and safe exposure in humans.
Rofecoxib was the most potent and selective compound in this analysis. However, IC_{80} values for PGE_2 inhibition *in vivo* (IC_{80} 693 ng ml^{-1}) were only three fold smaller than the estimates obtained for diclofenac and ketorolac. This finding is very pertinent to our investigation, since the *in vitro* selectivity of ketorolac for COX-1 is lost downstream in the inflammatory cascade. In fact, it has been shown that both COX-1 and COX-2 contribute to the synthetic pathway of prostaglandins. With regard to the TXB_2, inhibition was observed only after administration of diclofenac and ketorolac. Rofecoxib did not cause any inhibition at a dose of 10 mg kg^{-1}. Based on its *in vitro* potency for TXB_2 inhibition, the exposure to rofecoxib *in vivo* could have yielded up to 50% inhibition.

As expected, the PKPD relationships of the inflammatory markers showed large variability over time, which was also observed by Patrignani *et al.* (38). Such variability can be explained to some extent by assay factors and by circadian variation in circulating enzyme levels. Unfortunately, the experimental design (single dose per group, sparse sampling) does not allow us to carefully explore other influential covariates.

In vitro-in vivo correlations in rats and humans. Bearing in mind the objectives of drug screening during the early stages of drug development, it becomes evident that evidence for an *in vitro-in vivo* correlation would facilitate extrapolation from pre-clinical findings. However, careful consideration must be made to distinguish two key factors underlying an extrapolation. First, one needs to consider how differences in pharmacokinetics and pharmacodynamics within species, i.e., from *in vitro* to *in vivo* conditions. Second, one needs to account for differences in physiological function and disease between species, and between health and disease conditions.
The lack of evidence for such correlations between species would suggest a change to approach currently used for the screening of novel COX inhibitors, which rely primarily on selectivity estimates *in vitro* and on behavioural measures of analgesia *in vivo*. None of these two experimental settings provide a quantitative evaluation of pharmacological effects on the pathway of inflammatory response.

The graphical evaluation proposed in this manuscript addresses some of the issues arising from the first point. It does not encompass the potential impact of disease state and disease progression on pharmacokinetics and pharmacodynamics. A conclusive statement about the existence of a correlation between the *in vitro-in vivo* correlation in rats is not possible with the limited amount of data. Unfortunately, we were not able to identify literature data in rats for other selective and non-selective COX inhibitors. There were far more data available in humans. A clear correlation between *in vitro* and *in vivo* results was observed in humans, with the exception of ketorolac. This finding

suggests that a simple *in vitro* assay in human blood could used for the prediction of *in vivo* drug effects. Interestingly, inhibition of TXB_2 by ketorolac was >100 times more potent *in vitro* than *in vivo* both in rats and humans (figure 5). This can be explained by the fact that the analgesic dose in humans by far exceeds the *in vitro* potency values, preventing the accurate estimation of model parameters following a single dose *in vivo*.
A next step was to link *in vitro* data in rats to *in vivo* data in humans (figure 6). Evidence for such correlation would eventually provide further confidence about the predictive value of preclinical experiments, as early as lead optimisation. Again, given the limited amount of data no clear conclusion could be drawn from this observation.

One of our most interesting findings was to establish that the sensitivity of the cyclo-oxygenase system to drug inhibition is higher in humans than in rats. Differences in the Hill coefficient for the effects on of PGE_2 and TXB_2 were observed between species. Such differences have been observed in previous investigations (39), but have not been taken into consideration in the comparison of compounds. The implications of this finding is that based on potency estimates from rats, one will underestimate the overall inhibitory effect of a compound in human. Ignoring the steepness of the slope of the concentration-effect relationship when predicting the effective dose in patients, will results in a dose higher than necessary and potentially less selective. This is why we have defended the need for reparameterisation of pharmacodynamic models to indicate IC_{80} instead of IC_{50} values. The use IC_{80} does account for the influence of the Hill coefficient on a concentration response curve, allowing for appropriate comparison of data across compounds as well as across species.

In conclusion, we have anticipated that the assessment of drug effect on biomarkers may provide a better rationale for dose selection in phase I and phase II studies. The evidence for an *in vitro-in vivo* correlation in humans provides further support for this approach, as opposed to empirical extrapolation of findings in preclinical models of inflammatory pain. Our results cannot discard a similar correlation may exist in rats, but it is clear from these results that differences exist between species. Such and may be explained by physiological mechanisms and have been thus far ignored during the assessment of the analgesic effect in animals.

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