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Correlation between *in vitro* and *in vivo* concentration–effect relationships of naproxen in rats and healthy volunteers

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1 Understanding the mechanisms underlying the analgesic effect of new cyclooxygenase inhibitors is essential to identify dosing requirements in early stages of drug development. Accurate extrapolation to humans of *in vitro* and *in vivo* findings in preclinical species is needed to optimise dosing regimen in inflammatory conditions.

2 The current investigation characterises the inhibition of prostaglandin E2 (PGE_2) and thromboxane B2 (TXB_2) by naproxen *in vitro* and *in vivo* in rat and human blood. The inhibition of PGE_2 in the absence or presence of increasing concentrations of naproxen (10^{-8}–10^{-1} M) was measured by *ex vivo* whole blood stimulation with LPS, whereas inhibition of TXB_2 was measured in serum following blood clotting. In further experiments, inhibition of PGE_2 and TXB_2 levels was also assessed *ex vivo* in animals treated with naproxen (2.5, 10, 25 mg kg^{-1}). Subsequently, pharmacokinetic (PK)/pharmacodynamics (PD) modelling of *in vitro* and *in vivo* data was performed using nonlinear mixed effects in NONMEM (V).

3 Inhibition of PGE_2 and TXB_2 was characterised by a sigmoid E_{max} model. The exposure–response relationships *in vitro* and *in vivo* were of the same order of magnitude in both species. IC_{80} estimates obtained *in vitro* were similar for PGE_2 inhibition (130.8 ± 11 and 131.9 ± 19 10^{-6} M, mean ± s.d. for humans and rats, respectively), but slightly different for TXB_2 inhibition (103.9 ± 15 and 151.4 ± 40 10^{-6} M, mean ± s.d. for humans and rats, respectively, $P < 0.05$). These differences, however, may not be biologically relevant.

4 The results confirm the value of exposure–effect relationships determined *in vitro* as a means to predict the pharmacological activity *in vivo*. This analysis also highlights the need to parameterise concentration–effect relationships in early drug development, as indicated by the estimates of IC_{80} for PGE_2 and TXB_2 inhibition.
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Abbreviations: CV%, coefficient of variation; COX, cyclooxygenase; EIA, enzyme immunoassay; f_u, free fraction; LPS, lipopolysaccharide; MVOF, minimum value of the objective function; NSAIDs, nonsteroidal anti-inflammatory drugs; PD, pharmacodynamics; PG, prostaglandins; PGE_2, prostaglandin E2; PK, pharmacokinetics; SD, Sprague–Dawley; TXB, thromboxanes; TXB_2, thromboxane B2

Introduction

Naproxen is a nonselective cyclooxygenase (COX) inhibitor commonly used for the treatment of acute and chronic pain, rheumatoid arthritis and osteoarthritis. COX inhibitors act by inhibiting cyclooxygenase activity and consequently the formation of proinflammatory mediators like prostaglandins (PG) and thromboxanes (TXB) (Vane, 1971). Since the early 1990s, it has been generally accepted that COX exists in two isoforms. COX-1 (COX-1) is a housekeeping enzyme responsible for modulating physiological events and is present in most tissues including stomach, kidney and platelets, whereas COX-2 (COX-2) is highly induced in various cells by proinflammatory stimuli, mitogens and cytokines (Vane & Botting, 2001). Continuous COX-1 inhibition is thought to be principally responsible for gastrointestinal adverse effects following prolonged administration of nonselective COX inhibitors, whereas selective COX-2 inhibition accounts for the anti-inflammatory, antipyretic and analgesic efficacy (Vane, 1994). Recent investigations demonstrate that the roles of COX-1 and COX-2 are oversimplified (Egan *et al*., 2005; Lee *et al*., 2005; Patrignani *et al*., 2005). Data from those studies suggest that COX-2 is present under nonpathological conditions in tissues such as kidney, brain and the spinal cord, playing an important role in the maintenance of physiological homeostasis (Martinez *et al*., 2002).

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, it has been one of the major goals of clinical pharmacology to find systematic ways to identify dosing regimens that produce clinically relevant analgesia (Derendorf *et al*., 2000). An important question that remains to be answered is how much and how long COX-2 and COX-1 should be inhibited to ensure an optimal risk–benefit ratio,

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allowing for sustained analgesic response and appropriate safety margin. To date, the dose selection of COX inhibitors has been based primarily on clinical end points for analgesia, an approach which disregards the impact of maximum, long-lasting blockade of either enzyme systems (Huntjens *et al*., 2005).

The nature and complexity of the interaction between various factors that determine the analgesic response of COX inhibitors requires the identification of specific biomarkers to explain and understand variability in treatment effect. The use of a biomarker in pain measurements is an important step in the development of new COX inhibitors, as it can link pharmacokinetics (PK) to the analgesic effect and eventually provide a proxy for safety evaluation. Given the nature of the inflammatory response and the mechanism of action of COX inhibitors, a number of mediators can be used as an intermediate step between PK and analgesia. In conjunction with nonlinear mixed effect modelling, the relationship between biological marker, pain measurement and safety can then be characterised. Primary candidates for such a role are PG and TXB. In addition, the mechanisms of inflammation in rodents and humans bear similarities, which may facilitate the extrapolation of biomarkers from preclinical to clinical data (Huntjens *et al*., 2005).

In this study, we have assessed the PK–pharmacodynamic (PK/PD) relationship of naproxen *in vitro* and *in vivo* in rats and healthy volunteers. It was anticipated that *in vitro* PK/PD relationships can form the basis for scaling and predicting drug effects *in vivo*. Plasma prostaglandin E_2 (PGE_2) and serum thromboxane B_2 (TXB_2) concentrations were selected as biomarkers for the pharmacological effect and associated side effects. Furthermore, we have evaluated the relevance and requirements for the scaling of PD parameters from rats to humans.

Methods

The current investigation includes results from a PK study in cannulated animals (study 1) and the PK/PD modelling of TXB_2 and PGE_2 inhibition in noncannulated animals (study 2). Study 1 was performed to characterise the PK of naproxen using serial blood sampling and to enable subsequent analysis of the sparse PK data obtained in study 2. This set of experiments was required to accurately estimate naproxen concentrations associated with sampling the times for the biomarkers.

Animals

Experiments were performed on male Sprague–Dawley (SD) rats (Charles River BV, Maastricht, The Netherlands) weighing 308 ± 7 g (mean $\pm$ s.e.m., $n = 83$), upon approval of the study protocols 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-h day night^{-1} schedule (lights on 0700 hours) 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, 3 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 for infusions of naproxen and in the 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 serial collection of blood. The arterial cannula was filled with heparinised 25% (w v^{-1}) polyvinylpyrrolidone (PVP) (Brocacef, Maarssen, The Netherlands) in saline. Rats receiving an intraperitoneal (i.p.) injection of naproxen were implanted with only an arterial cannula. Cannulae were tunnelled subcutaneously to the back of the neck and exteriorised and fixed with a rubber ring. Before the experiment, the PVP solution was removed and the cannulae were flushed with saline containing 20 IU ml^{-1} heparin. The 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

Naproxen (mol weight = 230.26) was purchased from Sigma Alldrich BV (Zwijndrecht, The Netherlands). Naproxen was administered as an intravenously (i.v.) infusion at a dose of 25 mg kg^{-1} or as an i.p. bolus at a dose of 2.5, 10 or 25 mg kg^{-1}. Naproxen was dissolved in 0.9% NaCl. Naproxen was administered as an i.v. infusion primarily to enable the estimation of its relative bioavailability after i.p. administration.

Experimental design

Study 1 All experiments were started between 0830 and 0930 hours to exclude the influence of the circadian rhythms. Naproxen (25 mg kg^{-1}) was administered i.v. at a rate of 20 μl min^{-1} over 5 min using an infusion pump (Bioanalytical Systems Inc., Indiana, U.S.A.) or given as an i.p. injection (2.5 and 25 mg kg^{-1}) to conscious and freely moving rats. Serial arterial blood samples (100 μl) were taken at predefined time points (0, 5, 10, 15, 20, 25, 30, 35, 40, 45 min, 1, 1.5, 2, 4, 6, 9, 10, 12, 14 and 24 h) and the total volume of blood samples was kept to 2.0 ml during each experiment. The blood samples were immediately heparinised and centrifuged at 5000 r.p.m. for 10 min for plasma collection and were stored at −20°C until analysis. The same volume of collected blood was reconstituted with physiological saline solution.

Study 2 For the characterisation of the complete time course of the PD effects over a period of 48 h, experiments were started in the morning (0800 hours) or in the evening (1800 hours). Animals were administered naproxen i.p. (2.5, 10, 25 mg kg^{-1}). The drug was given in a dose volume of 1 ml kg^{-1}. Sampling from the tail vein was limited to seven blood samples per animal. Blood samples of 250 μl were taken at predefined time points up to 48 h after drug administration for the determination of naproxen, TXB_2 and PGE_2 concentrations. A blood sample for the estimation of baseline levels of PGE_2 and TXB_2 was taken between 15 and 45 min before dosing. 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 r.p.m. 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 h at 37°C 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 $\mu g\,ml^{-1}$ in methanol and heparin (10 IU) Blood samples were placed in tubes and 10 $\mu g\,ml^{-1}$ lipopolysaccharide (LPS) was added. Samples were incubated and stirred for 24 h 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 six male SD rats was collected *via* the right jugular vein. The surgical procedure was performed under anaesthesia with 0.1 $mg\,kg^{-1}$ i.m. dose of metetomidine 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 quantification. Before the experiment, tubes were prepared by evaporation of methanol containing fixed amounts of naproxen (0, 10^{-8}–10^{-1} M). Evaporated heparin (10 IU) and aspirin (10 $\mu g\,ml^{-1}$) 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 h 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 $\mu g\,ml^{-1}$ LPS was added. Samples were incubated for 24 h 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 in healthy volunteers, peripheral venous blood samples were collected by venous puncture of the cubital vein. Informed consent was obtained from the seven subjects enrolled. 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 were normal in routine haematological and biochemical studies. Smokers and subjects with a bleeding disorder, an allergy to aspirin or any other nonsteroidal anti-inflammatory drugs (NSAIDs), or a history of any gastrointestinal disease were excluded. Subjects abstained from the use of aspirin and other NSAIDs for at least 2 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.

Plasma protein binding

Plasma protein binding was determined *in vitro*. Naproxen (10^{-4} and 10^{-3} M) was added to 500 μl whole blood in heparin. After 30-min incubation at 37°C, plasma was separated and 50 μl was retained for analysis. The remaining plasma was subjected to ultracentrifugation using Centrifee micropartition devices (Millipore Corporation, Bedford, MA, U.S.A.). The plasma was filtered at 2000 × *g* at 37°C for 20 min, yielding 150 μl ultrafiltrate. After sample preparation, plasma and ultrafiltrate samples were analysed by HPLC. The free fraction (f_u) was calculated by dividing the free concentration in the ultrafiltrate by the total (bound and free) concentration in plasma.

Drug analysis

Drug concentrations were analysed based on a method by Satterwhite & Boudinot (1988). Briefly, plasma samples were spiked with 50 μl of internal standard (1.0 $g\,ml^{-1}$ ketoprofen in methanol). The pH was adjusted by addition of 0.2 ml 1 M phosphate solution at pH 2. After extraction with 5 ml diethyl ether, the residue was dissolved in 100 μl mobile phase, of which a volume of 50 μl was injected into the HPLC system. The HPLC system consisted of a Waters 501 Solvent pump, a Waters 717plus autosampler (both Millipore-Waters, Milford, MA, U.S.A.), Superflow 757 Kratus UV absorbance detector (Shimadzu, Kyoto, Japan). Chromatography was performed on a C_{18} 3 μm cartridge column (100 × 4.6 mm i.d., Chrompack, Bergen op Zoom, The Netherlands) equipped with a guard column. The mobile phase consisted of 0.02 M phosphate buffer (pH 7.0) and acetonitrile (82 : 18 v v^{-1}) with a flow rate of 1 $ml\,min^{-1}$. Detection was achieved by measuring the ultraviolet absorbance at a wavelength of 258 nm. Data acquisition and processing was performed using a Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan). The signal showed linearity over the range of 50–100,000 ng ml^{-1}. The within- and between-day coefficients of variation of the assay were 1.82 and 8.21%, respectively.

Analysis of TXB_2 and PGE_2

PGE_2 and TXB_2 were measured by a validated enzyme immunoassay (EIA) (Amersham Biosciences Europe GmbH, Freiburg, Germany). Briefly, samples were diluted in assay buffer (2–50 times for PGE_2, 200–2000 times for TXB_2) and a 50 μl sample was transferred into a coated well plate. After addition of 50 μl antibody and 50 μl peroxidase conjugate, samples were incubated for 1 h, washed four times and incubated for 15 min (TXB_2) or 30 min (PGE_2) when 150 μl substrate was added. The enzyme reaction was halted by addition of 100 μl 1 M sulphuric acid and optical density was measured in a plate reader at 450 nm.

Data analysis

The PK and PD of naproxen were assessed by nonlinear mixed effects modelling, as implemented in NONMEM version V, level 1.1 (Globomax, Ellicott City, U.S.A.). Final model parameters were estimated by the first order conditional estimation method with η–ε interaction (FOCE interaction). This approach allows the estimation of inter- and intraindividual variability in the 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, U.S.A.), NONMEM, was used for data processing, management (including bootstrap analysis) and graphical data display.

PK analysis

Naproxen disposition properties were characterised by compartmental models. One-, two- and three-compartment models with nonlinear or Michaelis-Menten elimination were tested for naproxen. Model selection and identification 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. Based on model selection criteria, a two-compartment model was identified to describe the PK of naproxen. The PK analysis was performed by use of the ADVAN6 routine in NONMEM. Owing to practical limitations, no plasma samples could be collected during the absorption phase after intraperitoneal injection. To overcome model parameter identifiability problems, two attempts were made to characterise naproxen absorption after i.p. dosing; namely, by modelling it as i.v. data (model A) or by fixing the absorption rate constant k_a to $10\,\text{min}^{-1}$ after exploring various rate constants between 0.5 and $15\,\text{min}^{-1}$ (model B). The PK parameters that were determined were clearance (CL), intercompartmental clearance (Q), and the volumes of distribution in the central (V_1) and peripheral compartments (V_2).

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

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

where θ is the population estimate for parameter P, P_i is the individual estimate and η_i is the normally distributed interindividual 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. On this basis, a combination of a proportional and an additive error model was proposed to describe residual error in the plasma drug concentration:

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

where $C_{obs,ij}$ is the jth observed concentration in the ith 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 PK 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 vs arterial blood sampling *via* cannulae and PK parameters.

To assess the precision and stability of the PK model and hence generate accurate predictions of the concentration–time course of naproxen, the final PK models were subjected to an internal validation (Ette *et al*., 2003). The validation consisted of a bootstrap procedure and posterior predictive check. For the bootstrap procedure, 1000 data sets were generated randomly sampled from the original data set with replacement. Subsequently, the final population PK models were fitted to the bootstrap replicates one at a time. Finally, the mean, standard error, CV% and 95% confidence intervals of all model parameters were calculated and compared to parameter values obtained from the original study. To assess the predictive performance of the population PK models, 1000 data sets were simulated from the final model parameter estimates. The mean and the 95% confidence interval were calculated from the simulated naproxen concentrations at the predefined time points.

PD analysis

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

$$Effect = I_0 - (I_0 - I_{max}) \times \frac{C^n}{(C^n + IC_{50}^n)} \tag{3}$$

where I_{max} represents the maximal inhibitory response to naproxen, I_0 the baseline production of PGE_2 or TXB_2 and n the Hill factor. This equation is equivalent to E_{max} and EC_{50}, but different symbols are used to indicate that this is referring to an inhibitory effect of the drug. The interpretation of *Effect* (and I_{max}) is a fractional change from baseline response I_0 in the absence of drug ($C=0$).

As no specific covariate was found for PK, population parameter estimates were used as input for estimating plasma concentration at the sampling times for PD.

Exploratory graphical analysis showed a correlation between clock time and I_{max}, which was described by the following equation:

$$I_{max} = \theta_i + \theta_j \times \text{clocktime} \tag{4}$$

where I_{max} is the maximal inhibitory response, and θ_i and θ_j are intercept and slope of the model parameter I_{max}, respectively.

The sigmoid I_{max} model (Equation 3) was used for data analysis of the *in vitro* data in rats and healthy volunteers. In rats, however, a correlation between I_0 and TXB_2 production without drug administration was observed and described by the following equation:

$$I_0 = \theta_i + \theta_j\,(\text{Blank}\,TXB_2 - \text{median}\,\text{Blank}\,TXB_2) \tag{5}$$

where I_0 is the baseline TXB_2 production, and θ_i and θ_j are intercept and slope of model parameter I_0, respectively. We have not found a correlation between baseline levels and TXB_2 production in human blood.

Results

Pharmacokinetics

A two-compartment model with combined proportional and additive errors best described the PK of naproxen. Based on the specified selection criteria, the model without an absorption phase was selected for characterising naproxen PK after i.p. administration (model A). A correlation was observed between ω^2CL, $\omega^2 V_1$ and $\omega^2 V_2$ and therefore the covariance of those parameters was added to the final model. The correlation coefficients were 0.65 for ω^2CL and $\omega^2 V_1$ ($P<0.001$), 0.72 for ω^2CL and $\omega^2 V_2$ ($P<0.001$) and 0.67 for $\omega^2 V_1$ and $\omega^2 V_2$ ($P<0.001$).

The observed and predicted concentration–time courses are depicted in Figure 1. The posterior predictive check showed model stability and consistency, as indicated by an accuracy prediction of >95% of the measured naproxen plasma concentrations over time. The final parameter estimates are summarised in Table 1. As indicated by CV%, the accuracy of model parameter estimates was within acceptable limits for the final model and bootstrap analysis.

Plasma protein binding showed fluctuation over the investigated naproxen concentration range, with larger unbound fractions at higher concentrations. The f_u increased from 1.86 ± 0.16 at 10^{-4} M to 11.39 ± 1.01% at 10^{-3} M naproxen in rats (mean$\pm$s.d.; $n=4$) and from 0.43 ± 0.33% at 10^{-4} M to 1.72 ± 0.44% at 10^{-3} M naproxen in healthy volunteers ($n=6$).

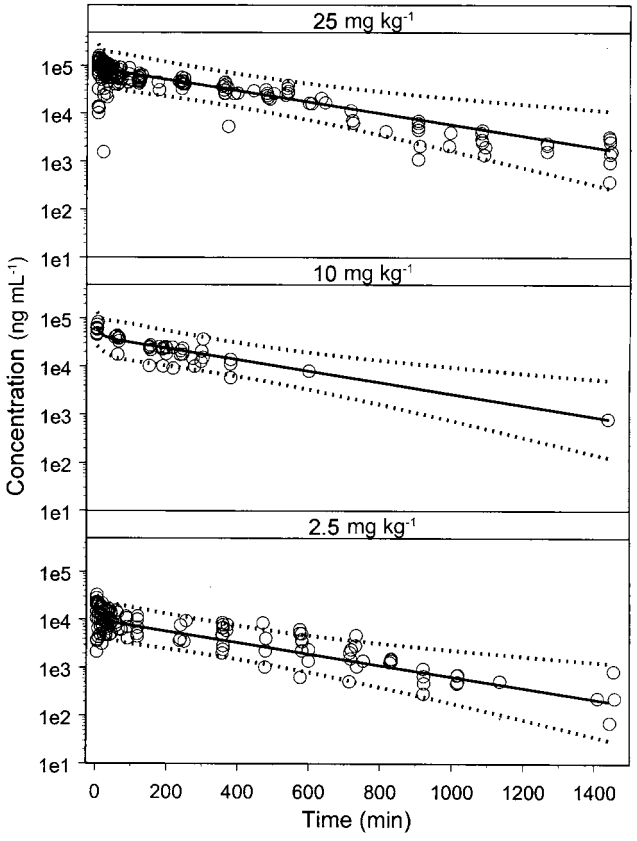

Figure 1 Population PK of naproxen after i.p. administration ($n=9$ per group). Open symbols represent individual data points. Solid black line indicates population prediction, dashed lines represent 95% confidence intervals.

Table 1 Population pharmacokinetic model and bootstrap analysis for naproxen

	Final model estimates Fixed effects	*Bootstrapping estimates Fixed effects*
CL (ml min^{-1})	0.211 (6)	0.210 (6)
V_1 (ml)	47.0 (14)	46.8 (13)
V_2 (ml)	28.7 (26)	28.6 (19)
Q (ml min^{-1})	1.70 (26)	1.71 (38)
Random effects	IIV[a]	IIV
ωCL (%)	41 (20)	42 (21)
ωV_1 (%)	51 (34)	53 (34)
ωV_2 (%)	69 (70)	75 (49)
Residual variability		
Exponentional error (%)	20 (20)	20 (23)
Additive error (ng ml^{-1}) or (μM)	146 (57) 0.63 (57)	169 (144) 0.73 (57)

Values in parentheses are relative standard errors (%) of the estimates.
[a]IIV = interindividual variability.

Naproxen PD in vivo

Before drug administration, LPS-induced PGE_2 production averaged 70 ± 27 ng ml^{-1} ($n=67$), whereas whole blood TXB_2 production averaged 314 ± 255 ng ml^{-1} ($n=34$). The inhibition of PGE_2 and TXB_2 production was very rapid, with maximal inhibition being achieved 2 min after dosing. Very large variability in the data was observed for both PGE_2 and TXB_2 production. The PK/PD relationship was best described by a sigmoid I_{max} model. The incorporation of clock time as a function of I_{max} significantly improved the fit. A clock time cycle of 24 h was defined with zero being set at 0800 hours. In contrast, there was no correlation between clock time and baseline levels of PGE_2 and TXB_2. No significant correlations between PD parameter estimates were observed. A summary of the model estimates is presented in Table 2. The IC_{50} values were 2951 and 1353 ng ml^{-1}, whereas IC_{80} values were 11,489 and 3496 ng ml^{-1} for PGE_2 and TXB_2 inhibition, respectively. The log IC_{50} ratio (COX-2/COX-1) of 0.34 indicates that naproxen *in vivo* is a nonselective COX inhibitor in rats. The concentration–effect relationships for PGE_2 and TXB_2 inhibition are depicted in Figure 2.

Naproxen PD in vitro

Under baseline conditions, LPS-induced PGE_2 production averaged 26 ± 4 ng ml^{-1} ($n=5$) in rats and 33 ± 19 ng ml^{-1} ($n=6$) in healthy volunteers. Whole blood TXB_2 production averaged 290 ± 236 ng ml^{-1} ($n=6$) in rats and 326 ± 64 ng ml^{-1} ($n=6$) in healthy volunteers. The *in vitro* PGE_2 and TXB_2 production in rats and humans was modelled by an inhibitory I_{max} model (Figure 3). A significant correlation ($r^2>0.99$) was observed between I_0 and blank TXB_2 production in rats ($P<0.001$). By implementing this relationship, MVOF was decreased by 72 units. All structural and stochastic model parameters are presented in Table 3. Reported IC_{80} values are calculated from the primary PD parameters. Even though IC_{50}

estimates for TXB_2 and PGE_2 inhibition were statistically different in rats and humans, PK/PD modelling reveals that IC_{80} estimates for PGE_2 inhibition are identical in rats ($1.32\ 10^{-4}$ M) and humans ($1.31\ 10^{-4}$ M). In Table 4, a comparison between *in vitro* and *in vivo* results for IC_{50} and IC_{80} values is presented. All values are presented in molar units (M) for comparison. *In vitro* and *in vivo* results are similar for PGE_2 inhibition in rats, whereas TXB_2 inhibition *in vitro* and *in vivo* in rats differ 10-fold in IC_{50} and IC_{80}.

Table 2 Population model estimates for the *in vivo* inhibitory effects of naproxen on PGE_2 and TXB_2 production in rats

Model parameter	*Population estimates*	*IIV*
PGE_2 inhibition	*Fixed effects*	*Random effects*
I_0 (ng ml^{-1})	65.9 (4)	(−)
Hill coefficient	1.02 (12)	45 (29)
IC_{50} (ng ml^{-1})	2951 (2)	107 (41)
IC_{80} (ng ml^{-1})	11489 (−)	(−)
I_{max} (ng ml^{-1})		
$\theta_{Intercept}$	2.68 (12)	(−)
θ_{Slope}	−0.247 (12)	(−)
TXB_2 inhibition		
I_0 (ng ml^{-1})	253 (14)	43 (51)
Hill coefficient	1.46 (15)	(−)
IC_{50} (ng ml^{-1})	1353(4)	66 (40)
IC_{80} (ng ml^{-1})	3496 (−)	(−)
I_{max} (ng ml^{-1})		
$\theta_{Intercept}$	12.2 (20)	(−)
θ_{Slope}	−0.98 (23)	(−)

Values in parentheses are relative standard errors (%) of the estimates.

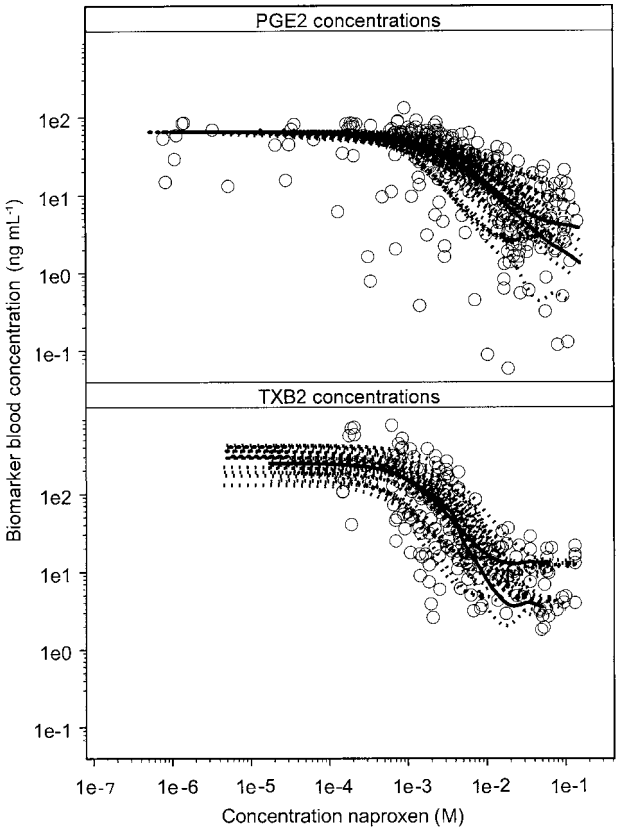

Figure 2 Naproxen effects *in vivo*. Upper panel: naproxen exposure vs PGE_2 concentrations ($n = 67$). Lower panel: naproxen exposure vs TXB_2 concentrations ($n = 34$). Open symbols represent individual data points. Solid black line indicates the population prediction, dashed lines represent individual predictions.

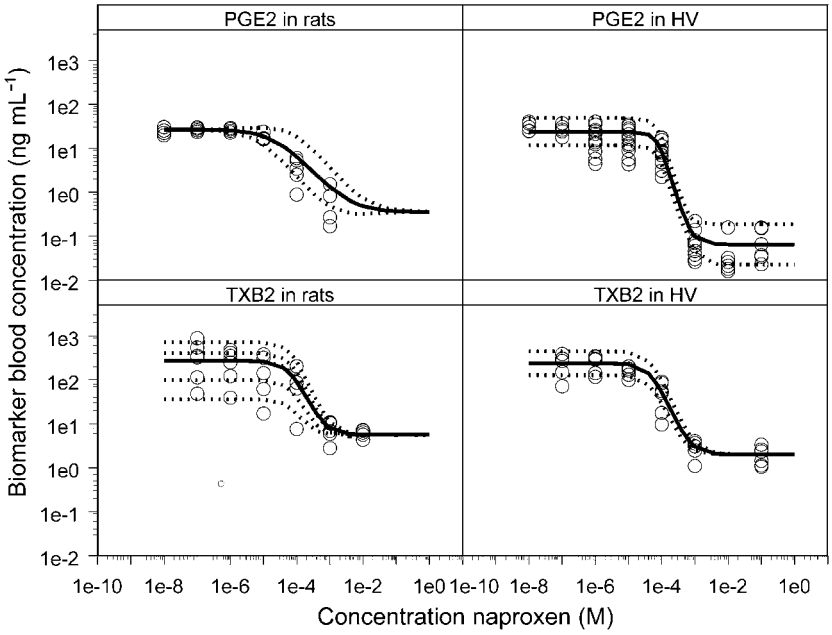

Figure 3 Naproxen effects *in vitro* ($n = 6$ per group). Open symbols indicate individual data points. Solid line shows population prediction and dashed lines show 95% confidence intervals obtained from the posterior predictive check. In the lower panel TXB_2 in rats, solid line indicates the population prediction of the median value of blank TXB_2 production (covariate of the concentration–effect relationship), dashed lines are the individual *post hoc* Bayesian predictions.

Table 3 Population model estimates for PGE_2 and TXB_2 inhibition by naproxen *in vitro* in rats and humans (HV)

	Model parameter estimates PGE_2 *Inhibition*	
Fixed effects	*Rats*	*HV*
I_0 (ng ml^{-1})	26.7 (5)	23.4 (13)
Hill coefficient	0.95 (2)	2.66 (15)
IC_{50} (μM)	30.7 (15)	79.5 (9)
I_{max} (ng ml^{-1})	0.35 (24)	0.07 (29)
Random effects		
ωI_0 (%)	(−)	38 (57)
ωIC_{50} (%)	94 (52)	(−)
ωI_{max} (%)	(−)	59 (83)
	TXB_2 inhibition	
Fixed effects	*Rats*	*HV*
I_0 (ng ml^{-1})		
$\theta_{Intercept}$	272 (5)	235 (13)
θ_{Slope}	1.04 (6)	(−)
Hill coefficient	1.88 (8)	1.81 (8)
IC_{50} (μM)	72.4 (26)	48.3 (14)
I_{max} (ng ml^{-1})	5.7 (10)	2.02 (16)
Random effects		
ωI_0 (%)	(−)	33 (68)
ωIC_{50} (%)	56	ND

ND = not determined.

Table 4 Comparison of population parameter estimates for the pharmacodynamic effects of naproxen *in vitro* and *in vivo*

	In vitro *in HV*	In vitro *in rats* PGE_2 *inhibition*	In vivo *in rats*
IC_{50} (μM)[a]	81.1	30.7	12.8
95% CI[b]	67.7–94.5	22.0–39.4	12.3–13.3
IC_{80} (μM)[c]	130.8	131.9	49.9
95% CI	109.2–152.4	94.4–169.4	47.9–51.9
		TXB_2 inhibition	
IC_{50} (μM)	48.3	72.4	5.9
95% CI	34.6–62.0	35.0–109.8	5.4–6.4
IC_{80} (μM)	103.9	151.4	15.2
95% CI	74.5–133.3	73.1–299.6	14.0–16.3

[a]All data are presented in μM for comparison. Molecular weight is 230.26 g mol^{-1}.
[b]CI = confidence interval.
[c]Secondary parameter.

Discussion and conclusions

Understanding how COX inhibitors affect markers of inflammation at an early stage of drug development may enable more accurate estimation of clinical doses and strengthen the rationale for dose selection. Furthermore, such markers may provide a better basis for translating anti-inflammatory activity into analgesic effect in experimental models as well as in patients. In the current study, we have assessed the PK–PD relationship of naproxen *in vitro* in rats and healthy volunteers and compared it to *in vivo* findings in rats, the commonly used species in experimental models of inflammatory pain. PGE_2 and serum TXB_2 concentrations were used as biomarkers for the anti-inflammatory response and side effects associated with the inhibition of COX.

Problems and limitations encountered in this research

Few technical limitations had to be overcome to address the underlying research question on the correlation between drug effect *in vivo* and *in vitro*, within and between species. Given that *in vivo* experiments cannot be performed in conjunction with simultaneous PK sampling, we have developed a population PK model to infer drug exposure at the sampling times in these experiments. Published literature describes the use of one-, two- and three-compartment models for the PK of naproxen, depending on sampling frequency and route of administration (Satterwhite & Boudinot, 1995; Josa *et al*., 2001). Our validation procedures demonstrated the precision and stability of the two-compartment model for the PK of naproxen. The population parameter estimates for CL (0.694 ml min^{-1} kg^{-1}) and V_{ss} (249 ml kg^{-1}) were in agreement with those published previously by Satterwhite & Boudinot (1991) and Josa *et al*. (2001).

Blood samples were obtained from the tail vein, which limited the number of samples that can be obtained from one animal. This sampling method was chosen to avoid the potential effects of arterial cannulation on PD, in particular the reduction in plasma albumin due to an acute phase reaction (Gabay & Kushner, 1999).

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 noncannulated animals or without quantitative evaluation of drug effect on markers of inflammation. Such an experimental setting is a major limitation to understand the relationship between dose, exposure and pharmacological activity. This also hampers any attempt to use preclinical findings to accurately identify efficacious and safe exposure in humans.

Protein binding seems to be a major determinant of the PK and PD of NSAIDs (Lin *et al*., 1987). In patients, naproxen f_u can vary up to eightfold in therapy, which can alter PD and consequently influence the occurrence and severity of side effects. Our findings for the f_u of naproxen in rats and humans are comparable to published data (Satterwhite & Boudinot, 1991; Borga & Borga, 1997) and suggest that protein binding in rats is lower than in humans. In patients, naproxen f_u can vary up to eightfold in therapy, which can alter PD and consequently influence the occurrence and severity side effects. To date, it remains unclear whether total or unbound plasma concentrations are better correlated with efficacy. This is partly due to the wide range of COX inhibitors with proven efficacy, which shows considerable differences in protein binding. In rats, changes in the f_u of naproxen should not have any relevant effect on PD because of the low level of binding. However, protein binding should be considered for scaling purposes across species.

The PK/PD relationships of the inflammatory markers showed large variability over time. Patrignani *et al*. (1997) have also shown that the inhibition of PGE_2 and TXB_2 by COX inhibitors display rather large interindividual variability. Such variability is often observed when measuring endogenous compounds and can be explained to some extent by the circadian variation in circulating enzyme levels. In fact, actual clock time was introduced as function of I_{max} to account for the differences observed in the PD profiles of experiments that started in the morning (0800 hours) and in the afternoon

(1800 hours). Our results showed that maximum inhibition increases during the day, indicating that the pool of COX enzyme may not be constant throughout the experiment. Nevertheless, the values for a change in I_{max} were relatively small and therefore cannot be considered physiologically relevant *in vivo*.

Gierse *et al*. (1999) have found that naproxen shows differential inhibitory effects on COX-1 and COX-2, acting, respectively, as a competitive and as mixed inhibitor. In fact, COX-2 inhibition was shown to be slow, reversible and weak. In addition, there seems to be no delay in binding to COX. However, we cannot exclude such phenomena based on the evidence from our experiments, as the measurement of PGE_2 levels is an indirect measure of COX inhibition. Baseline production levels of PGE_2 *in vitro* (26 ± 4 and $33 \pm 19\,ng\,ml^{-1}$, for rats and humans) and TXB_2 (290 ± 236 and $326 \pm 64\,ng\,ml^{-1}$, for rats and humans) are comparable with the literature data (Panara *et al*., 1998, 1999). Although I_{max} and I_0 vary for the different groups, naproxen inhibits TXB_2 and PGE_2 production levels by more than 97%. In addition, for TXB_2 inhibition we observed a correlation between I_0 and blank production of TXB_2 in rats.

In contrast to the model-based approach used in this study, most of the research on COX inhibitors in animal models of pain does not consider the parameterisation of results. This makes the comparison and extrapolation of data across species and between compounds rather difficult. In fact, there are barely any data available in the literature on the potency (IC_{50}) and intrinsic activity (I_{max}) of naproxen for the inhibition of PGE_2 and TXB_2 in rats. In a slightly different experimental setting based on human whole blood assay, mean estimates for IC_{50} and IC_{80} were 0.09, 1.10 and 0.28, 2.60 10^{-4} M, respectively, for PGE_2 and TXB_2 inhibition (Warner *et al*., 1999).

In vitro *correlations in rat and human blood*

When the *in vitro* IC_{50} values for PGE_2 inhibition in rats and humans are compared, these are significantly different, however, within a log unit range. This could be explained by the difference in the Hill coefficient in rats and humans, indicating distinct binding properties of the enzymes, even though COX-2 in rats and humans shows more than 80% homology (Mancini *et al*., 2001). On the other hand, *in vitro* TXB_2 inhibition in rats and humans is similar, with comparable IC_{50} and IC_{80} values, suggesting a possibility for the prediction of drug activity in humans from rat data. As COX-1 inhibition is associated with GI tract side effects, findings in toxicology experiments in rats could have predictive value for humans. In fact, clinical data suggest that recovery of gastric COX-1 activity is, like platelets, dependent on production of new cells rather than synthesis of new protein by extant cells (Feldman *et al*., 2000).

In addition, it is important to highlight that COX-1 activity in various systems (e.g., platelets and leucocytes) is different in rats and humans. In contrast to humans, COX-1 activity in rats also yields detectable amounts of PGE_2 (Giuliano & Warner, 2002). These differences in the homeostasis of COX-1 in the rat may contribute to the explanation of discrepancies in the slope of concentration–effect curve (Hill factor) and IC_{50} values for PGE_2 inhibition. Such differences seem to disappear when drug effect is assessed at a higher inhibition range, as parameterised by IC_{80}. *In vitro* data from different selective and nonselective COX inhibitors should be analysed to confirm similarities across compounds.

In vitro–in vivo *correlations in rats*

The doses of naproxen that were selected for the *in vivo* study included those used in experimental models of pain. The concentration-PGE_2 inhibition *in vivo* was found to be similar to the concentration–effect curves *in vitro* in rats, that is, IC_{80} estimates *in vitro* differed by only twofold from each other. On the contrary, for TXB_2 inhibition we observed a 10-fold difference in potency between *in vitro* and *in vivo* results. Naproxen is more potent *in vivo* based on either IC_{50} or IC_{80} values. It is difficult to elucidate the potential causes for such discrepancy. Earlier studies on the role of COX-1 on platelet aggregation have shown that both the rate and maximal extent of TXB inhibition largely depend on the rate of platelet turnover (Patrono *et al*., 1985). Hence, the observed discrepancy between *in vitro* and *ex vivo* could be explained by factors associated with the level of expression of COX-1, platelet turnover and the kinetics of prostanoids in plasma. These processes are altered *in vitro*. Yet, similar findings have been observed by Panara *et al*. in a first attempt to compare *in vitro* and *in vivo* PGE_2 and TXB_2 inhibition following administration of meloxicam to healthy volunteers. The authors estimated IC_{50} values for PGE_2 and TXB_2 inhibition *in vitro* and graphically presented *in vivo* data in conjunction with the *in vitro* predictions. The concentration–response curve for inhibition of PGE_2 appeared to be similar *in vitro* and *in vivo*, whereas inhibition of TXB_2 *in vivo* was a 10-fold less potent than *in vitro*. The observed differences seemed to have little clinical significance (Panara *et al*., 1999).

Recently, the relevance of IC_{80} estimates from the human whole blood assay *in vitro* to estimate the therapeutic analgesic dose in patients has been highlighted (Huntjens *et al*., 2005). From receptor pharmacology theory, it is known that antagonists and enzyme inhibitors usually require high level of occupancy or binding to yield meaningful pharmacological response and efficacy. Therefore, the use of IC_{80} values is preferred for comparison and extrapolation purposes. In that sense, IC_{80} not only reflects a parameterisation of the concentration–effect relationship but also provides information about the type of interaction between the drug and biological system, which is not captured by EC_{50} estimates.

This feature is particularly relevant for biological systems that have large receptor reserve capacity. In fact, clinically effective concentrations of naproxen, achieved after oral doses of 250 mg twice daily are associated with PGE_2 inhibition levels $\geqslant 80\%$ (Hassan-Alin *et al*., 2005). It is unclear, however, whether this is the level of inhibition at which analgesia occurs in animal models of inflammatory pain. Hence, translating preclinical findings *in vivo* without thorough understanding of the underlying mechanisms renders the dose selection of COX inhibitors in humans fraught with empiricism.

Conclusion

The relationship between naproxen concentrations and inhibition of PGE_2 and TXB_2 has been characterised *in vitro* and *in vivo* in rats and humans. Parameterisation of the concentration–response curve provided evidence that the PGE_2 inhibi-

tion in either species is comparable, whereas TXB_2 inhibition differs by 10-fold *in vivo*.

These differences should be carefully considered when evaluating the COX-1-related activity of compounds in early drug development. These biomarkers may therefore provide a scientific basis for selecting the clinical doses of COX inhibitors. In addition, our results also show the importance of an integrated PK/PD approach to overcome current limitations in experimental research of anti-inflammatory drugs.

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