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

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Chapter 4

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

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ABSTRACT

1. Understanding the mechanisms underlying the analgesic effect of new cyclo-oxygenase 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 pre-clinical 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, whilst 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, PK/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 ± SD for humans and rats, respectively), but slightly different for TXB_2 inhibition (103.9 ± 15 and 151.4 ± 40 10^{-6} M, mean ± SD 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.

INTRODUCTION

Naproxen is a non-selective COX inhibitor commonly used for the treatment of acute and chronic pain, rheumatoid arthritis and osteoarthritis. COX inhibitors act by inhibiting cyclo-oxygenase (COX) activity and consequently the formation of pro-inflammatory mediators like prostaglandins (PG) and thromboxanes (TXB) (1). Since the early 90s, it has been generally accepted that cyclo-oxygenase exists in two isoforms. Cyclo-oxygenase-1 (COX-1) is a housekeeping enzyme responsible for modulating physiological events and is present in most tissues including stomach, kidney and platelets, whereas cyclo-oxygenase-2 (COX-2) is highly induced in various cells by pro-inflammatory stimuli, mitogens and cytokines (2). Continuous COX-1 inhibition is thought to be principally responsible for gastrointestinal adverse effects following prolonged administration of non-selective COX inhibitors, whereas selective COX-2 inhibition accounts for the anti-inflammatory, anti-pyretic and analgesic efficacy (3). Recent investigations demonstrate that the roles of COX-1 and COX-2 are oversimplified (4-6). Data from those studies suggest that COX-2 is present under non-pathological conditions in tissues such as kidney, brain and the spinal cord, playing an important role in the maintenance of physiological homeostasis (7).

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 (8). 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, allowing for sustained analgesic response and appropriate safety margin. To date, the dose selection of COX inhibitors has been based primarily on clinical endpoints for analgesia, an approach which disregards the impact of maximum, long-lasting blockade of either enzyme systems (9).

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 non-linear 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 pre-clinical to clinical data (9).

In this study, we have assessed the pharmacokinetic-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 E2 (PGE_2) and serum thromboxane B2 (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.

MATERIALS AND 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 non-cannulated animals (study 2). Study 1 was performed to characterise the pharmacokinetics of naproxen using serial blood sampling and enable subsequent analysis of the sparse pharmacokinetic 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 B.V., Maastricht, The Netherlands) weighing 308 ± 7 g (Mean ± SEM, n=83), upon approval of the study protocols by the Ethical Committee on Animal Experimentation of the Leiden University. 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 07.00 a.m.) and a temperature of 21^0C. 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 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) polyvinylpyrrolidone (PVP) (Brocacef, Maarssen, The Netherlands) in saline. Rats receiving an intraperitoneal 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. Prior to the experiment, the PVP solution was removed and the cannulae were flushed with saline containing 20 I.U. 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 (molecular weight = 230.26 g/mol) was purchased from Sigma Alldrich BV (Zwijndrecht, The Netherlands). Naproxen was administered as an 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 intraperitoneal administration.

Experimental design

Study 1

All experiments were started between 08.30 and 09.30 a.m. to exclude the influence of the circadian rhythms. Naproxen (25 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 (2.5 and 25 mg kg^{-1}) to conscious and freely moving rats. Serial arterial blood samples (100 µl) were taken at pre-defined 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 rpm for 10 min for plasma collection and were stored at -20^{0}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 hours, experiments were started in the morning (08.00 a.m.) or in the evening (06.00 p.m.). Animals were administered naproxen intraperitoneally (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 7 blood samples per animal. Blood samples of 250 µl were taken at pre-defined time points up to 48 hours 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 prior to 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 rpm for 10 min. Plasma was stored at -20^{0}C until analysis. Blood samples for TXB_2 analysis were placed into tubes and allowed to clot for 1 hour at 37^{0}C in a stirring water bath. Serum was collected after centrifugation and stored at -20^{0}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^{0}C in a water bath. Plasma was separated by centrifugation and stored at -20^{0}C until analysis.

In vitro experiments. For the *in vitro* experiments in rats, blood from six male SD 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. Prior to the experiment, tubes were prepared by evaporation of methanol containing fixed amounts of naproxen (0, 10^{-8} - 10^{-1} M). 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^{0}C in a stirring water bath. Serum was collected after centrifugation and stored at -20^{0}C until analysis. Blood samples for the PGE_2 analysis were placed in tubes and 10 µg ml^{-1} lipopolysaccharide (LPS) was added. Samples were incubated for 24 hours at 37^{0}C in a stirring water bath. Plasma was separated by centrifugation and stored at -20^{0}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 non-steroidal 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 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.

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 37^0C, 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). The plasma was filtered at 2000 *g* at 37^0C 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 and Boudinot (10). 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 1M 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, USA), Superflow 757 Kratus UV absorbance detector (Shimadzu, Kyoto, Japan). Chromatography was performed on a C_{18} 3 µm cartridge column (100 x 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) 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 50-100000 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 hour, 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 1M sulphuric acid and optical density was measured in a plate reader at 450 nm.

Data analysis. The pharmacokinetics and pharmacodynamics of naproxen 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 the estimation of inter- and intra-individual 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, USA) - NONMEM was used for data processing, management (including bootstrap analysis) and graphical data display.

Pharmacokinetic analysis. Naproxen disposition properties were characterised by compartmental models. One-, two -and three compartment models with non-linear 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 pharmacokinetics of naproxen. The pharmacokinetic analysis was performed by use of the ADVAN6 routine in NONMEM. Due to practical limitations, no plasma samples could be collected during the absorption phase after intra-peritoneal injection. To overcome model parameter identifiability problems, two attempts were made to characterise naproxen absorption after i.p. dosing; namely, by modelling it as intravenous data (model A) or by fixing the absorption rate constant k_a to 10 min^{-1} after exploring various rate constants between 0.5 and 15 min^{-1} (model B). The pharmacokinetic parameters that were determined were clearance (Cl), inter-compartmental clearance (Q), and the volumes of distribution in the central (V_1) and peripheral compartments (V_2).

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:

$$P_i = \theta_i \cdot \exp(\eta_i) \quad (1)$$

where θ is the population estimate for parameter P, P_i is the individual estimate and η_i is the normally distributed inter-individual 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 inter-individual 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:

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

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.

To assess the precision and stability of the pharmacokinetic model and hence generate accurate predictions of the concentration-time course of naproxen, the final pharmacokinetic models were subjected to an internal validation (11). 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, coefficient of variation 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 pre-defined time-points.

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

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

where I_{max} represents the maximal inhibitory response to naproxen, I0 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 pharmacokinetics, population parameter estimates were used as input for estimating plasma concentration at the sampling times for pharmacodynamics.
Exploratory graphical analysis showed a correlation between clock time and I_{max}, which was described by the following equation:

$$I_{\max} = \theta_i + \theta_j \cdot clock\ time \quad (4)$$

where I_{max} is the maximal inhibitory response, 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:

$$I_0 = \theta_i + \theta_j * (Blank\_TXB_2 - median\_TXB_2) \quad (5)$$

where I_0 is the baseline TXB_2 production, 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 pharmacokinetics of naproxen. Based on the specified selection criteria, the model without an absorption phase was selected for characterising naproxen pharmacokinetics after intraperitoneal administration (model A). A correlation was observed between ω^2Cl, ω^2V_1 and ω^2V_2 and therefore the covariance of those parameters was added to the final model. The correlation coefficients were 0.65 for ω^2Cl and ω^2V_1 ($p < 0.001$), 0.72 for ω^2Cl and ω^2V_2 ($p<0.001$) and 0.67 for ω^2V_1 and ω^2V_2 ($p<0.001$).
The observed and predicted concentration-time courses are depicted in Figure 1. The visual 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 free fraction increased from 1.86 ± 0.16 % at 10^{-4} M to 11.39 ± 1.01 % at 10^{-3} M naproxen in rats (Mean ± SD; 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).
Naproxen pharmacodynamics in vivo. Prior to drug administration, LPS-induced PGE_2 production

averaged 70 ± 27 ng ml^{-1} (n=67), whilst 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 two minutes 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.

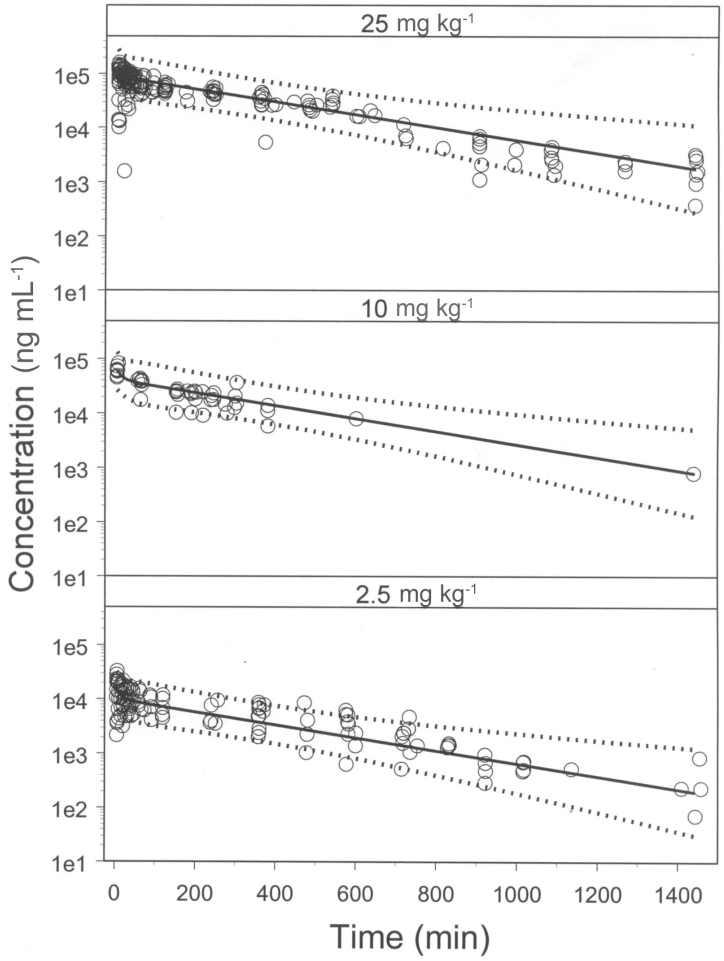

Figure 1. Population pharmacokinetics of naproxen after i.p. administration (n=9 per group). Open symbols represent individual data points. Solid black line indicates population prediction, dashed lines represent 95% confidence intervals.

The incorporation of clock time as a function of Imax significantly improved the fit. A clock time cycle of 24 hours was defined with zero being set at 08.00 a.m. 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}, whilst IC_{80} values were 11489 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 non-selective COX inhibitor in rats. The concentration-effect relationships for PGE_2 and TXB_2 inhibition are depicted in Figure 2.

Naproxen pharmacodynamics 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.

Table 1. Population pharmacokinetic model and bootstrap analysis for naproxen. Values in parentheses are relative standard errors (%) of the estimates.

	Final Model Estimates	Bootstrapping Estimates
	Fixed effects	*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*	IIV
ω Cl (%)	41 (20)	42 (21)
ω V_1 (%)	51 (34)	53 (34)
ω V_2 (%)	69 (70)	75 (49)
Residual variability		
Exponentional error (%)	20 920)	20 (23)
Additive error (ng mL^{-1})	146 (57)	169 (144)
or (10^{-6} M)	0.63 (57)	0.73 (57)

**IIV: inter-individual variability*

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

Model Parameter		Population estimates	IIV
		Fixed effects	*Random effects*
PGE_2 Inhibition	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)	
		Fixed effects	*Random effects*
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)	

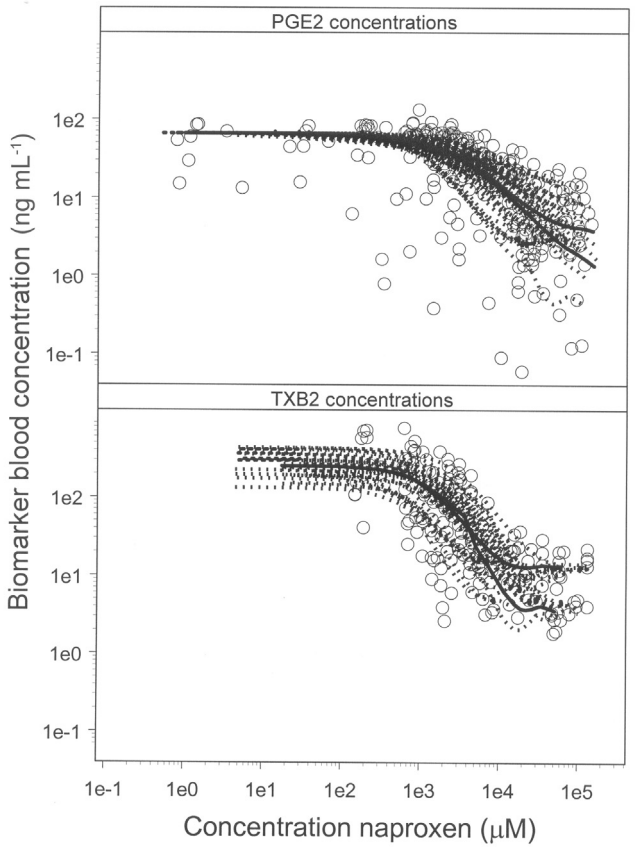

Figure 2. Naproxen effects *in vivo*. Upper panel: naproxen exposure versus PGE_2 concentrations (n=67). Lower panel: naproxen exposure versus TXB_2 concentrations (n=34). Open symbols represent individual data points. Solid black line indicates the population prediction, dashed lines represent individual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

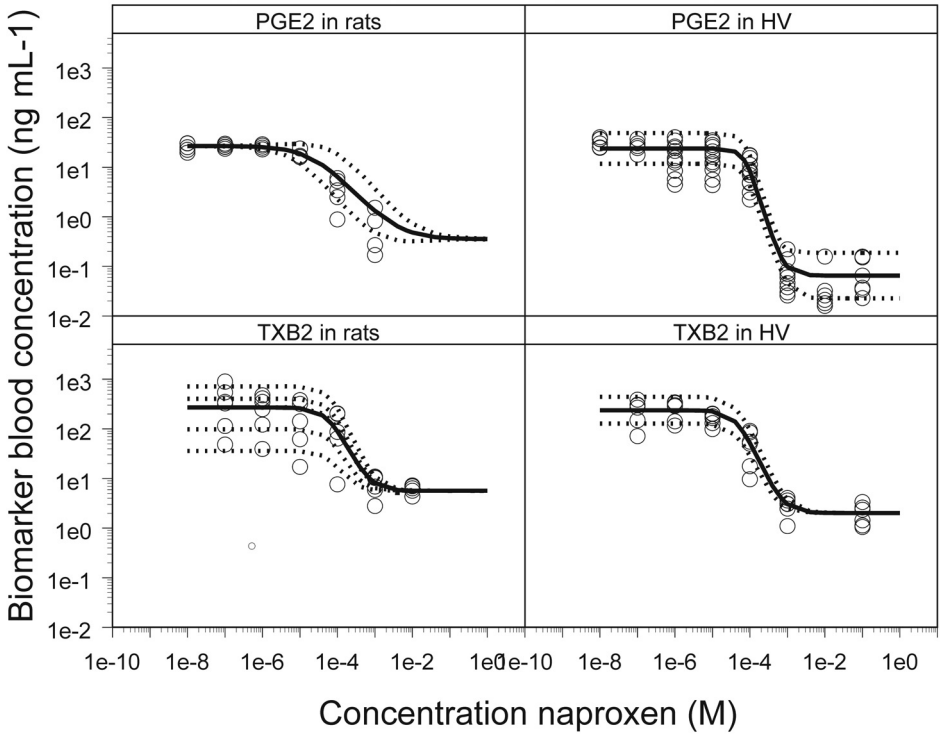

Figure 3. Naproxen effects *in vitro* (n=6 per group). Open symbols indicate individual data points. Solid line shows population prediction and dashed lines show 95% confidence intervals obtained from the visual predictive check. In the lower panel TXB_2 in rats, solid line indicates the population prediction 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 PGE2 and TXB2 inhibition by naproxen in vitro in rats and humans (HV).

Parameter	Parameter estimate	Parameter estimate
	PGE_2 blood concentration	
Fixed effects	Rats	HV
I_0 (ng mL^{-1})	26.7 (5)	23.4 (13)
Hill coefficient	0.95 (2)	2.66 915)
IC_{50} (10^{-4} M)	0.307 (15)	0.795 (9)
I_{max} (ng mL^{-1})	0.35 (24)	0.07 (29)
Random effects		
ω I_0 (%)		38 (57)
ω IC_{50} (%)	94 (52)	
ω I_{max} (%)		59 (83)
	TXB_2 blood concentration	
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} (10^{-4} M)	0.724 (26)	0.483 (14)
I_{max} (ng mL^{-1})	5.7 (10)	2.02 (16)
Random effects		
ω I_0 (%)		33 (68)
ω IC_{50} (%)	56	

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**	***In vivo* in rats**
	PGE_2 inhibition		
IC_{50} (10^{-6} M)*	81.1	30.7	12.8
95 % CI**	67.7-94.5	22.0-39.4	12.3-13.3
IC_{80} (10^{-4} M)***	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} (10^{-6} M)	48.3	72.4	5.9
95 % CI	34.6-62.0	35.0-109.8	5.4-6.4
IC_{80} (10^{-6} M)	103.9	151.4	15.2
95 % CI	74.5-133.3	73.1-299.6	14.0-16.3

*All data is presented in μM for comparison. Molecular weight is 230.26 g/mol.
**CI, Confidence interval
***Secundary parameter

DISCUSSION AND CONCLUSION

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 pharmacokinetic-pharmacodynamic 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. Prostaglandin E2 (PGE_2) and serum thromboxane B2 (TXB_2) concentrations were used as biomarkers for the anti-inflammatory response

and side effects associated with the inhibition of cyclo-oxygenase.

Problems and limitations encountered in this research. A 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 pharmacokinetic sampling, we have developed a population pharmacokinetic 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 pharmacokinetics of naproxen, depending on sampling frequency and route of administration (12;13). Our validation procedures demonstrated the precision and stability of the two-compartment model for the pharmacokinetics 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 and Josa *et al.*. (12;14).

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 pharmacodynamics, in particular the reduction in plasma albumin due to an acute phase reaction (15).

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 setting 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.

Protein binding seems to be a major determinant of the PK and PD of NSAIDs (16). In patients, naproxen free fraction can vary up to eight-fold in therapy, which can alter pharmacodynamics and consequently influence the occurrence and severity of side effects. Our findings for the free fraction of naproxen in rats and humans are comparable to published data (14;17) and suggest that protein binding in rats is lower than in humans. In patients, naproxen free fraction can vary up to eight-fold in therapy, which can alter pharmacodynamics 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 free fraction of naproxen should not have any relevant effect on pharmacodynamics 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.* have also shown that the inhibition of PGE_2 and TXB_2 by COX inhibitors display rather large inter-individual variability (18). 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 Imax to account for the differences observed in the PD profiles of experiments that started in the morning (08.00 a.m.) and in the

afternoon (06.00 p.m.). 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 Imax were relatively small and therefore cannot be considered physiologically relevant *in vivo*.

Gierse *et al.* have found that naproxen shows differential inhibitory effect 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 (19). 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 ± 19 ng ml^{-1}, for rats and humans) and TXB_2 (290 ± 236 and 326 ± 64 ng ml^{-1}, for rats and humans) are comparable with literature data (20;21). 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 is 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 (22).

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, they 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 show more than 80% homology (23). 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. Since 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 (24).

In addition, it is important to highlight that COX-1 activity in various systems (e.g., platelets and leukocytes) is different in rats and humans. In contrast to humans, COX-1 activity in rats also yields detectable amounts of PGE_2 (25). 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 non-selective 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, i.e., IC_{80} estimates *in vitro* differed by only 2-fold 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 thromboxane inhibition largely depend upon the rate of platelet turnover (26). 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 (21).

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 (9). 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} reflects not only 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 >=80% (27). It is unclear, however, whether this is the level of inhibition at which analgesia occurs in animal models of inflammatory pain. Hence, translating pre-clinical 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 inhibition in either species is comparable, while 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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