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

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Chapter 9

Unravelling the relationship between exposure, biomarker and analgesic response to COX inhibitors in chronic inflammatory pain

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ABSTRACT

Background. Despite considerable understanding about the mechanism of action of COX inhibitors, the doses taken into clinical trials are often based on estimates of the analgesic response observed in pre-clinical models of pain. Since blockade of the cyclo-oxygenase pathway underlies the pharmacological effect, it would be beneficial to integrate biomarkers of pharmacological activity in the evaluation of the effective dose range for chronic inflammatory conditions, rather than relying on behavioural measures of hyperalgesia. Establishing the correlation between biomarkers and analgesic response may contribute to the identification of an optimal dosing regimen, without the confounders of response, such as placebo effect, context and large inter-subject variability. In addition, it may also provide further evidence of the construct validity for animal models of pain.

Objectives. In the current investigation, we challenge the current development paradigm using a mechanism-based approach to unravel the relationship between exposure, biomarker and analgesic response.

Methods. Data on hyperalgesia was derived from a chronic experiment with Freund's complement adjuvant (FCA) in rats. Animals received oral b.i.d. doses of four different COX inhibitors. Drug exposure, biomarkers (PGE_2 and TXB_2) and hyperalgesia were measured before and after dosing. First, hyperalgesia was analysed by logistic regression. Secondly, a mechanistic model was built to describe the relationship between drug exposure, biomarker inhibition and hyperalgesia.

Results. Full analgesia was achieved after administration of all four COX inhibitors. However, the maximal duration of effect was different. Logistic regression showed that the PGE_2 levels versus the probability of analgesia were time-dependent. A PKPD model based on two indirect responses was developed that explained the time-dependencies by counter mechanisms that are activated due to the inhibition of COX.

Conclusions. The analgesic effect induced by PGE_2 inhibition is highly time dependent, but not altered by chronic treatment. Measures of the analgesia in animals do not reflect the underlying anti-inflammatory activity of COX inhibitors and may therefore lead to inaccurate estimate of the dose required to achieve analgesia in humans. PKPD modelling of the biomarkers enables further understanding of pain signalling and of the mode of action of drugs interacting with targets on this pathway.

INTRODUCTION

The anti-inflammatory, analgesic and antipyretic effects of selective COX-2 inhibitors have been intensively studied and numerous articles have been published on the pre-clinical pharmacology of NSAIDs. A variety of rodent models is used to demonstrate efficacy of the compound and results are displayed as ED_{50} values (1;2). In fact, under the current drug development paradigm, these experiments provide the basis for the selection of doses for subsequent investigation in humans. Yet, reported ED_{50} values vary greatly among the different models (3). Furthermore, no attention is paid to the extent of selectivity *in vivo*, in that drug activity on primary and secondary targets (COX-1 and COX-2) is not carefully considered in conjunction with the behavioural measures of analgesia.

This situation is in contradiction with the arguments for rational drug therapy, i.e., the assumption that there is a causal relationship between dose, dosing regimen, and/or exposure to a medication on one hand and its therapeutic response as well as adverse effects on the other. 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. Despite a clear analgesic effect upon administration of COX inhibitors, no direct correlation is observed between the drug concentrations in systemic fluids (blood, plasma) and the extent of the pharmacological activity. Lack of such a direct correlation does not mean one cannot identify the optimal dose required to achieve a predefined treatment response. Biomarkers that lay on the causal pathway between drug exposure and clinical response could be an intermediate step in understanding the relationship between exposure to COX inhibitors and their analgesic effect (4).

Prostaglandins are well known as pro-inflammatory mediators on the cyclo-oxygenase pathway. Levels of prostaglandin E2 (PGE_2), the key prostaglandin mediating the cardinal signs of inflammation, are increased in various states of inflammation. Whilst COX-2 inhibition is directly linked to PGE_2 levels, COX-1 inhibition affects thromboxane formation in humans. Thromboxane 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 effects (5). Based on receptor pharmacology theory and assuming no additional mechanism of action, it is possible to narrow down the effective dose range of a COX inhibitor to a minimum threshold of inhibition. In effect, we have shown a correlation between exposure at various therapeutic indications and IC_{80} for prostaglandins (6).

In this article, we have investigated the relationship between drug exposure and the effect on two biomarkers of the inflammatory response (PGE_2 and TXB_2) as well as on hyperalgesia in the rat adjuvant arthritis model using four COX inhibitors with varying degrees of selectivity. The selected COX inhibitors were diclofenac, naproxen, ketorolac and rofecoxib. Using pharmacokinetic-pharmacodynamic modelling we have evaluated (1) the relationship between drug exposure and inhibition of PGE_2 and TXB_2, (2) the relationship between PGE_2 inhibition and analgesia, with analgesia measured as a binary scale and (3) the relationship between PGE_2 inhibition and analgesia

based on a mechanism-based model.

The present work also highlights the prerequisites for accurate selection of the analgesic dose in humans. It is anticipated that a new paradigm is required that accounts for biomarkers of pharmacology, rather than relying solely on behavioural measures of pain and pain relief. In addition, our approach reveals how pharmacokinetic-pharmacodynamic (PKPD) modelling can be used to dissect the underlying components of inflammatory pain, thereby discriminating PGE_2-induced pain from other inflammatory mechanisms.

MATERIAL AND METHODS

Experimental design. All procedures were reviewed and approved by the UK Home Office and were carried out in accordance with the requirements of the project license. The details of the study design have been described previously (7 in press).

In brief, male Sprague Dawley rats (n=81, 10-11 per group) were injected with Freund's complete adjuvant (FCA) into the left hind paw. The non-injected contra-lateral paw of each animal served as control. After acclimatisation for three days prior to the start of the experiments, animals were subjected to the behavioural test as a training period. Paw pressure was used as a measure of pain intensity. Prior to FCA injection, a baseline measurement was obtained and a blood sample was collected via the tail vein to determine biomarker levels. Behavioural tests were performed, one hour post inoculation, and on day 4 or 5, day 7, day 9, 10 or 11, day 13, 14 or 15, day 17, 18 or 19, day 21, day 26, 27 or 28 post inoculation (PID).

Oral drug administration was started in the morning at day 7 PID. Naproxen was administered at a dose of 1 and 15 mg/kg, rofecoxib at a dose of 0.5 and 10 mg kg, ketorolac at a dose of 4 mg/kg and diclofenac at a dose of 10 mg/kg. An additional group of animals was tested pre-emptively. To evaluate the potential disease-modifying effects of rofecoxib, drug administration started five days prior to the FCA injection, according to a 10 mg/kg b.i.d. 12/12 h schedule up to day 5 PID.

Evaluation of the analgesic effect was performed upon acute and chronic dosing. Acute drug effects were assessed on day 7 before, 0.5-0.75 h, 1-1.5 h, 2-3 h, 4, 8 and 24 h after drug administration. Chronic dosing started at day 8 PID in the morning according to b.i.d. 12/12 hour dosing schedule up to day 19 PID. After a washout period of 48 hours, a single oral dose was administered on day 21 PID. Behavioural testing was performed at day 21 before, 0.5-0.75 h, 1-1.5 h, 2-3 h, 4, 8 and 24 h after drug administration.

Data analysis. Drug effects were assessed by nonlinear mixed effects modelling, as implemented in NONMEM version VI, (Globomax, Ellicott City, USA). Final model parameters were estimated by the first order conditional estimation method with η-ε interaction (FOCE interaction) and with the Laplacian estimation option with the "Likelihood" option for categorical data. 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. Model discrimination was based on goodness of fit plots, simulations and changes in the objective function value (OFV). For nested models, the more complex model was selected if the OFV decreased by more than 6.6 (one parameter difference). This decrease corresponds to a nominal statistical significance level of 0.01.

Pharmacokinetics. Separate satellite experiments were conducted to enable characterisation of the full time course profile of COX inhibitors in plasma. Data from the satellite animals were simultaneously analysed by nonlinear mixed effects modelling in NONMEM. For naproxen, rofecoxib and diclofenac, exposure profiles were derived using dense sampling schedule after oral, i.v. and i.p. administration in healthy and/or diseased animals (7;8). For ketorolac, exposure profiles were obtained from joint analysis of a satellite group with a dense sampling schedule and sparse sampling from the current experiments. A one compartment model with first order absorption was used to describe the data, as implemented in ADVAN2, TRANS1 subroutine in NONMEM. Population parameter estimates were clearance (Cl/F, 45 ml/h), central volume of distribution (V/F, 79.2 ml) and absorption rate constant (K_a, 600 1/h). K_a was fixed due to very rapid absorption phase.

Biomarkers. Inhibition of PGE_2 and TXB_2 was calculated according to previous PKPD modelling by Huntjens *et al.* (9 in prep). An overview of the parameter estimates is shown in table 1. Sparse samples obtained in this study were used to support previous parameter estimates (results not shown). As variability in pharmacodynamics is much larger than the variability in pharmacokinetics, predicted population values were used for the analysis of the analgesic response. Biomarker levels were estimated at the sampling times of the pain measurements.

Modelling of pain response. Paw pressure was used as pharmacodynamic endpoint for the analgesic effect. The difference between the inflamed and non-inflamed paw at each specific time point was used an input for the pharmacodynamic model (equation 1).

$$\%PI_{t(i)} = \left(1 - \frac{PP\_\inf lamed_{t(i)}}{PP\_non\inf lamed_{t(i)}}\right) * 100\% \qquad (1)$$

where %PI is the observed pain intensity at time i, expressed as percentage; $PP_{inflamed}$ is the measured paw pressure of the inflamed paw at time i, $PP_{non\text{-}inflamed}$ is the measured paw pressure of the contralateral paw at time i.

Logistic regression. Two different scenarios were investigated for the analgesic effect on a binary scale: the scenarios differ in their threshold for pain relief (1) and no pain relief (0). The following transformation was applied to convert the continuous data into binary response:

Scenario 1:

-If %PI 20%, then observation score is 1 (complete pain relief) else 0 (no pain relief)

Scenario 2:

- If %PI 35%, then observation score is 1 (complete pain relief) else 0 (no pain relief)

The choice for these thresholds was based on previous publications, which show the magnitude in pain intensity required to achieve meaningful pain relief. The difference between scenarios is small, since less than 6% of the data was added to the pain free status (1) in scenario 2, as compared to scenario 1. Given that the relationship between pain and biomarker should be independent of treatment type, data from all compounds were analysed simultaneously. Intriguingly, rofecoxib was shown to produce maximum inhibition of PGE_2 of only 30%, whereas 100% suppression of PGE_2 was observed for all other COX inhibitors. The explanation for these findings have been previous discussed by Huntjens *et al.*., which are most likely caused by the role of COX-1 in PGE_2 formation in rats (7).

A logistic regression was used for the analysis of the binary response variables according to the approach described by Staab *et al.* and Yano *et al.* (10;11). In brief, the logistic regression is represented as:

$$P_{Y_{ij}} = \frac{e^L}{e^L + 1} \qquad (2)$$

where P_{Yij} corresponds to the probability of getting an observation (Y) equal to a score m in the i^{th} individual at the j^{th} time point. Each individual response was modelled as a conditional probability $P(Yij=m|\eta_I)$, where I is the individual random effect. η_I is assumed to be 0 with variance ω^2. The logit (L) within eq. 2 is given by the following equation:

$$L = \theta_1 + \theta_2 * PGE + \eta_i \qquad (3)$$

where θ_1 and θ_2 corresponds to the fixed effects parameters for slope and intercept and PGE is the PGE_2 level. The intercept is given by $\log(EC_{50})$ x slope. Model development proceeded sequentially from a model with only an intercept to a model with in the inclusion of the PGE_2 response and subsequently any other significant covariate. Covariate effects such as TXB_2 concentrations were tested by inclusion one at the time.
The predictive performance of the model was checked by comparing the predicted population mean probability with the mean probability derived from the raw data, which was computed by dividing the number of observed scores equal to m by the total number of scores of pre-defined range of PGE_2 values (0<=15 ng/ml, 15<=75 ng/ml and >75 ng/ml). To obtain the predicted population mean probabilities, 100 data sets were simulated with the final model in such a way that the numbers of animals, dosing events, observations, and covariates were the same as in the original experiment. The predicted probability was obtained by dividing the number of scores equal to m by the total number of predicted scores.

The final model was subjected to bootstrapping as an internal validation procedure. Five hundred data sets were generated randomly sampled with replacement. Subsequently, the final pharmacodynamic model was fitted to the bootstrap replicates one at the time. The mean, standard

error, coefficient of variation and 95% confidence intervals of all parameters were then calculated and compared to the parameter values obtained from the original study.

Mechanism-based pharmacodynamic model. The initial dichotomisation of the data made evident the disconnection between pharmacological activity and analgesic response, supporting our hypothesis that a pathway analysis is required to fully characterise analgesic response. Therefore, a mechanism-based PKPD model was subsequently developed which included the effect of feed-back mechanisms down stream in the inflammatory cascade. The interaction between the algesic effect of PGE_2 and other inflammatory mediators is shown in figure 3. It is hypothesized that long-term inhibition of COX leads to the activation of counter regulatory mechanisms. Such counter regulatory mechanisms include for instance the formation of leukotrienes (LTs).

The pharmacodynamic model describes pain response (assessed by a behavioural endpoint such as anti-hyperalgesia) as a process with a given turnover rate, which characterises the appearance, maintenance and waning of pain under experimental conditions. Whilst PGE_2 has a concentration-dependent effect on pain response, its inhibition indirectly initiates the production of other inflammatory mediators, such as LTB_4, which also induce pain. Mathematically, the mechanism-based model proposed here consists of two indirect response models, which capture the interaction between the mechanisms underlying pain signalling and pain response (Figure 1).
Pain response was described as follows:

$$d(Pain)/dt = k_{in} * (1 - f(PGE2_1)) - k_{out} * Pain * (1 - f(mediators)) \quad (4)$$

where Pain is the analgesic effect, k_{out} is the first order rate constant for the degradation of the pain response, k_{in} is the zero-order rate constant for the production of the pain response due to FCA-induced inflammation, $f(PGE_{2\,1})$ is a function for the effect of PGE_2 on pain response and f(mediators) is a function for the effect of the endogenous mediators, which counteract pain response.

The emergence of counter-regulatory mechanisms over time was described as follows:

$$d(mediators)/dt = k_{production} * (f(PGE2_2)) - k_{degration} * mediators \quad (5)$$

where mediators are downstream mediators in the inflammatory cascade which appear according to zero and first-order rate constants kproduction and kdegredation, indicating the rate of synthesis and degradation, respectively. $f(PGE_{2\,2})$ is a function for the effect of PGE_2 on the synthesis of these mediators.

According to the proposed model, PGE_2 levels can modulate pain response by two distinct mechanisms: 1) by a direct effect on the formation of pain (i.e., the algesic effect); 2) by its modulatory action on the formation of other inflammatory mediators. This interaction is described

according to the following equations:

$$f(PGE2_1) = slope_1 * S(PGE_2) \quad (6)$$

$$f(PGE2_2) = slope_2 * S(PGE_2) \quad (7)$$

where $S(PGE_2)$ is a function for the stimulus resulting from the inhibition of PGE_2 and $slope_1$ and $slope_2$ are the individual rates for the production of response.

Given the nature of the data, a continuous function could not be used to describe the PGE_2-induced effect on pain response and on inflammatory mediators. Instead, it was dichotomised as follows:

$$S(PGE_2) = inhibitionPGE_2 \quad (8)$$

$$S(PGE_2) = \alpha * (\frac{inhibitionPGE_2}{80})^{\gamma} \quad (9)$$

where equation 8 was used when PGE_2 levels were below 80% inhibition and equation 9 was used for levels above 80% inhibition. This threshold is based on the evidence from previous findings supporting the strong correlation between IC_{80} and analgesic effect in humans (12). a and γ are scaling factors for the relationship.

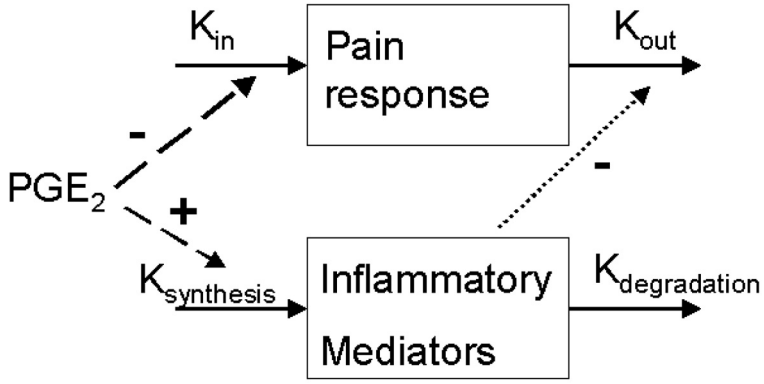

Figure 1: Schematic representation of the mechanistic model for the contribution of PGE2 to hyperalgesia (pain response). Interactions are symbolised by dashed lines, where '+/-' indicates a positive or negative effect, respectively. An explanation of the model parameters is given in the text.

RESULTS

Exploratory analysis. Initially, drug exposure versus analgesic effect profiles was plotted for a closer evaluation of the correlation between systemic drug concentrations and response (figure 2). Intriguingly, maximum analgesic effect was observed for low and high dose levels of naproxen and rofecoxib. Since the differences in exposure were proportional to the ratio between the dose levels, a single concentration-effect relationship could not be warranted for these compounds. The discrepancies between analgesic effect, pharmacokinetics in plasma and PGE_2 levels are evident from the time course of drug concentrations and PGE_2 inhibition shown in Figure 3.

Logistic regression analysis. A first attempt to model pain relief was based on a stochastic approach, which correlates the probability of pain response to PGE_2 levels. Assessment of such a correlation represents an important step in the use of a biomarker of drug effect. Pain intensity data was therefore dichotomised in 0 and 1. A histogram of the data (figure 4) clearly shows distinct distributions, indicating that the analgesic effect wanes within three hours after dosing, despite sustained inhibition of PGE_2 and relatively high circulating drug concentrations. Hence, a time-dependency exists in pain response in addition to the confounding effect of dose observed after administration of different dose levels for naproxen and rofecoxib.

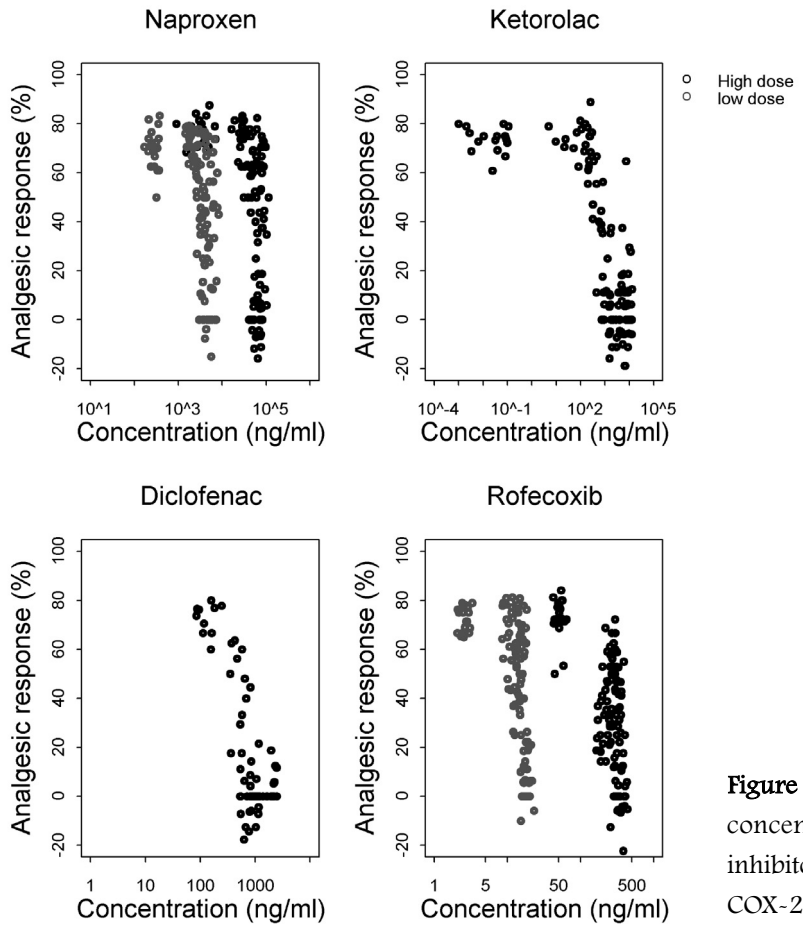

Figure 2: Lack of correlation between plasma concentrations and analgesic effect for COX-inhibitors with varying degrees of selectivity for COX-2.

The time-dependency in pain response is so striking that a direct correlation between pain intensity and drug concentration or PGE_2 levels could not be identified when all data are analysed together (i.e., from 0 to 24 h post-dose). Therefore, an analysis of the probability of pain relief associated with PGE_2 inhibition was performed taking into account the time after drug administration (TAD).

Data was clustered into three groups for the assessment of probability of treatment effect, namely TAD<3 hours, 3<TAD<8 hours and TAD>8 hours. As shown in figure 5, a clear relationship is observed only for the first group (TAD<3 hours). Surprisingly, there was no correlation between pain relief and PGE_2 levels at a later time span. The pharmacodynamic model included therefore only the response variable in the first group. The relationship between PGE_2 and pain relief was described by a linear model (Table 2). Even though an E_{max} model seemed more plausible, it did not result in a significant improvement in the goodness of fit as reflected by the -2LL value. In spite of the

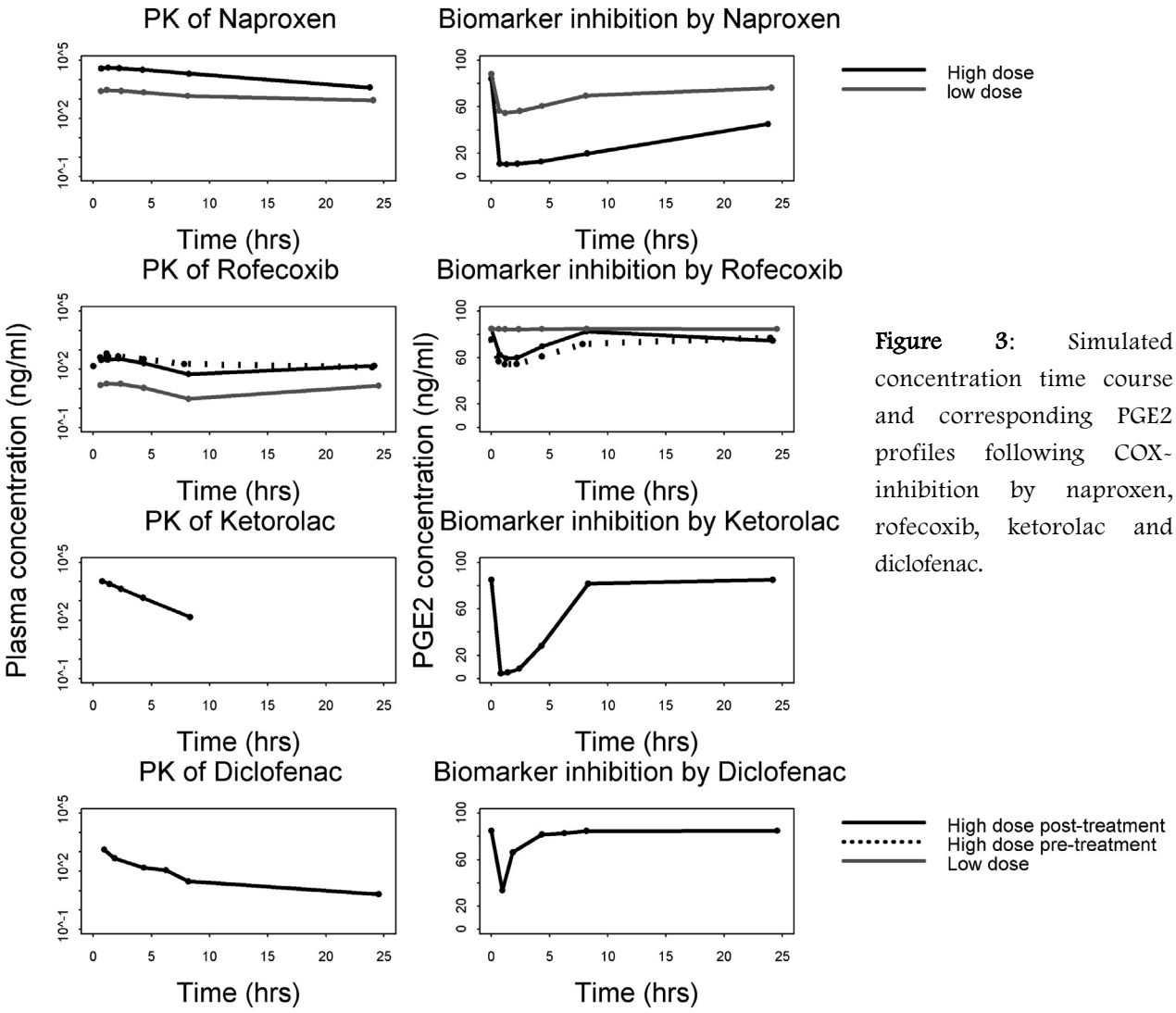

Figure 3: Simulated concentration time course and corresponding PGE2 profiles following COX-inhibition by naproxen, rofecoxib, ketorolac and diclofenac.

assumption of a drug-independent relationship between biomarker and analgesic effect, rofecoxib data was found to behave distinctly from the other treatments, yielding different estimates for EC_{50} of PGE_2 in that treatment group (Table 2).

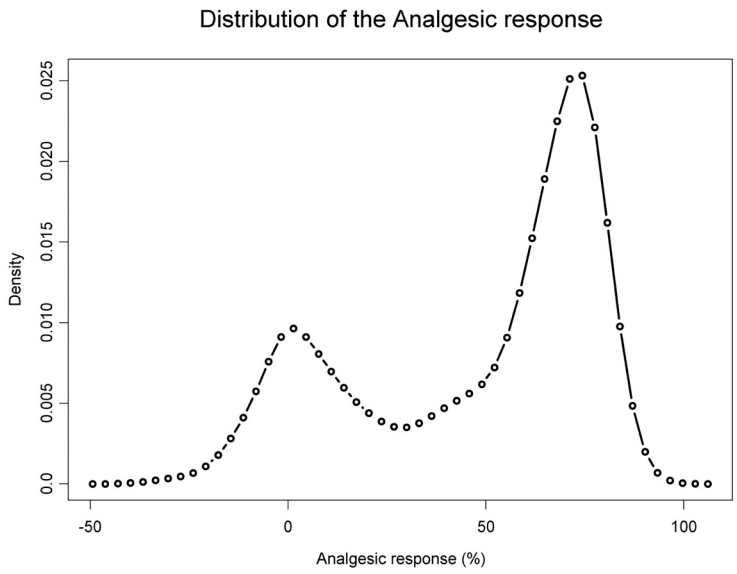

Figure 4: Distribution of the pain scores as percentage pain intensity described by equation 1. See text for further details.

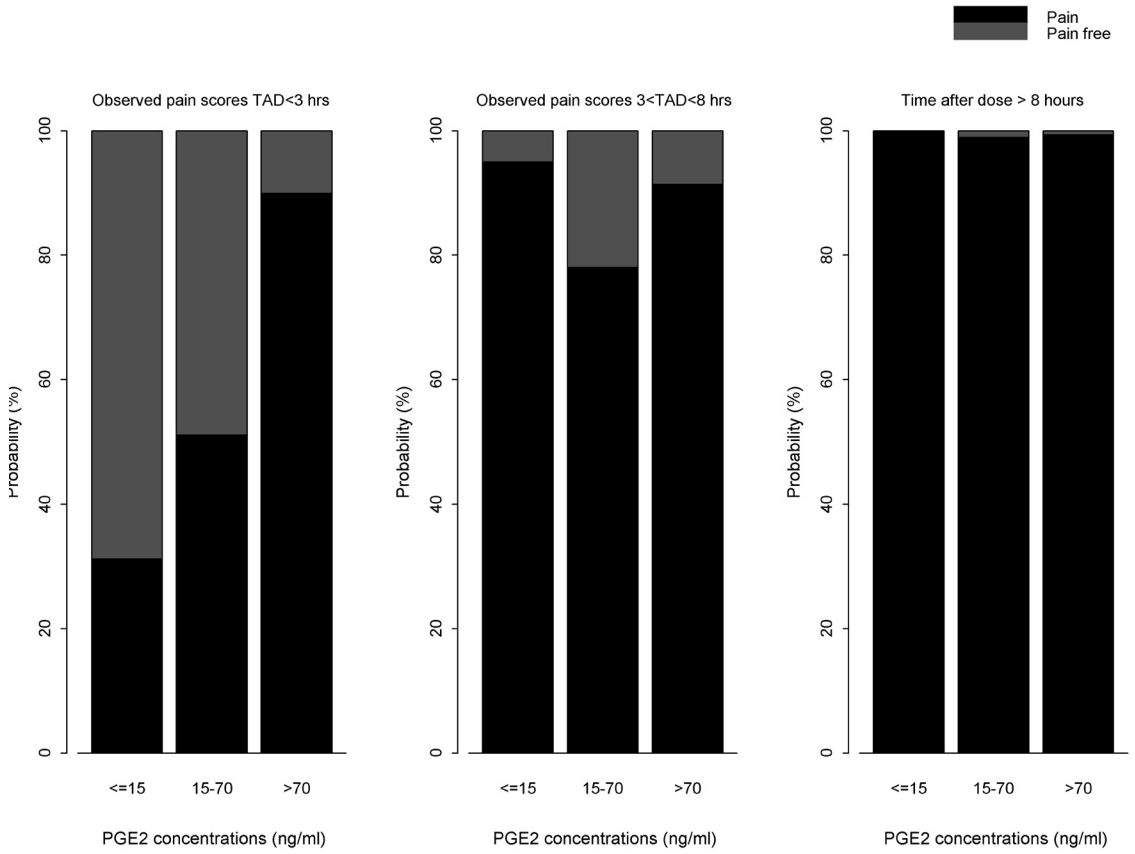

Figure 5: Probability of pain response for different degrees of PGE_2 inhibition. Data has been split into three clusters to account for time-dependencies in response. Clustering of the data reveals the short-lasting analgesia associated with PGE2 inhibition. Each bar depicts the proportion of scores for varying degrees of inhibition (PGE_2 < 15 ng/ml, 15 -75 ng/ml and > 75 ng/ml).

TXB_2 levels were found to have no effect on the relationship between PGE_2 and analgesic effect for non-selective COX inhibitors. Inter-individual variability (IIV) could not be estimated despite the wide variation in the data.

As shown in table 2, the standard errors indicated that model parameters were estimated with good precision (Table 2). Table 2 also provides a summary of the results of the bootstrap procedure, presented as mean and coefficient of variation (% CV). The mean values of the bootstrap procedure were comparable to the parameter estimates from the original dataset, giving further evidence of the robustness of the model. Good agreement between predicted and observed probabilities was demonstrated for the relationship between biomarker and pain relief relationship (figure 6). The

Table 2: Model parameter estimates for the relationship between PGE_2 levels and pain response in the FCA-model of inflammatory pain. Data were fitted by a logistic model with the dichotomisation of response into pain and pain relief.

	Original dataset		500 bootstrap replicates	
	Parameter	**CV%**	**Parameter**	**CV%**
Base	-1.31	10	-1.34	12
EC_{50} (ng/ml)	19.3	12	19.8	16
EC_{50} (ng/ml) rfx*	41.7	12	41.9	7

median prediction error was 1.3%, whilst the median absolute prediction error was 34%. These figures indicate the lack of bias and acceptable degree of precision.

Mechanism-based model. The mean time course of PGE_2 levels and the corresponding analgesic response for each compound is shown in figure 7. It is clear that PGE_2 inhibition outlasts the analgesic effect after administration of naproxen and rofecoxib, whilst the opposite occurs for diclofenac. On the other hand, for ketorolac a comparable profile is observed for both PGE_2 inhibition and analgesic effect. It is also worth noting that maximum inhibition of PGE_2 was less than 50% after administration of rofecoxib.

Initially, a basic model was proposed that describes pain signalling in hyperalgesia not only via PGE_2 production, but also accounts for downstream mediators. Figure 8 shows simulated profiles using naproxen data. The parameter estimates are summarised in table 3. Model stability and plausibility was subsequently assessed by a sensitivity analysis, during which each parameter was tested at a time for wide range of possible values (figure 9). Of particular interest is the evidence for the role of a counter-regulation component, without which the time course of observed analgesic effect cannot be explained.

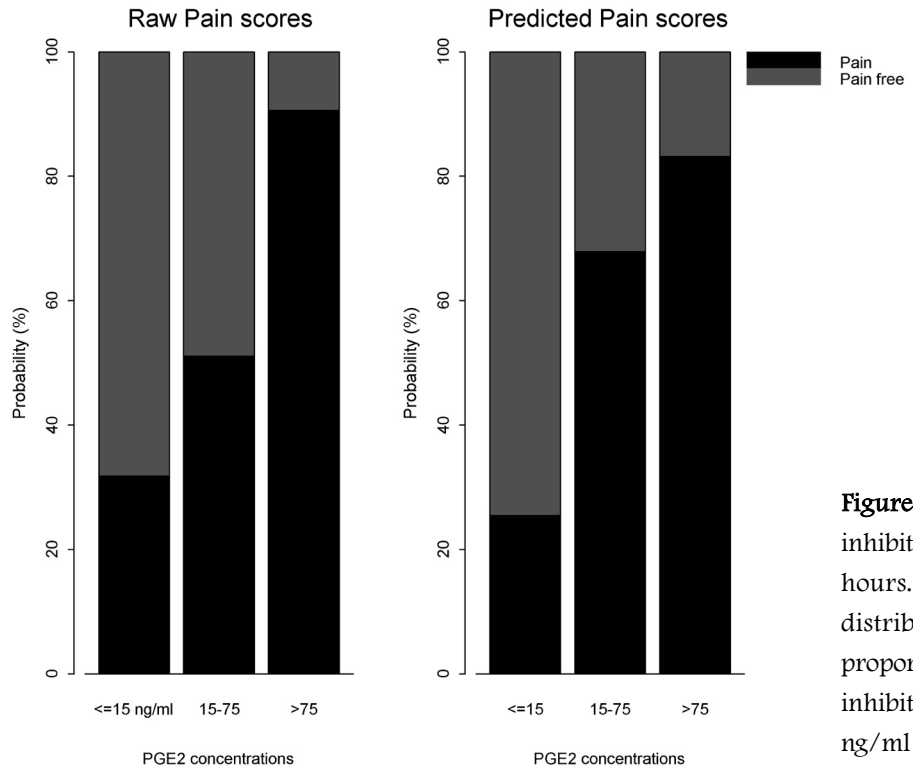

Figure 6: Correlation between PGE_2 inhibition and analgesic effect at TAD<3 hours. Observed and predicted score distribution. Each bar depicts the proportion of scores for varying degrees of inhibition (PGE_2 < 15 ng/ml, 15 -75 ng/ml and > 75 ng/ml).

A next step in the analysis was to assess the suitability of the model for different compounds. As shown in figure 10 from the predicted and observed time course of the analgesic effect, it is evident that counter-regulatory mechanisms are required to explain the discrepancies between PGE_2 inhibition and hyperalgesia. Interestingly, during model building we have found that rofecoxib data could also be fitted with the parameters describing counter-regulation set to zero. It is also worth noting that in contrast to the other three COX inhibitors, diclofenac shows long analgesic effects and relatively short inhibition for PGE_2, which suggests the involvement of other mechanisms for anti-hyperalgesia.

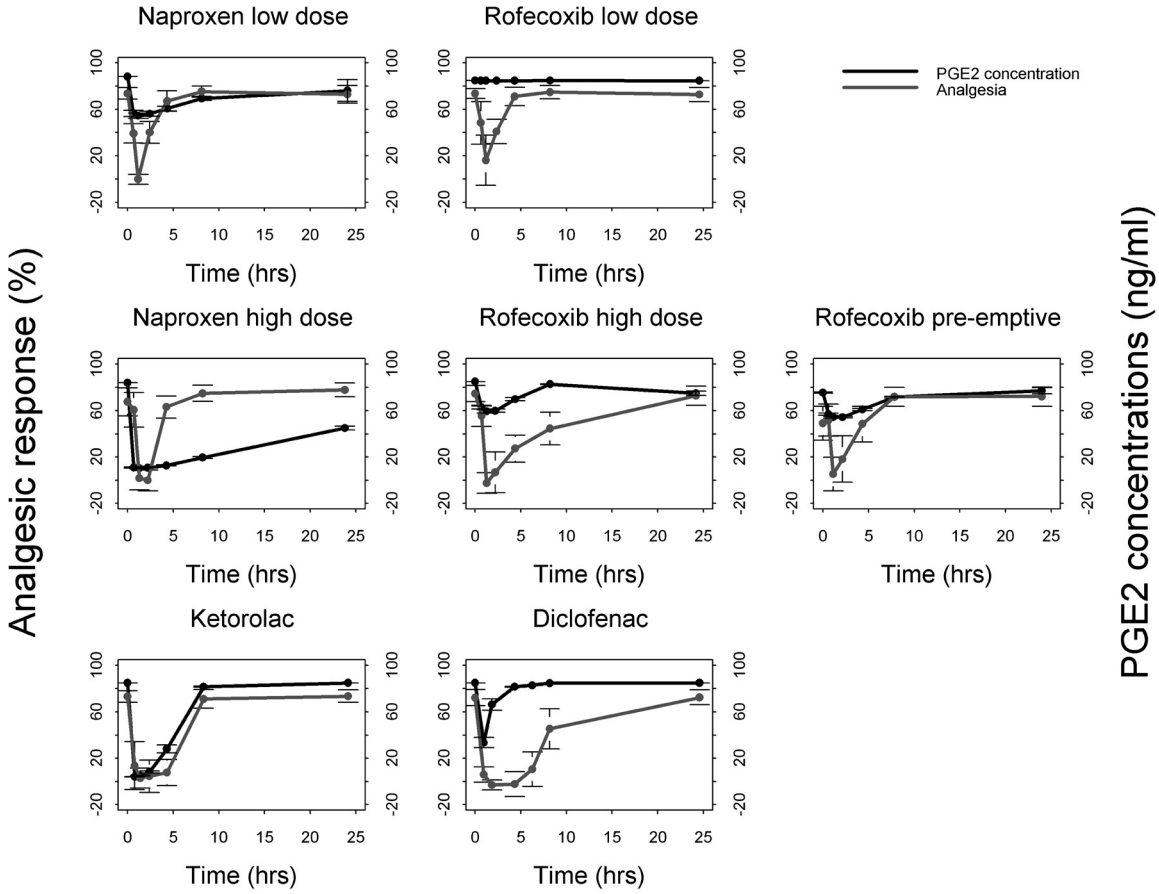

Figure 7: Time course of predicted PGE_2 levels and corresponding analgesic response profiles for naproxen, rofecoxib, ketorolac and diclofenac. Data is presented as mean ± stdev.

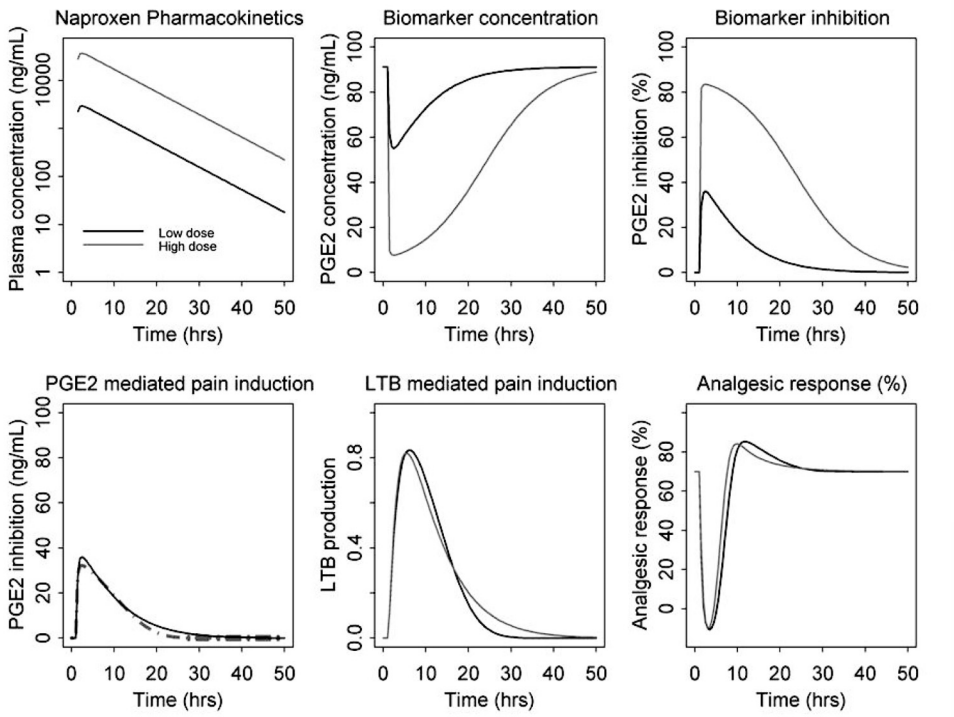

Figure 8: Set-up of the mechanistic model for the contribution of PGE_2 inhibition to the analgesic effect. Each panel represents a step in the cascade from plasma concentration to the analgesia. Upper panels (from left to right) show the steps from systemic exposure to the inhibition of the biomarker PGE_2 over time. Lower panels show the putative inflammatory cascade (including pain induction by PGE_2 and other mediators) and the time course of the analgesic effect.

DISCUSSION AND CONCLUSION

Understanding of the exposure-response relationship is an important requisite for accurate dose selection in clinical development. Our investigation shows the relevance of a pathway analsysis to fully characterise analgesic drug effects and support the assessment of the relationship between drug

Table 3: Parameter estimates for the analgesic effect after administration of naproxen, rofecoxib, ketorolac and diclofenac. The proposed mechanism-based model for FCA-induced hyperalgesia takes into account pain signalling by PGE_2 and downstream mechanisms in the inflammatory cascade.

Parameter	Naproxen*	Diclofenac	Ketorolac	Rofecoxib**
k_{in} (%/hr)	186	48.8	100.421	272.3-62.6
k_{out} (1/ht)	2.67	0.676	1.37	3.63-1.21
$K_{synthesis}$ (%/hr)	1.42		1.2	0.1-1.27
$K_{degradation}$ (1/hr)	0.85-15.8*		7.02	1.48-0.079
$slope_1$ (%)	0.191	0.0401	0.0523	0.2-0.107
$slope_2$ (%)	0.0131		0.0111	0.0012-0.015
α	2.67		20.5	
γ	51.7		0.998	

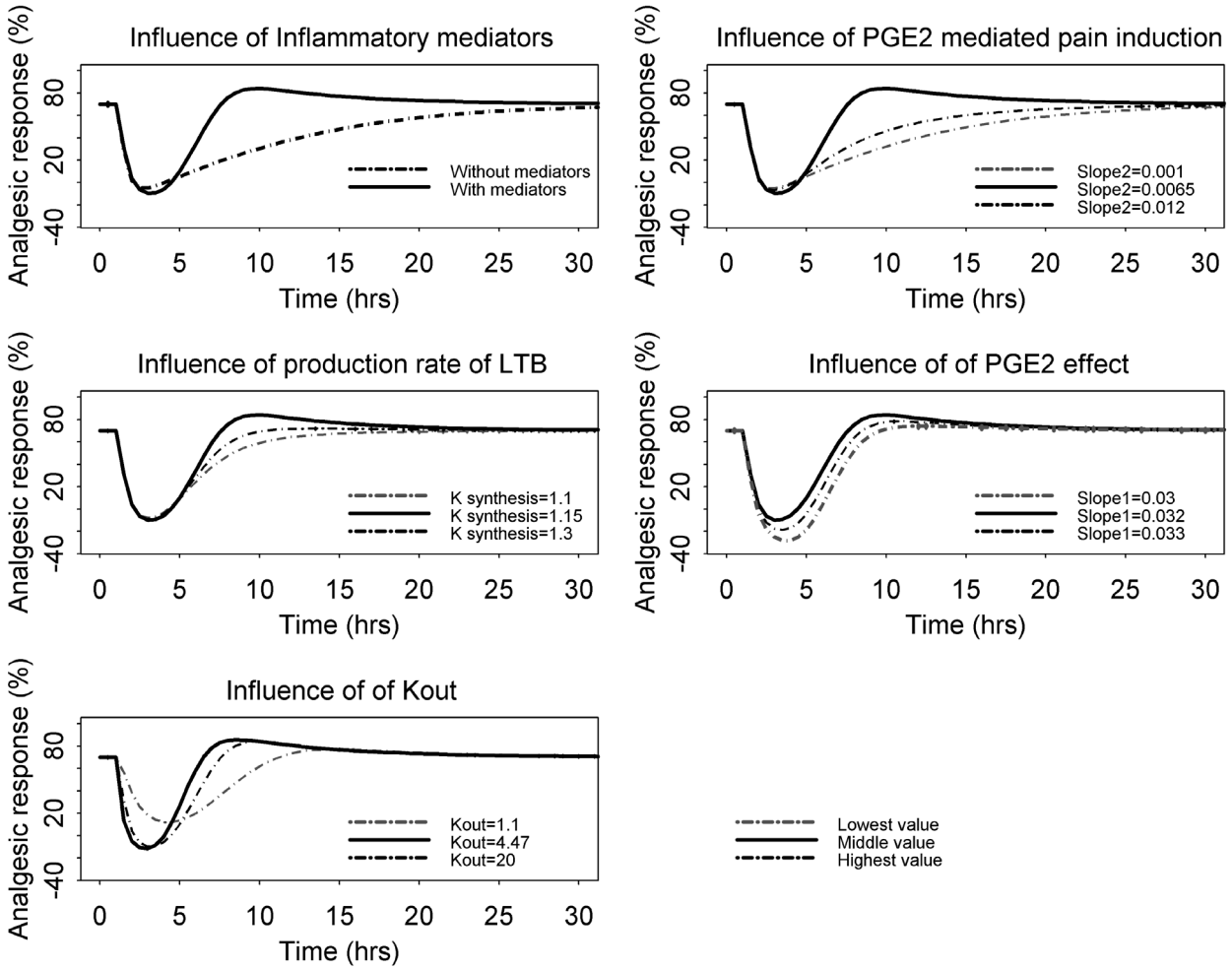

Figure 9: Sensitivity analysis for modelling of hyperalgesia induced by PGE_2 and other downstream mediators of the inflammatory cascade.

exposure and response. The incorporation of (a simplified representation of) the complex downstream mechanisms in the inflammatory cascade forms a essential step in this analysis. It is evident from our results that information on *in vitro* target selectivity only is not sufficient to predict the duration of the analgesic effect *in vivo*. The proposed approach shows how to dissect the underlying components of inflammatory pain, thereby discriminating target occupancy-related effects (i.e., PGE_2 inhibition) from other inflammatory mechanisms (13). In addition, the findings also raise the question about the construct validity of the models currently used for the pre-clinical evaluation of analgesic drugs, which disregard differences between species in pain perception. Obviously, a new paradigm is required that accounts for biomarkers of pharmacology, rather than relying solely on behavioural measures of pain and pain relief. In particular, if one wants to make inferences about pharmacologically related safety events arising from inappropriate dosing regimen or prolonged drug exposure.

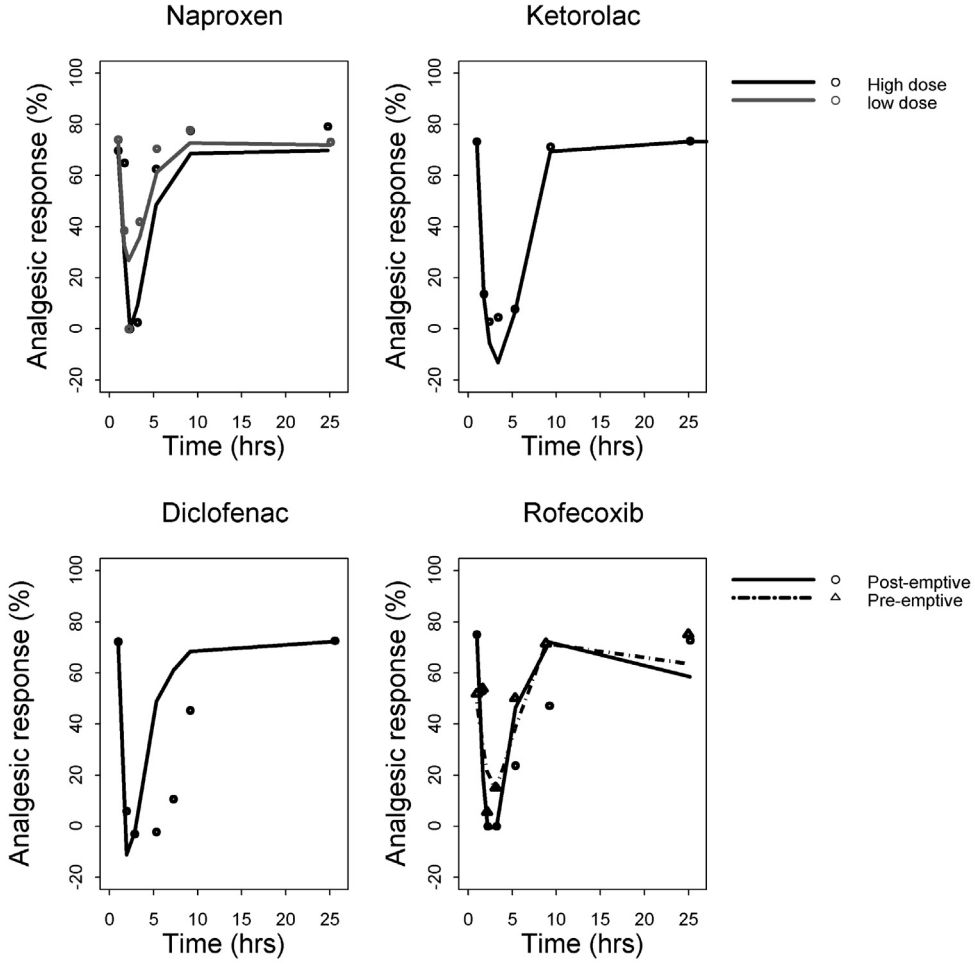

Figure 10: Predicted and observed time course of the analgesic effect after administration of four different COX-inhibitors based on a mechanism-based model for FCA-induced hyperalgesia in rats. The model takes into account pain signalling by PGE_2 and downstream mechanisms in the inflammatory cascade. Lines are the predicted values; symbols represent the observed data.

Logistic regression analysis. Whilst different behavioural measures have been devised in pre-clinical models of pain, none of them conceptually captures what in clinical terms is described as pain relief. In fact, pain relief is often characterised as a binary measure in the clinical evaluation of analgesic

drugs. In our investigation, logistic regression was used as a first step in the development of a model to assess the relationship between biomarker and analgesia. By defining a threshold for a minimum change in pain intensity (hyperalgesia), a probability can be estimated for relief relative to the circulating levels of PGE_2. This approach was chosen for a couple of reasons: 1) the method that can be easily implemented, allowing for further evaluation of the mechanisms underlying analgesia; 2) limited understanding of the relationship between biomarkers and analgesia. Despite evidence from previous investigation, it is not completely clear if a threshold is sufficient to establish the link between biomarker (PGE_2 inhibition) and effect (analgesia) or whether it is the extent or gradient of inhibition that drives changes in pain signalling and subsequent pain perception. A logistic regression also provides a first impression of the magnitude of time-dependency in the cascade of events which determine the inflammatory reaction in peripheral tissues as well as centrally (14).

This approach was based on the assumption that the relationship between PGE_2 levels and analgesic effect is independent of a drug's mechanism of action or upstream effects. The data was divided into three groups based on visual inspection of the response profiles over time, irrespective of treatment type. A clear biomarker response relationship was observed only within three hours post-dose. The presence of such a time dependency was unanticipated. We cannot discriminate whether the dynamics of pain signalling is specific to this animal model or reflects inflammation dynamics under chronic conditions. In contrast to previous investigations in acute inflammatory pain in humans, which show how the constitutive COX-1 activity at the site of tissue injury is followed by enhanced PGE_2 formation with the expression of COX-2 over the first 2 to 3 hours after injury, there is to this date no published data correlating biomarkers to analgesia in pre-clinical models (15).

In our analysis, we also found that a significant improvement of the model fit (-19 points in objective function ($p<0.001$)) was seen by using a separate value of the EC_{50} for rofecoxib. This distinction is explained by the partial PGE_2 inhibition achieved *in vitro* and *in vivo* after administration of rofecoxib (a maximal inhibition of approximately 50-60% was observed). Recently, it has been reported that differently from humans, the production of PGE_2 might be governed by both COX-1 and COX-2 enzymes (16). Even in the presence of suppression of COX-2 activity by rofecoxib PGE_2 can still be produced by COX-1. This represents a clear divergence between animal and human pharmacology. In fact, the potency of rofecoxib was a two-fold lower compared to the other compounds (EC_{50} is 41.7 ng/ml for rofecoxib versus 19.3 ng/ml for naproxen, diclofenac and ketorolac). A thorough evaluation of potential explanatory factors for the short-lasting effect of analgesia was not in the scope of our investigation. However, we did investigate whether TXB_2 levels played a role on the extent and duration of response. No correlation was found between TXB_2 and drug effect on PGE_2 and analgesia. This finding is of interest in view of the fact that the contribution of TXB_2 in pain suppression is still under debate (17).

After three hours the relationship between PGE_2 levels and analgesic response fades out, presumably due to counter-regulatory mechanisms. This raises further questions about the inadequacy of

behavioural measures to predict the required dose range in humans. In this respect, it is clear that continuous suppression of PGE_2 will occur in humans, leading to possible cardiovascular safety issues which would not be observed in pre-clinical species due to the differences in the role of COX-1 and COX-2 in humans (18;19). An approach that accounts for the effects downstream in the inflammatory cascade is therefore required to anticipate how pharmacological properties may or may not yield pharmacology-related adverse events.

Mechanism-based modelling. The second step in the analysis of the effects of COX inhibitors to FCA-induced inflammatory pain was to develop a model based on parameterisation of pain signalling, which included triggers for counter-regulatory mechanisms (figure 1). It is hypothesised that long-term inhibition of COX leads to the formation of other mediators in the inflammatory cascade, such as leukotrienes (LTs), interleukins and TNF-α. Of particular interest is the possible role of LTs, which are synthesised through the lipo-oxygenase (LOX) pathway and seem to be closely involved in the maintenance of inflammation. In fact, published data by Martel-Pelletier *et al.* (20) support the theory that COX inhibition can lead to a switch to leukotriene B4 (LTB_4) production and induction of catabolic factors. The pharmacodynamic model used in our analysis consisted in the inclusion of a modulating mechanism to the underlying PGE_2-mediated hyperalgesia, which links the formation of secondary inflammatory mediators, such as LTB_4, to the circulating level of PGE_2. This association is not only physiologically plausible, but also desirable from a modelling perspective since the number of parameters can be reduced. Evidence supporting this model structure can be derived from animal experiments showing that the complex biochemical interactions of short-lived inflammatory mediators, combined with the neural release of substance P and the process of plasma extravasation, result in a positive feedback loop continually refueling the inflammatory process. The continued synthesis or release of these mediators contributes to the prolonged time course of inflammation (21;22).

It is worth mentioning that similar approaches have been used for the characterisation of drug effect in disease areas. For instance, numerous mechanistic models have been reported for type 2 diabetes mellitus (23). In contrast to pain physiology, the mechanisms underlying glucose homeostasis involve important biomarkers such as insulin, fasting plasma glucose and β-cell function, which can be objectively measured. Moreover, correct prediction of anti-hyperglicaemic dose requires incorporation of the homeostatic feedback mechanism between insulin and glucose. This interaction can only described if data on these biomarkers is available. Exploratory sampling of mediators downstream in the inflammatory cascade was not the primary scope of our experiments. Hence, we have attempted to use literature findings to support the proposed interactions between PGE_2 and other mediators. An interesting finding was that parameter estimates describing counter-regulation were significant for naproxen and ketorolac, whereas these were negligible for rofecoxib. In contrast, diclofenac-induced analgesia outlasted PGE_2 inhibition. Given the range of concentrations used for each compound, these findings seem to suggest that COX selectivity may determine the extent effect, but not its duration. These discrepancies may also point out to the contribution of additional targets

or mode of action of these two compounds. In fact, published data have shown that these drugs also have specific effects on the spinal cord and central nervous system, which may be COX independent (22). Another possible explanation for the differences in response could originate from different pathways exhibiting both pro-and anti-inflammatory activities (depending on organs and disease stage) (24).

The prediction of the therapeutic dose range is an important objective of pre-clinical research. As our results show, the differences in the analgesic effects of naproxen are very small for a 15-fold increase in dose (1 to 15 mg/kg). A limitation to our experimental setting is the lack of a dose range for each compound, so that one could further evaluate how the increase in exposure correlates with analgesia. Nevertheless, Prospectively, this situation highlights the risk of strong experimental bias, given that dose selection in the evaluation of novel compounds is rather arbitrary in at that stage of drug development.

In conclusion, we have shown that a pathway analysis is required to describe the correlation between drug exposure, biomarker and analgesic effects. A new paradigm for the evaluation of COX inhibitors is required to warrant accurate prediction of efficacy and safety in humans. Our results reveal that currently accepted animal models may lack construct validity, in that COX selectivity and downstream effects upon COX inhibition may not reflect drug action in humans. It is also clear that COX selectivity may be a determinant for the extent of PGE_2 inhibition, but does not govern the duration of the effect, as demonstrated for compounds with varying degrees of selectivity. The sole use of behavioural measures in pain models overlooks the differences in the pharmacology of COX inhibitors that ultimately drive response and adverse events.

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