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

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Chapter 8

Differences in the sensitivity of behavioural measures of pain to the selectivity of cyclo-oxygenase inhibitors

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ABSTRACT

Objectives. Freund's complete adjuvant (FCA) is an animal model of inflammatory pain commonly used in the screening of COX inhibitors. However, there is little understanding of how behavioural measures of the anti-inflammatory effect in the FCA model correlate to differences in mechanism of action and whether such endpoints equally reflect drug activity in humans. In the current investigation we evaluate the time course of the analgesic effect for different endpoints after treatment with drugs with varying degrees of selectivity for COX-1 and COX-2. We also assess prostaglandin (PGE₂) and thromboxane (TXB₂) inhibition to establish the correlation between behavioural measures and the degree of selectivity for COX 1 and COX-2.

Methods. Sprague-Dawley rats were treated with FCA by intra-plantar injection. On post-inoculation day (PID) 7, rats received a single oral dose of naproxen, diclofenac, ketorolac or rofecoxib. Drug treatment continued until PID 21. A control group received placebo only. Behavioural endpoints for inflammatory pain and blood samples for biomarkers were obtained at various time points before and after dosing to characterise the time course of drug effect and disease progression.

Results. COX inhibitors showed no effect on the dynamic plantar test. In contrast, full analgesia was observed after drug administration for weight bearing capacity (WBC) and paw pressure (PP), with varying duration of the effect for each of the endpoints. No tolerance to drug effect was observed up to 14 days of chronic treatment. Rofecoxib showed an increase in baseline pain threshold values after chronic treatment, which may be related to its pharmacokinetic characteristics.

Conclusions. Changes in paw pressure threshold seem to best reflect the anti-hyperalgesic properties of COX inhibitors with enough sensitivity to enable estimation of the dose-exposure-response curve.

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) act by inhibiting cyclo-oxygenase (COX) activity and consequently the formation of pro-inflammatory mediators like prostaglandins (PG) and thromboxanes (TXB) (1). Whilst cyclo-oxygenase selectivity *in vitro* has been considered an important factor for differentiating compounds in terms of their anti-inflammatory, anti-pyretic and analgesic effects (2), the screening and dose selection of new, highly selective COX inhibitors relies largely on a variety of pre-clinical models which provide behavioural measures of analgesia *in vivo*. Furthermore, it is assumed that disease conditions that mimic inflammation are required for the assessment of the analgesic effect. In fact, these investigations presuppose that behavioural measures are sufficient evidence to support the selection of doses in clinical studies, irrespective of how a drug modulates biomarkers of inflammatory response.

Based on these assumptions, one ought to concur that the choice of which animal model to use is essential for the correct extrapolation to the clinic. Frequently used models in rodents include carrageen-induced inflammation, Freund's complete adjuvant (FCA) model, formalin injection, capsaicin injection (3-7). Various endpoints have been derived, such as paw withdrawal threshold, thermal nociception, static and dynamic plantar tests (4;7-11). Recent publications compare the pharmacological activity of compounds in these models and results are displayed as ED₅₀ values (12;13). However, reported ED₅₀ values vary greatly among these models and no explanation is provided for the underlying differences and potential implication for the pharmacological effect in humans (14). Moreover, such estimates disregard differences in the pharmacokinetic properties of the drugs under investigation, which makes extrapolation of the findings rather difficult.

From a drug development perspective, the use *in vivo* animal models should ensure the scalability of findings across species. In addition, endpoints should be sensitive enough to enable the assessment of concentration-effect relations, which can then be used to guide the rationale for dosing in humans. However, often attempts fail to establish the correlation between drug concentrations in systemic fluids and the analgesic effect. The aforementioned situation leads to ambiguity about the selection of the therapeutic dose for analgesia.

We hypothesise that at least two aspects contribute to the explanation of the failure in identifying characterising pharmacokinetic-pharmacodynamic (PKPD) relations for COX inhibitors. The first aspect is the (lack of) sensitivity of the behavioural measures to discriminate between compounds with different pharmacological properties. In other words, it is still unclear whether one finds the same magnitude of differences in the measurements of drug effect *in vivo*, as identified by measurements *in vitro*. Hence, it is essential to understand which endpoints best mirror pharmacological differences, including pharmacokinetic properties. The second aspect involves better understanding of how inhibition of inflammatory mediators relate to pain perception and anti-inflammatory effects *in vivo*. Of particular relevance is the disconnection between prolonged inhibition of PGE₂ and apparently short-lasting anti-hyperalgesic effect of drugs tested in acute and

chronic models of inflammatory pain. Characterisation of the latter is critical to accurately design experiments and interpret the resulting dose-response curve.

Among the models mentioned above, the FCA model has long been used to evaluate the analgesic efficacy of NSAIDs in rodents (4;9;15;16). Moreover, in contrast to most pain models, a stable chronic inflammatory condition is established at several days post injection. In the current investigation we compare the analgesic effect in the FCA model of four COX inhibitors with varying degrees of selectivity *in vitro*. Based on this comparison, it was our objective to investigate the role of disease progression drug effect and to assess the correlation between drug-induced changes in behavioural measures and the corresponding effect on the biomarkers of selectivity for COX-1 and COX-2. As behavioural pharmacodynamic endpoints, mechanical hyperalgesia, weight-bearing capacity and static allodynia were selected. All drugs were administered at doses known to yield pharmacologically active concentrations over the course of the experiment. Given the current interest in the evaluation of prophylactic treatments for inflammatory pain, a treatment arm with rofecoxib was also included in which dosing started prior to the inoculation of FCA.

MATERIALS AND METHODS

Chemicals. Naproxen, diclofenac and ketorolac were purchased from Sigma Aldrich BV (Zwijndrecht, The Netherlands). Rofecoxib was synthesised by Pharmaceutical Development (GlaxoSmithKline, UK). All compounds were suspended and dissolved in 1% methylcellulose solution.

Animals. Male Sprague-Dawley rats (n=81) were purchased from Harlan (UK) and housed at 22⁰C with a 12-h light/dark cycle for seven days before the start of the experiments. Animals were housed in groups of six per cage and food and water were available *ad libitum*. All procedures were reviewed and approved by the UK Home Office and were carried out in accordance with the requirements of the project licence. One animal died unexpectedly before completion of the experiments.

Inflammation. Freund's complete adjuvant (FCA 100 µl of a 1 mg/kg m. tuberculosis, Sigma Alldrich BV) was injected intraplantarly with a 27-gauge needle into the left hind paw (n=10 per group). The non-injected contra-lateral paw of each animal served as a normal control. The FCA injection produced an area of localised erythema and oedema that did not disturb grooming, sleep-wake rhythm or social interactions.

Study design. The diagram in figure 1 depicts the dosing and sampling schedules for PK, biomarker and PD data. Animals (n=80) were randomly assigned to 8 groups. After an acclimatisation period of seven days, three days before the start of the experiments, animals were subjected to a training phase on the three different behavioural tests. Prior to the FCA injection, a baseline measurement was obtained and a blood sample was collected via the tail vein to assess biomarker levels and drug concentrations. Behavioural tests were performed one hour post inoculation and on seven different

occasions on days 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). Biomarker and drug concentrations were determined at day 1, day 7, day 8, day 21 and day 22.

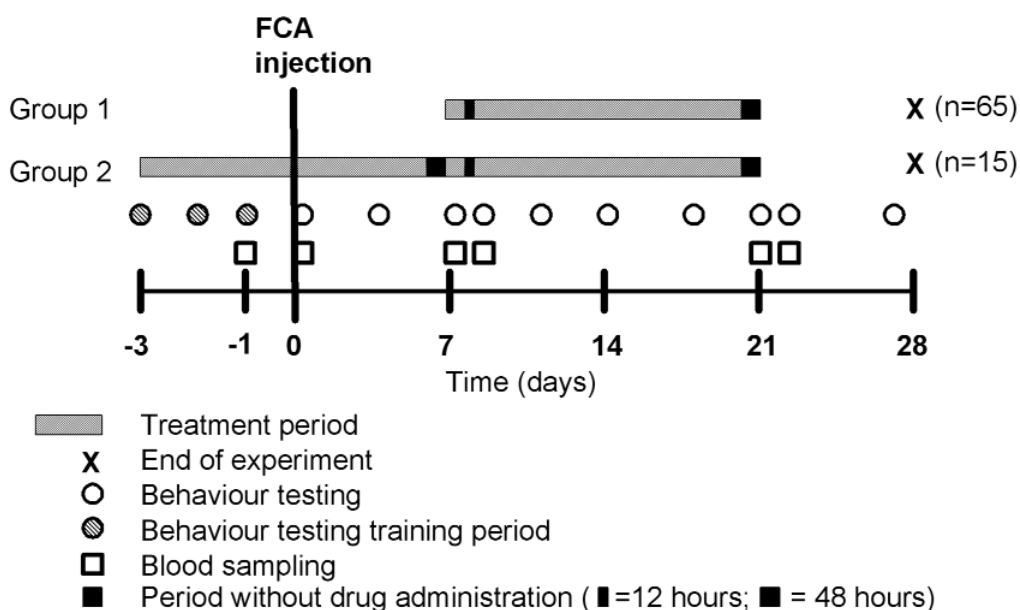


Figure 1. Schematic representation of the experimental setting for the simultaneous evaluation of COX-induced changes on behavioural measures and biomarkers of anti-inflammatory response in the FCA model.

With the exception of the pre-emptive treatment group, drug administration was started on day 7 PID, after the establishment of a chronic inflammatory condition. Dosing time was restricted between 8 and 9 AM. A single oral dose was administered as oral gavage. Naproxen was administered at a dose of 1 and 15 mg/kg, diclofenac at a dose of 10 mg/kg, ketorolac at a dose of 4 mg/kg and rofecoxib at a dose of 0.5 and 10 mg/kg. The relevance of pre-emptive treatment was explored for rofecoxib by dosing animals with 10 mg/kg bid 12/12 h five days prior to the FCA injection. Rofecoxib administration was given until day 5 PID in the morning to allow complete wash-out of drug in plasma at the time of the behavioural tests. Behaviour testing was performed at day 7 PID before drug administration, and 0.5-0.75 h, 1-1.5 h, 2-3 h, 4, 8 and 24 h thereafter.

The influence of disease progression on anti-inflammatory drug activity was evaluated from day 8 PID at 8 PM until day 19 PID at 8 AM by resuming twice-daily administration of naproxen, ketorolac, rofecoxib and placebo. Following a wash-out period of 48 hours at the end of the treatment period, an additional single oral dose was administered on day 21 PID between 8-9 AM. The wash-out of drug in plasma (withdrawal period) and "re-challenge" with a final dose was deemed necessary to assess whether drug treatment had a modifying effect on the time course the inflammatory response. Behavioural testing was performed at day 21 PID, before drug administration, and 0.5-0.75 h, 1-1.5 h, 2-3 h, 4, 8 and 24 h thereafter.

Behavioural tests. The endpoints listed below were measured at various time points according to a fixed schedule, starting with the least noxious stimulus. First weight bearing capacity was measured, followed by dynamic plantar test and paw withdrawal threshold.

Weight bearing capacity (WBC) as a measure of touch sensitivity. Animals were placed in a Perspex box such that their hind paws were positioned on two mechanotransducers and the weight borne on each paw was averaged over a 3 sec period. The % weight distribution was calculated using the following equations:

% weight distribution in inflamed paw = weight borne by ipsilateral/total weight borne by both paws
*100 %

and for the contralateral paw:

% weight distribution in non-inflamed paw = 100 - % weight distribution in inflamed paw.

Dynamic plantar test (DPT) as a measure of static allodynia. The response to a non-noxious mechanical stimulus was tested using the dynamic plantar test (Ugo Basile). Animals were placed in raised Plexiglas cubicles that had mesh wire bottoms. After allowing the animals to adapt to the environment for 10 minutes, a filament with a dull end was applied to the hind paw. An increasing force (0-50 g) was applied to the paw until a withdrawal reflex was elicited. The threshold was recorded in grams.

Paw pressure (PP) as a measure of mechanical hyperalgesia. Paw withdrawal thresholds of both ipsilateral and contralateral paws were determined as previously described using an algometer (Ugo Basile, Italy) (26). An increasing weight was applied to each paw until a withdrawal reflex was elicited. The force at which a rat withdrew its paw was multiplied by 10, as recommended by the manufacturer, and recorded as the withdrawal force (g).

Biomarker and pharmacokinetic assessments. Blood samples of 250 µl were taken from the tail vein at pre-defined time points for the determination of drug concentrations (PK), TXB₂ and PGE₂ concentrations. Blood samples were taken between 1.5 and 4 hours post dosing on day 7 and on day 21. For the assessment of pharmacokinetics in plasma, samples were taken at approximately 5 hours after administration of diclofenac, at 2 hours after administration of ketorolac, between 1.5-4 hours after administration of naproxen and between 1.5 and 3 hours after administration of rofecoxib. The aforementioned sampling times were based on the pharmacokinetic properties of the compounds. Following collection, samples were split into aliquots of 100 µl (for PK and PGE₂) and 50 µl (for TXB₂). Blood samples for PK were placed into heparinised tubes and centrifuged at 5000 rpm for 10 min. Plasma was stored at -80°C until analysis by LC-MSMS. Blood samples for TXB₂ analysis were placed into tubes and allowed to clot for 1 hour at 37°C in a stirring water bath. Serum was collected after centrifugation and stored at -20°C until analysis. Tubes for the analysis of PGE₂ were prepared by evaporating aspirin (10 µg/ml in methanol and heparin (10 I.U.) Blood samples were placed in these tubes and 10 µg/ml lipopolysaccharide (LPS) was added. Samples were incubated and stirred

for 24 hours at 37°C in a water bath. Plasma was separated by centrifugation and stored at -20°C until analysis.

Analysis of TXB₂ and PGE₂. PGE₂ and TXB₂ 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 PGE₂, 200-2000 times for TXB₂) 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 (TXB₂) or 30 min (PGE₂) after which 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. For the sake of clarity, this manuscript focuses on the analysis of the correlation between behavioural measures and biomarkers. Details of the pharmacokinetic-pharmacodynamic analysis will be presented elsewhere.

Statistical analysis. Results are presented as mean and corresponding standard deviations, except for figure 9 for which the SEM was depicted. All variables under evaluation showed normal distribution as assessed by Kolmogorov-Smirnoff test. Comparison of the observed responses between ipsilateral and contralateral paws was based on a paired t-test. Comparison between drug treatment groups and placebo were done using ANOVA analysis with Dunnett's multiple comparison *post-hoc* test. The statistical significance level was set at $p < 0.05$.

RESULTS

Baseline response. The baseline response before FCA injection for each of the three behavioural tests was stable and showed no significant variation. The mean WBC was 82.1 ± 12.8 g and 79.8 ± 11.0

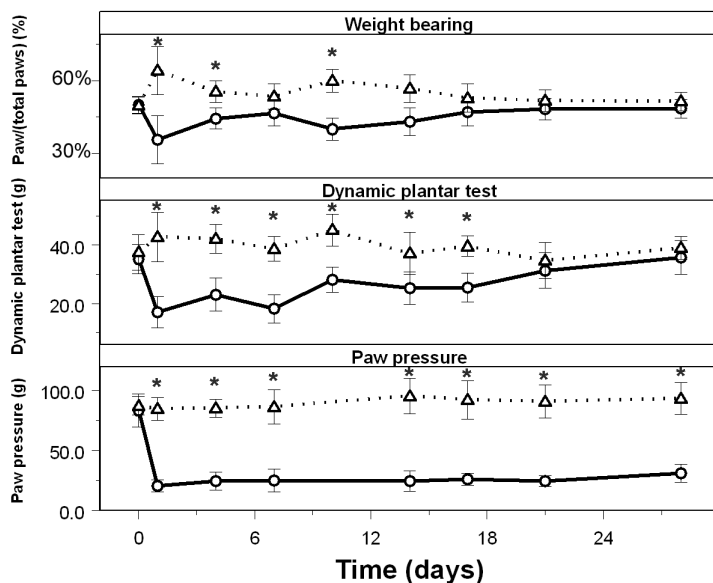


Figure 2. Time course of WBC, DPT and PP threshold after unilateral intraplantar FCA injection (n=10, mean \pm stdev). WBC is shown as % weight-bearing capacity per paw. Symbols denote the inflamed (circles) and non-inflamed (triangles) paw. *Indicates that differences between ipsi- and contralateral paw are statistically significant at that sampling time ($p < 0.05$).

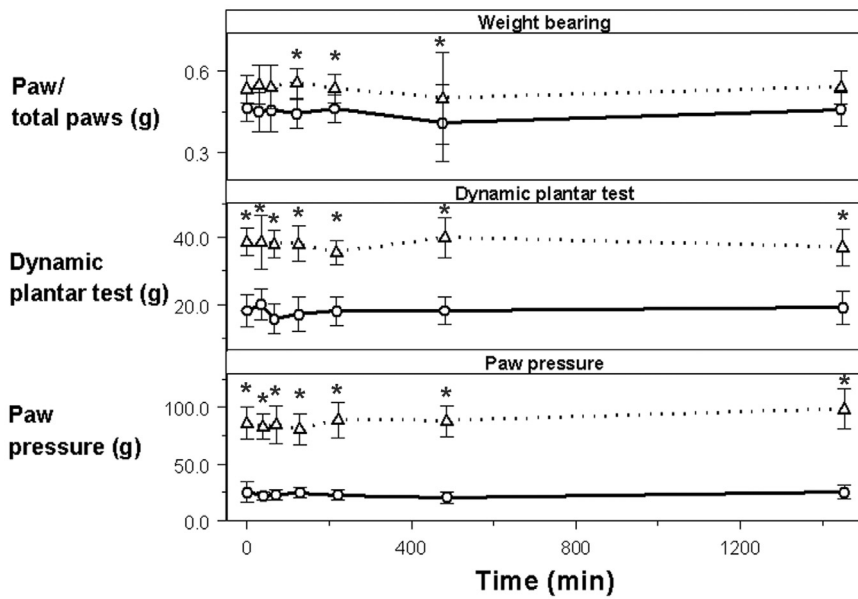


Figure 3. Time course of WBC, DPT and PP on day 7 after unilateral intraplantar FCA injection (n=10, mean \pm stdev). The relevance of repeated measures on PID 7 and subsequent treatment days is shown. Symbols denote the inflamed (circles) and non-inflamed (triangles) paw. *Indicates that differences between ipsi- and contralateral paw are statistically significant at that sampling time ($p < 0.05$).

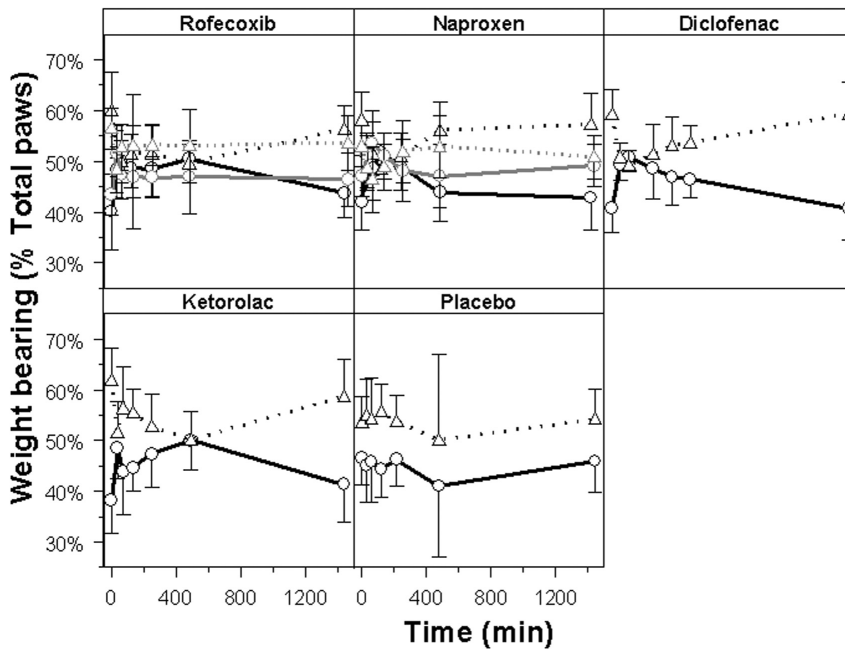


Figure 4. Time course of the effect of rofecoxib (0.5 and 10 mg/kg), naproxen (1 and 15 mg/kg), diclofenac (10 mg/kg) and ketorolac (4 mg/kg) on WBC after single oral administration on day 7 PID (n=10-11, mean \pm stdev). Animals were assessed for change in hind paw weight distribution before drug administration. Symbols denote the inflamed (circles) and non-inflamed (triangles) paw. Comparison between treatment and placebo was based on the ANOVA analysis with Dunnett's multiple comparison *post-hoc* test. * indicates statistically significant difference compared to placebo value ($P < 0.05$) for the specific time point.

g for the ipsilateral and contra-lateral paw, respectively ($n=71$, $p=0.07$). The mean latency for the DPT was 35.1 ± 7.1 g and 35.4 ± 6.8 g for ipsilateral and contralateral paw, respectively ($n=71$, $p=0.69$). The mean withdrawal threshold to PP was 95.4 ± 18.6 g and 97.3 ± 18.9 g for ipsilateral and contralateral paw, respectively ($n=71$, $p=0.21$).

Time course of response following administration of placebo. A statistically significant difference between inflamed and non-inflamed paw was observed for the weight-bearing response until day 14 PID, with the exception of the measurement on day 7 PID, which did not reach statistical significance level ($p=0.06$). In fact, the maximum difference of 28% between ipsi- and contralateral paw was observed on day 1 PID. In contrast, statistically significant differences were observed until day 21 PID and until day 28 PID for the DPT and for the PP threshold, respectively (figure 2). The maximum difference between ipsi- and contralateral paw was 60% on day 1 PID for the DPT and 70% for the PP threshold. Similarly, as shown in figure 3, a stable response was observed for all three dynamic measures on day 7 PID i.e., the first treatment day under chronic inflammatory conditions. Maximum differences between ipsi- and contralateral paw were 20%, 58% and 77% for weight bearing, DPT and PP threshold, respectively.

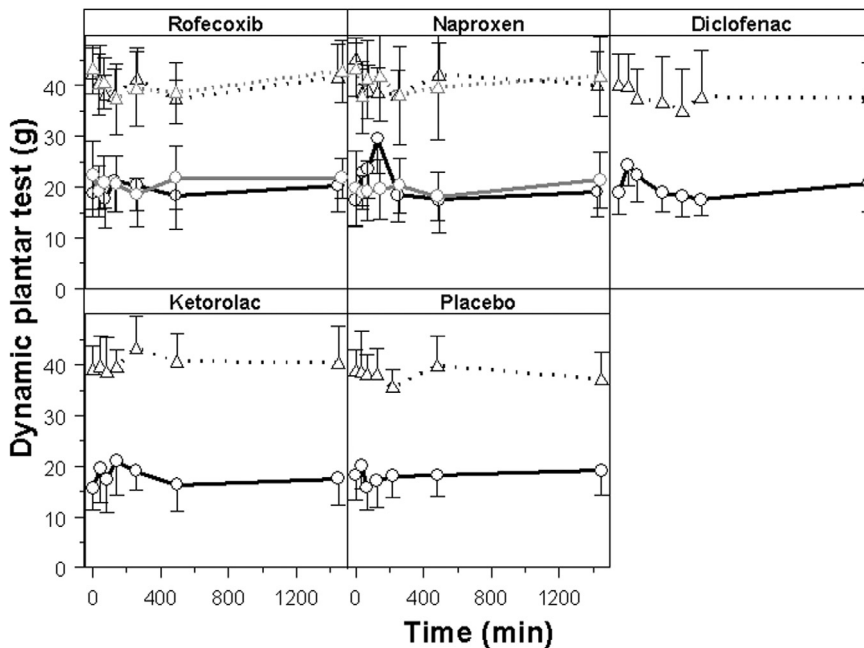


Figure 5. Time course of the effect of rofecoxib (0.5 and 10 mg/kg), naproxen (1 and 15 mg/kg), diclofenac (10 mg/kg) and ketorolac (4 mg/kg) on the DPT after single oral administration on day 7 PID ($n=10$, mean \pm stdev). Baseline measurements were carried out before drug administration. Symbols denote the inflamed (circles) and non-inflamed (triangles) paw. Comparison between treatment and placebo was based on the ANOVA analysis with Dunnett's multiple comparison *post-hoc* test. * indicates statistically significant difference compared to placebo value ($P<0.05$) for the specific time point.

Time course of response following drug treatment. Similar to the placebo group described above, on day 7 PID animals were administered a single dose of each COX inhibitor. Drug effect on WBC was observed for all 4 compounds and lasted for at least 4 hours post dosing (figure 4). In contrast to the

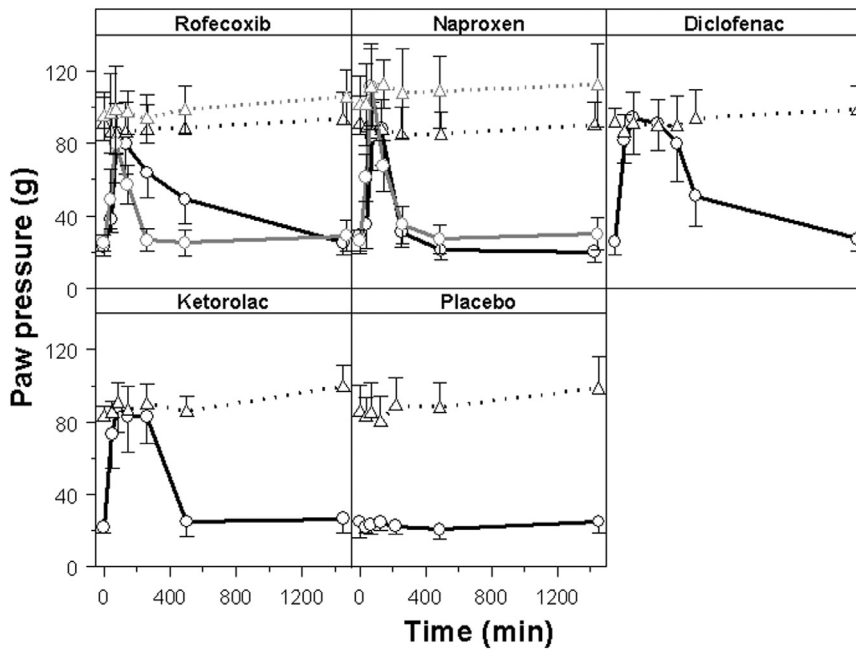


Figure 6. Time course of the effect of rofecoxib (0.5 and 10 mg/kg), naproxen (1 and 15 mg/kg), diclofenac (10 mg/kg) and ketorolac (4 mg/kg) on the PP threshold after single oral administration on day 7 PID ($n=10$, mean \pm stdev). Baseline measurements were carried out before drug administration. Symbols denote the inflamed (circles) and non-inflamed (triangles) paw. Comparison between treatment and placebo was based on the ANOVA analysis with Dunnett's multiple comparison post-hoc test. * indicates statistically significant difference compared to placebo value ($P<0.05$) for the specific time point.

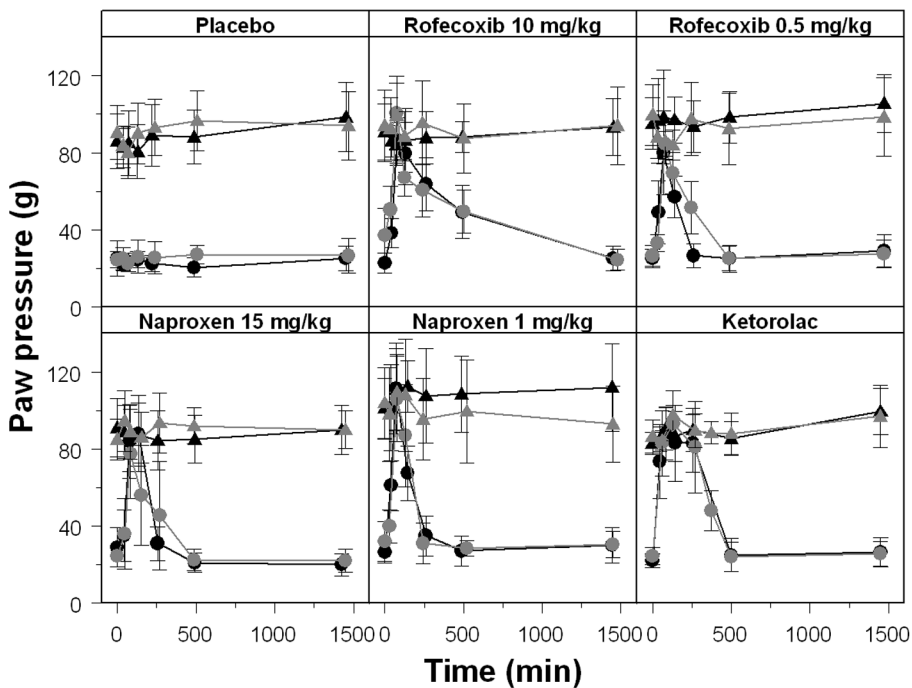


Figure 7. Comparison of the time course of the effect of placebo, rofecoxib (0.5 and 10 mg/kg), naproxen (1 and 15 mg/kg), and ketorolac (4 mg/kg) on PP threshold after single (day 7 PID) and twice-daily chronic administration (day 21 PID) ($n=10$, mean \pm stdev). Black line denotes analgesic response on day 7 PID whereas grey lines depict results on day 21 PID. No significant differences between day 7 and day 21 were found.

effects on weight bearing, none of the compounds produced a statistically significant change on the DPT (figure 5). Differently from the effects on WBC, assessment of the PP threshold yielded distinct time course of the analgesic effect for each drug with clear separation from placebo (figure 6). Diclofenac shows the longest analgesic response, up to 8 hours after administration, whereas naproxen (1 mg/kg) and rofecoxib (0.5 mg/kg) show the shortest duration in effect. These results are summarised in table 1. The results of the four compounds on WBC show a broad scattering over time, e.g. ketorolac differs significantly from placebo at baseline and at 8 hours after dosing. For the DPT, only naproxen 15 mg/kg shows a short lasting effect.

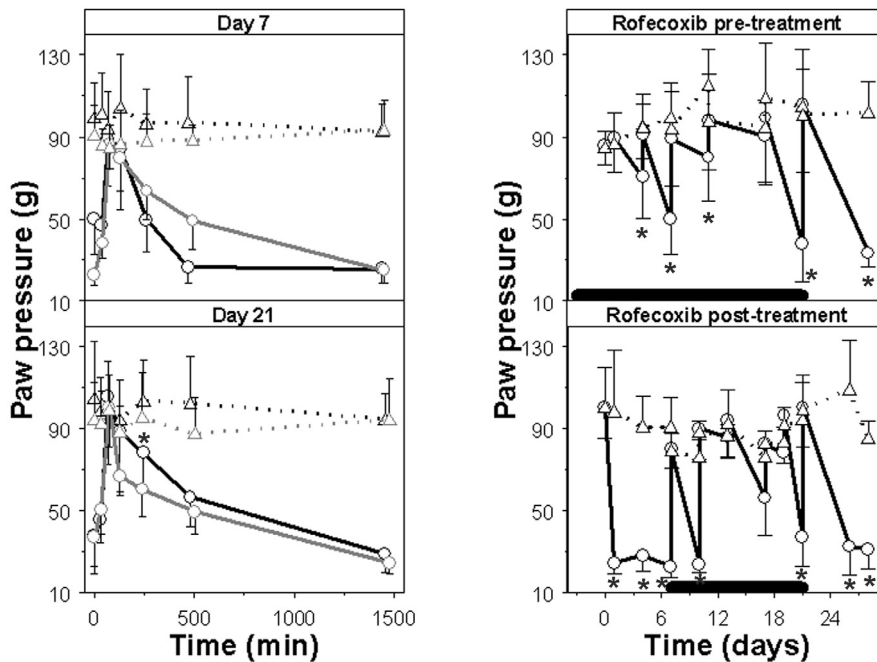


Figure 8. Relevance of pre-emptive (black line) vs. post-emptive (grey line) treatment on the course of inflammatory response in the FCA model as assessed by changes in the PP threshold after administration of rofecoxib (10 mg/kg). Left panel shows the time course of the analgesic effect on PP at day 7 and day 21 for rofecoxib (10 mg/kg bid). Right panel shows the time course of the behavioural response throughout the experiment from 5 days prior to inoculation until the last assessment on day 28 PID. Black bar indicates treatment days with rofecoxib. During the course of drug administration, two consecutive measurements were performed; the first one at 12 h post-dosing followed by a second measurement 1 h after administration. *Indicates that differences between ipsi- and contralateral paw are statistically significant at the specific time point ($p < 0.05$).

Based on the findings described above for placebo and drug treatment, it seemed unlikely that any drug-induced effect would be discernible by weight-bearing capacity or by the DPT following chronic dosing. In fact, on day 21 PID, no statistically significant differences were detected between the ipsi- and contralateral paw for WBC or DPT before drug administration (data not shown). Similarly to the results obtained on day 7 PID, figure 7 shows that a clearly distinct time course of the analgesic effect was also observed on day 21 PID for the PP threshold. Interestingly, drug effects on day 21 PID were not statistically different from day 7 PID.

Pre-emptive drug treatment and development of FCA-induced inflammation. As indicated in figure 1, administration of rofecoxib started five days prior to FCA injection and continued until day 5 PID. A statistically significant difference between ipsi- and contralateral paw for the pre-treatment baseline response for PP was observed on PID 7 ($p < 0.001$). A significant analgesic effect was also observed for PP on day 7 and 21. Again, no effects were observed in the DPT (data not shown). The time profiles for rofecoxib pre-treatment versus post-treatment are shown in the right panel of figure 8. Full analgesia is observed in the first days post FCA injection. After the initial washout, the baseline value is comparable to post-treatment values. After chronic administration, i.e., around day 14 (following 6 days of b.i.d. dosing) both pre-treated and post-treated animals show higher values at 12 hours post dosing compared to day 7. This increase in the response is observed until the washout period at day 19.

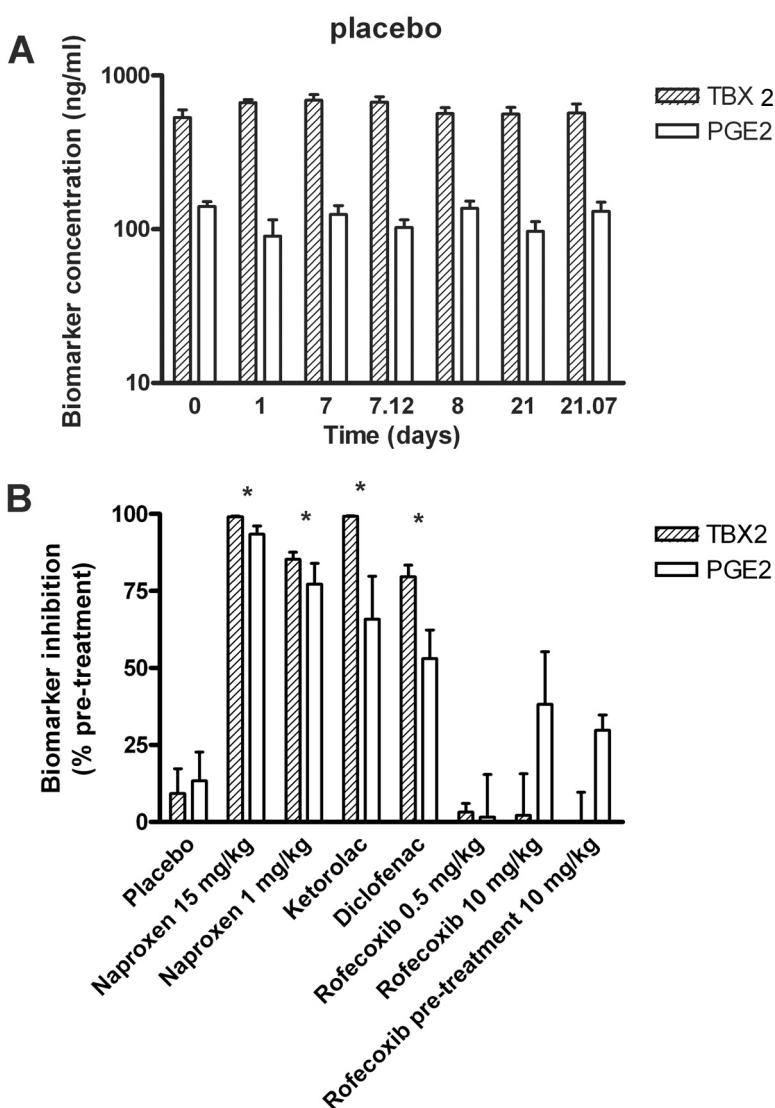


Figure 9. Biomarker levels in the FCA model before and after treatment with COX-inhibitors. Panel A displays the time course of PGE₂ and TXB₂ production in placebo treated animals ($n=10-11$, mean \pm SEM). Differences between pre- and post-inoculation were not statically significant. Values at time zero refer to biomarker levels before inoculation with FCA. + symbol on days 7 and 21 indicates biomarker levels at the end of dosing interval for twice-daily administration. Panel B displays PGE₂ and TXB₂ inhibition levels after placebo and drug administration ($n=5-10$, mean \pm SEM). Blood samples were taken between 1.5 and 4 hours post dose. *Indicates statistically significant differences between drug and placebo ($p < 0.05$).

Biomarker and pain response. The baseline PGE₂ and TXB₂ concentrations were 120 ± 60 ng ml⁻¹ and 514 ± 161 ng ml⁻¹ before the FCA injection, respectively (n=59). After placebo the PGE₂ and TXB₂ concentrations over time were stable and not significantly different compared to pre-FCA values (figure 9). PGE₂ and TXB₂ concentrations after drug treatment on day 7 were determined at different time points (between 1.5-4 hours post dose) for the different compounds (figure 9, lower panel). Diclofenac, ketorolac and naproxen showed significant TXB₂ inhibition after drug administration, whereas rofecoxib did not inhibit TXB₂ concentrations. Diclofenac, ketorolac and naproxen also produced significant PGE₂ inhibition. In contrast, the effect of rofecoxib 10 mg/kg was limited and did not statistical significance. No inhibition was observed during pre-treatment or following the lower dose of rofecoxib (0.5 mg/kg).

Table 1. Overview of the duration of the effects of rofecoxib, naproxen, diclofenac and ketorolac on weight bearing capacity, dynamic plantar test and paw pressure threshold. The time interval (hours post-dose) indicates the period for which significant differences ($p > 0.05$) were observed between inflamed and non-inflamed paw. Data refer to treatment effect observed on day 7 PID.

Compound	Weight bearing capacity	Dynamic plantar test	Paw pressure
Rofecoxib 0.5 mg/kg	0.5-8 h	No effect	1 h
Rofecoxib 10 mg/kg	0.5-8 h	No effect	1-2 h
Naproxen 1 mg/kg	0.5-4 h	No effect	1 h
Naproxen 15 mg/kg	0.5-4 h	No effect	1-2 h
Diclofenac 10 mg/kg	0.75-6 h	No effect	2-6 h
Ketorolac 4 mg/kg	0.5-1.25 & 4-8 h	No effect	0.75-4 h

DISCUSSION AND CONCLUSION

The aim of our study was to investigate the sensitivity of different behavioural endpoints to the effects of COX inhibitors with varying degrees of selectivity in a chronic inflammation model in rats. The relevance of *in vivo* pharmacodynamic responses assessed in pre-clinical models is a key issue in clinical pharmacology, in that estimates of the analgesic effect in animals are assumed to predict drug action in patients and consequently provide the rationale for dose selection. This reasoning contrasts with research efforts in achieving high degree of COX selectivity during drug design and pre-clinical screening of compounds, which postulates the use of selectivity as basis for differentiation of drug effect *in vivo*.

Increasing evidence from publish literature seems to arise suggesting that behavioural endpoints used in conjunction with animal models of pain represent a qualitative rather than quantitative measure of drug effect *in vivo*, most of which bear no correlation with the underlying mechanisms of action (17). In this context, the terms qualitative and quantitative refer to the sensitivity and specificity of the measurements used to characterise drug action. A sensitive endpoint enables not only differentiation of drug effect from placebo, but also reflects pharmacological differences between compounds, including their exposure-response relations.

Currently, results from animal models of pain do not provide accurate estimates of the exposure

levels required for analgesia. Furthermore, there is no consensus about how to rank the relevance of the various behavioural measures that can be used as endpoints in pre-clinical experiments. We have characterised the time course of chronic paw inflammation as assessed by WBC, DPT and PP threshold, as commonly used in pre-clinical development programs and deemed an appropriate tool to assess drug effect on touch sensitivity, static allodynia, and hyperalgesia. Thus far, their value in predicting the extent of the analgesic effect in humans as well as their correlation with the corresponding biomarker response has not been established.

Sensitivity of behavioural measures. In our experiments, static allodynia was measured by the DPT and touch sensitivity by WBC. The difference between the DPT and WBC is the magnitude of applied stimulus. In both cases, increased pressure is applied onto the animal skin, but the stimulus after WBC is much larger, as compared to DPT. (18). Specific data on the pathophysiology of allodynia in rats is not available for FCA-induced pain. However, Field *et al.* reported distinct effects of morphine in neuropathic pain. Morphine was able to block static allodynia but failed to block dynamic allodynia, which indicates that the mechanism for both types of allodynia is different. The authors concluded that A β -fibres are responsible for dynamic allodynia, whereas A δ - and A β -fibres are involved in static allodynia in neuropathic pain in rats (18). As shown in the placebo group, both static and dynamic allodynia developed during chronic inflammation, indicating that A δ and A β fibres are involved in FCA-induced pain. Interestingly, longitudinal data do not hint towards a habituation effect in the DPT, since there was no clear trend in latency to response as time progressed. With regard to touch sensitivity, our findings are comparable with those published by Hay *et al* and Wilson *et al.* (4). However, reported differences between inflamed and non-inflamed paw are approximately 50% and stable over 14 days, which is much larger than observed in our data, which could be related to different strains of rats. In this respect it is important that Hay *et al.* and Wilson *et al* used Glaxo-bred random hooded rats, whereas we used Sprague-Dawley rats. Random hooded rats do not gain as much weight as Sprague-Dawley rats, and therefore their bodyweight is more stable throughout the experiment. The increase in body weight renders the use of weight borne by each hind paw inaccurate. Therefore, the correction based on total weight borne by the two paws together was deemed necessary for unbiased evaluation of drug effect.

Mechanical hyperalgesia was clearly detectable 24 hours after FCA inoculation. In fact, this finding confirms the results published by Nagakura *et al.*, who showed rapid onset of joint hyperalgesia after FCA injection (9). In contrast to the waning time course of allodynia and touch sensitivity, as assessed by WBC and DPT, hyperalgesia was persistent until day 28 PID. The aforementioned differences underlying the aetiology of allodynia and hyperalgesia and the clear distinction in the magnitude and time course of each behavioural endpoint substantiates the need to understand the role of the inflammatory process to interpret drug effect in chronic pain conditions.

COX inhibitor mediated analgesia. COX inhibitors had no effect on static allodynia, as measured by the DPT, whilst their effect on weight-bearing capacity was rather variable, without a clear correlation between the time course of response and pharmacokinetics. Furthermore, this effect

showed no dose-dependency for either naproxen or rofecoxib. No publications could be found to compare or confirm this finding in the FCA model. The only available evidence for drug effect on static allodynia has been reported Fox *et al.*, who showed significant effect of lumiracoxib and valdecoxib in a rat model of bone cancer pain (19). The effects of COX inhibitors on WBC could be related to the central sensitization via A β -fibres, whereas the static allodynia is related to A β and A δ afferents. Given that the drug levels were sufficiently high to suppress COX-activity, it can be hypothesised that the inhibition of PGE₂ and/or TXB₂ by selective and non-selective COX inhibitors does not completely prevent the signal transduction associated with static allodynia. Another explanation could be the occurrence of very high levels of inducible COX-2, in which case considerably higher dose levels would be required to deactivate the A β and A δ -fibre nociceptors in the dorsal horn of the spinal cord.

On the other hand, all four COX inhibitors showed significant effect on hyperalgesia, with a dose-dependent effect for naproxen and rofecoxib, as indicated by the increase in PP threshold with increasing dose. In addition, no differences were observed in the magnitude of response after single administration at day 7 PID or after chronic twice-daily administration until day 21 PID. These results strongly suggest that COX inhibitors do not have disease modifying properties, nor do they lead to the development of tolerance (figure 7). The main difference between compounds was the duration of the anti-hyperalgesic effect, which could be explained by difference in pharmacokinetics and consequently on the course and extent of COX inhibition. The observed increase in PP threshold, 12 hours post dose after chronic administration of rofecoxib in post- en pre-emptively treated rats (figure 8), is likely to be related to rofecoxib pharmacokinetics. Neither naproxen nor ketorolac showed any residual effect at 12 hours post dose, as compared to single administration. Rofecoxib is subject to enterohepatic recycling in rats, which may lead to multiple peaks in the plasma concentration-time profile and increased drug exposure following chronic administration (19;20). The discrepancies in the drug response profile for different behavioural endpoints corroborate our initial concerns about the need to carefully consider the specificity and selectivity of measures used pre-clinically to evaluate and differentiate COX inhibitors. In addition, the lack of evidence for disease modifying properties following chronic dosing raises the question about the relevance of differences in histology markers at cellular and tissue level, as compared to changes in physiological mediators on the transduction pathways for pain perception.

Biomarkers of inflammation and pharmacology. Whole blood concentrations of PGE₂ and TXB₂ showed no significant variation over time in placebo-treated animals. This finding can be partly explained by the limited repercussion of FCA-induced inflammatory response. In contrast to systemic levels, PGE₂ is increased and COX-2 protein is significantly up-regulated at the site of inflammation and spinal cord (4;21). In fact, based on physiological mechanisms and taking into account the fast turnover of PGE₂, one would not expect to observe increased PGE₂ levels in the systemic circulation. Moreover, *ex vivo* LPS-induced PGE₂ production by monocytes obscures the detection of changes in PGE₂ concentrations caused by the inflammatory response associated with FCA inoculation. As well-established in previous investigations, complete inhibition of PGE₂ and TXB₂ was attained

following administration of efficacious doses of non-selective COX inhibitors (naproxen, diclofenac and ketorolac). Paradoxically, at the measured sampling times we have observed limited inhibition of PGE₂ and TXB₂ after administration of the selective COX-2 inhibitor (rofecoxib) (22). These results can be explained by the sampling schedule used for the biomarkers, which occurred three hours after peak concentrations. For COX inhibitors, maximum inhibition of PGE₂ and TXB₂ is observed at the time of maximum plasma concentration. Moreover, in a recent article by Giuliano *et al.*, it was demonstrated that in rats PGE₂ is produced by COX-2 and COX-1 (23). In contrast to non-selective compounds, only COX-2 activity is suppressed by a selective COX-2 inhibitor and COX-1 is still able to produce PGE₂. Based on *in vitro* data of rofecoxib in rats (unpublished observations) we found that even very high concentrations of rofecoxib do not inhibit PGE₂ production beyond 70%, which is in line with the current results *in vivo*. We also discard any potential issues with the enzyme immunoassay or cross-reactivity with rofecoxib, which could mask the inhibition of whole blood PGE₂.

Any effort to correlate the differences in pharmacological properties of COX inhibitors with behavioural measures of analgesia will benefit from understanding of the mechanisms underlying the inhibition of cyclo-oxygenase. Such developments are however very scarce. Recently, Matson *et al.* showed how the reduction of spontaneous activity by adjuvant (RSAA) can be used as a measure of the effect of different analgesic drugs in rats with inflamed knee joints. An advantage of this new behavioural model is that it does not use an evoked stimulus to measure hypersensitivity. Yet again, the authors disregard the sensitivity of the endpoint in relation to the time course of drug effect and pay no attention to how their findings correlate with the underlying pharmacological activity (24).

Our findings in the FCA model clearly demonstrate that the behavioural endpoints associated with static allodynia and touch sensitivity are insensitive to COX-inhibition or lack sensitivity to varying degree of COX-inhibition. Furthermore, the results for rofecoxib suggest differential systemic effects between non-selective and selective COX-2 inhibitors, raising questions about the relevance of drug distribution (biophase equilibration) as well as the role of central inhibition of COX-2, compared to drug action at the site of inflammation. In fact, Warner *et al.* have hypothesised that differences in protein binding kinetics can alter the selectivity of competitive inhibitors of COX *in vivo*; implying that drug selectivity in one body compartment may not be taken as evidence of selectivity and potency at the site of action (25). Irrespective of the answers to these questions, our findings show that only hyperalgesia, as assessed by changes in PP threshold, seems to reflect differences in systemic pharmacokinetics rather than quantitatively differentiate the pharmacodynamic effects of selective and non-selective COX inhibitors.

Recommendations for in vivo screening of COX inhibitors. Our results support the use of PP threshold in the FCA model as the only suitable behavioural measure in the screening of COX inhibitors. Changes in PP threshold seem to reflect the anti-hyperalgesic properties of these compounds with enough sensitivity to enable the estimation of a dose-exposure-response curve.

However, allometric scaling of behavioural responses based on weight differences across species will not provide accurate estimates of a clinically effective and safe dose range. The rationale for dose selection in humans must also consider the extent and duration of drug effect on both biomarkers PGE₂ and TXB₂. In fact, a mechanism-based modelling approach is required to further characterise the relationship between pharmacokinetics, biomarker inhibition and analgesic effect.

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