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Cyclooxygenase selectivity of non-steroid anti-inflammatory drugs in humans: ex vivo evaluation

Francesco Giuliano [a], José Geraldo P. Ferraz [b], Renata Pereira [c], Gilberto de Nucci [c,d], Timothy D. Warner [a,*]

[a] *Department of Cardiac, Vascular and Inflammation Research, The William Harvey Research Institute, St. Bartholomew's and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1M 6BQ, UK*

[b] *Discipline of Gastroenterology, Department of Internal Medicine, Faculty of Medical Sciences, State University of Campinas, Rua Carlos Chagas 420, 13081-970 Campinas, Brazil*

[c] *Department of Pharmacology, Faculty of Medical Sciences, State University of Campinas, Rua Carlos Chagas 420, 13081-970 Campinas, Brazil*

[d] *Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, Av. Prof. Lineu Prestes 1524, 05508-900 São Paulo, Brazil*

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Abstract

We have recently described a novel assay to assess ex vivo the activity and selectivity on cyclooxygenase-1 and -2 (EC 1.14.99.1) of non-steroid anti-inflammatory drugs (NSAID) administered to rats [Br. J. Pharmacol. 126 (1999) 1824.]. Here, we have extended these studies to humans. Healthy male volunteers were given orally one of the following drugs (mg) for 5 days: etodolac (200 or 400 b.i.d.), meloxicam (7.5 or 15 q.d.), nimesulide (100 or 200 b.i.d.), nabumetone (500 or 1000 b.i.d.) or naproxen (500 b.i.d.). Blood samples were withdrawn from the volunteers before and up to 24 h after the last dose. Plasma obtained from the blood was tested for its ability to inhibit prostanoid formation in interleukin-1β-treated A549 cells (cyclooxygenase-2 system) and human washed platelets (cyclooxygenase-1 system). Plasma from etodolac-treated subjects demonstrated a slight selectivity towards the inhibition of cyclooxygenase-2. This effect was more prominent in plasma from subjects receiving meloxicam or nimesulide. Plasma from nabumetone-treated subjects showed no or little selectivity towards cyclooxygenase-1 depending on the dose of drug administered, while plasma taken from subjects receiving naproxen was more active at inhibiting cyclooxygenase-1 than cyclooxygenase-2. In conclusion, we have demonstrated that this assay can be used to assess ex vivo the relative activity against cyclooxygenase-1 and cyclooxygenase-2 of NSAIDs consumed by human volunteers. It is to be hoped that data from such systems will aid in our understanding of the relationships between the differential inhibition of cyclooxygenase-1 and cyclooxygenase-2 by NSAIDs and their reported efficacies and (gastrointestinal) toxicities.

Keywords: Cyclooxygenase-1; Cyclooxygenase-2; Nonsteroid anti-inflammatory drug selectivity; Ex vivo evaluation; Etodolac; Meloxicam; Nabumetone; Naproxen; Nimesulide

1. Introduction

Non-steroid anti-inflammatory drugs (NSAID) are the agents of choice for the treatment of a variety of inflammatory conditions. However, their use is often associated with gastrointestinal side effects that range from asymptomatic to severe (see Bjorkman, 1999). For example, it is estimated that 2000–2500 people die each year in the UK due to NSAID-induced ulcer bleeding or perforation (Blower et al., 1997; Tramèr et al., 2000). As it has appeared for NSAIDs that it is their ability to inhibit the constitutive "house-keeping" enzyme, cyclooxygenase-1, that underlies their side effects and inhibition of inducible cyclooxygenase-2 that accounts for their therapeutic effects (Mitchell et al., 1993; Mitchell and Warner, 1999) there has been much interest in determining the cyclooxygenase-selectivity of NSAIDs. As is well known, this concept has fuelled the development of newer, rationally designed cyclooxygenase-2 inhibitors, such as celecoxib and rofecoxib (Mitchell and Warner, 1999). There are, however, also a number of more recently developed NSAIDs such as meloxicam and nimesulide that are selective towards the inhibition of cyclooxygenase-2 in vitro (Warner et al., 1999). However, the demonstration of cyclooxygenase-2-selectivity in in vitro assays still only allows cautious projections of NSAIDs' activities and selectivities in whole

* Corresponding author. Tel.: +44-20-7882-6100; fax: +44-20-7882-6104.

E-mail address: t.d.warner@mds.qmw.ac.uk (T.D. Warner).

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organisms to be made (see Davies, 1997), if not only because NSAIDs have complex and varying pharmacokinetic patterns in vivo. For this reason, we have developed a novel assay to determine ex vivo the activity of NSAIDs given in vivo to rats (Giuliano and Warner, 1999). The results from this study led us to hypothesise that similar ex vivo investigations would be feasible in humans and could help to better understand the correlation between NSAIDs differential inhibition of cyclooxygenase and their reported gastrointestinal toxicity index. Others have previously published analyses of the ex vivo activities of a number of NSAIDs in systems examining the activity of cyclooxygenase-1 and cyclooxygenase-2 present or inducible within the blood samples (Patrignani et al., 1994; Panara et al., 1999; Van Hecken et al., 2000). Here, we have used our alternate approach to examine the activities in our systems of four drugs, etodolac, meloxicam, nabumetone and nimesulide, associated with reports of selectivity towards cyclooxygenase-2 in vitro and/or evidence of a reduced risk of gastrointestinal toxicity (see Lanza, 1993; Bjarnason and Thjodleifsson, 1999; Schoenfeld, 1999). For comparison we included the widely used, classical NSAID, naproxen (see McCarthy, 1999).

2. Material and methods

2.1. Materials

Volunteers were administered etodolac (Lodine®, Wyeth-Ayerst), meloxicam (Mobic®, Boehringer Ingelheim), nabumetone (Reliflex®, SmithKline Beecham), naproxen (Naprosyn®, Hoechst Marion Roussel) or nimesulide (Scaflam®, Schering-Plough) obtained from the pharmacy.

All chemicals and media were obtained from Sigma (Poole, UK) except where otherwise stated.

For the radioimmunoassays, antisera to thromboxane B_2 and prostaglandin E_2 were purchased from Sigma; [^{3}H]-thromboxane B_2 and [^{3}H]prostaglandin E_2 were from Amersham (Little Chalfont, UK).

2.2. Subjects

Nine healthy volunteers aged between 18 and 40 years and weighing 55–90 kg participated in this study. The study was approved by the Ethics Committee of the Faculty of Medical Sciences, State University of Campinas, and was conducted according with the Declaration of Helsinki. The volunteers' health status was determined through their medical history, physical examination, blood chemistry, urinalysis and electrocardiogram. Upper gastrointestinal endoscopy was also performed in order to exclude abnormalities of the gastroduodenal mucosa. Volunteers with normal clinical, laboratorial and endoscopic profiles were included in the study. All volunteers signed a written informed consent prior to entering the study.

Table 1
Drugs and relative doses (mg) given to subjects randomised into two study groups

NSAIDs	Group			
	Low dose	*n*	High dose	*n*
Etodolac	200 b.i.d.	4	400 b.i.d.	5
Meloxicam	7.5 q.d.	4	15 q.d.	4
Nabumetone	500 b.i.d.	4	1000 b.i.d.	5
Nimesulide	100 b.i.d.	3	200 b.i.d.	5
Naproxen	500 b.i.d.			9

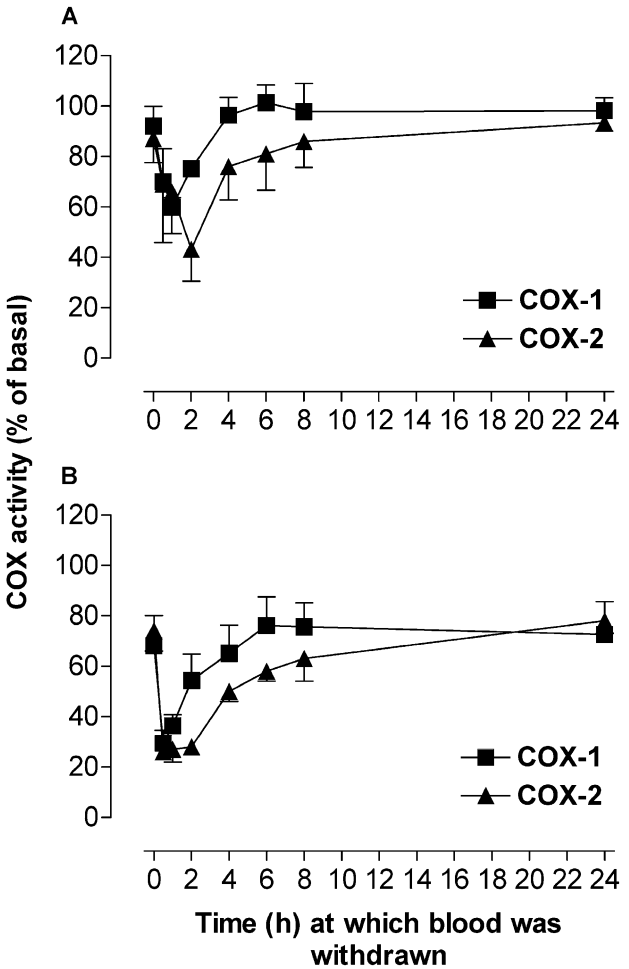

Fig. 1. Effect of plasma from etodolac-treated volunteers on cyclooxygenase-1 and cyclooxygenase-2 activity. Plasma samples from volunteers receiving low (A; 200 mg) or high dose (B; 400 mg) of etodolac produced a small selective inhibition of cyclooxygenase-2 over cyclooxygenase-1. Etodolac showed a peak of activity between 0.5 and 2 h after administration that quickly returned to control levels. Data are expressed as percent of basal and are represented as mean ± S.E.M. ($n = 4–5$).

The volunteers were randomised into two study groups receiving low or high doses of NSAIDs. The full daily dose of drugs was administered as shown in Table 1 for 4 days, on the fifth day all the NSAIDs were only given once. Individuals from each study group were then allowed a 21-day wash out interval before commencement of the next study period. To address concerns expressed by the

Table 2
Area contained within the effect–time curves (AWECs) $t = 0$ h to $t = 8$ h, values obtained from the curves shown in Figs. 1–5. Increasing number indicates increasing inhibition of prostaglandin formation. Comparison of AWECs by unpaired *t*-test were to ascertain drugs' selectivities (cyclooxygenase-1 vs. cyclooxygenase-2) and dose-dependent effects on cyclooxygenase-1 and cyclooxygenase-2 activity

NSAIDs	Dose (mg)	AWECs (% h)		Statistics		
		Cyclooxygenase-1	Cyclooxygenase-2	Cyclooxygenase-1 vs. cyclooxygenase-2	Cyclooxygenase-1 low dose vs. high dose	Cyclooxygenase-2 low dose vs. high dose
Etodolac	200	92 ± 29	230 ± 92	ns	a	ns
	400	301 ± 67	429 ± 30	ns		
Meloxicam	7.5	144 ± 42	393 ± 74	a	ns	ns
	15	215 ± 60	481 ± 32	b		
Nabumetone	500	173 ± 63	242 ± 53	ns	a	ns
	1000	401 ± 35	162 ± 62	b		
Nimesulide	100	103 ± 23	612 ± 4	c	a	ns
	200	169 ± 14	613 ± 19	c		
Naproxen	500	768 ± 16	485 ± 36	c	–	–

ns = not significant. a = $P < 0.05$, b = $P < 0.01$, c = $P < 0.001$.

Ethics Committee, lansoprazole (Zoton®, Lederle) 30 mg q.d. was co-administered with the test drugs in order to reduce the chances of gastrointestinal disturbances or damage.

On the first day of each study period, blood samples (15 ml) were collected in tri-sodium citrate (10 mM, final) from all volunteers prior to the administration of the drugs. These samples will be referred to as "basal". On the fifth day, blood samples were collected via a butterfly cannula inserted in a convenient forearm vein immediately before (0) and 0.5, 1, 2, 4, 6, 8 and 24 h following the final single administration of the drugs. Blood samples were spun and the plasma obtained snap frozen. Plasma samples were stored at −80 °C until further analysis.

2.3. Cell culture

A549, a human epithelial carcinoma cell line (ECACC Ref. No. 86012804), expresses cyclooxygenase-2 when exposed to interleukin-1β (Mitchell et al., 1994). Production of prostaglandin E_2 by this cell line can therefore be used as an index of cyclooxygenase-2 activity. A549 cells were maintained in a humidified atmosphere of 5% CO_2–95% air at 37 °C and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum. For the experimental procedures, cells were seeded into 96-well plates and grown to confluence (approximately 5×10^4 cells/well) before use. In order to induce cyclooxygenase-2 expression, cells were incubated for 24 h in fresh DMEM supplemented with 10% foetal bovine serum and interleukin-1β (R&D Systems, Oxon, UK) 10 ng ml^{-1}. Before the experimental procedure, the medium was replaced with 50 μl/well fresh DMEM plus Ca^{2+}-free modified Krebs–Ringer solution (4:1, v/v; see below) at 37 °C.

2.4. Washed platelets

The production of thromboxane B_2 by platelets was used as an index of cyclooxygenase-1 activity. Blood from

Table 3
Area contained within the effect–time curves (AWECs) $t = 0$ h to $t = 24$ h, values obtained from the curves shown in Figs. 1–5. Increasing number indicates increasing inhibition of prostaglandin formation. Comparison of AWECs by unpaired *t*-test were to ascertain drugs' selectivities (cyclooxygenase-1 vs. cyclooxygenase-2) and dose-dependent effects on cyclooxygenase-1 and cyclooxygenase-2 activity

NSAIDs	Dose (mg)	AWECs (% h)		Statistics		
		Cyclooxygenase-1	Cyclooxygenase-2	Cyclooxygenase-1 vs. cyclooxygenase-2	Cyclooxygenase-1 low dose vs. high dose	Cyclooxygenase-2 low dose vs. high dose
Etodolac	200	126 ± 129	394 ± 173	ns	a	a
	400	755 ± 204	906 ± 126	ns		
Meloxicam	7.5	340 ± 115	1163 ± 223	b	ns	ns
	15	565 ± 187	1266 ± 145	a		
Nabumetone	500	403 ± 169	688 ± 238	ns	b	ns
	1000	1133 ± 140	393 ± 186	b		
Nimesulide	100	219 ± 79	1356 ± 70	c	ns	ns
	200	270 ± 85	1508 ± 131	c		
Naproxen	500	2083 ± 221	1335 ± 149	c	–	–

ns = not significant. a = $P < 0.05$, b = $P < 0.01$, c = $P < 0.001$.

healthy volunteers, who had not taken NSAIDs for at least 2 weeks, was collected by venepuncture into plastic tubes containing tri-sodium citrate (10 mM, final). The blood was centrifuged at $200 \times g$ for 7 min to produce platelet rich plasma. Prostacyclin (300 ng ml^{-1}) was then added to the platelet rich plasma to prevent platelet aggregation, followed by centrifugation at $1000 \times g$ for 15 min to sediment the platelets. The resulting supernatant was removed and replaced with an equal volume of Ca^{2+}-free modified Krebs–Ringer solution at 37 °C (10 mM HEPES, 20 mM $NaHCO_3$, 120 mM NaCl, 4 mM KCl, 2 mM Na_2SO_4, 0.1% glucose, 0.1% bovine serum albumin). The pellet was gently resuspended and further prostacyclin (300 ng ml^{-1}) added. The platelets were pelleted again and resuspended in Ca^{2+}-free modified Krebs–Ringer buffer at 37 °C to match one-fourth of the initial plasma volume. Thirty minutes later, the platelet suspension was diluted in DMEM (1:5, v/v) supplemented with 10% foetal bovine serum and plated into 96-well plates (50 μl/well).

2.5. Evaluation of NSAIDs activity on cyclooxygenase-1 and cyclooxygenase-2

To assay NSAID activity in plasma collected from the volunteers, 50 μl of plasma was added to medium bathing either pre-induced A549 cells or washed platelets. After incubation for 30 min at 37 °C, calcium ionophore A23187 (50 μM) was added as a common stimulus and the cells or platelets incubated for a further 15 min at 37 °C. At the end of the incubation, plates containing the platelet suspension were centrifuged for 5 min at $1500 \times g$ (4 °C) and the supernatant removed and snap frozen until analysis by radioimmunoassay. Medium from A549 plates was also removed and frozen. The activity of different plasma series from single volunteers was measured on the same experimental day. Each set of plasma samples was used on only one occasion.

2.6. Data analysis

Data is presented as mean ± S.E.M. The degree of cyclooxygenase-1 and cyclooxygenase-2 inhibition caused by plasma samples was calculated as a percentage of the activity measured in control wells (basal). The areas within the effect–time curves (AWECs) were calculated by GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA) using the trapezoid rule; i.e. the area contained by each curve is made up of a series of trapezoids (because data points are connected with straight lines) so the sum of the trapezoid areas equals the area contained by the curve. Larger AWECs correspond to greater levels of COX inhibition. Statistical differences between AWECs were assessed by unpaired t test, as appropriate. A P value of less than 0.05 was considered statistically significant. All analyses, as for AWEC, were performed using GraphPad Prism 3.0.

3. Results

3.1. Prostanoid production

In the presence of control plasma samples (basal), the production of thromboxane B_2 by platelets (cyclooxygenase-1) and of prostaglandin E_2 by A549 cells (cyclooxygenase-2) was 87 ± 7 and 100 ± 10 ng ml^{-1} ($n = 42$), respectively.

3.2. Evaluation of NSAIDs on cyclooxygenase-1 and cyclooxygenase-2

3.2.1. Etodolac

At $t = 0$ h, plasma taken from volunteers treated with etodolac 400 mg, but not 200 mg, caused a significant reduction in the activities of both cyclooxygenase-1 and cyclooxygenase-2. Following oral dosing with the final

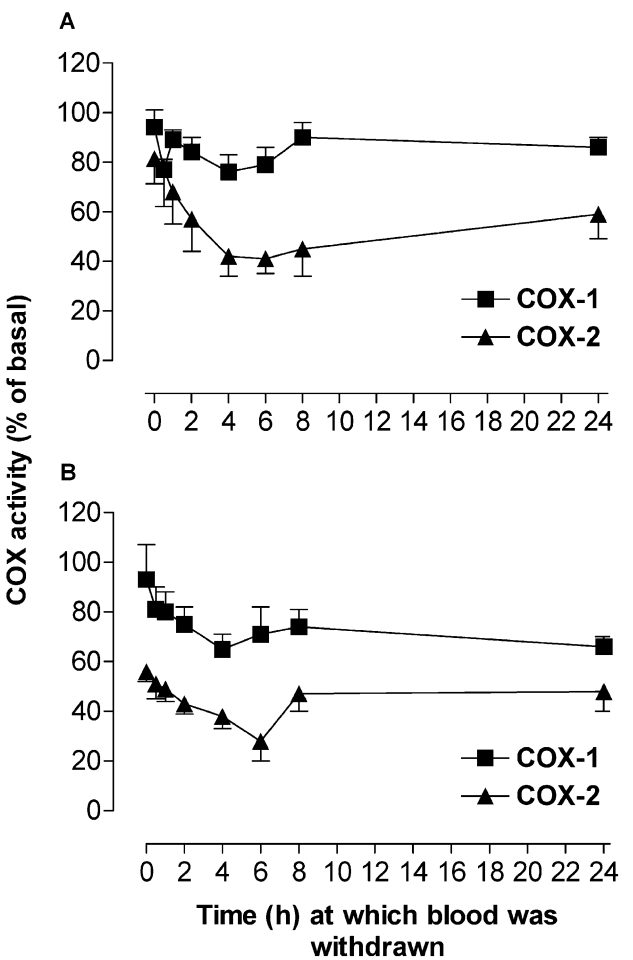

Fig. 2. Effect of plasma from volunteers administered meloxicam 7.5 (A) or 15 mg (B) on the activity of cyclooxygenase-1 and cyclooxygenase-2. Meloxicam produced a selective inhibition of cyclooxygenase-2 over cyclooxygenase-1 both at high and low dose. Plasma from meloxicam-treated volunteers produced a stable inhibition of both cyclooxygenase isoforms during the sampling period. Data are expressed as percent of basal and are represented as mean ± S.E.M. ($n = 4$).

dose of etodolac, plasma samples from patients given either high or low dose etodolac showed an increased inhibitory activity that peaked between 0.5 and 2 h. Activity returned to pre-administration levels at $t = 24$ h. Despite there being a suggestion at both low and high doses of etodolac being selective towards the inhibition of cyclooxygenase-2, but this was not confirmed by statistical analysis (Fig. 1a,b; Tables 2 and 3). Plasma from patients given etodolac 400 mg did, however, produce a stronger inhibition of cyclooxygenase-1 and cyclooxygenase-2 than did plasma from patients receiving etodolac 200 mg (Table 2 and 3).

3.2.2. Meloxicam

Of the drugs tested, meloxicam was the only one to be administered once daily. Plasma from volunteers given meloxicam 7.5 or 15 mg showed a significant selective inhibition of cyclooxygenase-2 (Fig. 2; Tables 2 and 3). Plasma samples taken at $t = 0$ h from subjects receiving the higher dose of meloxicam (Fig. 2b) also demonstrated notable and selective inhibition of cyclooxygenase-2 relative to cyclooxygenase-1. This observation suggests that 24 h after its fourth administration meloxicam was still present in the blood at inhibitory concentrations. Although this effect was less evident for the lower dose of meloxicam (Fig. 2a), it is worthy of note that the inhibitions of cyclooxygenase-1 or -2 measured at $t = 0$ and $t = 24$ were not significantly different ($P > 0.05$, unpaired t-test).

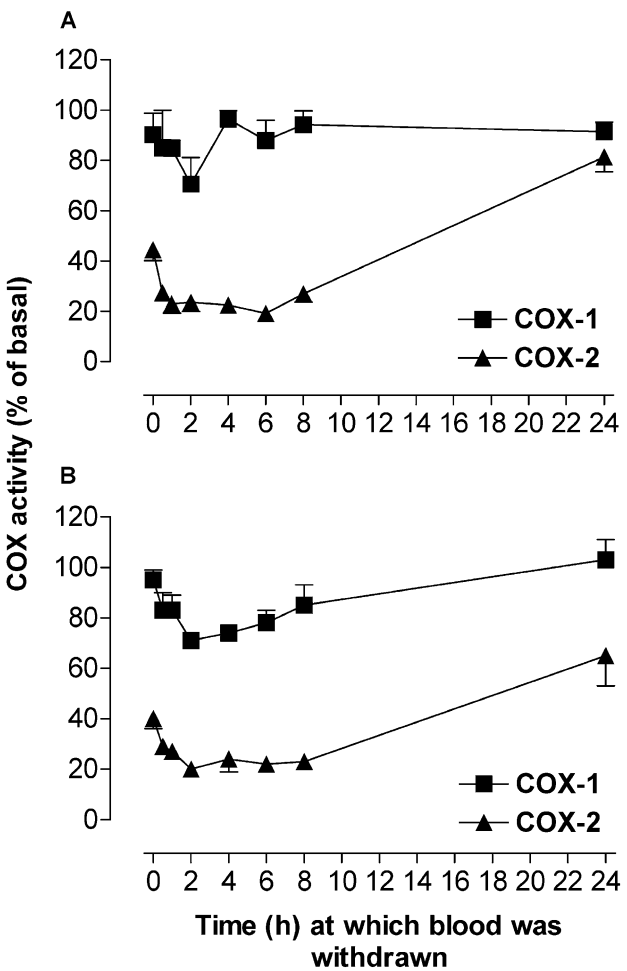

Fig. 3. Effect of plasma from nimesulide-treated volunteers on cyclooxygenase-1 and cyclooxygenase-2 activity. Plasma from volunteers given nimesulide 100 (A) or 200 mg (B) showed a wide selectivity towards the inhibition of cyclooxygenase-2 both at low and high doses. In contrast with prediction from published pharmacokinetics, nimesulide activity was relatively long lived during the sampling period. Data are expressed as percent of basal and are represented as mean ± S.E.M. ($n = 3$–5).

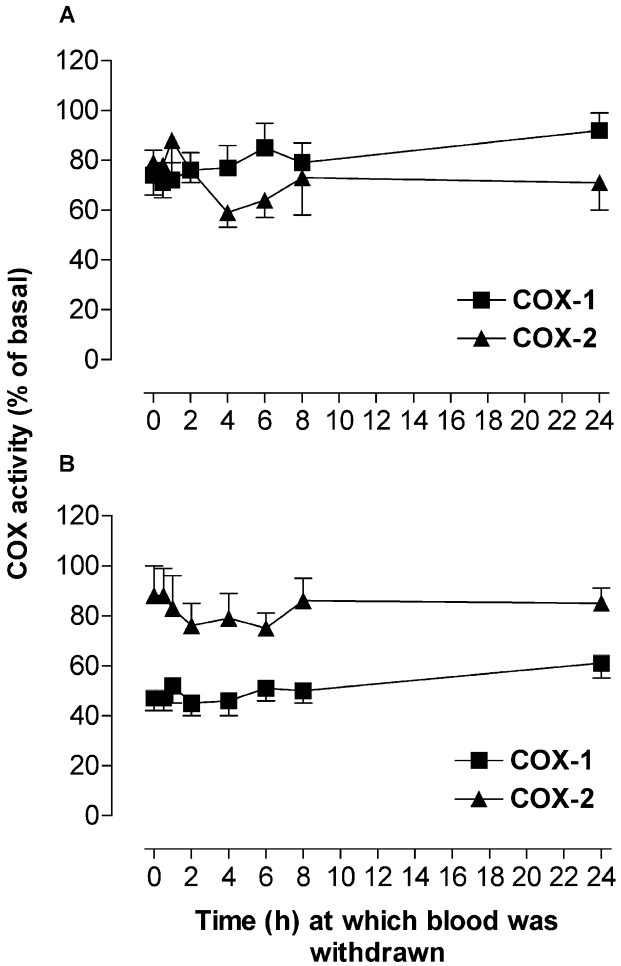

Fig. 4. Effect of plasma from volunteers given nabumetone 500 (A) or 1000 mg (B) on the activity of cyclooxygenase-1 and cyclooxygenase-2. At the low dose nabumetone inhibited weakly and non-selectively both cyclooxygenase-1 and cyclooxygenase-2. However, plasma samples from volunteers given the higher dose of nabumetone produced a stronger inhibition of cyclooxygenase-1. Data are expressed as percent of basal and are represented as mean ± S.E.M. ($n = 4$–5).

3.2.3. Nimesulide

Following administration of nimesulide at 100 mg, plasma from study subjects produced a marked inhibition of cyclooxygenase-2 with little effect on cyclooxygenase-1 (Fig. 3a; Tables 2 and 3). Administration of nimesulide 200 mg produced a stronger inhibition of cyclooxygenase-1 without any significant extra reduction in the activity of cyclooxygenase-2 (Fig. 3b; Tables 2 and 3). Moreover, similarly to what we observed for meloxicam, plasma

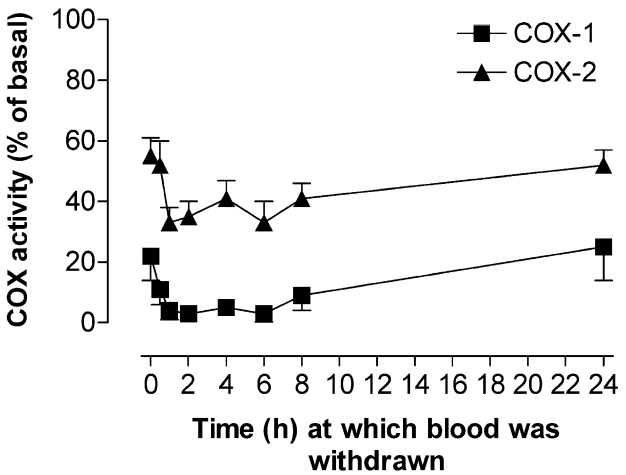

Fig. 5. Effect of plasma from naproxen-treated volunteers on cyclooxygenase-1 and cyclooxygenase-2. All the subjects included in the study received naproxen 500 mg. Plasma from the volunteers produced a strong inhibition of both cyclooxygenase isoforms with a marked selectivity towards cyclooxygenase-1. Data are expressed as percent of basal and are represented as mean ± S.E.M. ($n = 9$).

collected from volunteers just preceding the final administration of nimesulide ($t = 0$ h) still inhibited cyclooxygenase-2 effectively. Interestingly, plasma samples from nimesulide-treated volunteers produced a stable inhibition of cyclooxygenase-2 for up to 8 h after the administration of the drug.

3.2.4. Nabumetone

Plasma collected from patients given nabumetone 500 mg caused a weak, non-selective inhibition of cyclooxygenase-1 and cyclooxygenase-2 (Fig. 4a). Similarly to nimesulide, administration of the higher dose of nabumetone produced a stronger inhibition of cyclooxygenase-1 without any accompanying reduction in cyclooxygenase-2 activity (Fig. 4b; Tables 2 and 3). Consistent with nabumetone's plasma half-life (see Davies, 1997), the inhibitory activity of plasma from nabumetone-treated subjects remained constant throughout the 24-h sampling period.

3.2.5. Naproxen

Of the drugs tested, naproxen produced the greatest inhibition of cyclooxygenase-1 (Fig. 5; Tables 2 and 3) and at $t = 0$ h was already effective in inhibiting both cyclooxygenase-1 and cyclooxygenase-2. More importantly, following the last administration of naproxen the inhibitory activity in the plasma samples increased sharply such that cyclooxygenase-1 was almost completely inhibited by plasma samples collected at $t = 1$ h.

4. Discussion

Here, we have applied a novel procedure for the ex vivo assessment of the activity and selectivity of NSAIDs given to humans. This assay differs from other ex vivo procedures (e.g. Patrignani et al., 1994; Panara et al., 1999; van Hecken et al., 2000) in that it reports directly the inhibitory activity present in the plasma at the time of sampling only. This novel approach also lessens the operational complexity that may deter researchers from measuring cyclooxygenase-2 inhibition and/or favour the assessment of only a small number of blood samples. In fact, plasma samples can be easily collected during a clinical trial, stored, and later assessed for their activity on cyclooxygenase-1 and/or cyclooxygenase-2.

In our study, we chose to include those NSAIDs that, in recent years, have been suggested to possess an improved gastrointestinal safety profile over traditional NSAIDs (see Lanza, 1993; Bjarnason and Thjodleifsson, 1999; Schoenfeld, 1999), namely etodolac, meloxicam, nimesulide and nabumetone. We also included naproxen, as this drug is often used as a comparator in clinical trials and also because its gastrointestinal safety profile is well documented. It is worthy of note that all the drugs used in this study were administered at recommended clinical doses. In accordance with its use in clinical practice, meloxicam was administered only once daily, which is consistent with its circulating half-life of approximately 20 h (see Davies and Skjodt, 1999) to which our results correlated well. More importantly, in our experiments, meloxicam produced a selective inhibition of cyclooxygenase-2 as predicted by our previous in vitro analyses (Warner et al., 1999). Both low and high doses of meloxicam produced a substantial and long lasting inhibition of cyclooxygenase-2 while having much less activity against cyclooxygenase-1. Plasma from patients given meloxicam 7.5 or 15 mg inhibited cyclooxygenase-1 by an average of 15 ± 2% and 25 ± 3%, respectively. The maximum inhibition of COX-1 we found following dosing with meloxicam 15 mg was approximately half that reported by de Meijer et al. (1999) or Van Hecken et al. (2000), despite similarities in the inhibition noted at 24 h. The reason for this difference is not clear, but may relate to our assay being 'snapshot' in character, whereas in these other two studies platelets were used after longer-term exposure in vivo to meloxicam.

Nimesulide demonstrated a high degree of cyclooxygenase-2 selectivity. At both low and high dose, it was poorly active against cyclooxygenase-1 (12 ± 3% and 16 ± 2% inhibition, respectively) while producing a sustained inhibition of cyclooxygenase-2 for up to 8 h after its last administration. Interestingly, pharmacokinetic studies report nimesulide to be short lived within the circulation. However, this apparent incongruity may be explained by the fact that 4-hydroxynimesulide, the only metabolite of nimesulide found in plasma, has anti-inflammatory properties and an elimination half-life that is 1.5–2-fold longer than that of the parent drug (see Bernareggi, 1998). Notably, we found few significant differences between the effects of low and high dose meloxicam or nimesulide (Tables 2 and 3). Possibly, this may be explained by the

observation that 2-fold changes in drug concentration would translate to relatively small shifts along the pharmacological concentration response curves, and such small shifts may well not be detected with the relatively low number of samples involved in our assays.

Similarly to meloxicam and nimesulide, etodolac too inhibited cyclooxygenase-1 and cyclooxygenase-2. However, etodolac only showed a small, statistically not significant, selectivity towards cyclooxygenase-2 at both high and low doses. In contrast to meloxicam and nimesulide, this result does not fully support the predictions based on our previous in vitro data (Warner et al., 1999). Nevertheless, the same in vitro data predict well the properties of nabumetone and naproxen. Nabumetone at the clinically recommended doses used here was found to be a weak inhibitor of both cyclooxygenase-1 and cyclooxygenase-2 with selectivity towards inhibition of cyclooxygenase-1. In detail, plasma collected from volunteers given the low dose of nabumetone (500 mg) equally inhibited both isoforms by approximately 25% throughout the 24-h sampling period. However, at the high dose (1000 mg) nabumetone did not reduce cyclooxygenase-2 activity further, although it did inhibit cyclooxygenase-1 by an additional 25%. At the dose used here, 500 mg, naproxen was a strong and selective inhibitor of cyclooxygenase-1. Plasma samples collected 2 h after the administration of naproxen, inhibited cyclooxygenase-1 and cyclooxygenase-2 activity by $97 \pm 1\%$ and $65 \pm 5\%$, respectively. More importantly, 22 h later, plasma from naproxen-treated volunteers still produced a sustained inhibition of cyclooxygenase-1 and cyclooxygenase-2.

Although a direct comparison between the test drugs is not advisable, we can use our results to investigate the link between cyclooxygenase-2 selectivity and the safety profile of each of the drugs we tested. It is worth pointing out that the selective inhibition of cyclooxygenase-2 represents one of the factors that amongst others contributes to the improved gastrointestinal safety of newly developed NSAIDs. Etodolac and nabumetone exemplify well this point. Both drugs inhibit cyclooxygenase-1 as well as cyclooxygenase-2, and while etodolac shows a very small selectivity towards cyclooxygenase-2, nabumetone is non-selective at the low dose (500 mg) and slightly selective for cyclooxygenase-1 at the high dose (1000 mg). Yet, both nabumetone and etodolac appear to have excellent gastrointestinal safety profiles (Singh et al., 1997). With regard to nabumetone, its good gastrointestinal safety may be explained on the basis of some physiochemical and pharmacokinetic properties such as a non-acidic pro-drug chemical structure, a favourable partitioning coefficient for the pro-drug and the active metabolite (6-methoxy-2-naphthylacetic acid), and an absence of enterohepatic recirculation for the active metabolite (Rainsford, 1999). Etodolac, with the exception of displaying minimal enterohepatic recirculation, does not share many features with nabumetone. It is, therefore, possible that the small selectivity for cyclooxygenase-2 together with a short half-life and minimal enterohepatic recirculation all contribute to etodolac's good gastrointestinal safety (Rothstein, 1998). Meloxicam and nimesulide do not seem to possess physiochemical or pharmacokinetic "advantages" over other NSAIDs, yet they deliver anti-inflammatory activity with a safer gastrointestinal profile. It is, therefore, highly likely that this may be primarily accounted for by the drugs' selectivity for cyclooxygenase-2. Although other factors are bound to contribute to the safety profile of meloxicam, nimesulide and, in general, all NSAIDs, one cannot ignore the correlation existing between differential inhibition of cyclooxygenase-1/-2 and gastrointestinal safety (see Mitchell and Warner, 1999). However, with regard to this last point, it needs to be stressed that previous comparisons of drug safety have not taken into account the actual degree of cyclooxygenase inhibition when drugs are used at recommended doses. Indeed, our results show that the doses of drugs we tested, which are normally used in clinical practice, are not equiactive against either cyclooxygenase-1 or cyclooxygenase-2. It appears more reasonable that only the comparison of equiactive drug dosages would allow the true comparison of drugs' safety (Furst, 1999). To this end, our assay could be of some utility in predicting drugs' in vivo activity and possibly as an additional tool in dose-ranging studies. Indeed, our system may represent an answer to the concern of some authors that is necessary to integrate better clinical pharmacokinetics and pharmacological data (see Davies, 1997; Davies and Anderson, 1997).

We should expand upon the observation we made above, that our assay differs from other ex vivo procedures (e.g. Patrignani et al., 1994; Panara et al., 1999; van Hecken et al., 2000) in that it reports only the cyclooxygenase inhibitory activity present in the plasma at the time of sampling. The difference in assay outcome can be best appreciated by considering two particular examples: a drug that irreversibly inhibits cyclooxygenase (e.g. aspirin) and a drug that inhibits the expression of cyclooxygenase (e.g. an anti-inflammatory steroid). Determining the level of cyclooxygenase-1 activity by looking at the production of thromboxane A_2 in whole blood in standard ex vivo procedures would show that those receiving aspirin as a test drug had a much reduced thromboxane A_2 production. This is accounted for by aspirin's ability to irreversibly inhibit cyclooxygenase-1. However, at the time of sampling the aspirin levels in the plasma may be very low, as aspirin is rapidly metabolised (Patrono et al., 1985). In contrast to the standard procedures our modified assay would supply this direct report, i.e. a lack of inhibitory activity (as we have demonstrated in rats; Giuliano and Warner, 1999). The cyclooxygenase-2 arm of our assay takes place over the same fixed 1 h period as the cyclooxygenase-1 arm, and unlike other ex vivo assays (Patrignani et al., 1994; Panara et al., 1999; van Hecken et al., 2000) there is no requirement for the ex vivo induction of

cyclooxygenase-2 activity. For example, if subjects had ingested a test compound such as an anti-inflammatory steroid and cyclooxygenase-2 activity was determined by the ex vivo induction of prostaglandin E_2 accumulation a false positive could be recorded, because steroids inhibit the induction of cyclooxygenase-2 (Masferrer and Seibert, 1994). In our assay, the drug is not present during the period of cyclooxygenase-2 induction and expression and this source of error is avoided. So, the modified assay we present here shows the level of inhibitory NSAID or metabolite(s) at fixed times—it is providing 'biological pharmacokinetics'.

In conclusion, we have developed a new assay to measure ex vivo the activity and selectivity of NSAIDs following standard dosing in humans. This assay may represent a useful complement for existing systems as it allows simple and direct measure of the relative activity against cyclooxygenase-1 and cyclooxygenase-2 of NSAIDs consumed by human volunteers. The method we have described may also represent an efficient tool for reassessing and comparing the relative potencies of traditional NSAIDs as well as those of newly developed cyclooxygenase-2 inhibitors. On the basis of dosage correction for equipotencies, this system may aid, for example, in the better understanding of the links between inhibition of cyclooxygenase-1 and cyclooxygenase-2 by NSAIDs and their reported gastrointestinal toxicities; although of course our own study did not provide such information because subjects took the test drugs for a relatively short period of time and also received lansoprazole. Furthermore, our assay approach permits the dissociation in time and place of human sample generation and cyclooxygenase activity measurements, making it an excellent adjunct to clinical studies from which plasma samples may be readily available.

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References

Bernareggi, A., 1998. Clinical pharmacokinetics of nimesulide. Clin. Pharmacokinet. 35, 247–274.

Bjarnason, I., Thjodleifsson, B., 1999. Gastrointestinal toxicity of non-steroidal anti-inflammatory drugs: the effect of nimesulide compared with naproxen on the human gastrointestinal tract. Rheumatology 38, (Suppl. 1), 24–32.

Bjorkman, D.J., 1999. Current status of nonsteroidal anti-inflammatory drug (NSAID) use in the United States: risk factors and frequency of complications. Am. J. Med. 107, 3S–8S.

Blower, A.L., Brooks, A., Fenn, G.C., Pearce, M.Y., Morant, S., Bardhan, K.D., 1997. Emergency admissions for upper gastrointestinal disease and their relation to NSAID use. Aliment. Pharmacol. Ther. 11, 283–291.

Davies, N.M., 1997. Clinical pharmacokinetics of nabumetone. The dawn of selective cyclo-oxygenase-2 inhibition? Clin. Pharmacokinet. 33, 404–416.

Davies, N.M., Anderson, K.E., 1997. Clinical pharmacokinetics of naproxen. Clin. Pharmacokinet. 32, 268–293.

Davies, N.M., Skjodt, N.M., 1999. Clinical pharmacokinetics of meloxicam. A cyclo-oxygenase-2 preferential nonsteroidal anti-inflammatory drug. Clin. Pharmacokinet. 36, 115–126.

De Meijer, A., Vollaard, H., De Metz, M., Verbruggen, B., Thomas, C., Novakova, I., 1999. Meloxicam, 15 mg/day, spares platelet function in healthy volunteers. Clin. Pharmacol. Ther. 66, 425–430.

Furst, D.E., 1999. Pharmacology and efficacy of cyclooxygenase (COX) inhibitors. Am. J. Med. 107, 18S–22S.

Giuliano, F., Warner, T.D., 1999. Ex vivo assay to determine the cyclooxygenase selectivity of non-steroidal anti-inflammatory drugs. Br. J. Pharmacol. 126, 1824–1830.

Lanza, F.L., 1993. Gastrointestinal toxicity of newer NSAIDs. Am. J. Gastroenterol. 88, 1318–1323.

Masferrer, J.L., Seibert, K., 1994. Regulation of prostaglandin synthesis by glucocorticoids. Receptor 4, 25–30.

McCarthy, D.M., 1999. Comparative toxicity of nonsteroidal anti-inflammatory drugs. Am. J. Med. 107, 37S–46S.

Mitchell, J.A., Warner, T.D., 1999. Cyclo-oxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. Br. J. Pharmacol. 128, 1121–1132.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J., Vane, J.R., 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc. Natl. Acad. Sci. U. S. A. 90, 11693–11697.

Mitchell, J.A., Belvisi, M.G., Akarasereenont, P., Robbins, R.A., Known, O.-J., Croxtall, J., Barnes, P.J., Vane, J.R., 1994. Induction of cyclooxygenase-2 by cytokines in human pulmonary epithelial cells: regulation by dexamethasone. Br. J. Pharmacol. 113, 1008–1014.

Panara, M.R., Renda, G., Sciulli, M.G., Santini, G., Di Giambernardino, M., Rotondo, M.T., Tacconelli, S., Seta, F., Patrono, C., Patrignani, P., 1999. Dose-dependent inhibition of platelet cyclooxygenase-1 and monocyte cyclooxygenase-2 by meloxicam in healthy subjects. J. Pharmacol. Exp. Ther. 290, 276–280.

Patrignani, P., Panara, M.R., Greco, A., Fusco, O., Natoli, C., Iacobelli, S., Cipollone, F., Ganci, A., Créminon, C., Maclouf, J., Patrono, C., 1994. Biochemical and pharmacological characterisation of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. J. Pharmacol. Exp. Ther. 271, 1705–1712.

Patrono, C., Ciabattoni, G., Patrignani, P., Pugliese, F., Filabozzi, P., Catella, F., Davi, G., Forni, L., 1985. Clinical pharmacology of platelet cyclooxygenase inhibition. Circulation 72, 1177–1184.

Rainsford, K.D., 1999. Profile and mechanisms of gastrointestinal and other side effects of nonsteroidal anti-inflammatory drugs (NSAIDs). Am. J. Med. 107, 27S–35S.

Rothstein, R., 1998. Safety profiles of leading nonsteroidal anti-inflammatory drugs. Am. J. Med. 105, 39S–43S.

Schoenfeld, P., 1999. Gastrointestinal safety profile of meloxicam: a meta-analysis and systematic review of randomized controlled trials. Am. J. Med. 107, 48S–54S.

Singh, G.H., Terry, R., Ramey, D.R., Fries, J.F., Triadafilopoulos, G., Halpern, J., Brown, B.W., 1997. Comparative GI toxicity of NSAID. Arthritis Rheum. 40, S115 (suppl.).

Tramèr, M.R., Moore, R.A., Reynolds, D.J.M., McQuay, H.J., 2000. Quantitative estimation of rare adverse events which follow a biological progression: a new model applied to chronic NSAID use. Pain 85, 169–182.

Van Hecken, A., Schwartz, J.I., Depre, M., De Lepeleire, I., Dallob, A.,

Tanaka, W., Wynants, K., Buntinx, A., Arnout, J., Wong, P.H., Ebel, D.L., Gertz, B.J., De Schepper, P.J., 2000. Comparative inhibitory activity of rofecoxib, meloxicam, diclofenac, ibuprofen, and naproxen on COX-2 versus COX-1 in healthy volunteers. J. Clin. Pharmacol. 40, 1109–1120.

Warner, T.D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J.A., Vane, J.R., 1999. Nonsteroid drug selectivities for cyclo-oxygenase-1 rather than cyclo-oxygenase-2 are associated with human gastrointestinal toxicity: a full in vitro analysis. Proc. Natl. Acad. Sci. U.S.A. 96, 7563–7568.