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Ex vivo assay to determine the cyclooxygenase selectivity of non-steroidal anti-inflammatory drugs

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1 In this study we describe experiments to establish *ex vivo* the selectivity of non-steroidal anti-inflammatory drugs (NSAIDs) given *in vivo*.

2 Anaesthetised (Inactin, 120 mg kg^{-1}) male Wistar rats (220–250 g) received an i.v. dose of one of the following compounds (dose mg kg^{-1}): aspirin (20), diclofenac (3), L-745,337 (30), nimesulide (15), salicylate (20), sulindac (10). Blood samples were taken before and up to 6 h after dosing and the plasma obtained from it was tested for its ability to inhibit prostanoid formation in IL-1β-treated A549 cells (COX-2 system) and human washed platelets (COX-1 system). For control the same compounds were also added directly to the assay systems.

3 All drugs, except sodium salicylate, inhibited COX-1 and COX-2 when added directly to the test systems. Plasma from aspirin-treated rats was without effect on either COX-1 or COX-2, consistent with the rapid *in vivo* metabolism to salicylate. Conversely, plasma from sulindac-treated rats inhibited COX-1 and COX-2 with potencies according with *in vivo* metabolism to sulindac sulphide. Diclofenac was COX-1/2 non-selective when tested *in vitro*, but a slightly preferential inhibitor of COX-2 when tested *ex vivo*. Nimesulide was confirmed as preferential inhibitor of COX-2 both *in vitro* and *ex vivo*. L-745,337 was a selective COX-2 inhibitor when tested *in vitro* or *ex vivo*.

4 In conclusion, our experiments show clearly (a) NSAIDs inactivation, (b) activation of pro-drugs, and (c) NSAIDs selectivity. Our assay provides useful information about the selectivity of NSAIDs that could be extended by the analysis of plasma samples taken from humans similarly treated with test drugs.

Keywords: COX-1; COX-2; NSAIDs selectivity; NSAIDs inactivation; NSAIDs activation; *ex vivo* evaluation

Abbreviations: COX, cyclooxygenase; DMEM, Dulbecco's modified Eagle medium; FBS, foetal bovine serum; IL-1β, interleukin-1β; NSAIDs, non-steroidal anti-inflammatory drugs; PG, prostaglandin; PRP, platelet rich plasma; TX, thromboxane

Introduction

Prostanoids are formed initially by the conversion of arachidonic acid to a common intermediate, prostaglandin (PG) H_2, by the enzyme prostaglandin endoperoxide synthase (PGHS) also known as cyclooxygenase (COX) (see Vane *et al*., 1998). This enzyme carries out two distinct reactions. The cyclooxygenase activity forms PGG_2 from arachidonic acid, while the peroxidase activity of PGHS converts PGG_2 to PGH_2. The subsequent formation of particular prostanoids depends on the actions of specific isomerases acting upon PGH_2 (see Hershman, 1996). Following the discovery in the early 1990s of an inducible isoform of COX (Xie *et al*., 1991; Kujubu *et al*., 1991; Wong *et al*., 1991; O'Banion *et al*., 1991) it is now known that COX exists in at least two isoforms. A constitutive 'house-keeping' enzyme, COX-1, which is responsible for homeostatic functions (Moncada *et al*., 1976; Whittle *et al*., 1980) and an inducible isoform, COX-2, associated with mitogenic events and inflammatory states (Raz *et al*., 1988; Fu *et al*., 1990; Masferr *et al*., 1990; O'Banion *et al*., 1992; Lee *et al*., 1992; Xie *et al*., 1992). As a refinement of the mechanism proposed by Vane in 1971 it has therefore been suggested that inhibition of COX-2 underlies the therapeutic efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) whilst inhibition of COX-1 underlies their side effects (Mitchell *et al*., 1993). The potential therapeutic benefit of COX-2 selective inhibitors has encouraged the development of numerous *in vitro* test systems in which to define and develop both classical and novel NSAIDs. The numerous *in vitro* test systems may be classed into three main groups, purified/recombinant enzymes (Meade *et al*., 1993), cultures of intact cells (Cromlish *et al*., 1996) and human whole blood (Patrignani *et al*., 1994; 1997). Although all of these test systems have provided information about the abilities of NSAIDs to inhibit COX-1 and -2, they also have shortcomings that limit their application to the preliminary testing of a drug. For example, assays employing broken cells cannot account for cell penetration and intracellular drug distribution. Many cell-based assay systems use non-human cells and fail to account for plasma binding. Human blood-based assays, therefore, have clear advantages. They do, however, still have drawbacks. In particular, different incubation times for assays of COX-1 and COX-2 activity reduce the ability of these systems to predict with accuracy the *in vivo* efficacy and selectivity of NSAIDs.

Here, therefore, we have investigated the possibility of establishing an assay to assess *ex vivo* the activity of NSAIDs given *in vivo*. To achieve this goal we injected rats intravenously with NSAIDs and then withdrew blood at different time points. The NSAID activity in the plasma was determined by its ability to inhibit COX-1 and COX-2 in, respectively, human washed platelets and IL-1β-treated A549 cells. The NSAIDs used in this study for the *in vivo* experiments were chosen to represent the four functionally

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defined classes of NSAIDs: COX-1 selective (aspirin, salicylate and sulindac), COX-1/-2 non-selective (diclofenac), COX-2 preferential inhibitors (nimesulide) and COX-2 selective compounds (L-745,337). From this assay system we have obtained good predictive data with regard to the activities of NSAIDs and novel COX-2 selective compounds.

Methods

In vivo

Male Wistar rats (220–250 g; Tuck, Rayleigh, U.K.) were anaesthetised by intraperitoneal injection of thiobutabartibal sodium (Inactin; 120 mg kg^{-1}, i.p.; RBI, Natick, U.S.A.). Body temperature was maintained at 37°C by means of a homeothermic blanket connected to a rectal probe. The trachea was cannulated to facilitate ventilation. The right carotid artery was cannulated and connected to a pressure transducer (type 4-422-0001, Transamerica Instruments) for the monitoring of systemic blood pressure displayed on a Graphtec Linearcorder. The jugular vein was also cannulated to allow injection of drugs and infusion of saline. Animals were left for 30 min following surgery to stabilise after which time a control plasma sample was withdrawn ($t=-60$). One hour later ($t=0$) the rats received an i.v. bolus of aspirin (20 mg kg^{-1}; $n=4$), diclofenac (3 mg kg^{-1}; $n=4$), L-745,337 (30 mg kg^{-1}; $n=5$), nimesulide (15 mg kg^{-1}; $n=4$), sodium salicylate (20 mg kg^{-1}; $n=4$) or sulindac (10 mg kg^{-1}; $n=5$). Blood samples of $\cong 300$ μl were taken from the carotid artery at $t=-60$, 5, 60, 120, 180, 240, 300 and 360 min. The samples were centrifuged 12000 g for 2 min at 4°C and the plasma removed supplemented with heparin (15 U ml^{-1}, National Veterinary Supplies, Stoke on Trent) and snap frozen in liquid N_2. Three hundred μl of warm saline was injected i.v. after each withdrawal of blood. At the end of the time course, animals were killed by an overdose of anaesthetic. Individual drugs were administered to rats on at least 4 different study days.

Cell culture

A549, a human epithelial carcinoma cell line (ECACC Ref. No. 86012804), expresses COX-2 when exposed to IL-1β (Mitchell *et al*., 1994). Production of PGE_2 by this cell line can therefore be used as an index of COX-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; Sigma, Poole, U.K.) supplemented with 10% foetal bovine serum (FBS; Sigma, Poole, U.K.). For the experimental procedures, cells were seeded into 96-well plates and grown to confluence before use. In order to induce COX-2 expression, cells were incubated for 24 h in fresh DMEM supplemented with 10% FBS and IL-1β 10 ng/ml. Before the experimental procedure, the medium was replaced with fresh DMEM: Ca^{2+}-free modified Krebs-Ringer solution (see below) (4 : 1, v/v) at 37°C.

Washed platelets

The production of thromboxane (TX) B_2 by platelets was used as index of COX-1 activity. Blood from healthy volunteers, who had not taken NSAIDs for at least two weeks, was collected by venepuncture into gelatine-coated (0.1% porcine gelatine in H_2O, 1–3 h at 37°C) plastic tubes containing tri-sodium citrate 3.15% (1 : 9, v/v). The blood was centrifuged at $200\times g$ for 7 min to produce platelet rich plasma (PRP). Prostacyclin (300 ng ml^{-1}) was then added to the PRP 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 half of the initial plasma volume. Thirty minutes later the platelet suspension was diluted 1 : 5 in DMEM supplemented with 10% FBS and plated into gelatine-coated 96-well plates (100 μl $well^{-1}$).

Evaluation of NSAIDs activity on COX-1 and COX-2

To assay NSAIDs activity in plasma collected from the rats, 10 μl of plasma was added to medium bathing either pre-induced A549 cells or washed platelets. Concentration response curves to aspirin ($n=4$) or salicylate ($n=4$) (0.1 nM to 1 mM), diclofenac ($n=4$), L-745,337 ($n=5$), nimesulide ($n=4$), sulindac ($n=4$) or sulindac sulphide ($n=5$) (0.01 nM to 0.1 mM) were also constructed on the same culture plates on which the corresponding plasma series were tested. After incubation for 30 min at 37°C, calcium ionophore A23187 (50 μM) was added 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. Each plate of cells and platelets included two sets of control wells, one incubated with vehicle (0.1% dimethyl sulfoxide) the other receiving control plasma withdrawn at $t=-60$. Inhibition of COX-1 and COX-2 by test compounds or plasma samples was calculated as percentage of the activity measured in the corresponding control wells. The effects of vehicle, plasma series and NSAIDs on COX activity were measured in at least three separate determinations (wells) on at least four different experimental days. The same set of plasma samples or drug dilutions were not used on more than one experimental day.

Materials

For the *in vivo* procedure, all the drugs were dissolved in a 5% bicarbonate - 2.5% glucose buffer in H_2O and sonicated until a clear solution or a very fine suspension were obtained. The required amount of drug was dissolved in 1 ml and injected over a period of 100 s. For the *in vitro* experiments, all the drugs were dissolved in DMSO to form stock solutions of 0.1 to 1 M before being further diluted in DMEM.

All compounds used were obtained from Sigma (Poole, U.K.) unless otherwise stated. L-745,337 was a gift from Merck Frosst, Canada. Sulindac sulphide was purchased from Affiniti (Exeter, U.K.). For the radioimmunoassay, antisera to PGE_2 and TXB_2 were obtained from Sigma (Poole, U.K.); [^{3}H]-PGE_2 and [^{3}H]-TXB_2 were purchased from Amersham (Little Chalfont, U.K.). IL-1β was obtained from Genzyme (Kings Hill, U.K.).

Data analysis

Results are expressed as mean ± s.e.m. Concentration response curves were fitted using a sigmoidal regression with variable slope.

Two-way analysis of the variance (ANOVA) was used to evaluate statistical differences between dose response curves. IC_{50} values were compared using the *t*-test for unpaired observations. A one-sample *t*-test was performed to determine significant differences from normalized controls. A *P* value less than 0.05 was considered statistically significant. All analysis and regressions were performed using GraphPad Prism (GraphPad Software, San Diego, CA, U.S.A.).

Results

In vivo

The mean arterial blood pressure of the rats at $t=-70$ and $t=360$ min was, respectively, 94.3 ± 2.2 mmHg and 75 ± 2 mmHg with no significant differences between treatment groups ($P>0.05$, one way ANOVA; $n=25$). A transient increase in blood pressure of 15 ± 5 mmHg, lasting 5–20 min, was recorded after the i.v. injection of each drug. Again, no significant difference between drugs was noted.

Prostanoid production

The stimulated release of PGE_2 by A549 cells in the presence of drug vehicle or control plasma was 60.3 ± 3.1 ng ml^{-1} ($n=30$) and 62.3 ± 3.9 ng ml^{-1} ($n=25$), respectively. Thromboxane B_2 production by washed platelets was 25 ± 2 ng ml^{-1} in the presence of drug vehicle ($n=30$) and 21.2 ± 1.3 ng ml^{-1} in the presence of control plasma ($n=25$).

Evaluation of NSAIDs activity on COX-1 and COX-2

Diclofenac Of the compounds tested, diclofenac was the most potent inhibitor of COX-1 and COX-2, although it was non-selective. Plasma taken from diclofenac-treated rats inhibited both COX-1 and COX-2 with a small but significant selectivity for COX-2 ($P<0.05$, two-way ANOVA) (Table 1, Figure 1a). Notably, the inhibitory activity of the plasma from diclofenac-treated rats decreased throughout the *in vivo* experimental period (Figure 1b).

Nimesulide *In vitro*, nimesulide showed a preferential inhibition of COX-2 (IC_{50} 0.071 μM) over COX-1 (IC_{50} 0.392 μM) (Table 1, Figure 2a). Consistently, in the *ex vivo* assay, nimesulide showed a significant selectivity ($P<0.05$, two-way ANOVA) towards the inhibition of COX-2. Plasma samples from nimesulide-treated rats also demonstrated little change in activity over the 6 h sampling period (Table 1, Figure 2b).

Aspirin and salicylate *In vitro*, aspirin was approximately six times more potent as an inhibitor of COX-1 (IC_{50} 1.88 μM) than COX-2 (IC_{50} 12.34 μM) (Table 1, Figure 3a). However plasma taken from aspirin-treated rats was without any significant effect ($P>0.05$, one sample *t*-test) on the activities of either COX-1 or COX-2 as early as 5 min after administration *in vivo* (Figure 3b). Similarly salicylate, the metabolite of aspirin, was without activity when added directly to the assay systems in concentrations of up to 1 mM (Table 1, Figure 4a), or when plasma from salicylate-treated animals was tested (Figure 4b).

Sulindac and sulindac sulphide Plasma taken from sulindac-treated animals inhibited COX-1 to a markedly greater extent than COX-2 throughout the sampling period (Figure 5b). In a similar fashion, when added directly to the assay systems, sulindac and in particular its active metabolite, sulindac sulphide, inhibited both isoforms of COX with selectivity towards COX-1 (Table 1, Figure 5a and c). Importantly, it should be noted that sulindac sulphide inhibited COX-1 355 times more potently than the parent drug.

L-745,337 The novel COX-2 selective drug L-745,337 clearly showed the greatest selectivity for COX-2 over COX-1 both *in vitro* (1100 fold) (Table 1, Figure 6a) and in the *ex vivo* assay (Figure 6b). In this latter assay, L-745,337 (30 mg kg^{-1}) was

Table 1 IC_{50} values for NSAIDs on COX-1 and COX-2 activity in washed human platelets and A549 cells. I = inactive

	IC_{50} (μM)		
NSAIDs	*COX-1*	*COX-2*	*COX-2/COX-1*
Aspirin	1.88	12.34	6.56
Diclofenac	0.0027	0.0016	0.59
L-745,337	23.45	0.027	0.0011
Nimesulide	0.392	0.071	0.18
Sodium salicylate	I	I	–
Sulindac	13.85	196	14.1
Sulindac sulphide	0.017	0.55	32.35

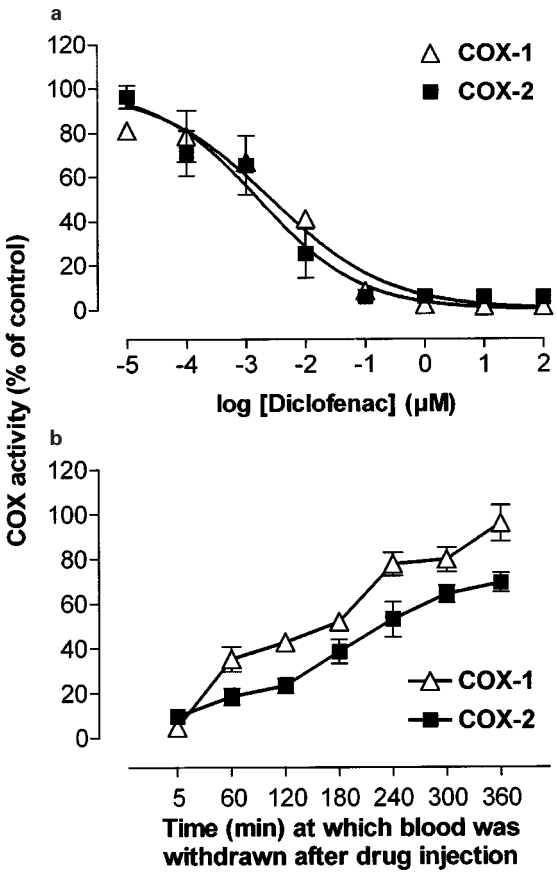

Figure 1 Effect of diclofenac (a) and plasma samples from diclofenac-treated rats (b) on COX-1 and COX-2 activity. Platelets (COX-1 system) and A549 (COX-2 system) cells were exposed to diclofenac or plasma samples for 30 min and then challenged with calcium ionophore A23187 (50 μM) for 15 min. When added directly to the assay system, diclofenac was COX-1/-2 non-selective whereas plasma samples from diclofenac-treated animals showed a small but significant selectivity towards COX-2. Data are expressed as mean ± s.e.m. from three determinations from four separate experimental days.

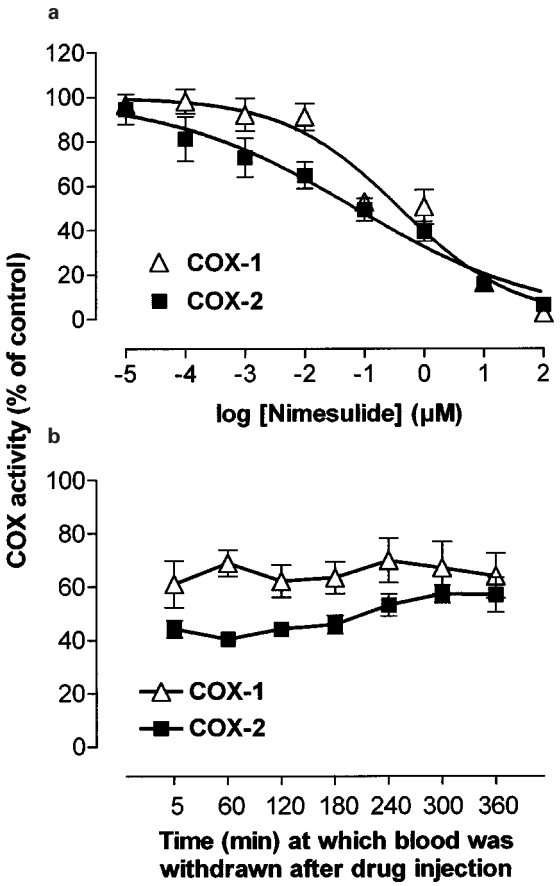

Figure 2 Effect of nimesulide (a) and plasma samples from nimesulide-treated rats (b) on COX-1 and COX-2 activity. Nimesulide showed preferential inhibition of COX-2 both when added directly to the assay system and when plasma samples from nimesulide-treated rats were used. Data are expressed as mean ± s.e.m. from three determinations from four separate experimental days.

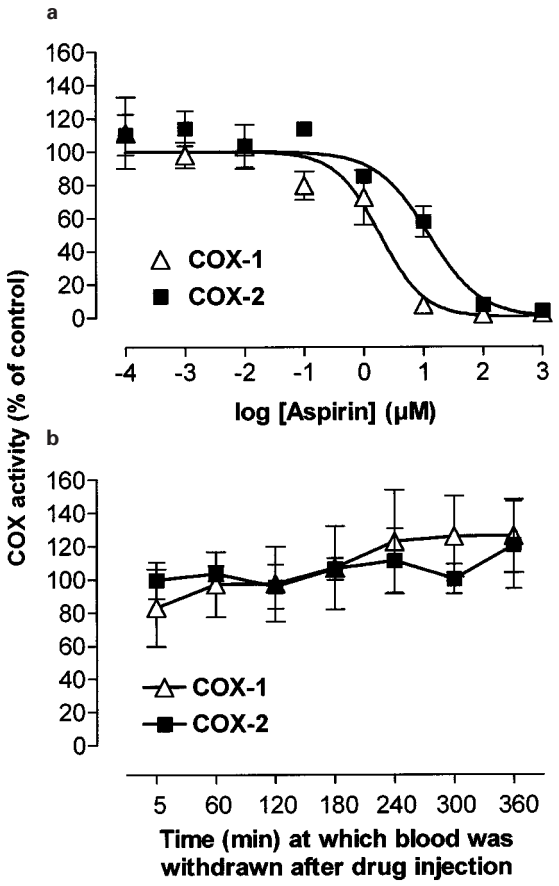

Figure 3 Effect of aspirin (a) and plasma samples from aspirin-treated rats (b) on COX-1 and COX-2 activity. Aspirin added directly to the assay system inhibited COX-1 more potently than COX-2 whereas plasma samples from aspirin-treated animals failed to show any significant activity on both isoforms. Data are expressed as mean ± s.e.m. from three determinations from four separate experimental days.

without any significant effect on COX-1 ($P > 0.05$, one sample *t*-test) whereas it caused a strong inhibition of COX-2.

Discussion

Here we report a novel approach to assessing the activity of NSAIDs against COX-1 and COX-2. Our data demonstrate selectivity and metabolism of both classical NSAIDs and, more importantly, for a novel COX-2 selective compound.

NSAIDs produce their beneficial effects by inhibiting COX-2 and their deleterious effects by inhibiting COX-1 (see Vane *et al*., 1998). Thus, predictive *in vitro* and *in vivo* assays of NSAIDs selectivity for COX-1 vs COX-2 are essential both to rationalize the use of existing NSAIDs and to lead to the production of the next generation of NSAIDs. The most widely used test system for the evaluation of NSAIDs efficacy and selectivity is currently the whole human blood assay. In this system, the clotting of whole human blood and the measurement of the TXB_2 formed is the basis for measuring COX-1 activity whereas the formation of PGE_2 or TXB_2 in blood incubated with bacterial lipopolysaccharide for 5–24 h is the basis for measuring COX-2 activity (Brideau *et al*., 1996; Patrignani *et al*., 1994; 1997; Young *et al*., 1996). This assay has the clear advantage of using easily accessible human COX-producing cells and of taking into account drug binding to plasma proteins. However, the need to induce COX-2 *ex vivo* introduces an important variable which in particular leads to a considerable time discrepancy between the COX-1 and COX-2 assays. Here we have established a test system that avoids this problem. Significantly, it also allows the analysis of not only COX selectivity but also NSAIDs activation and inactivation. Drugs were administered intravenously to limit variations caused by differences in absorption. However, the assay could be applied following oral administration of NSAIDs.

Aspirin added directly to the test systems caused inhibition of COX-1 and COX-2 with IC_{50} values of, respectively, 1.88 and 12.34 μM. Conversely, plasma taken from aspirin-treated rats had no effect on the activity of either COX isoform. This observation is in accordance with aspirin being rapidly hydrolysed in the blood stream to salicylate (Higgs *et al*., 1987) which is a weak inhibitor of COX. Under our assay conditions, where high levels of free arachidonic acid are achieved, these concentrations of salicylate were without effect on COX activity (Mitchell *et al*., 1997).

In contrast to aspirin, plasma from sulindac-treated animals inhibited both COX-1 and COX-2 with selectivity towards COX-1. Analysis of the control concentration response curves indicates that sulindac could only produce such an inhibition of COX-1 and COX-2 at concentrations greater than 0.1 mM. This is greater than that achievable following the dose given

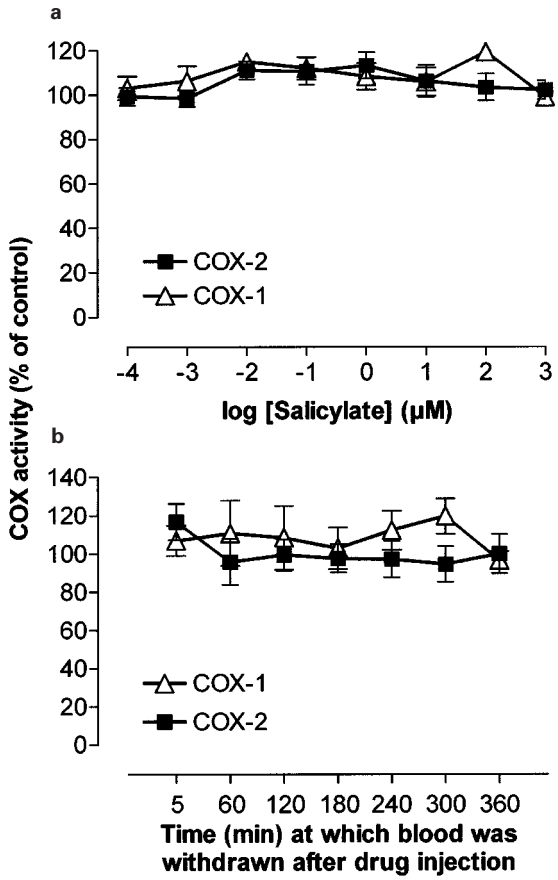

Figure 4 Effect of salicylate (a) and plasma samples from salicylate-treated rats (b) on COX-1 and COX-2 activity. Salicylate failed to show any activity either when added directly to the assay system or when plasma from salicylate-treated animals was used. Data are expressed as mean±s.e.m from three determinations from four separate experimental days.

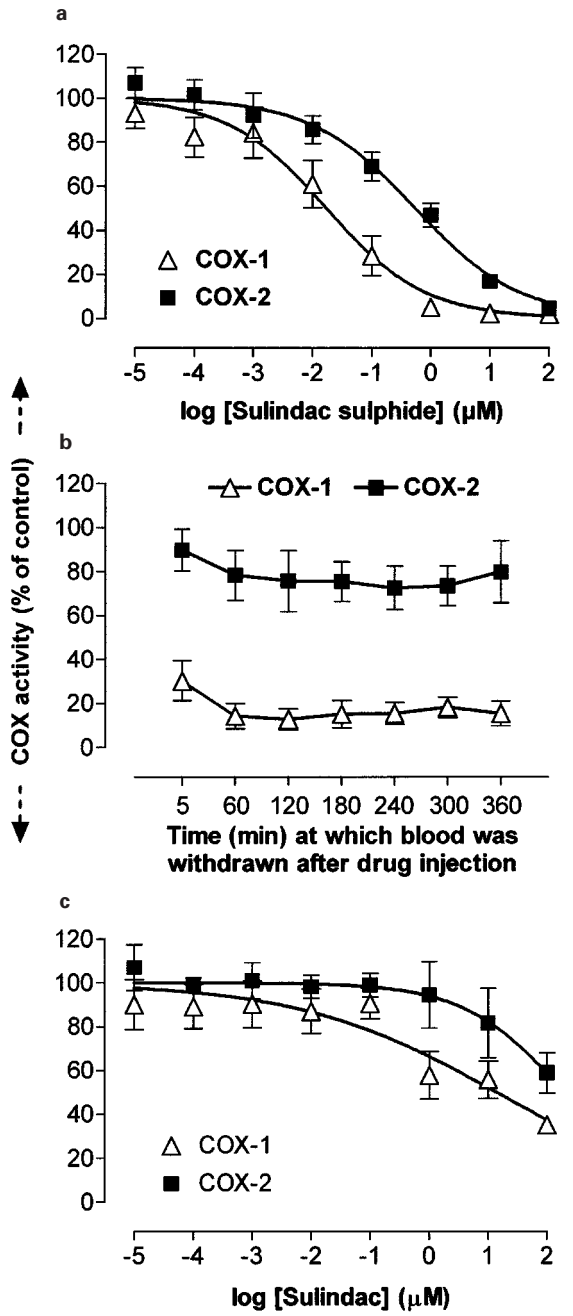

Figure 5 Effect of sulindac sulphide (a), plasma samples from sulindac-treated rats (b) and sulindac (c) on COX-1 and COX-2 activity. Sulindac sulphide and sulindac both showed selectivity towards the inhibition of COX-1. Plasma samples from sulindac-treated rats also selectively inhibited COX-1 with potency according to the *in vivo* metabolism of sulindac to sulindac sulphide. Data are expressed as mean±s.e.m. from three determinations from at least four separate experimental days.

and indicates metabolic activation of sulindac, probably to sulindac sulphide (Kwan & Duggan, 1977). Indeed, our control curves indicated sulindac sulphide to produce inhibition of COX-1 and COX-2 at concentrations that accord much more closely with the dose given. Thus, this test system effectively allows the testing and characterization of pro-drugs. Moreover, the selectivity towards COX-1 shown by sulindac sulphide *in vitro* was clearly reproducible *ex vivo*.

To substantiate the hypothesis that our test system would be suitable for evaluating *ex vivo* the selectivity of NSAIDs administered *in vivo*, we tested three more drugs. Having already tested COX-1 selective drugs, we chose diclofenac as representative of COX-1/-2 non-selective NSAIDs (Mitchell, 1993), nimesulide as representative of preferential COX-2 inhibitors (see Famaey, 1997), and L-745,337, a COX-2 selective compound (Chan *et al*., 1995).

When tested *in vitro*, diclofenac produced approximately equipotent inhibition of COX-1 and COX-2. In the *ex vivo* assay, diclofenac produced a slightly preferential inhibition of COX-2. It is tempting to propose that the mild selectivity of diclofenac towards the inhibition of COX-2 could account in part for the low relative risk of upper gastrointestinal toxicity reported by several authors for this drug (Henry *et al*., 1996; Garcia Rodriguez *et al*., 1998). The *ex vivo* assay may, therefore, provide a better indication of gastrointestinal risk. In the *ex vivo* assay diclofenac also showed a decrease in the inhibitory activity throughout the *in vivo* experimental period in accordance with its short half-life (Willis *et al*., 1979). Conversely, nimesulide activity in the plasma samples from nimesulide-treated rats was approximately stable throughout the *in vivo* experimental period. However the lack of reliable intravenous pharmacokinetic studies following intravenous administration of this drug (Bernareggi, 1993) does not allow any validation of our findings. The selectivity of nimesulide as an inhibitor of COX-2 was confirmed both *in vitro* and *ex vivo*. Nimesulide's preferential inhibition of COX-2 over COX-1

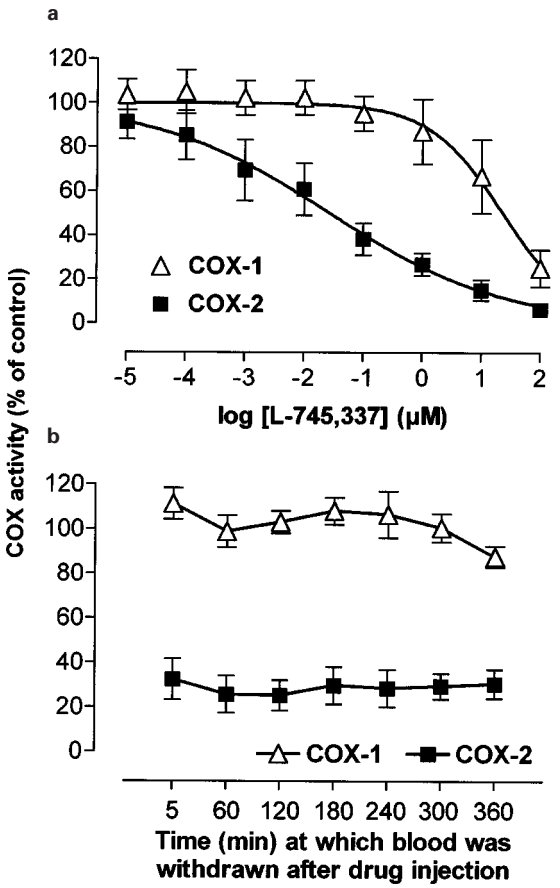

Figure 6 Effect of L-745,337 (a) and plasma samples from L-745,337-treated rats (b) on COX-1 and COX-2 activity. When tested directly, L-745,337 inhibited COX-2 1100 fold more potently than COX-1. Plasma samples form L-745,337-treated rats also selectively inhibited COX-2 activity without any significant effect on COX-1. Data are expressed as mean ± s.e.m. from three determinations from five separate experimental days.

well correlates with the high gastric tolerability observed at low dosage (100 or 200 mg twice daily) for short treatment periods (Marini & Spotti, 1993). However, as the dose and/or administration period increase, the gastric tolerability of nimesulide is reduced (Warrington *et al.*, 1993; Garcia Rodriguez *et al.*, 1998). The safety margins shown by COX-2 preferential inhibitors are lower than those for COX-2 selective inhibitors. Here, in fact, the novel COX-2 selective drug L-745,337 when tested *in vitro* was 1100 fold more active against COX-2 than COX-1. In the *ex vivo* assay, L-745,337 was also confirmed to be highly selective COX-2 inhibitor, for plasma from L-745,337 treated rats was without any effect on COX-1. This observation is consistent with the anti-inflammatory and gastric sparing profile of L-745,337 (Chan *et al.*, 1995). The *ex vivo* assay also showed that L-745,337 activity was very stable throughout the *in vivo* experimental period.

The data above suggest that the *ex vivo* assay system we have developed has a very promising utility both for profiling the actions of existing NSAIDs and for the characterization of new compounds. It can be used to study selectivity and metabolism under defined conditions for COX-1 and COX-2. It does, however, have two disadvantages. Firstly the use of rat plasma in combination with human cells and platelets produces a biological heterogeneity that limits the potential for predictivity in humans. Secondly, because of the limited availability of plasma from drug-treated rats it was possible to test *ex vivo* only small volumes (10 μl) of plasma. The resulting 10 fold dilution produces an underestimation of the real drug activity in the plasma. Nevertheless, it is clear that this assay provides information valuable in predicting the *in vivo* efficacy and selectivity of old and new NSAIDs.

From the studies we have performed, however, it is not possible to draw conclusions about the relative effects of NSAIDs against COX-1 and COX-2 when used, for instance, as anti-inflammatories. In particular, as we were interested in demonstrating *in vivo* activation and/or inactivation, NSAIDs were administered as i.v. boluses. Clinically, of course, these drugs are generally administered orally. Further studies in which blood samples are taken from animals receiving NSAIDs in orally active anti-inflammatory and analgesic doses would of course clarify this issue. More importantly, this assay could be of much usefulness in the analysis of plasma samples taken from humans treated with NSAIDs. The use of pure plasma *ex vivo* in association with human cells or platelets would provide a high quality standard from which to predict NSAIDs' *in vivo* efficacy and selectivity. This test system may also represent a new approach for the study of NSAID pharmacokinetic interactions with other drugs. Generally, in most of the investigations dealing with pharmacokinetic interactions the total blood concentration of the drug is determined. However the clinical value of differences in plasma concentrations is questionable (Brouwers & de Smet, 1994). The evaluation of the drug activity in plasma rather than its total blood concentration could represent a much better clinical index.

In conclusion, we have established a test system that is capable of assessing *ex vivo* the activity of NSAIDs administered *in vivo*. In particular, using aspirin and sulindac, we have shown that our assay can unveil phenomena of drug inactivation as well as drug activation. More importantly, using diclofenac, nimesulide and the novel COX-2 inhibitor L-745,337, we have demonstrated the possibility of studying the differential inhibition of COX isoforms with improved accuracy in predicting NSAIDs selectivity *in vivo*. Therefore, the application of this system to the analysis of human-derived samples may help to better understand the correlation between NSAIDs differential inhibition of COX and their reported gastrointestinal toxicity index. Studies on the pharmacokinetics interactions of NSAIDs with other drugs may also benefit from the application of this test system.

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