Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 68 (2004) 1957–1969
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Elucidation of the mechanism of inhibition of cyclooxygenases by acyl-coenzyme A and acylglucuronic conjugates of ketoprofen

Nicolas Levoin[a,1], Céline Blondeau[a], Cécile Guillaume[a], Line Grandcolas[a], Françoise Chretien[b], Jean-Yves Jouzeau[a], Etienne Benoit[c], Yves Chapleur[b], Patrick Netter[a], Françoise Lapicque[a,*]

[a]*UMR 7561 CNRS-UHP, Physiopathologie et Pharmacologie Articulaires, Faculté de Médecine—BP 184, F-54505 Vandoeuvre les Nancy, France*
[b]*UMR 7565 CNRS-UHP, Groupe SUCRES, Faculté des Sciences, Vandoeuvre les Nancy, France*
[c]*Laboratoire de Biochimie, Ecole Nationale Supérieure Vétérinaire de Lyon, France*

Received 1 April 2004; accepted 15 July 2004

Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the cyclooxygenase (COX) isoforms which accounts for their clinical effects. The differential inhibition of COX-1 and COX-2 is not sufficient to explain the absence of a correlation between in vitro and in vivo effects, especially for 2-aryl-propionates, thus indicating the participation of metabolites. Conjugates to glucuronic acid and to coenzyme-A are mainly produced, and have been shown to be chemically reactive. Therefore, we studied the interaction of the ketoprofen metabolites with the COX enzymes. After incubation with bovine pulmonary artery endothelial cells (BPAEC), COX-1 was inhibited stereoselectively by S-ketoprofen acylglucuronide, and more significantly by CoA-thioester. After washing-out the medium, COX-1 activity was essentially recovered, indicating a reversible inhibition. In LPS-stimulated J774.2 cells, COX activity (mainly inducible COX-2) was inhibited reversibly and stereospecifically by S-ketoprofen glucuronide, whereas it disappeared totally and was not recovered after incubation with CoA-thioester. Correspondingly, inhibition of purified COX-2 with this compound was observed to be rapid and irreversible. Using an anti-ketoprofen antibody, COX immunoprecipitated from cells exhibited adduct formation for COX-2 but not for COX-1. This was observed after incubation with CoA-thioester, and, surprisingly, also with glucuronide. Molecular docking gave support to explain this discrepancy: the glucuronide was found to establish a strong interaction with Y115 located in the membrane binding domain, whereas the thioester was preferentially bound to the active site of the enzyme. Overall, our results suggest a contribution of CoA-thioester metabolites of carboxylic NSAIDs to their pharmacological action by irreversibly and selectively inhibiting COX-2.

Keywords: Chiral nonsteroidal anti-inflammatory drugs; COX inhibition; Metabolite; Acylglucuronide; Coenzyme A

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of articular and inflammatory diseases. It is generally accepted that NSAIDs exert their clinical effects by inhibiting cyclooxygenase (COX), thereby blocking the synthesis of prostaglandins. COX exists in at least two different isoforms: COX-1 is expressed constitutively and is present in most cells under physiological conditions, whereas COX-2 is mainly induced in response to inflammatory stimuli. Over the last few years, the inhibition of the inducible enzyme has then been thought to be responsible for the beneficial properties of NSAIDs, while blocking the housekeeping functions of COX-1 would contribute to their gastro-intestinal side-effects. This explanation appears today overly simple, since COX-1 is also implicated in inflammation whereas COX-2 is involved in intestinal homeostasis and in the resolution of inflammation [1]. A number of in vitro assays have been published comparing the potencies of NSAIDs

Abbreviations: COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drug; CoA, coenzyme A; LPS, lipopolysaccharide; UGT, UDP-glucuronosyltransferase; KPF, ketoprofen; Glc, glucuronide; NAK, non-acylating analogue of KPF–CoA; BPAEC, bovine pulmonary arterial endothelial cells; DMEM, Dulbeccos modified Eagles medium; PG, prostaglandin; MBD, membrane binding domain; SD, steepest descents; CG, conjugate gradient.

* Corresponding author. Tel.: +33 383 68 39 50; fax: +33 383 68 39 59.
E-mail address: Francoise.Lapicque@medecine.uhp-nancy.fr (F. Lapicque).
[1] Present address: Bioprojet-Biotech, F-35762 Saint-Grégoire, France.

0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.07.015

against COX-1 and COX-2, and several recent reports have dealt with structural aspects of COX selectivity using X-ray analysis after crystallisation. However, no definitive correlation has been found between isoform selectivity and clinical effects, and led to a search for other mechanisms of action.

Chiral 2-arylpropionic acid NSAIDs are generally marketed as a racemic mixture of R- and S-enantiomers. Most of their clinical effects have been attributed to the S-enantiomer, as highly stereoselective inhibitor of COX in vitro. However, although being a poor COX inhibitor, the R-form possesses significant analgesic activity, as demonstrated for ketoprofen (KPF) in humans [2]. Since little chiral inversion from R- to the S-antipode occurred in these conditions, it can be suggested that NSAIDs act via both COX-dependent and COX-independent mechanisms.

This discrepancy may also originate from the participation of intermediate products of metabolism in COX inhibition, which could not be expected from in vitro studies. 2-Arylpropionic acids are extensively metabolised as acylglucuronides by UDP-glucuronosyltransferases (UGT), mainly in liver, before elimination into urine. Several NSAIDs undergo thioesterification with coenzyme A (CoA) by acyl-CoA synthetases (EC 6.2.1.3), leading to glycine conjugation [3], lipid incorporation [4], and chiral inversion of the R-enantiomer into S-antipode, in the case of asymmetric molecules. Both acylglucuronides and thioesters are chemically activated carboxylic acids and can therefore be expected to react with nucleophilic groups contained in proteins.

Acylglucuronides of mefenamate [5] and KPF [6,7] for example, were effectively shown to be responsible for adduct formation on albumin. Diflunisal was also observed to be capable to form adducts in perfused rat liver via glucuronidation [8]. KPF glucuronides bound UGT2B1 isoform covalently and were thus responsible for irreversible inhibition of the enzyme [9].

Endogenous fatty acyl-CoAs are high energy intermediates in the lipid pathway: they are involved in numerous biochemical processes such as calcium ion fluxes, membrane trafficking, membrane targeting and association [10]. They participate in gene regulation, interacting with transcription factors, such as yeast Fad R [11], HNF4α [12]. They are also inhibitors of numerous enzymes, e.g., UGT [13], mitochondrial adenosine translocase and citrate transporter, glucokinase, glucose-6-phosphatase, pyruvate dehydrogenase, acetyl-CoA carboxylase, 3-methylglutaryl-CoA reductase [14]. Acyl-CoAs from xenobiotics have been far less studied. However, some of them have been shown to be inhibitors of acetyl-CoA carboxylase [15], carnitine palmitoyl transferase I [16], glutathione S-transferases [17] and COX [18]. Since acyl-CoAs are chemically activated carboxylic acids, these effects may be due to their acylating properties and occur by covalent binding. As a matter of fact, chemical modification of unknown proteins has been effectively observed by fibrate

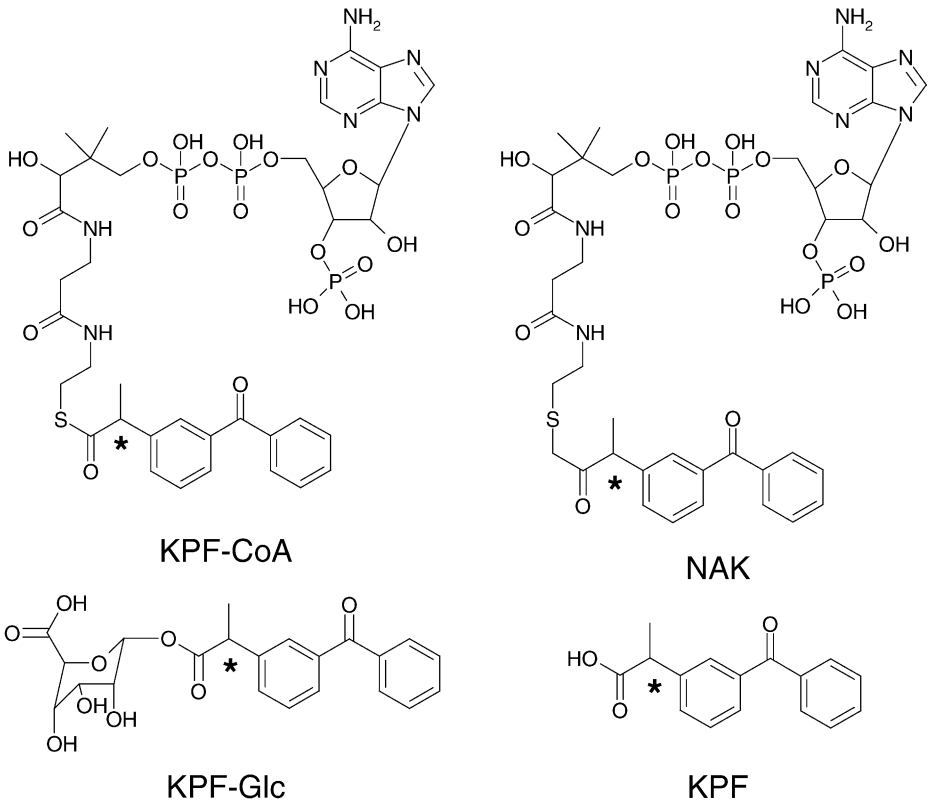

Fig. 1. Ketoprofen (KPF) and conjugates. (KPF–Glc: glucuronide of KPF, KPF–CoA: acyl-CoA of KPF, NAK: non acylating analogue of KPF–CoA: (*) denotes chiral centre of KPF).

derivatives [19], and of human serum albumin by naproxen-CoA [20].

Therefore, the aim of the present study was to compare acylglucuronides and acyl-CoA conjugates to the parent drug for their potency to inhibit COX. KPF was chosen as an example of chiral NSAID, and enantiomers were evaluated separately. Experiments were done using intact cells expressing preferentially COX-1 or COX-2, and purified enzymes. The mechanism of inhibition was elucidated by complementary assessments: COX recovery after exposure to the conjugates, formation of adducts with the enzymes, behaviour of a non-acylating analogue of KPF–CoA, NAK (Fig. 1).

2. Materials and methods

2.1. Chemicals and reagents

Racemic, R- and S-KPF were kindly offered by Rhone Poulenc Rorer. Arachidonic acid was obtained from Cayman Chemicals. All other chemicals and reagents were purchased from Sigma.

2.2. Synthesis and analysis of KPF conjugates

The glucuronide, KPF–Glc, was prepared from phenobarbital-treated rat liver microsomes immobilised on alginate beads as described previously [21]. R- or S-KPF was used as the substrate to obtain pure R- or S-KPF–Glc. Glucuronides were then purified by semi-preparative HPLC (7 μm Lichrosorb RP18 column 250 mm × 10 mm, Merck), with a mobile phase consisting of acetonitrile/H_2O/trifluoro-acetic acid (40:60:0.06) at a flow rate of 2.5 mL/min.

KPF–CoA and NAK were synthesised chemically as described previously [22]. Elementary analysis, HRMS, ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra were in accordance with the proposed structure. Assignments were confirmed by double irradiation or two-dimensional spectroscopy. Acyl-CoA of both KPF enantiomers was obtained. They gave two peaks in preparative HPLC, which were purified stereospecifically. However, owing to an epimerisation reaction occurring during the reaction [22], the assignment of one configuration to one eluate was not possible at this stage. Therefore, for the present work, acyl-CoA of KPF enantiomers will be named CoA-1 and CoA-2 following their elution order, without knowing if CoA-1 corresponds to R-KPF–CoA or S-KPF–CoA and vice versa.

Purity and stability of KPF conjugates were tested by analytical HPLC, using a C18 column (Radial Pack 80 mm × 10 mm in RCM module, Waters), and a mobile phase of methanol/9 mM phosphate buffer pH 5.5 (45:55) at a flow rate of 1.5 mL/min. Elution was monitored at 254 nm, using a 996 photodiode array detector and a 510 pump (Waters).

2.3. Inhibition of COX activity in cultured cells

Bovine pulmonary arterial endothelial cells (BPAEC) and murine macrophages (J774.2 cells) (The European Collection of Animal Cell Culture, Salisbury, UK) were grown to confluence in 24-well culture plates with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 4 mM glutamine, 50 μg/mL penicillin/streptomycin and 0.25 μg/mL amphothericin B. J774.2 cells were activated 14 h with LPS (1 μg/mL) in DMEM medium containing 2.5% foetal calf serum for induction of COX-2 [23]. Both cell cultures were incubated at 20 °C for 30 min with one inhibitor at varying concentrations. Arachidonate (30 μM) was then added and the cells were incubated for a further 15-min period. The supernatants were assayed for 6-keto-prostaglandin-F1α, 6kPGF1α, (BPAEC) or PGE2 (J774.2) content by ELISA (Assay Designs). Recovery activity of COX was then determined as reported by Laneuville et al. [24]. Briefly, the cells were washed twice for 45 min to remove the inhibitor, arachidonate (30 μM) was added for further 15-min incubation, and prostaglandins were quantified as previously. The supernatants were analysed by HPLC for KPF–Glc, KPF–CoA and KPF content to assess the possibility of conjugate hydrolysis.

2.4. Preparation of permeabilised cells

Cells prepared as described above were incubated with each inhibitor in the presence of 0.02% saponine. Preliminary trypan blue tests revealed that cell permeabilisation occurred under these conditions.

2.5. Kinetics of inhibition of purified COX enzymes

Purified ovine COX-1 and COX-2 enzymes were obtained from Cayman Chemicals. Enzyme activity was determined by measuring PGF2α production as reported by Janusz et al. [25]. Briefly, 30 μL enzyme (6.8×10^{-13} mol) and 30 μL inhibitor to a final concentration of either 5×10^{-5}, 5×10^{-6}, or 5×10^{-7} M was added to 250 μL of 50 mM phosphate buffer pH 7.4 containing 1 mM phenol, 1 μM hematine and 0.5 mg/mL $SnCl_2$, and incubated at 20 °C for a period of time of 0, 2, 5, 10 or 15 min. Then 30 μM arachidonate was added for 5 min at 37 °C. Incubation was stopped by adding 20 μL of 1 N HCl and neutralised with 1 N NaOH before prostaglandin assay by ELISA. Controls were done for each experimental concentration of inhibitor and incubation time.

2.6. Analysis of COX adducts by western blotting

Purified COX enzyme (6×10^{-11} mol) in 50 mM pH 7.4 phosphate buffer containing 1 μM hematine, 300 μM diethyl dithiocarbamate and 0.1% Tween 20®, were incu-

bated for 1 h at 20 °C with KPF–CoA (2×10^{-10} mol). Samples were mixed with Laemmli reagent (final concentration 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue in Tris buffer). SDS–PAGE was performed using 9% acrylamide for separating gel and 4% for stacking gel. Proteins were transferred onto Immobilon P membrane (Millipore) by electroblotting, using a glycine/Tris/0.1% MeOH buffer adjusted to pH 7.4 for 1 h at 12 V. Blots were saturated with 3% BSA in 0.04% Tween 20® in PBS. Immunodetection was performed by colorimetry using an anti-KPF polyclonal antibody obtained from rabbits immunized against KPF-thyreoglobuline by the method described by Maire-Gauthier et al. [26]. The anti-KPF antibody was diluted at 1/6000 and the secondary goat anti-rabbit IgG alkaline phosphatase conjugate at 1/5000.

2.7. Immunoprecipitation of COX

BPAEC and adherent activated J774.2 cells (5×10^6 cells) were incubated with KPF–CoA or KPF–Glc to a final concentration of 10^{-3} or 10^{-4} M for 2 h at room temperature. The cells were then washed twice with PBS to remove KPF conjugates. They were scrapped and centrifuged for 10 min at 24,000 × *g*, and the resulting pellet was taken up in 1 mL 150 mM NaCl, 1% Igepal (CAS registry No. [26571-11-9]), 0.5 mM deoxycholate, 50 mM pH 7.4 phosphate buffer solution (WB). Cells were lysed by thermal shocks and ultrasonication, then membrane proteins were solubilised (1.5 h at 0 °C) and the samples were ultracentrifuged at 130,000 × *g* for 1 h at 4 °C. The supernatant was added to 10 μL protein G-agarose 0.2 g/L and incubated for 2 h at 4 °C on a rotating platform for preclearing. The suspension was then centrifuged for 1 min at 24,000 × *g*. The supernatant was incubated with 10 μL of 1/100 anti-COX-1 or anti-COX-2 for 2 h before addition of 10 μL protein G-sepharose overnight at 4 °C. The suspension was then centrifuged at 24,000 × *g* for 1 min and the supernatant carefully removed. The beads were once more suspended in 1 mL WB and incubated for 20 min at room temperature on a rocking platform. Washing was repeated with 500 mM NaCl, 0.1% Igepal, 0.05% deoxycholate then with 0.1% Igepal, 0.05% deoxycholate in phosphate buffer. The last pellet was taken up in gel loading buffer and analysed by Western blot. In preliminary experiments, the specificity of immunoprecipitation was assessed using selective antibodies for COX-1 and COX-2, and further staining by Ponceau red ensured the absence of any other protein after the purification.

The presence of KPF adducts was evaluated as described above, using anti-KPF antibody. To check the presence of immunoprecipitated COX-1, the membrane was washed after revelation by the anti-KPF antibody and reblocked with HSA, before overnight incubation with an anti-COX-1 antibody at 1/750.

2.8. Molecular modelling

The postulated binding site of NSAID in COX-2 membrane binding domain (MBD) was studied from crystal structure of apo COX-2 (PDB entry 5cox [27]). Docking at the active site was studied from holo COX-2 (PDB entry 3pgh) complexed with flurbiprofen, using the entire protein monomer.

The initial docking was obtained using LigandFit (Cerius2, Accelrys). The active site grid was defined with reference to a manually docked probe consisting of co-crystallized ligand with sp3 carbon substitutes filling the free space around the ligand in the MBD. Ligand fitting was performed with flexible ligand and 150,000 Monte Carlo trials, a nonbond cutoff distance of 12 Å, and a distance-dependent dielectric constant ($\varepsilon = 4R$).

Then the best scored conformation was energy-optimised using Discover 3 software (InsightII, Accelrys). The backbone and side-chains of residues beyond 12 Å of the docked ligand were tethered using a quadratic potential. A nonbond cell-multipole method was used with a distance-dependent dielectric constant ($\varepsilon = 4R$), for 2000 steps of a SD/CG (steepest descents/conjugate gradient) protocol until convergence within 0.001 kcal/mol/Å.

Short molecular dynamics was performed to test the stability of this final complex. Since the precise environment of the internal MBD at the interface between water and lipids was not known, molecular dynamics was performed in vacuo, with the same atoms as previously in frozen state. The integration time step was set to 1 fs, and calculations were performed at constant volume and temperature. A heating stage of 5 ps was used starting from 10 to 300 K. Once the system was equilibrated (150 ps), data were collected for a further 300 ps. CFF force field was used for the entire protocol (Accelrys).

The trajectory was drawn to follow the motion of the ligand, showing distances between the two centroids of Y355 phenyl ring and of the distal phenyl ring of KPF–Glc ($d1$), between NH of R120 and the centroid of the proximal phenyl ring of KPF–Glc ($d2$), between NZ of K83 and carboxylate of KPF–Glc ($d3$), and between the centroids of Y115 phenyl ring and of the osidic ring of KPF–Glc ($d4$).

The docking of KPF conjugates in the cyclooxygenase active site was started using a manual superimposition of KPF with flurbiprofen. The molecular structure was then optimised using 2000 steps of a SD/CG protocol as previously described, keeping the protein in frozen state. Finally, the complex was minimized again in the same conditions, keeping only the backbone frozen.

2.9. Data analysis and modelling

The inhibition of prostaglandin production by KPF and conjugates was calculated as the percentage of the decrease of activity in the same conditions. The inhibition expressed by the sigmoidal concentration-response curve was mod-

elled using the following equation mathematically analogous to the Hill function:

$$Y = \frac{a - d}{[1 + (X/c)^b] + d}$$

where X and Y represent the molar concentration of inhibitor and the percentage of inhibition, respectively; a, b, c and d are the equation parameters, as described by Blain et al. [28]. Fitting of the experimental data to the above model was carried out by minimisation of the objective function, defined as the sum of square deviations between predicted and experimental response. The best fit was obtained with a weight at $1/Y$. Use of this equation also yields estimate for the IC_{50} values from the values of the parameters.

3. Results

3.1. Validation of the cellular system

BPAEC and LPS-activated J774.2 cells were used for testing COX-1 and COX-2 activity, respectively, by the amount of 6kPGF1α and PGE2 produced after the addition of 30 μM arachidonate. LPS-treatment increased the production of PGE2 from 13 ± 5 ng/mL to 100 ± 7 ng/mL (n = 6). The concentration of PGE2 can therefore be considered representative of the COX-2 activity, since PGE2 is the main eicosanoid produced through the COX-2 pathway [29].

Owing to the low lipophilicity of the metabolites, with calculated log P values at 0.6 and −2.4 for KPF–Glc and KPF–CoA, respectively, to be compared to 3.0 for KPF, we intended first to facilitate their intracellular diffusion by permeabilisation of the cell membranes. Therefore, in preliminary experiments, intact cells and cells permeabilised by 0.02% saponine were compared. The amount of prostaglandins produced was increased by about 25% in saponine-treated cells. This increase is likely due to the intracellular production released by this treatment, as described by Horton et al. [30]. However, the extent of inhibition was not affected by the presence of saponine, irrespective of the compound studied, namely S-KPF, glucuronide (S-KPF–Glc) or acyl-CoA (data not shown). Since the metabolites were not hydrolysed even after a 2-h incubation at 37 °C, as confirmed by HPLC assay of supernatants (data not shown), it was concluded that they were able to penetrate the intact cells and to inactivate intracellular COXs by themselves. In addition to passive diffusion, the occurrence of other transport mechanisms can be suggested for these compounds. Besides, intact cells could be used in the following experiments.

3.2. Inhibition of COX activity in intact cells

KPF and conjugates were assessed for their capacity to inhibit COX activity in BPAEC and in activated J774.2 cells, immediately after 30-min incubation. Inhibition versus concentration curves were drawn (Fig. 2) and corresponding IC50 were calculated (Table 1).

As expected, KPF inhibited both COXs stereoselectively in favour of the S-enantiomer, with R/S IC50 ratios at about 240 for COX-1 and 15 for COX-2 (Table 1). S-KPF–Glc inhibited COX-1 and COX-2, with IC50 values at 2 and 1 orders of magnitude higher than the parent drug, respectively, whereas its diastereoisomer was a poor inhibitor of COX-1 with nil effect on COX-2. Both COXs were inhibited by KPF–CoA (CoA-1 and CoA-2), with IC50 values comparable to that of S-KPF–Glc. The non-acylating analogue NAK inhibited COX-1 with the same potency as KPF–CoA, but was a weak inhibitor of COX-2.

After incubation with one of these compounds, the cells were washed thoroughly until their presence was not detected in the supernatant, and COX activity was determined again, for assessment of its recovery. The remaining inhibition was plotted versus the initial concentration of the compound (Fig. 2), and allowed the IC50 value of recovery to be calculated (Table 1).

Under these conditions, COX activity was totally restored in the case of KPF and NAK in both cell lineages, expressing substantial reversible inhibition (Fig. 2). In activated J774.2 cells COX activity was also restored after incubation with S-KPF–Glc, whereas activity was not recovered after KPF–CoA, as indicated by the perfect superimposition of the curves of immediate and recovery inhibition. The COX

Table 1
IC50 (M) values of COX activity by KPF and conjugates in BPAEC (mainly COX-1) and in stimulated J774.2 macrophages (mainly COX-2)

Compound	COX-1		COX-2	
	Immediate inhibition	Recovery	Immediate inhibition	Recovery
R-KPF	6.95×10^{-7}	$>10^{-3}$	8.02×10^{-5}	$>10^{-3}$
S-KPF	2.88×10^{-9}	$>10^{-3}$	5.46×10^{-6}	$>10^{-3}$
R-KPF–Glc	9.89×10^{-5}	7.92×10^{-4}	$>10^{-3}$	$>10^{-3}$
S-KPF–Glc	1.89×10^{-7}	4.58×10^{-5}	5.7×10^{-5}	$>10^{-3}$
CoA1	4.31×10^{-7}	6.47×10^{-5}	7.09×10^{-5}	1.07×10^{-4}
CoA2	8.37×10^{-8}	1.4×10^{-5}	5.5×10^{-5}	1.08×10^{-4}
NAK	3.17×10^{-7}	$>10^{-3}$	5.02×10^{-3}	$>10^{-3}$

COX-1 and COX-2 were assessed in BPAEC by assay of the production of 6kPGF1α, and in LPS-stimulated J774.2 by PGE2, respectively, immediately after 30-min incubation with the compounds (immediate inhibition) and after washing-out (recovery). Calculations were done with Hill function.

activity in BPAEC exhibited an intermediate behaviour. However, with IC50 values for recovery at about 100 times higher than for immediate inhibition, the inhibition can be supposed to be mainly reversible.

3.3. Time-dependent inhibition of purified COX by KPF–CoA

Use of isolated enzymes showed that KPF–CoA was actually a direct COX inhibitor. This effect was not due to hydrolysis of KPF–CoA, since no released KPF was detected in these conditions. The incubation of both COXs with KPF–CoA led to an inactivation, increasing with time and concentration (Fig. 3), as depicted for time-dependent or irreversible inhibitors. The activities of COX-1 and COX-2 were reduced by 30 and 20%, respectively, after 2-min incubation with 50 μM KPF–CoA, and by 55 and 40% after 15 min. The effect of time was particularly important within the first 2 min, expressing a fast mechanism, affecting both COX isoforms to a similar extent. On the contrary, the evolution of inhibition by S-KPF versus time was expressed by a plateau attained from the beginning of incubation, as expected from a reversible inhibitor.

3.4. Acylation of COX by ketoprofen conjugates

The incubation of purified COX enzymes with KPF–CoA resulted in the formation of KPF-adducts, as revealed by Western blotting with anti-KPF antibody (Fig. 4). Both isoforms were found to be subjected to this chemical modification. In the presence of arachidonate, the amount of adducts was decreased significantly for both COXs, suggesting that acylation occurs in the active site of the enzymes. Adduct formation was also observed with KPF–Glc, but not with KPF.

We next investigated whether acylation occurred in the cells after incubation with KPF metabolites. Therefore, COX-1 and COX-2 were selectively immunoprecipitated

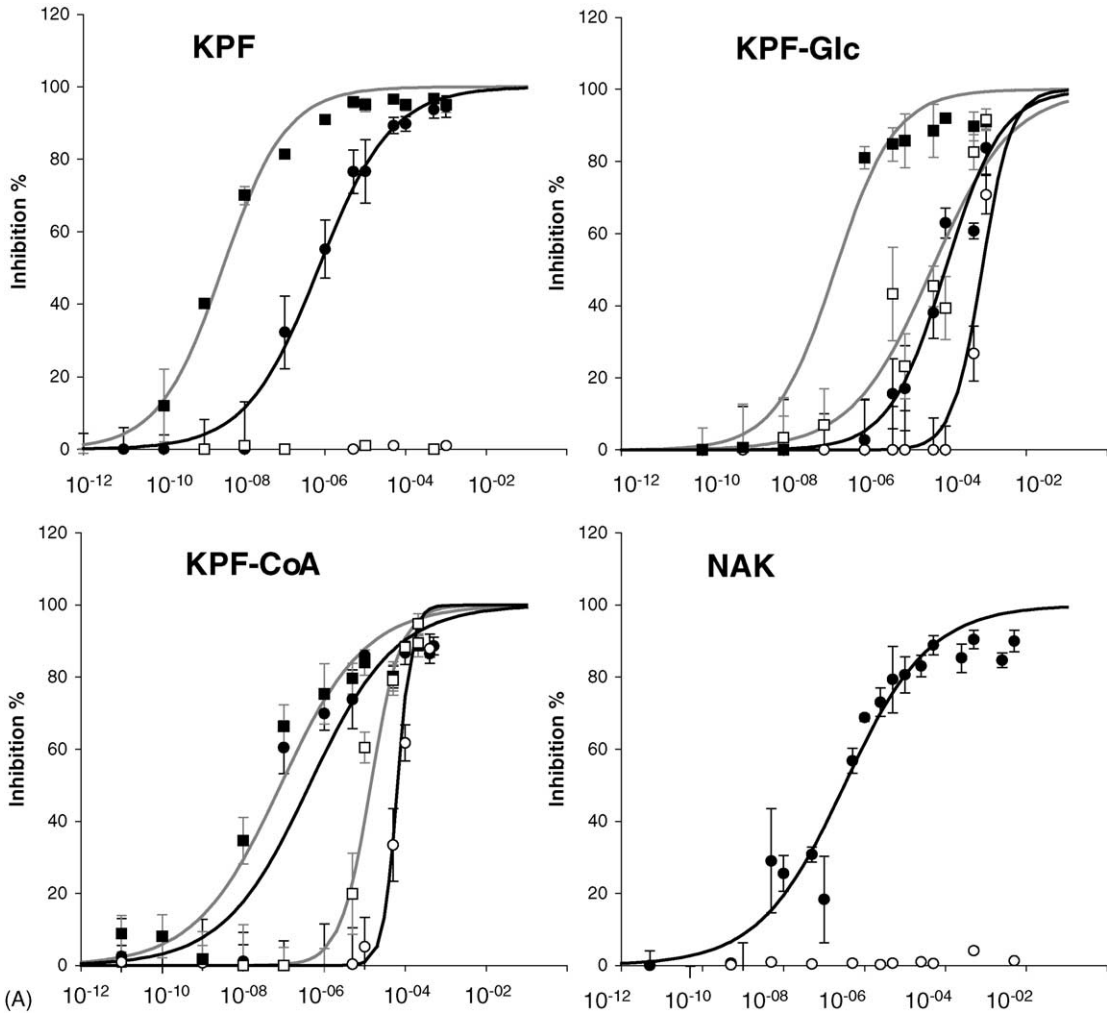

Fig. 2. Inhibition of COX activity by KPF and conjugates in BPAEC (mainly COX-1) (A) and in LPS-stimulated J774.2 macrophages (mainly COX-2) (B). Activity of COX-1 and COX-2 was assessed by the production of 6kPGF1α in BPAEC, and of PGE2 in LPS-stimulated J774.2 cells, respectively, immediately after a 30-min incubation with the compounds (immediate inhibition, ●, ■) and after washing-out (recovery, ○, □). Inhibition was determined for R-KPF, R-KPF–Glc, KPF–CoA-1, NAK (●, ○), and for S-KPF, S-KPF–Glc, KPF–CoA-2 (■, □); data are mean values of 3 and 4 experiments and were fitted by Hill function (——, ——).

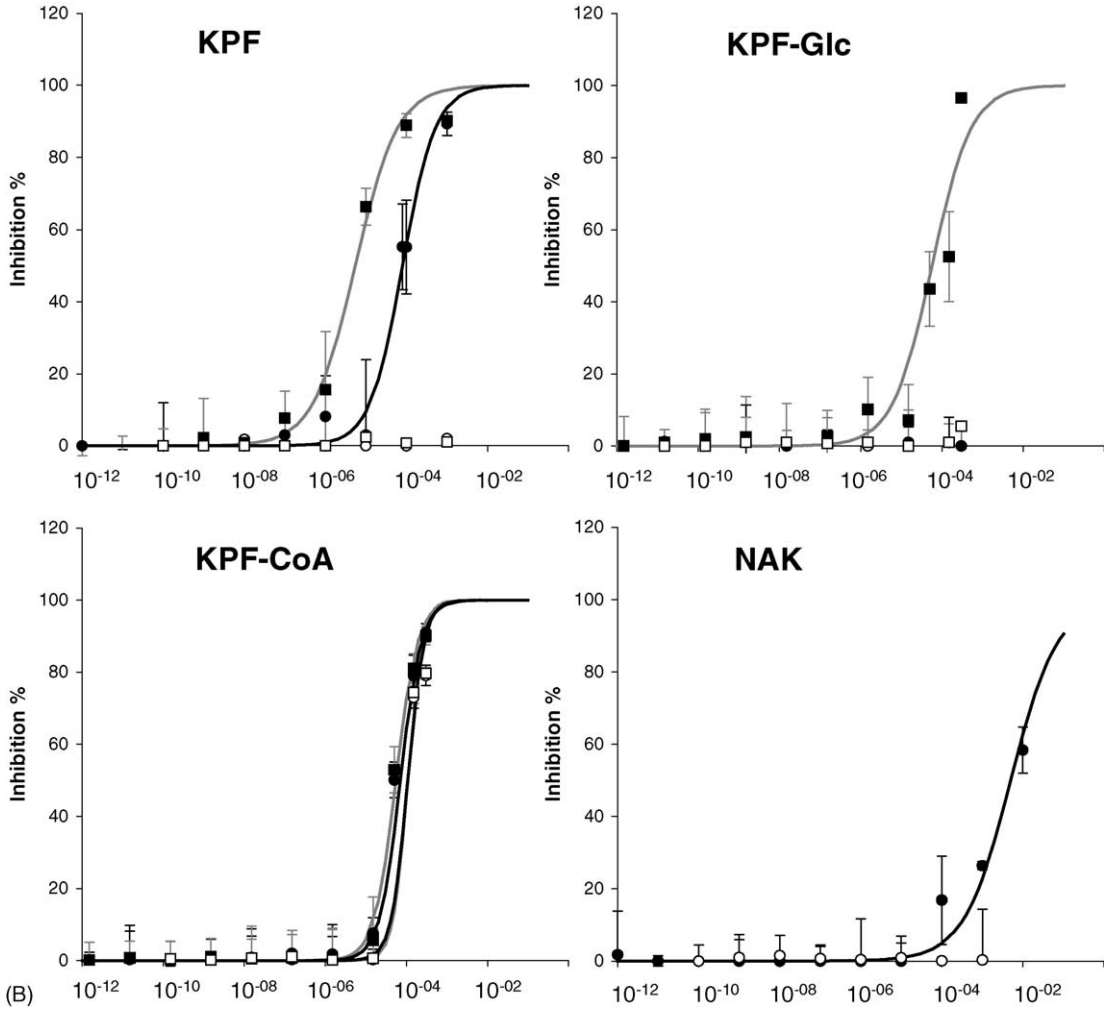

Fig. 2. (*Continued*).

from incubated BPAEC and J774.2 cells, respectively, and adducts were evaluated on Western blot revealed with anti-KPF antibody (Fig. 5). Adduct formation was observed for COX-2 in J774.2 cells with the two metabolites, and was more significant with KPF–CoA than with the glucuronide. On the contrary, no adduct was detected with COX-1 from BPAEC, whereas the presence of the protein was confirmed by a further incubation of the membrane with an anti-COX-1 antibody.

4. Discussion

The contribution of metabolites to the pharmacological activity of drugs has been considered either as beneficial as for morphine glucuronide [31], or conversely as responsible for side-effects [32]. We report here that KPF conjugates with glucuronide and with coenzyme A are potent inhibitors of COX, although with a differential behaviour towards the COX isoforms.

In BPAEC, COX-1 was inhibited by KPF–Glc. The well-known enantioselectivity of the parent molecule for COX-1 was even enhanced by the osidic moiety, the R/S IC50 ratio increasing from 240 to 520 (Table 1). KPF–CoA was a more potent inhibitor than the other metabolite, but exhibited no difference between the two enantiomers. Using ibuprofenyl-CoA, Neupert et al. [18] have also reported this loss of stereoselectivity: the R/S IC50 ratio was 10 and 0.5 for ibuprofenyl-CoA on COX-1 and COX-2, respectively, instead of 16 and over 250 for ibuprofen. This could be explained by intracellular inversion of the configuration due to epimerase activity, leading to a unique enantiomer. However, no epimerisation in the culture medium was observed as far as KPF–CoA or KPF was concerned.

After washing-out the metabolites from the BPAEC medium, COX-1 activity was recovered to a large extent, expressing the inhibition as essentially reversible. Accordingly, after selective immunoprecipitation of COX isoenzymes and Western blot analysis with an anti-KPF antibody, no adduct of KPF was found. Paradoxically, the inactivation of purified COX-1 enzyme by KPF–CoA was time-dependent and corresponded to the covalent binding of KPF, as revealed by Western blot. This dis-

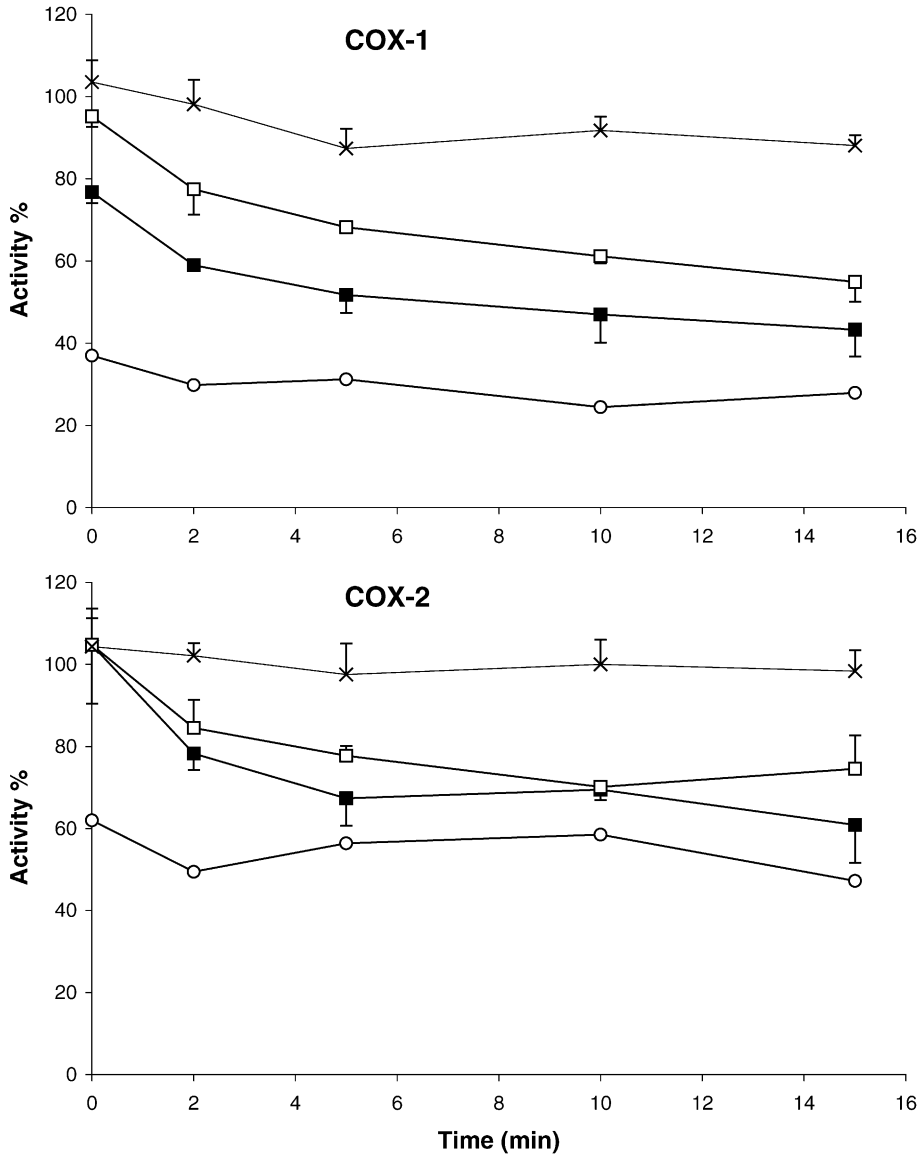

Fig. 3. Kinetics of inhibition of purified COXs by KPF and KPF–CoA. Enzymatic activity was measured by PGF2α production after pre-incubation of COX with KPF at 7×10^{-8} M (○), or KPF–CoA at 5×10^{-5} M (■), 5×10^{-6} M (□), or 5×10^{-7} M (×), then adding 30 μM arachidonate.

crepancy may be explained by (i) a difference in protein behaviour between cells and purified enzyme, due to purification and/or to an unidentified role of the cell membrane in the function of the enzyme, as previously observed [33], or by (ii) a low solubilisation yield of COX-1, whose concentation can be sufficient for detecting the protein by using an anti-COX-1 antibody, but probably too low for detecting KPF adducts using an anti-KPF antibody.

COX-2 activity in J774.2 cells was inhibited by both KPF–CoA conjugates, and stereospecifically by S-KPF–Glc, its antipode having no effect. COX-2 activity was totally restored after elimination of the glucuronide. On the contrary, no catalytic capacity was recovered in the case of KPF–CoA. The curve of the inhibition extent with the initial product concentration is even perfectly superimposable with that determined for immediate inhibition. This expresses the fact that for a given metabolite concentration, COX-2 remained inactivate after its elimination as much as in its initial presence. This conserved inhibition is thus different from that of time-dependent inhibitors, such as indomethacin, a reversible inhibitor, which dissociates slowly from COX but losses 25% of its inhibition capacity in similar conditions [24]. Western blot data demonstrate unambiguously that KPF–CoA is irreversibly bound to COX-2, and competition experiments with arachidonate, the natural substrate of COX, suggest that COX acylation occurs in the active site. It can therefore be proposed that KPF–CoA inhibits COX-2 activity via covalent modification of the enzyme. This hypothesis is further supported by the complete recovery after incubation with NAK, the non-acylating analogue of KPF–CoA, in which the reactive thioester function is replaced by a thiomethylene keto

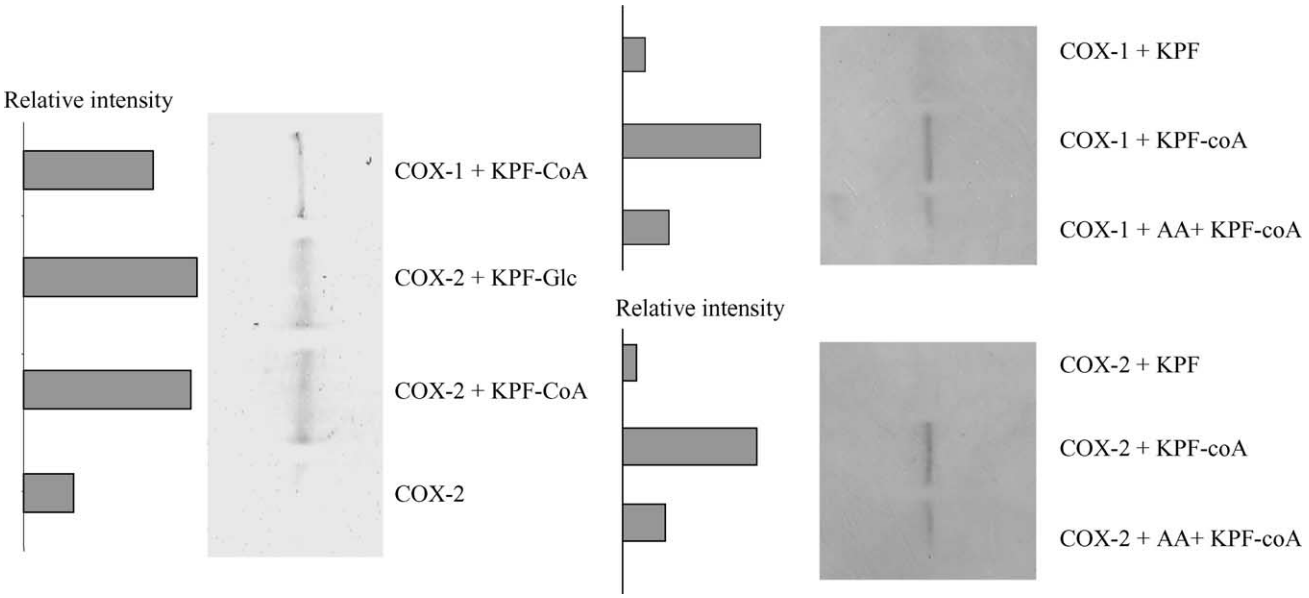

Fig. 4. KPF-acylation of purified COXs by KPF metabolites. COX-1 and COX-2 (6×10^{-11} mol) were incubated for 1 h with KPF, KPF–CoA, or KPF–Glc (2×10^{-10} mol). Arachidonate, AA (5×10^{-10} mol), was added 5 min before KPF–CoA. Western blot was analysed using anti-KPF antibody.

group, and by the time-dependent inhibition observed for the purified enzyme.

KPF–Glc was also covalently bound to COX-2, both in cells and to isolated enzyme. However, this binding had no effect on enzyme activity after wash-out, contrary to KPF–CoA. It can then be supposed that KPF binding occurs in domain of the protein different from the catalytic site.

Molecular modelling was then used to facilitate interpretation of the above results. KPF–Glc and KPF–CoA were thus found to bind to the cyclooxygenase active site quite in a similar manner to KPF, their respective ester and thioester functions interacting with the triad R120, E524, Y355. The modelled complex clearly argues in favour of Y355 as the nucleophilic amino-acid responsible for COX acylation, since its distance to the thioester of KPF–CoA and to the ester of KPF–Glc is at 3.7 Å (Fig. 6), and 4.4 Å (data not shown), respectively. Interestingly, Y355 was

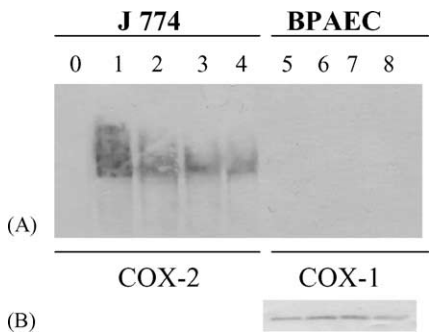

Fig. 5. Immunoprecipitation of COX from cells treated with KPF metabolites. BPAEC and J774.2 were incubated 2 h with KPF metabolites before immunoprecipitation of COX-1 and COX-2, respectively. Proteins were then analysed using anti-KPF (A) or anti-COX-1 antibody (B). 0: control; 1, 5: KPF–CoA 10^{-3} M; 2, 6: KPF–CoA 10^{-4} M; 3, 7: KPF–Glc 10^{-3} M and 4, 8: KPF–Glc 10^{-4} M.

previously shown to potentially participate in the time-dependent inhibition through interaction with the substrate and the inhibitor [34] and to play a significant role in COX-2 stereoselectivity towards chiral NSAIDs [35].

Both metabolites can bind to the active site, their reactive function interacting with Y355. But as suggested by Llorens et al. [36], they should interact with the membrane binding domain (MBD) prior to entering the active site. Analysis of the crystal structure revealed a hydrophobic cluster in this domain in the vicinity of W100, V103, and I102, which we identified by molecular docking to constitute a speculative binding site for KPF, as previously shown for flurbiprofen [36]. The hydrophilic moiety of KPF metabolites does not inhibit this proposed fixation, even though slightly affecting the binding mode of the benzophenone. The KPF moiety of the molecule is in the proximity of V116, V349, L359, F357, Y355, I112 and V89, while, for glucuronide, the osidic part is in a more polar environment, with a salt bridge between the carboxylic function and K83 (Fig. 7). Molecular dynamics was used to test the stability of the complex by following the possible motion of the ligand. As shown in Fig. 8, the key distances of its trajectory were constant during the 300 ps simulation. This supports the hypothesis of a strong interaction between KPG-Glc and the site, with π-stacking for $d1$, cation-π for $d2$ and salt bridge for $d3$. As far as adducts are concerned, the only postulated residue is Y115, which is in compatible orientation and distance (4 Å) from the ester function of the glucuronide, as shown in Fig. 8. Here, KPF–Glc could adopt a fixed and packed conformation allowing the interaction between the ester and Y115, then leading to a possible acylation of the residue. Molecular dynamics performed with KPF-acylated Y115 resulted in fast reorientation of KPF outside the site, and

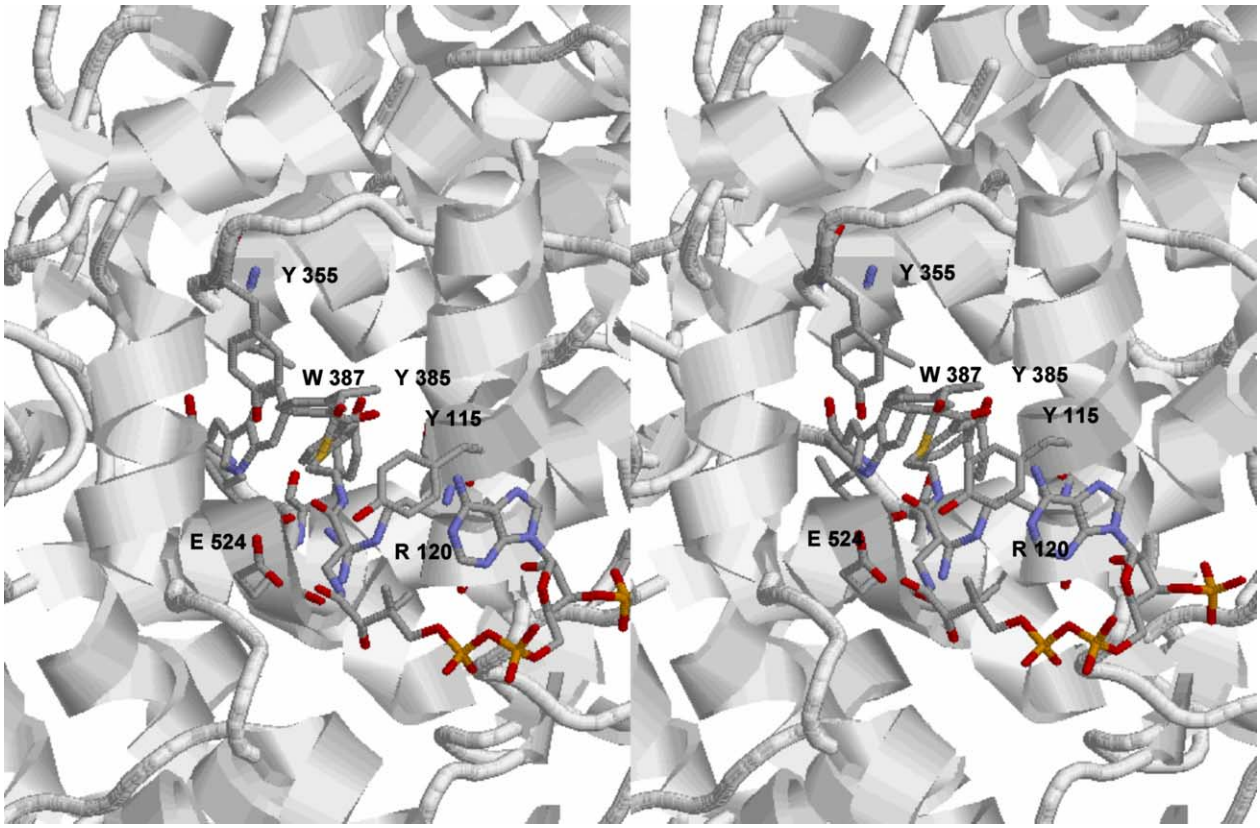

Fig. 6. Proposed complex of KPF–CoA with COX-2 active site view from the membrane binding domain: detail showing the close contact between Y355 and KPF–CoA (distance between OH of Y355 and thioester group of KPF–CoA: 3.7 Å). Stereoimage prepared with Rasmol.

the alignment with the lipophilic helix of the MBD. Therefore, Fig. 9 suggests that the chemical modification of Y115 does not hinder access to the active site, and that COX-2 would be chemically modified but only reversibly inhibited by KPF–Glc.

The same study was done for KPF–CoA. However, in this case, the long hydrophilic chain may adopt a number of conformations: packed in the MBD, unfolded in the medium, or interacting with polar residues outside the core of this domain. Due to this high mobility allowing numerous

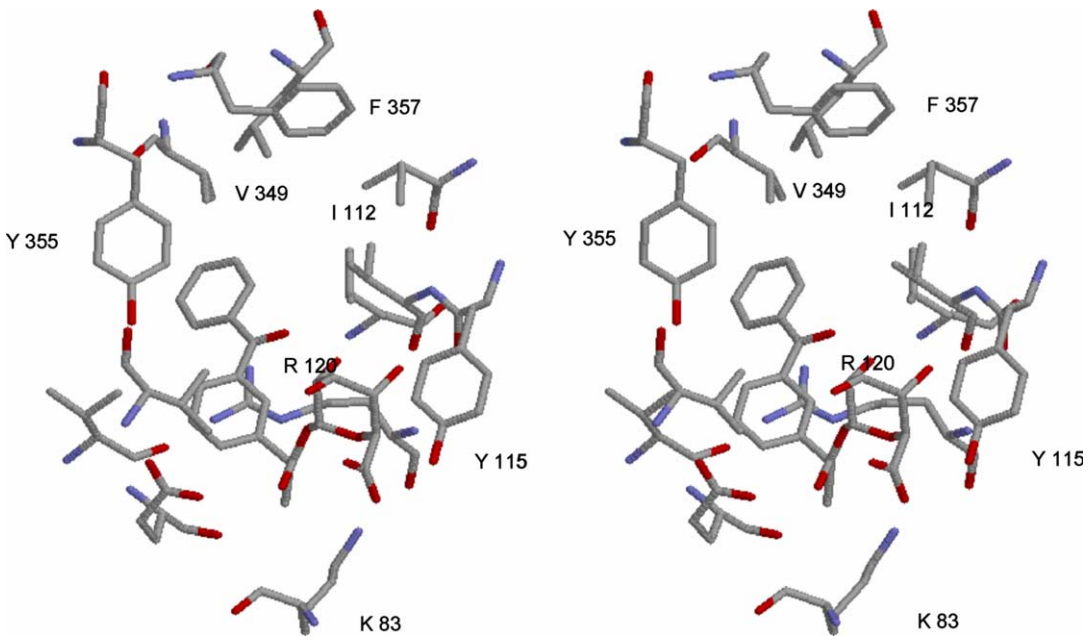

Fig. 7. Proposed complex of KPF–Glc with COX-2 membrane binding domain binding site (distance between OH of Y115 and ester group of KPF–Glc: 3.5 Å).

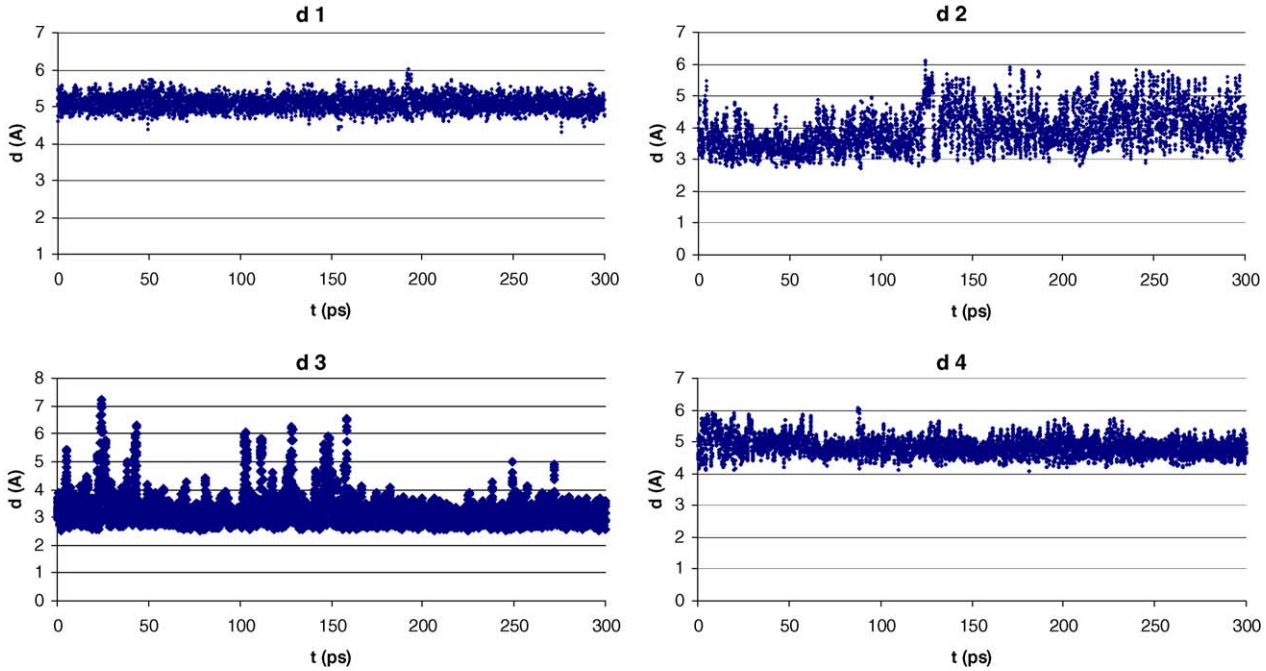

Fig. 8. Distances of key contact points between COX-2 and KPF–Glc during MD. Details for definition of distances are given in M and M.

different structures, attempts were made in orienting manually the CoA moiety towards polar residues. Multiple conformers appeared with comparable probability, and made it difficult to conclude on a precise binding mode. The very flexible packing in the MBD does not allow a strong interaction with Y115, as required for chemical reactions. KPF–CoA will then be able to join the active site and to acylate Y355, leading thus to the irreversible inhibition of activity by obstruction of the site.

Taken together, these experiments with intact cells have shown the glucuronide of KPF to be a reversible inhibitor of both COXs, whereas inhibition by CoA thioester was either reversible for COX-1, or irreversible for COX-2. Docking experiments actually provided a

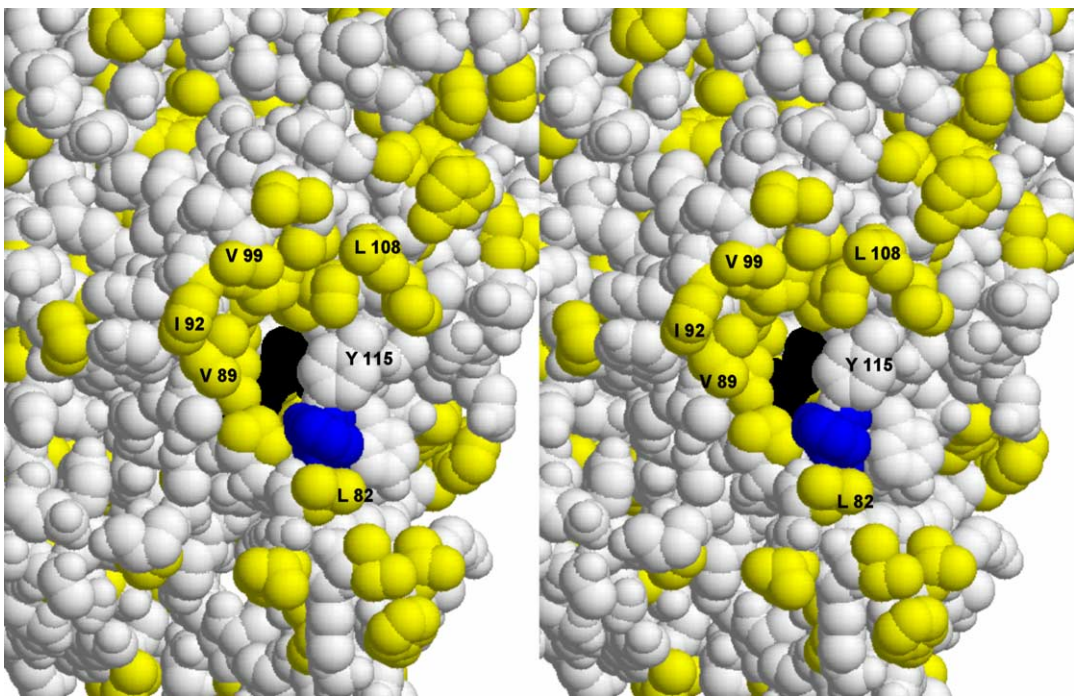

Fig. 9. Proposed structure for acylated COX-2 by KPF–Glc. Side-chains of hydrophobic residues are in yellow. The triad R120, E524, Y355 of the entrance of the site is shown in black and KPF covalently bound to Y115 in blue.

molecular explanation for the interaction of KPF metabolites with COX-2. Nevertheless, further studies will be necessary to characterise the exact protein domain of covalent binding. Peptide mapping and site-directed mutagenesis are in progress to define the amino acids involved in this mechanism.

With respect to glucuronides, inhibition of enzyme activity was only described for UGT [9] after acylation of the protein. Until now, their reactivity was mainly considered to be responsible for immuno-allergic side-effects. These phase II metabolites are hydrophilic conjugates, so their physico-chemical properties may be rate-controlling for their penetration into the cells, contrary to the parent drug, which penetrates the cells mainly by passive diffusion. In this study, the effect of glucuronide was almost the same using either intact or permeabilised cells, and let us suppose them able to diffuse through the plasma membrane of cells in target tissues after plasma transport. This study provides then the first example of their contribution to pharmacological effects of drugs. Therefore, glucuronide may explain interindividual differences in NSAID response, through variations in UGT activity [37].

Acyl-CoAs do not seem to exist outside of cells, so their inhibition effect is to be limited to inside the cells, where they are formed. However, acyl-CoA synthetases are expressed in various types of cells, and particularly in the cells expressing also COXs. Inhibition of COX activity was previously observed for CoA thioesters of ibuprofen [18], of salicylate in macrophages [38], and also of fatty acids in microsomes [39], but the mechanism was not elucidated. The present work demonstrates that KPF–CoA is a selective irreversible inhibitor of COX-2 and that its acylation is responsible for durable inhibition. Interestingly, conversion of NSAIDs into ester and amide derivatives generates highly selective COX-2 inhibitors, for which interaction was shown with Y355 [40].

To conclude, this study suggests a novel mode of action of NSAIDs with active metabolites. They are probably more COX-2 selective than estimated previously from the parent drug, due to the selective acylation of the enzyme by their CoA metabolite.

Acknowledgements

“Sur l’onde calme et noire où dorment les étoiles . . .”. This paper is dedicated to Céline, who left us so precociously by a sombre spring morning.

This work was supported by the Association de Recherche sur la Polyarthrite Rhumatoïde (ARP), Paris, the Centre Hospitalier Régional Universitaire de Nancy (CPRC grant), the Centre National de la Recherche Scientifique (CNRS) and the Ministère de l’Education Nationale, de la Recherche et de la Technologie (thesis grant of NL).

References

[1] Parente L, Perretti M. Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. Biochem Pharmacol 2003;65:153–9.
[2] Cooper SA, Reynolds DC, Reynolds B, Hersh EV. Analgesic efficacy and safety of (R)-ketoprofen in postoperative dental pain. J Clin Pharmacol 1998;38:11S–8S.
[3] Kasuya F, Igarashi K, Fukui M. Participation of a medium chain acyl-CoA synthetase in glycine conjugation of the benzoic acid derivatives with the electron-donating groups. Biochem Pharmacol 1996;51:805–9.
[4] Sallustio BC, Meffin PJ, Knights KM. The stereospecific incorporation of fenoprofen into rat hepatocyte and adipocyte triacylglycerols. Biochem Pharmacol 1988;37:1919–23.
[5] McGurk KA, Remmel RP, Hosagrahara VP, Tosh D, Burchell B. Reactivity of mefenamic acid 1-O-acyl glucuronide with proteins in vitro and ex vivo. Drug Metab Dispos 1996;24:842–9.
[6] Dubois N, Lapicque F, Maurice MH, Pritchard M, Fournel-Gigleux S, Magdalou J, et al. In vitro irreversible binding of ketoprofen glucuronide to plasma proteins. Drug Metab Dispos 1993;21:617–23.
[7] Dubois-Presle N, Lapicque F, Maurice MH, Fournel-Gigleux S, Magdalou J, Abiteboul M, et al. Stereoselective esterase activity of human serum albumin toward ketoprofen glucuronide. Mol Pharmacol 1995;47:647–53.
[8] Wang M, Dickinson RG. Disposition and covalent binding of diflunisal and diflunisal acyl glucuronide in the isolated perfused rat liver. Drug Metab Dispos 1998;26:98–104.
[9] Terrier N, Benoit E, Senay C, Lapicque F, Radominska-Pandya A, Magdalou J, et al. Human and rat liver UDP-glucuronosyltransferases are targets of ketoprofen acylglucuronide. Mol Pharmacol 1999;56:226–34.
[10] Ford DA, Horner CC, Gross RW. Protein kinase C acylation by palmitoyl-coenzyme A facilitates its translocation to membranes. Biochemistry 1998;37:11953–61.
[11] Raman N, DiRusso CC. Analysis of acyl-coenzyme A binding to the transcription factor FadR and identification of amino acid residues in the carboxyl terminus required for ligand binding. J Biol Chem 1995;270:1092–7.
[12] Hertz R, Magenheim J, Berman I, Bar-Tana J. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4alpha. Nature 1998;392:512–6.
[13] Yamashita A, Watanabe M, Tonegawa T, Sugiura T, Waku K. Acyl-CoA binding and acylation of UDP-glucuronosyltransferase isoforms of rat liver: their effect on enzyme activity. Biochem J 1995;312:301–8.
[14] Faergeman NJ, Knudsen J. Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. Biochem J 1997;323:1–12.
[15] Kemal C, Casida JE. Coenzyme A esters of 2-aryloxyphenoxypropionate herbicides and 2-arylpropionate antiinflammatory drugs are potent and stereoselective inhibitors of rat liver acetyl-CoA carboxylase. Life Sci 1992;50:533–40.
[16] Kiorpes TC, Hoerr D, Ho W, Weaner LE, Inman MG, Tutwiler GF. Identification of 2-tetradecylglycidyl-coenzyme A as the active form of methyl 2-tetradecylglycidate (methyl palmoxirate) and its characterization as an irreversible, active site-directed inhibitor of carnitine palmitoyltransferase A in isolated rat liver mitochondria. J Biol Chem 1984;259:9750–5.
[17] Silva C, Loyola G, Valenzuela R, García-Huidobro T, Monasterio O, Bronfman M. High-affinity binding of fatty acyl-CoAs and peroxisome proliferator-CoA esters to glutathione S-transferases. Effect on enzymatic activity. Eur J Biochem 1999;266:143–50.
[18] Neupert W, Brugger R, Euchenhofer C, Brune K, Geisslinger G. Effects of ibuprofen and its coenzyme A thioesters on human prostaglandin endoperoxide synthases. Br J Pharmacol 1997;122:487–92.

[19] Sallustio BC, Nunthasomboon S, Drogemuller CJ, Knights KM. In vitro covalent binding of nafenopin-CoA to human liver proteins. Toxicol Appl Pharmacol 2000;163:176–82.
[20] Olsen J, Bjørnsdottir I, Tjørnelund J, Hansen SH. Chemical reactivity of the naproxen acyl glucuronide and the naproxen coenzyme A thioester towards bionucleophiles. J Pharm Biomed Anal 2002;29: 7–15.
[21] Chakir S, Maurice MH, Magdalou J, Leroy. Dubois N, Lapicque F, et al. High-performance liquid chromatographic enantioselective assay for the measurement of ketoprofen glucuronidation by liver microsomes. J Chromatogr 1994;654:61–8.
[22] Levoin N, Chrétien F, Lapicque F, Chapleur Y. Synthesis and biological testing of acyl-CoA-ketoprofen conjugates as selective irreversible inhibitors of COX-2. Bioorg Med Chem 2002;10:753–7.
[23] Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ, Vane JR. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc Natl Acad Sci USA 1993;90:11693–7.
[24] Laneuville O, Breuer DK, Dewitt DL, Hla T, Funk CD, Smith WL. Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. J Pharmacol Exp Ther 1994;271:927–34.
[25] Janusz JM, Young PA, Ridgeway JM, Scherz MW, Enzweiler K, Wu LI, et al. New cyclooxygenase-2/5-lipoxygenase inhibitors. 1.7-*tert*-Butyl-2,3-dihydro-3,3-dimethylbenzofuran derivatives as gastrointestinal safe antiinflammatory and analgesic agents: discovery and variation of the 5-keto substituent. J Med Chem 1998;41:1112–23.
[26] Maire-Gauthier R, Buronfosse T, Magdalou J, Herber R, Besse S, Delatour P, et al. Species-dependent enantioselective glucuronidation of carprofen. Xenobiotica 1998;28:595–604.
[27] Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, et al. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. Nature 1996;384: 644–8.
[28] Blain H, Boileau C, Lapicque F, Nedelec E, Loeuille D, Guillaume C, et al. Limitation of the in vitro whole blood assay for predicting the COX selectivity of NSAIDs in clinical use. Br J Clin Pharmacol 2002;53:255–65.
[29] Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E2. J Biol Chem 1999;274:11660–6.
[30] Horton JK, Williams AS, Smith-Phillips Z, Martin RC, O'Beirne G. Intracellular measurement of prostaglandin E2: effect of anti-inflammatory drugs on cyclooxygenase activity and prostanoid expression. Anal Biochem 1999;271:18–28.
[31] Paul D, Standifer KM, Inturrisi CE, Pasternak GW. Pharmacological characterization of morphine-6 beta-glucuronide, a very potent morphine metabolite. J Pharmacol Exp Ther 1989;251:477–83.
[32] Wieland E, Shipkova M, Schellhaas U, Schütz E, Niedmann PD, Armstrong VW, et al. Induction of cytokine release by the acyl glucuronide of mycophenolic acid: a link to side-effects? Clin Biochem 2000;33:81–159.
[33] Eling TE, Glasgow WC, Curtis JF, Hubbard WC, Handler JA. Studies on the reduction of endogenously generated prostaglandin G2 by prostaglandin H synthase. J Biol Chem 1991;266:12348–55.
[34] So OY, Scarafia LE, Mak AY, Callan OH, Swinney DC. The dynamics of prostaglandin H synthases. Studies with prostaglandin H synthase 2 Y355F unmask mechanisms of time-dependent inhibition and allosteric activation. J Biol Chem 1998;273:5801–7.
[35] Bhattacharyya DK, Lecomte M, Rieke CJ, Garavito RM, Smith WL. Involvement of Arginine 120, Glutamate 524, and Tyrosine 355 in the binding of arachidonate and 2-phenylpropionic acid inhibitors to the cyclooxygenase active site of ovine prostaglandin endoperoxide H synthase-1. J Biol Chem 1996;271:2179–84.
[36] Llorens O, Perez JJ, Palomer A, Mauleon D. Structural basis of the dynamic mechanism of ligand binding to cyclooxygenase. Bioorg Med Chem Lett 1999;9:2779–84.
[37] Strassburg CP, Kneip S, Topp J, Obermayer-Straub P, Barut A, Tukey RH, et al. Polymorphic gene regulation and interindividual variation of UDP-glucuronosyltransferase activity in human small intestine. J Biol Chem 2000;275:36164–71.
[38] Hinz B, Kraus V, Pahl A, Brune K. Salicylate metabolites inhibit cyclooxygenase-2-dependent prostaglandin E2 synthesis in murine macrophages. Biochem Biophys Res Commun 2000;274:197–202.
[39] Fujimoto Y, Nakajima T, Murakami Y, Takami K, Sakuma S, Fujita T. Effect of fatty acyl-coenzyme A esters on prostaglandin synthesis in rabbit medulla microsomes. Prostaglandins Leukot Essent Fatty Acids 1992;47:265–8.
[40] Kalgutkar AS, Marnett AB, Crews BC, Remmel RP, Marnett LJ. Ester and amide derivatives of the nonsteroidal anti-inflammatory drug, indomethacin, as selective cyclooxygenase-2 inhibitors. J Med Chem 2000;43:2860–70.