Pergamon

Bioorganic & Medicinal Chemistry 10 (2002) 753–757

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Biological Testing of Acyl-CoA–Ketoprofen Conjugates as Selective Irreversible Inhibitors of COX-2

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Received 11 July 2001; accepted 17 September 2001

Abstract—Ketoprofenoyl-CoA thioester **3** was synthesized by coupling ketoprofen to coenzyme A using the mixed anhydride method. Diastereoisomeric compounds **3a** and **3b** corresponding to the enantiomers of ketoprofen, were obtained in optically pure form by preparative HPLC. A non-acylating analogue, *rac*-3-(3-benzoylphenyl)-2-oxo-butanoyl-CoA (**7**) was also prepared. The biological evaluation suggested that **3a** and **3b** are reversible inhibitors of COX-1 and irreversible inhibitors of COX-2. Compound **7** appears to be a poor but selective inhibitor of COX-1.

Introduction

Prostaglandins are active mediators of inflammatory responses and also provide cytoprotection in the stomach and intestine. The key enzyme of their biosynthesis is prostaglandin H2 synthase (PGHS or cyclooxygenase, COX), which converts arachidonic acid to PGH2, further metabolised by specific synthases and isomerases to give various prostanoids. COX exists as two isoforms: in most tissues, COX-1 is expressed constitutively and COX-2 is transiently up-regulated by pro-inflammatory mediators and down-regulated by corticosteroids. Numerous works in this area suggest that constitutive COX-1 protects the GI tract, whereas inducible COX-2 mediates inflammation. However, COX-2 is also involved in ulcer healing, renal physiology, female reproductive process,[1] and even in the resolution of the inflammatory response.[2,3]

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for the treatment of inflammation and articular diseases. Their pharmacological activity is mainly attributed to a competitive inhibition of COX. As many other xenobiotics, NSAIDs undergo two metabolic phases following their absorption: functionalisation, for example oxidative phase, and conjugation, consisting of the addition of various small hydrophilic molecules. For most drugs, these metabolic pathways are hydroxylation and glucuronic acid conjugation. Conjugation to glycine,[4] lipids incorporation,[5] and inversion of configuration[6] are observed in vivo for certain NSAIDs, and point out a particular conjugation to coenzyme A, acting as an intermediate for these reactions.

Acyl-CoA from diverse drugs have been shown to inhibit several enzymes like acetyl-CoA carboxylase,[7] glutathione S transferase [8] and COX.[9] Since acyl-CoA are chemically activated carboxylic acids, their effects may be due to their acylating properties and occur by covalent binding. Adduct formation has been actually demonstrated for fibrate derivatives which acylate unknown proteins.[10,11] This ability of adduct formation is particularly important in the case of carboxylic acid-containing xenobiotics, since their acyl chain retains the pharmacological activity and carries the reactive molecule to definite pharmacological targets.

The aim of this study was to prepare ketoprofenoyl-CoA **3** and its analogue **7** in order to study their effects on COX. Pharmacological activity often depends on the absolute configuration of chiral centres in the molecule, particularly for NSAIDs for which (*S*) enantiomer is a more potent inhibitor of COX than its (*R*) antipode.[12,13] Therefore, we describe in this paper the synthesis of **3** starting from ketoprofen, and the separation step giving pure (*S*) and (*R*) diastereoisomers. In order to highlight the inactivation mechanism of COX by acyl-CoA, an analogue of ketoprofenoyl-CoA was designed. Thus the replacement of the thioester group

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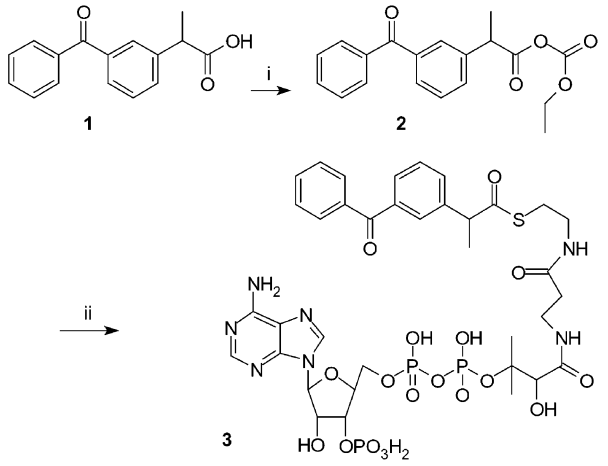
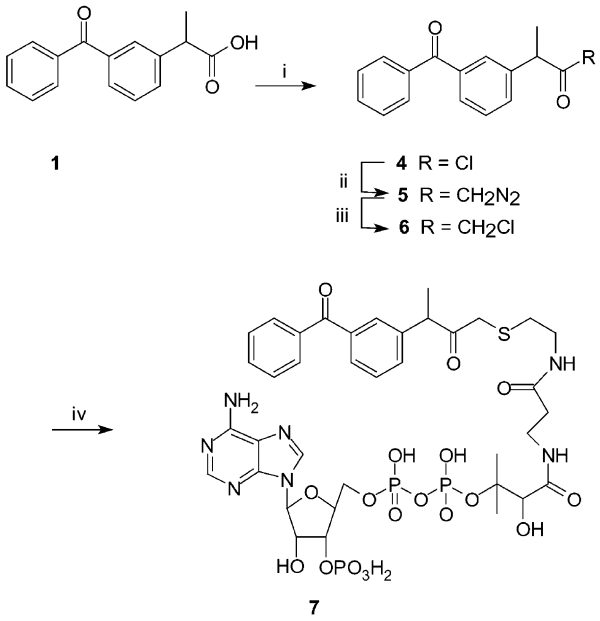
0968-0896/02/$ - see front matter
PII: S0968-0896(01)00330-3



Scheme 1. Reagents: (i), 2,6-lutidine, CH_2Cl_2 then $ClCO_2Et$, rt, 1 h; (ii), THF, CoASH, H_2O, Ph = 6.6, rt, 2 h.



Scheme 2. Reagents: (i), $ClCO_2Cl$, rt, 1 h; (ii), diazald, 0 °C, 2 h; (iii), anhydrous HCl in Et_2O; (iv), CoASH, H_2O/DMF, $CsCO_3$, rt, 24 h.

by a thiomethylene keto group would provide a non-acylating species. It was assumed that such a slight modification would not affect the recognition of the inhibitor by COX. The compound selected was *rac*-3-(3-benzoylphenyl)-2-oxo-butanoyl-CoA (**7**).

Results

The synthesis of compound **3** was performed by coupling *rac*-2-(3-benzoylphenyl)propionic acid (ketoprofen [KPF] **1**) to coenzyme A (CoASH) using the mixed anhydride method[14] (Scheme 1). Formation of the rather unstable compound **2** was almost achieved quantitatively within 1 h. When at most 10% of the starting compound **1** was detectable by HPLC monitoring, CoASH was added to the reaction mixture. The formation of compound **3** was monitored by HPLC. After 2 h of reaction, compound **2** completely disappeared. The organic solvent was evaporated under reduced pressure and unreacted **1** was extracted from the aqueous layer using hexane. Diastereoisomeric compounds **3a** and **3b** were obtained in optically pure form after two preparative HPLC steps. The first one allowed the separation of the mixture of diastereoisomers **3** from residual reactants; the second one gave pure **3a** and **3b**. In order to assign the absolute configuration to compounds **3a** and **3b**, the same synthesis was carried out starting from optically pure *R*-KPF. However both diastereoisomers were obtained, and the same result was obtained starting from optically pure *S*-KPF.

Therefore, an epimerisation reaction occurred during the overall process, as reported by Carabaza.[15] As roughly the same diastereoisomeric mixture was obtained whatever the KPF enantiomer used as the starting material, assignment of one configuration to one chromatographic eluate of **3** could not be done at this stage. Microsomial biosynthesis with rac-**1** using R-AINS-specific Acyl-CoA synthetase [16] produced only compound **3b**, but incubation of **3a** or **3b** with the microsomes resulted in the presence of the only **3b**, suggesting an epimerisation, due to epimerase activity.[15] It cannot, therefore, be concluded that compound **3b** is (*R*) ketoprofenoyl-CoA. Crystallization of each compound **3a** and **3b** is currently under investigation to provide a definitive answer to this issue.

The synthesis of compound **7** was based on the final alkylation of coenzyme A using the chloromethylketone analogue **6** of KPF. The synthesis of **7** is outlined in Scheme 2. Ketoprofenyl chloride **4**, obtained by reaction of oxalyl chloride with **1**, was treated with diazomethane to give *rac* 3(-3-benzoylphenyl)-2-oxo-diazobutane (**5**). Conversion of the crude resulting diazo group to the corresponding chloride by reaction of an anhydrous ethereal solution of hydrogen chloride, gave the compound **6** in 95% overall yield. Alkylation of coenzyme A by the compound **6** was performed in a water–dimethylformamid mixture in the presence of caesium carbonate. An optimised H_2O/DMF ratio of 3:14 gave the best result. Contrary to **3**, neither analytic nor semi-preparative HPLC allowed diastereoisomeric purification of **7**.

Biological Evaluation

The biological specificity of the two isoforms of COX reported before can be used to obtain more efficient NSAIDs without gastro-intestinal side effects. Most classical NSAIDs are reversible or time-dependent reversible inhibitors, except aspirin which acetylates Ser530 of COX-1 (Ser516 of COX-2). They generally inhibit both COX-1 and COX-2 to different extents; for this reason, extensive studies were conducted in recent years for the development of new molecules that selectively inhibits COX-2.[17] These studies were based on crystal structure determinations of the enzymes:[12,18]

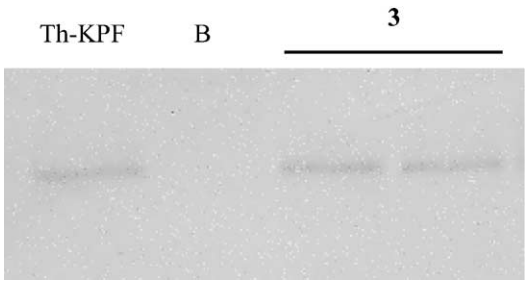


Figure 1. Detection of KPF adducts after 1.5 h incubation of COX-2 with **3**. Th-KPF: chemical adduct of KPF on Thyreoglobulin (positive control), **B:** COX-2 + KPF, **3:** COX-2 incubated with compound **3**.

Table 1. IC_{50}(μM) values of ketoprofen derivatives for COX-1 and COX-2[a]

Compd	COX-1[b,c]	Rec COX-1[b,d]	COX-2[b,c]	Rec COX-2[b,d]
(*R*) **1**	0.695	>10,000	80	>10,000
(*S*) **1**	0.003	>10,000	5	>10,000
3a	0.043	900	71	107
3b	0.083	140	55	108
7	0.317	>10,000	5000	>10,000

[a]COX inhibitions were performed as described in biological assays.
[b]Average of four determinations.
[c]Immediate inhibition.
[d]Remaining inhibition after recovery.

the most striking difference between the isoforms is that the active site of COX-1 is more stable and less bulky than COX-2. [19,20] Therefore, most selective inhibitors of COX-2 display steric groups that hinder their binding on the active site of COX-1.

The results reported in Table 1 show that acyl-CoA **3a** and **3b** are good inhibitors of both COX-1 and COX-2 with an IC_{50} one order of magnitude higher than the *S* enantiomer of the parent drug. It is worth noting that they do not present the marked enantioselectivity of KPF, probably due to epimerisation. Reversibility of the inhibition was tested by recovery experiments, where inhibitors were carefully removed before measuring the enzymatic activity. COX incubated with (*R*) or (*S*) **1** recovered thus, almost all their catalytic capacity (IC_{50} > 10,000), as expected from reversible inhibitors. In contrast, COX incubated with **3a** or **3b** were still inhibited: very weakly for COX-1, since IC_{50} values were higher in recovery experiment than during immediate inhibition, but strongly for COX-2, with comparable values of IC_{50} in both conditions. Thus, **3a** and **3b** appear essentially reversible inhibitors of COX-1 and completely irreversible inhibitors of COX-2.

Compound **7** was shown to be a slight inhibitor of both COX and to be COX-1 selective. As expected, it is a reversible inhibitor and this argues for an acylation mechanism responsible for the irreversible inhibition of COX-2 by KPF acyl-CoA. In fact, incubation of COX-2 with **3** resulted in the formation of KPF adducts, as revealed by Western blot experiments, using anti-KPF antibody (Fig. 1). The maximum rate of acylation was achieved within 1 h and adducts were stable since no clear difference in labelling was observed by varying the incubation time at room temperature from 1 to 11 h (Fig. 2). This confirms that acylation is certainly at the origin of the irreversible inhibition of COX-2 by ketoprofenoyl-CoA.

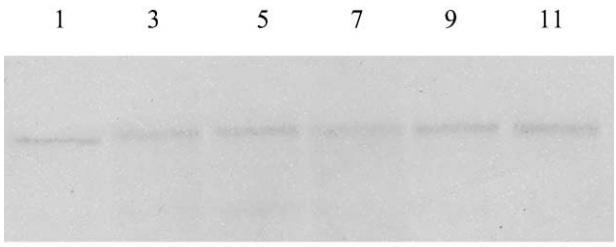


Figure 2. Kinetics of KPF adducts formation. COX-2 was incubated for 1, 3, 5, 7, 9, and 11 h with **3** before Western blotting.

Conclusion

The present work reports the synthesis and the enantiomeric purification of ketoprofenoyl-CoA (**3a**, **3b**) and its non-acylating analogue **7**. The biological evaluation of compound **3** showed that it is an irreversible selective inhibitor of COX-2. This result contributes to better understand the mechanism of action of NSAIDs and their anti-inflammatory properties. It is also meaningful for the research in articular therapeutics, and the development of more efficient drugs without side effects.

Besides, acyl-CoA are key intermediates in many biochemical pathways, acting as cofactors of enzymes and as intermediates of protein modification, for example palmitoylation. A convenient way to provide these intermediates and their non-acylating analogues is of large interest to elucidate biological processes.[21,22]

Experimental

General indications

FTIR spectra were recorded on Perkin-Elmer Spectrum 1000 on NaCl windows or KBr pellets. ^{1}H NMR and ^{13}C spectra were recorded on a Bruker AC 250 or on a Bruker DRX 400 spectrometer. Unless otherwise stated, all spectra were recorded in D_2O. Attribution of ^{13}C signals are based on the *J*-modulated spin-echo sequence and/or heteronuclear two-dimensional techniques. Mass spectra were recorded on a Trio 1000 Thermo Quest spectrometer in the electron impact mode or a Platform Micromass in the electro spray mode. Elemental analyses were obtained from the Service Central de Microanalyse du CNRS, Vernaison (France). Analytical thin-layer chromatography was performed on Merck 60F 254 pre-coated silica gel plates. Preparative chromatography was performed on silica gel 60 (230–40 mesh ASTM). Analytic reverse phase HPLC was performed on a C18 column (Waters RadialPak 8×10 mm in Waters RCM module), using at flow rate of 1.5 mL min^{-1} and a mobile phase of MeOH (45%), 9 mM phosphate buffer pH = 5.5 (55%). Elution was monitored at 254 nm, using Waters 996 photodiode

array detector and Waters 510 pump. Semi-preparative reverse phase HPLC was performed on a Merck Lichrosorb RP-18 column, (7 μm, 250×10 mm), at a flow rate of 2.5 mL min-1, and a mobile phase of MeOH (35%), 9 mM phosphate buffer pH = 5.5 (65%) by injections of 700 μL and monitoring at 290 nm.

2-(3-Benzoylphenyl)propionyl-CoA (3). To a solution of *rac*-2-(3-benzoylphenyl)propionic acid [ketoprofen (KPF)] (**1**) (122 mg, 0.48 mmol) in anhydrous dichloromethane (10 mL) was added 2,6 lutidine (56 μL, 0.48 mmol) and ethyl chloroformate (46 μL, 0.48 mmol) in anhydrous dichloromethane (3 mL). The reaction mixture was stirred at room temperature under a nitrogen atmosphere. The reaction was monitored by analytical HPLC. After 1 h of reaction compound **1** (retention time 9.5 min) was no longer detectable and only compound **2** (retention time 6.4 min) was present. The solution was concentrated to dryness and the crude residue was diluted in anhydrous THF (9 mL). A solution of coenzyme A (36.8 mg, 0.048 mmol) in water (9 mL) was then added. The pH of the resulting solution was adjusted to 6.2 using NaOH (5M) and stirred under a nitrogen atmosphere at room temperature. The reaction was monitored by analytical HPLC. After 2 h, CoASH (retention time 1.3 min) disappeared and two peaks corresponding to the diastereoisomers of ketoyl-CoA **3** appeared (retention time 4.7 for **3a** and 5.2 min for **3b**). The mixture was then concentrated under reduced pressure to give a milky liquid. The aqueous layer was extracted with hexane (10×10 mL) and concentrated. The residue was purified by semi-preparative HPLC to yield the compounds **3a** and **3b** as amorphous white powder. The compound obtained after chromatography was washed ten times with MeOH in order to remove inorganic phosphate. Compound **3a**: ^{1}H NMR (400 MHz): δ 0.55 (s, 3H, CH_3), 0.69 (d, 3H, J = 2.5Hz, CH_3), 1.32 (d, 1H, J = 7 Hz, CH_3), 2.11 (br m, 2H), 2.79 (m, 1H), 2.86 (m, 1H), 3.15, (br m, 4H), 3.37 (dd, 1H, J_{gem} = 9.5 Hz, $^3J_{HP}$ = 5 Hz, CH_2OP), 3.66 (dd, 1H, CH_2OP), 3.84 (s, 1H, *C*HOH), 3.90 (m, 1H, C*H*CH$_3$), 4.09 (br m, 2H, *H*-5rib, *H*5′rib), 4.43 (br m, 1H, *H*-4rib), 5.07 and 5.20 (br m, 2H, *H*-2rib, *H*-3rib), 6.08 (d, 1H, J = 6 Hz, *H*-1rib), 7.40 (m, 9H, Ph), 7.99 (s, 1H, *H*-2ad), 8.21 (s, 1H, *H*-8ad), ^{31}P NMR: δ −11 (d, 1P, ^2J = 20 Hz),−10.5 (d, 1P), 2.73 (s, 1P). Compound **3b**: ^{1}H NMR (400 MHz): δ 0.46 (s, 3H, CH_3), 0.65 (s, 3H, CH_3), 1.00 (d, 1H, J = 7 Hz, CH_3), 2.07 (br m, 2H), 2.58 (br m, 2H), 2.95, (br m, 2H), 3.34 (br m, 1H, CH_2OP), 3.53 (m, 1H, C*H*CH$_3$), 3.64 (br m, 1H, CH_2OP), 3.81 (s, 1H, C*H*OH), 4.06 (br m, 2H, *H*-5rib, *H*5′rib), 4.37 (br m, 1H, *H*-4rib), 4.80 (br m, 2H, *H*-2rib, *H*-3rib), 5.97 (d, 1H, J = 6 Hz, *H*-1rib), 7.10 (m, 9H, Ph), 7.91 (s, 1H, *H*-2ad), 8.27 (s, 1H, *H*-8ad). ^{31}P NMR: δ −10.8 (d, 1P, ^2J = 20 Hz), −10.4 (d, 1P), 2.68 (s, 1P).

3-(3-Benzoylphenyl)-2-oxo-chlorobutane (6). KPF (**1**) (1.27 g, 5 mmol) was dissolved in oxalyl chloride (3 mL). The solution was stirred at room temperature for 1 h, and then concentrated to dryness. The crude residue of rac-2-(3-benzoylphenyl)propionyl-chloride (**4**) was coevaporated twice with toluene. The dried residue was dissolved in diethyl ether and added dropwise to freshly prepared diazomethane (generated from 7.2 g diazald in diethyl ether at 0 °C). The resulting solution was allowed to stand for 2 h at 0 °C. The reaction mixture was quenched with heptane and the solution was concentrated to dryness, and led to the crude compound **5**, which was used without purification in the next step. To a solution of the crude compound **5** in anhydrous diethyl ether was added dropwise a saturated etheral solution of hydrogen chloride (8 mL of a solution obtained by extraction of 12 M HCl with diethyl ether and drying over magnesium sulphate). The resulting solution was stirred for 1 h at room temperature and the solvent was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel using hexane/diethyl ether 3:2 (v:v) to give 1.35 g of compound **6**. An analytical sample was recrystallised from ethyl ether. R_f 0.17 (hexane/diethyl ether 3:2); IR: 1734,1657 cm^{-1} ; ^{1}H NMR (250 MHz, CDCl$_3$): δ 1.48 (d, 3H, J = 7 Hz, CH_3), 4.05 (d, 1H, J_{gem} = 15 Hz, CH_2Cl), 4.12 (d, 1H, CH_2Cl), 4.15 (q, 1H, C*H*), 7.63 (m, 9H, Ph); ^{13}C NMR (62.9 MHz, CDCl$_3$): δ 17.81 (*C*H$_3$), 47.34 (*C*H$_2$Cl), 49.69 (*C*H), 128.43, 129.16, 129.52, 130.07, 131.69, 132.73 (9C, *C*H Ph), 137.26, 138.45, 139.69 (3C, *C* Ph), 196.21, 201.78 (2C, *C*O); EIMS (*m*/*z*): 288 (7%) (M + 2)$^+$, 286 (19%) (M)$^+$. Anal. calcd for $C_{17}H_{15}ClO_2$: C,71.21; H,5.27; Cl,12.36. Found: C,71.06; H,5.17; Cl, 12.56.

3-(3-Benzoylphenyl)-2-oxo-butanoyl-CoA (7). Co-enzyme A disodium salt (90 mg, 0.12 mmol) was dissolved in water (300 μL) then diluted with DMF (1.4 mL). The resulting solution was added dropwise to a suspension of compound **6** (40 mg, 0.14 mmol) and Cs_2CO_3 (52 mg, 0.16 mmol) in DMF (1 mL). The reaction mixture was stirred for 24 h at room temperature. The reaction was monitored by analytic HPLC. The retention time for **7** was 3.7 min. The solvent was evaporated under reduced pressure and the crude residue was purified by semi-preparative HPLC. ^{1}H NMR: δ 0.53 (s, 3H, CH_3), 0.72 (s, 3H CH_3), 1.06 (d, 1H, J = 7 Hz, CH_3), 2.22 (br m, 4H), 2.96 (m, 2H), 3.23 (br m, 2H), 3.39 (dd, 1H, J_{gem} = 9.5 Hz, $^3J_{HP}$ = 5 Hz, CH_2OP), 3.71 (dd, 1H, CH_2OP), 3.87 (m, 2H), 4.12 (br m, 2H, *H*-5rib, *H*5′rib), 4.44 (br m, 1H, *H*-4rib), 4.66 (br m, 2H, *H*-2rib, *H*-3rib), 5.97 (d, 1H, J = 6 Hz, *H*-1rib), 7.18 (m, 9H, Ph), 7.96 (s, 1H, *H*-2ad), 8.35 (s, 1H, *H*-8ad); ^{13}C NMR: δ 19.66 (*C*H$_3$keto), 20.80, 23.73 (2C, *C*H$_3$CoA), 33.44, 38.08, 38.17, 40.7 (5C, *C*H$_2$), 41.16 (*C*(CH$_3$)$_2$), 51.10 (*C*HOH), 53.10 (*C*HCH$_3$), 68.37 (d, ^2J = 4 Hz, *C*-5rib), 74.68 (d, ^2J = 6 Hz, CH$_2$OP), 76.58 (d, ^2J = 3.5 Hz, *C*-3rib), 77.03 (d, ^3J = 3.5 Hz, *C*-2rib), (m, *C*-4rib), 89.37 (*C*-1rib), 121.18 (*C*-5ad), 131.20, 131.88, 132.21, 132.73, 135.73, 135.45, 136.15 (9C, *C*HPh), 139.00, 139.89 (2C), 142.43 (*C*-8ad), 142.68 (*C*-6ad), 151.85 (*C*-2ad), 155.44, 158.08 (2C), 176.17, 177.41 (2C, *C*Oamide), 201.26, 211.67 (2C, *C*O); ^{31}P NMR: δ −11 (d, 1P, ^2J = 20 Hz), −10.5 (d, 1P), 2.73 (s, 1P); ESMS negative mode (*m*/*z*): 1016.2 (M−H)$^-$.

Biological assays

COX assays were conducted using intact cells expressing preferentially COX-1 or COX-2: BPAEC cells for COX-1

and LPS stimulated J774.2 for COX-2 (1μg/mL for 14 h).[23] We checked for reversibility of inhibition by recovery experiments.[24] All tests were performed in 12 wells plates (27×10^4 cells per well). After 30 min pre-incubation of cells and tested compounds, arachidonic acid 30 μM was added for 15 min, then supernatants analysed for 6-keto-PGF 1 α (BPAEC) and PGE2 (J774.2) contents. Cells were then washed twice for 40 min, and arachidonic acid 30 μM was added for 15 min to test COX activity recovery, quantifying prostaglandins as previously. 6-keto-PGF 1 α and PGE2 concentrations in supernatants were determined by EIA from Assay Designs, Ann Arbor, MI, USA.

Analysis of COX-2 adducts by SDS-PAGE and Western blot

Purified COX-2 (6×10^{-11} mol) (Cayman Chemicals, Ann Arbor, MI) in 1 μM hematine, 300 μM diethyl dithiocarbamate and Tween 20 0.1% 50 mM pH = 7.4 phosphate buffer were incubated for various times at 20 °C with **3** (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% stacking gel. Proteins were transferred onto Immobilon P membrane (Millipore, Bedford, MA, USA) by semi-dry electroblotting, using a 0.75 M glycine, 0.1 M Tris, 10% 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 using rabbit anti-KPF polyclonal antibody diluted 1/6000 and secondary goat anti-rabbit IgG alkaline phosphatase conjugated 1/5000. Anti-KPF antibody and KPF-thyreoglobuline were kind gift of Pr. E. Benoit (Ecole Nationale Vétérinaire, Lyon, France).

Acknowledgements

N.L. and F.L. wish to thank the Association de Recherche sur la Polyarthrite (ARP 1999-2000) and the Contrat de Programme en Recherche Clinique du CHU de Nancy (CPRC 1999) for financial support. Warmly dedicated to Pr Gérard Descotes on occasion of his retirement.

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