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Mechanistic Studies on the Selective Inhibition of Cyclooxygenase-2 by Indanone Derivatives

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ABSTRACT. The cyclooxygenase step in the conversion of arachidonic acid is a key point in the biosynthesis of prostanoids, managed by two enzymatic isoforms. In the following study we focused on the mechanism of the inhibitory action of CGP 28238 and structurally-related indanone derivatives using purified enzymes. Consistent with our earlier studies on cell systems, CGP 28238 revealed selective inhibition of cyclooxygenase-2. The process affects the bisoxygenase subunit time-dependently, and is reversible in the early phase of inhibition. From structure-activity relationships, we propose the formation of a Schiff base between the oxo-groups of CGP 28238 and an amino group at the active site providing additional binding forces for an effective inhibition of cyclooxygenase-2. BIOCHEM PHARMACOL 51;3:285–290

KEY WORDS. cyclooxygenase; nonsteroidal anti-inflammatory drugs; methansulfonilide; rat mesangial cells; arachidonic acid; prostanoids

COX‡ activity originates from two distinct and independently regulated isoenzymes (COX-1 and COX-2) encoded by two different genes [1]. Both isoforms are key enzymes in the AA cascade catalysing, as they do, the committed step in the biosynthesis of prostanoids. Excessive production of AA metabolites, mainly thought to be based on the induction of COX-2 by proinflammatory stimuli, has been suggested to play a major role in the pathophysiology of inflammation and arthritis, and during differentiation [2–4]. Activation of COX-1, the constitutively expressed enzyme, leads to prostaglandins acting in a more physiological way for cytoprotection and antithrombogenity [5].

Since the investigations of Vane, it has become apparent that the anti-inflammatory activities of NSAID such as aspirin, ibuprofen, and indomethacin can be related to their ability to inhibit the biosynthesis of prostaglandins from arachidonic acid [6]. However, exposure to these drugs is associated with an increased risk of gastrointestinal bleeding and renal complications by affecting the synthesis of prostaglandins essential for physiological functions [7]. Both cyclooxygenases are important targets for NSAID, and the spectrum of activities of commonly available standard NSAID ranges from a high selectivity towards COX-1 through equipotent activity on both isoforms, explaining their variations of side effects [8]. Thus, the development of specific inhibitors of COX-2 offers the prospect for a new generation of more selective nonsteroidal anti-inflammatory drugs without side effects attributable to inhibition of the constitutive enzyme.

Selective COX-2 inhibitors assumed to act therapeutically in minimising ulcers in stomach and intestine are NS-398 [9] and SC-5360 [10], but their inhibitory mechanisms are not fully understood. Recently we reported on a group of indanone derivatives that exhibited a selective inhibition of COX-2 with no effect on COX-1, and thereby offering excellent gastrointestinal safety [11, 12].

MATERIALS AND METHODS

COX isoenzymes (ovine) were purchased from Cayman Chemicals; [^{14}C]-CGP 28238 (spec. activity: 1236kBq/mg), CGP 28237, the metabolites of CGP 28238 (6-(2,4-difluorophenoxy)-5-methyl-sulfonylamino-1-indanone): M1(2-hydroxy), M2 (3-hydroxy), M3 (1,2-cis-dihydroxy), and M4 (1-hydroxy), as well as the prodrug M5 N-6-(2,4-difluorophenoxy-5-indanylmethansulfonamide), were synthesized by Dr. P. G. Ferrini, Ciba-Geigy, Basel, Switzerland; indomethacin, fluribiprofen, and sodium borohydride were purchased from Sigma (Deisenhofen, FRG). Stock solutions were prepared in DMSO, with the final concentration kept below 1%. [^{14}C]-arachidonic- and linolenic acid (53 mCi/mmol) were products of DuPont (Dreieich, FRG); radiolabelled rainbow markers were from Amersham (Braunschweig, FRG); and organic solvents were obtained from Merck (Darmstadt, FRG).

Cell Culture

Rat mesangial cells were cultured as described in [11].

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‡ *Abbreviations:* COX, cyclooxygenase; NSAID, nonsteroidal anti-inflammatory drugs; RMC, rat mesangial cells; AA, arachidonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MES, 2-(N-morpholino)ethanesulfonic acid.

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Measurement of Bisoxygenase Activity [Cyclooxygenase] by Oxygen Consumption

Bisoxygenase activity was assayed at 37° using a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, CO, U.S.A.) in a cuvette containing 200 µL Tris-HCl pH 8.0, 2 mM phenol 1 µM hematin, and 40 U COX-2 enzyme [13]. Reactions were initiated by addition of 80 µM arachidonic acid as substrate, and O_2 consumption was monitored. Calibration was done by injection of 10 nmol oxygen from a saturated solution.

Measurement of Peroxidase Activity of Cyclooxygenase

Peroxidase activity was assayed spectrometrically [13]. The reaction cuvette contained 100 mM Tris-HCl pH 8.0, 5.6 mM guaiacol, 1 µM hematin, and 40U COX-2 isoenzyme. After 2 min preincubation with inhibitors as indicated, the reaction was initiated by adding H_2O_2 in a final concentration of 400 µM. The increase in absorbance at 436 nm was monitored.

Measurement of Cyclooxygenase Activity by Product Analysis

COX isoenzymes were preincubated for the indicated timepoints in the presence of the inhibitors at variable concentrations, and then stimulated with either 25 µM [^{14}C]arachidonic acid or 25 µM [^{14}C]linoleic acid for 2 min. The generated radioactive products PGE_2 and PGD_2 or 9-HODE (9-hydroxyoctadecaenoic acid and 13-hydroxyoctadecaenoic acid), respectively, were extracted into ethylacetate (4 vol) pH 3, gently evaporated under N_2, and subsequently separated by thin-layer chromatography on silica gel plate developed in ethylacetate/H_2O/isooctane/acetic acid (90:100:50:20) for prostanoids and diethylether/petrolether/acetic acid (50:50:1, by vol) for lipoxygenase metabolites. The amount of prostanoid production was quantified by autoradiography, following visualisation with a phosphoimaging system (Image Quant; Molecular Dynamics), and expressed as % radioactivity of the total extracted radioactivity. Thereafter, the location of authentic prostanoids was visualized with iodine vapour, and the radioactive zones identified.

Cyclooxygenase Activity of Intact Rat Mesangial Cells

After stimulation with interleukin-1β (1 nM; 20 hr), cells were rinsed twice with PBS and the cyclooxygenase inhibitors added. Following 10 min incubation, the cells were washed and stimulated with [^{14}C]labelled arachidonic acid (final concentration 5 µM) at 37°C for 2 min. The reaction was stopped by scraping off the cells with a rubber policeman, and the cell suspension was transferred to 4 v/v ethylacetate. Further analysis was done as described above.

UV Cross-Linking of CGP 28238 to COX Isoenzymes

[^{14}C]CGP 28238 was used in a 100-fold molar excess (790 nCi) over COX isoenzymes, each 5.5×10^{-10} mol and cross-linked for 15 min by irradiation at 340–360 nm (Xenon, 100W) using a filter system (Balzer UV, Schott KG3) with a transmission of 70%. Subsequently, non-bound radioactivity was precipitated with 2% activated charcoal, saturated with 0.1% bovine serum albumin. The remaining supernatant was separated by a 10% denaturing SDS-PAGE, and the radioactivity detected after one week exposure by a phosphoimaging system (Image Quant, Molecular Dynamics).

Analysis of Schiff Base Formation

Radioactive [^{14}C]CGP 28238 (20-fold molar excess) was incubated with purified COX-2 enzyme (5.5×10^{-10} mol) for 1 hr at room temperature to reach equilibrium. After removal of nonbound radioactivity by activated charcoal, the samples (50 µL) were treated with 50 µL of freshly made sodium borohydride (80 mM in a 200 mM MES, pH 6.0) for 10 min on ice. Radiolabelled proteins were separated by 10% SDS-PAGE and subsequently blotted on a nitrocellulose membrane. The membrane was dried, and after two weeks' exposure was visualised by phosphoimaging. The identity of radiolabelled COX-2 was verified by Western-blot analysis as described [11], and quantified by the phosphoimaging system described above.

RESULTS

In our previous studies on the selective inhibition of COX-2 by CGP 28238, we used cellular systems such as IL-1-treated rat mesangial cells as a source of COX-2, and human platelets as a COX-1-dependent formation of thromboxane A_2 [11]. The respective IC_{50} values of the inhibition of the enzymatic activities were 15 nM and 72 µM, demonstrating a more than 1000-fold higher sensitivity of this compound for COX-2.

However, it should be noted that these values were obtained after a period of 15 min preincubation with the cells. In the present study, we tested the inhibitory potency of CGP 28238 on purified COX-1 and COX-2, allowing a preincubation time of 2 min (Table 1).

With arachidonic acid as substrate, the IC_{50} value for COX-2 was 3.1 µM, whereas COX-1 was not affected up to a concentration of 100 µM. Indomethacin as a control reached 320 nM for COX-1 and 20 µM for COX-2. Using the conversion of linoleic acid to 9 and 13-HODE as an additional

TABLE 1. IC_{50} Values of CGP 28238 and indomethacin on COX isoenzymes with arachidonic or linoleic acid as substrates

Substrate	Arachidonic acid		Linoleic acid	
Isoenzyme	COX-1	COX-2	COX-1	COX-2
CGP 28238	Ø 100 µM	3.1 µM	Ø 100 µM	3.8 µM
Indomethacin	320 nM	20 µM	n.d.	n.d.

Ø, No effect at this concentration; n.d., not done.

COX isoenzymes were incubated for 2 min with various concentrations of the inhibitors, and subsequently stimulated either with arachidonic or linoleic acid. IC_{50} is defined as the concentration required to lower the formation of PGD_2 and PGE_2 or 9/13-HODE to 50%, relative to control.

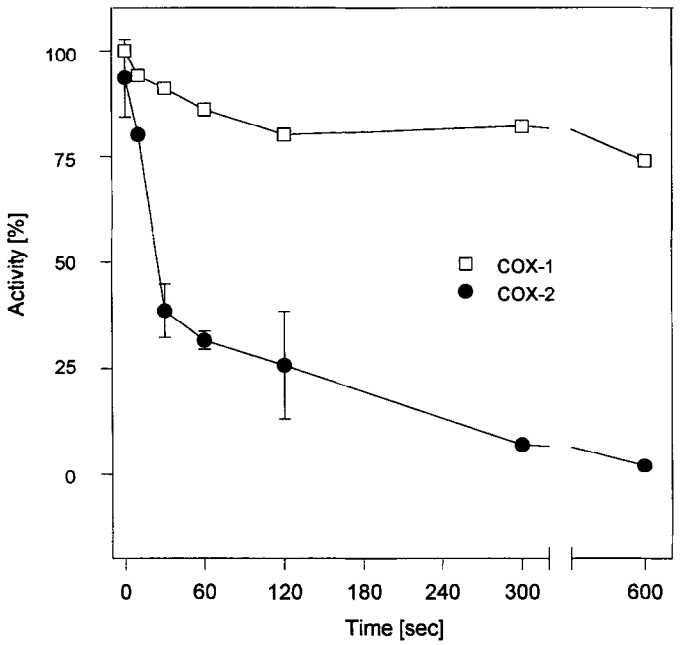


FIG. 1. Time-dependence of CGP 28238 on COX-1 and COX-2 activity. Purified COX isoenzymes were preincubated for the indicated times with 20 μM CGP 28238. Activity was measured as described under Material and Methods.

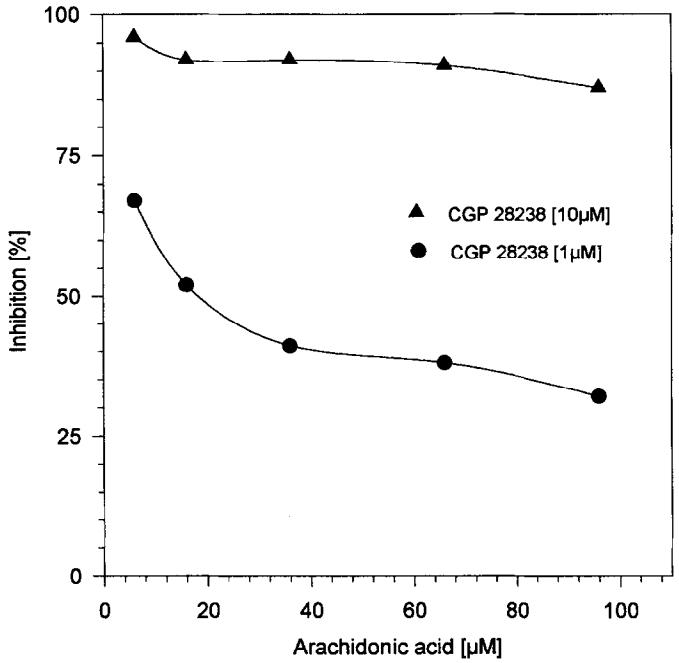


FIG. 2. The effect of substrate increment on the inhibition of COX-2 by CGP 28238. COX-2 was preincubated with a low suboptimal [1 μM] or a high [10 μM] concentration of CGP 28238 for 10 min. COX activity was determined using the indicated substrate concentrations. COX-2 inhibition was evaluated by comparison to the control incubation (without CGP 28238).

measurement of COX-2 activity, we obtained similar values as for prostanoid formation.

Assuming that the incubation time could have caused the large difference between the cellular and the enzyme systems we varied the time of preincubation and obtained the dependence shown in Fig. 1 and Table 4. Accordingly, the IC_{50} values decreased to about 750 nM for COX-2, and clearly demonstrated a dependence on time, but also showed that the low concentration needed to elicit an inhibition in intact mesangial cells could not be achieved with the purified enzyme.

Further experiments were designed to clarify the mode of action of CGP 28238 to explain these results. We first investigated the effect of CGP 28238 on the two catalytic properties inherent in the cyclooxygenase protein, the bisoxygenase and peroxidase activity of the COX-2 isoform. The peroxidase function of the enzyme was only slightly enhanced by increasing drug concentrations, whereas the bisoxygenase function was obviously affected (Table 2).

TABLE 2. Influence of CGP 28238 on cyclooxygenase and peroxidase activity of COX-2

Subunit activity of COX-2 (%)	CGP 28238 [μM]			
	0	6	60	600
Bisoxygenase	100	41.3 ± 4.1	10.2 ± 2.4	7.41 ± 3.1
Peroxidase	100	100 ± 3	105 ± 3	110 ± 8

Bisoxygenase activity was assayed by oxygen consumption after addition of 80 μM AA (100% activity = 5.8 nmol O_2/min/10 μg protein). The peroxidase activity was measured spectrophotometrically as the increase at 436 nm after initiating the reaction with 400 μM H_2O_2. The unaffected enzyme is given as 100%.

A second approach involved the nature of this inhibition being either reversible or irreversible. At a suboptimal concentration of 1 μM CGP 28238 and preincubation for 10 min, the subsequent addition of increasing levels of arachidonic acid caused a decrease in the inhibitory effect, indicating that at least part of the inhibition was reversible (Fig. 2). This tendency was also confirmed by dilution experiments reported in Table 3. The recovery of enzyme activity following 1:15 dilution of CGP 28238 demonstrated potent inhibition, even in the remaining concentration of 130 nM. In the case of an irreversible component, one may assume a covalent binding of the drug to the enzyme or a conformationally modified enzyme. However, a modification of COX-2 by [^{14}C]labelled CGP 28238, as has been reported for aspirin [14], could not be observed.

Thus, if a conformational change were the reason for an irreversible part of the inhibition, it would be of interest to

TABLE 3. Recovery of COX-2 after dilution experiments

	Inhibition (%)
CGP 28238 2 μM	84.6 ± 4.6
CGP 28238 130 nM (1:15)	66.1 ± 6.2

Cyclooxygenase-2 (40 U) was incubated in the presence of CGP 28238 [2 μM] for 10 min. A 1:15 dilution (resulting in a remaining inhibitor concentration of 130 nM) of the sample was done prior to stimulation with exogenous arachidonic acid. The data are means ± SE from three separate experiments where the recovery of enzyme activity was given as % to untreated controls considering the dilution conditions.

determine whether the drug had to enter the active site or could act through a remote site. Realising the carbonyl function in CGP 28238 in conjugation with the aromatic ring, we attempted irradiation of radiolabelled CGP 28238 at its absorption maximum in the presence of COX isoenzymes. In this experiment, illustrated in Fig. 3A, COX isoenzymes were cross-linked to [^{14}C]CGP 28238 at 340–360 nm wavelength. Pursuing separation by SDS electrophoresis and autoradiography, a unique radioactive band of COX-2 was seen (Fig. 3A, arrow). The control incubation with COX-1 was negative, and served as a control excluding a nonspecific photoactivation of unbound inhibitor.

Since these results pointed to an active site interaction of CGP 28238 with COX-2, we used different derivatives (Table 4) of this drug to understand the chemistry of the inhibitory mechanism.

The drugs M1–M4 were synthesised after they had been identified as metabolites of CGP 28238 in human phase I studies. M5 is a prodrug to CGP 28238 and CGP 28237 is a nonhalogenated analogue. All have been shown to be inactive in inhibiting COX-1 (data not shown), but have different inhibitory potencies against COX-2. CGP 28237 was equipotent to the parent compound, and M1 and M2 followed in descending order. M3 and M4 were relatively weak inhibitors in rat mesangial cells. This inhibitory pattern in the cellular system is reflected by inhibition of the purified COX-2, but with reduced potency.

On the basis of these findings, it became evident that the cyclopentanone ring was essential for an efficient inhibition of COX-2 suggesting to us the formation of a Schiff base intermediate between the keto group of CGP 28238 and an amine residue in the active site of cyclooxygenase-2. Sodium borohydride [15], known to be capable of reducing an imine to give an alkylated amine, was used to test this hypothesis. We incubated the labelled drug with COX-2 for 1 hr and then added borohydride to detect a Schiff base. We observed a labelled COX-2 band after separation on SDS-gel and blotting on a nitro-cellulose membrane (Fig. 3B). In a control incubation coincubated in the presence of flurbiprofen [100 μM], the intensity of CGP 28238 labelling of COX-2 was observed to be 5 times less (quantification by image quants, diagram Fig. 3B) than in the corresponding lane without flurbiprofen. The identity of COX-2 was proven by Western blot analysis with a polyclonal COX-2 antibody (data not shown). Radiolabelled protein standards are indicated on the left.

DISCUSSION

Our results with the purified proteins of COX isoforms confirm the selectivity of CGP 28238 as an inhibitor for COX-2, although the high inhibitory potencies seen in cellular systems could not be obtained. However, this is not unusual, and similar discrepancies between NSAID inhibition profiles in intact cells, cell homogenates, and purified enzymes have been reported by others [16]. Considering the extremely lipophilic compartments of COX localisation [17] in endoplasmatic reticulum and nuclei, distribution or transport effects as well as the complex inhibitory mechanisms involved may be responsible.

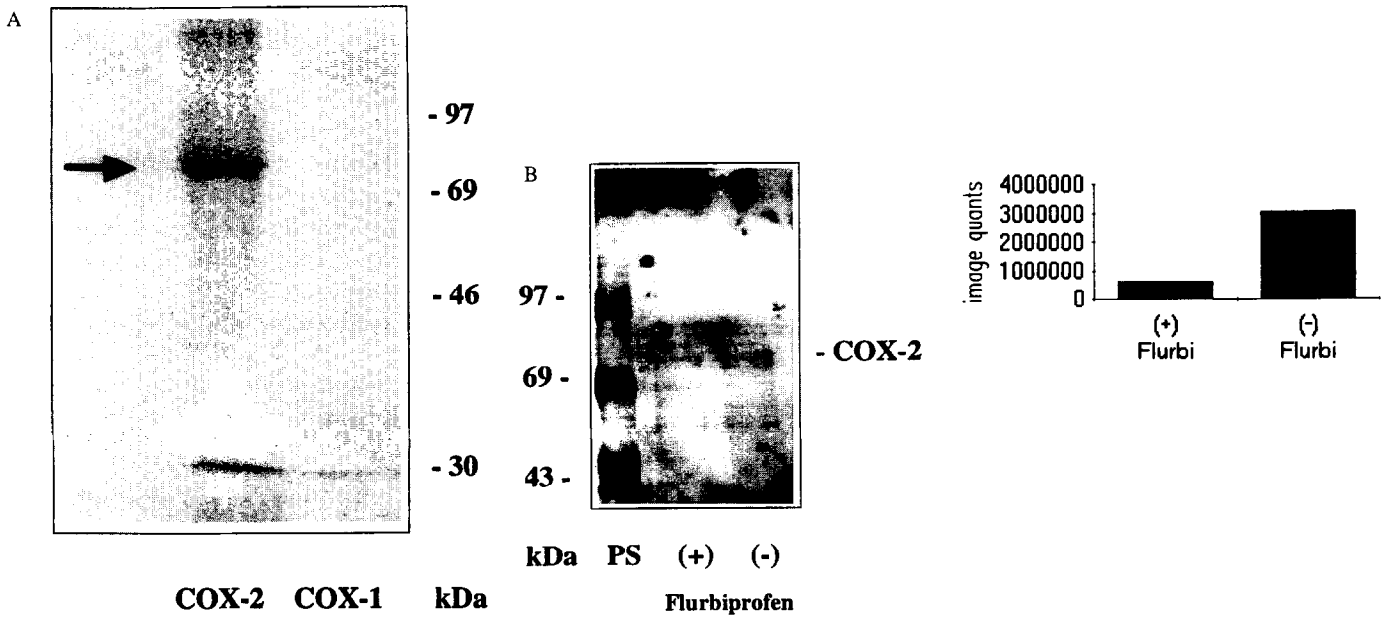
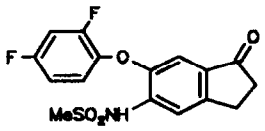
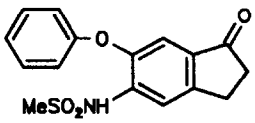
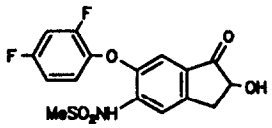
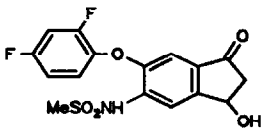
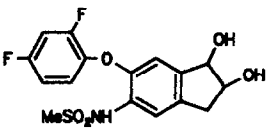
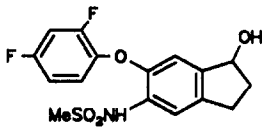
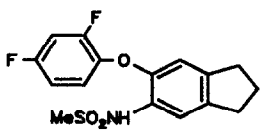


FIG. 3. (A) UV cross-linking of [^{14}C]labelled CGP 28238 to COX-2. The COX isoenzymes (COX-1, COX-2) were incubated in the presence of radioactive CGP 28238 and UV-cross-linked as described under Methods, following separation on 10% SDS-PAGE. The migration of unlabelled standards is given on the right. (B) Test of Schiff base formation between [^{14}C]labelled CGP 28238 and COX-2. Reduction of suggested Schiff base intermediate between an amino group of COX-2 and the cyclopentanone moiety of CGP 28238 by sodium borohydride in the presence (+) or absence (−) of 100 μM fluribiprofen. The positions of several labelled protein standards are indicated on the left.

TABLE 4. The effect of various CGP 28238 derivatives on COX-2 activity of the purified enzyme or in IL-1 induced rat mesangial cells (RMC)

Compound	Structure	COX-2 IC_{50} (2 min)	COX-2 IC_{50} (10 min)	RMC IC_{50} (10 min)
CGP 28238		3.1 μM	750 nM	15 nM
CGP 28237		4 μM	620 nM	20 nM
M1		Ø 50 μM	~12 μM	46 nM
M2		Ø 50 μM	~16 μM	91 nM
M3		Ø 50 μM	Ø 50 μM	10 μM
M4		Ø 50 μM	Ø 50 μM	10 μM
M5		Ø 50 μM	Ø 50 μM	toxic (at 1 μM)

Comparison of different CGP 28238 analogues on their inhibitory action on purified or cellular COX-2 enzyme. After preincubation for 2 min and 10 min with different concentrations of the indicated inhibitors, the enzyme was stimulated with [^{14}C]AA. The IC_{50} values were calculated from the conversion of AA to PGE_2 and PGD_2 (purified COX-2), or generation of 6-keto-$PGF_{1\alpha}$ for cellular COX-2 (RMC).

Ø, No effect up to this concentration.

Our data clearly point to a time-dependent increase in the inhibitory potency of CGP 28238 on the purified enzyme, where 10 min are required to reach maximal inhibition. There is definitely a competitive and reversible low-affinity binding of the drug in the initial phase that shifts to a high-affinity binding with time.

This property is shared with other inhibitors such as indomethacin and flurbiprofen [18]. The competition with arachi-

donic acid already suggests that the drugs enter the active site, and this is corroborated by the cross-linking experiment, the cyclooxygenase subunit interaction, and the covalent binding after reduction with borohydride.

Schiff base formation can contribute to a high-affinity but still reversible binding of carbonyl-containing drugs, with reduction to the secondary amine leading to covalent binding. Thus, the carbonyl group of CGP 28238 and that of its carbonyl-containing derivatives may lead to the high-affinity binding in the 10–100 nM range, which is supported by the results in Table 4. This suggests that the carbonyl group in CGP 28238 is an important feature for its inhibitory potency, but can be attenuated by steric hindrance as seen for M1 and M2, which bear an additional hydroxy group. On the other hand, halogenation of the phenyl residue has only marginal effect at the enzyme level, whereas the presence of the methane sulfonamide moiety is essential [19].

Since covalent binding of CGP 28238 was not verified under physiological conditions, the increased affinity with time may indeed be a consequence of a conformational change, as suggested by others [20, 21], and seems to be promoted by a possible formation of a Schiff base. Such changes in protein conformation are not unusual, since the mechanism of cyclooxygenase activity requires a transfer of PGG_2 as an intermediate of the bisoxygenase subunit to the peroxidase binding site; this site is distinct from the COX binding site, as evidenced by X-ray crystallographic data [22]. Based on the results of this study, it should be possible to accelerate the synthesis of improved COX-2 inhibitors.

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